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Risk assessment for peri- and post-menopausal women taking food supplements containing isolated isoflavones

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)

Abstract

The EFSA ANS Panel was asked to deliver a scientific opinion on the possible association between the intake of isoflavones from food supplements and harmful effects on mammary gland, uterus and thyroid in peri- and post-menopausal women. Isoflavones are naturally occurring substances which can be found in, among other sources, soy, red clover and kudzu root. The main isoflavones are genistein, daidzein, glycitein, formononetin, biochanin A and puerarin. Their chemical structures are related to 17β-oestradiol and they possess oestrogenic properties. Furthermore, isoflavones may interact with the synthesis of thyroid hormone. Food supplements targeted at peri- and post-menopausal women typically provide a daily dose of isoflavones in the range of 35–150 mg/day. A systematic review was performed to investigate whether an association could be found between intake of isoflavones from food supplements and adverse effects on the three target organs in peri- and post-menopausal women. The human data did not support the hypothesis of an increased risk of breast cancer from observational studies nor of an effect on mammographic density nor on proliferation marker Ki-67 expression in interventional studies. No effect was found on endometrial thickness and histopathological changes in the uterus up to 30 months of supplementation with 150 mg/day of soy isoflavones. After 60 months some non-malignant histopathological changes were reported. Thyroid hormones levels were not changed following intake of isoflavones from food supplements. The background exposure from the diet in the general European population was estimated to be lower than 1 mg/day, whereas in consumers of soy-based foods it could be higher. The Panel concluded that it was not possible to derive a single health-based guidance value for the different preparations in post-menopausal women. However the doses used in the intervention studies and their duration could serve as guidance for the intake of food supplements.

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Keywords: isoflavones, phytoestrogens, food supplements, mammary gland, uterus, thyroid, systematic review

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Summary

In accordance with Article 29 (1) of Regulation (EC) No 178/2002, the Bundesinstitut für Risikobewertung (BfR) asks the European Food Safety Authority to provide a scientific opinion on the possible health risks associated with the intake of isolated isoflavones in food supplements by peri- and post-menopausal women.

Isoflavones are a class of naturally occurring substances, present in a number of plants, especially in soybeans, red clover and kudzu root. Isolated isoflavones used in dietary supplements are defined as extracts from soybeans containing a mixture of predominantly glycosylated genistein, daidzein and glycitein and isolated forms of these soy-based compounds and extracts from red clover containing a mixture of predominantly glycosylated formononetin and biochanin A. Kudzu essentially contains the oestrogenic glycosides of daidzein and genistein in a ratio of two-thirds to one-third, usually as well as puerarin.

The scientific opinion requested by the BfR should address the relevant available scientific evidence on the potential adverse effects associated with intake of isolated isoflavones in food supplements by peri- and post-menopausal women, including data from both human and animal studies. The scientific opinion should focus on possible harmful effects on mammary gland, uterus and thyroid. If adverse effects are identified, it should provide indication of the underlying potential modes of action. The scientific opinion should also provide an estimate of exposure of the target populations to the isolated isoflavones from food supplements and, if possible, give advice on a safe intake level of isolated isoflavones from dietary supplements.

Thus, the assessment is a focused assessment concerning the population of interest (peri- and post-menopausal women) as well as the target organs of interest (breast, uterus, thyroid).

The isoflavones (and some of their unconjugated metabolites) considered relevant for this risk assessment were daidzein, genistein, glycitein, biochanin A and formononetin, and their glycosides daidzin, genistin, glycitin and puerarin. The potential hazards of concern for this assessment are those expected from the interaction of isoflavones with endocrine pathways. Interactions are known with the oestrogen receptors ERα and ERβ. Apart from the inhibition of thyroid peroxidase, several other mechanisms have been described from *in vitro* and animal experiments, which could indicate an effect of isoflavones on thyroid function.

A review of the association between isoflavones intake from food supplements and possible effects on mammary gland, uterus and thyroid was performed in accordance with the principles of systematic review, based on an *a priori* established protocol.The criteria defined for the selection of the studies limited inclusion to studies conducted in the relevant population of interest and to ovariectomised animals. All the studies included in the systematic review were appraised for validity and risk of bias using pre-defined criteria agreed before the start of the assessment. The literature on kinetics and metabolism of isoflavones was reviewed by a narrative approach. Published literature on genotoxicity, retrieved by a focused literature search, was conducted using the search strings detailed in the protocol.

For the systematic review, a total of 7 841 hits were retrieved. After screening for relevance, 43 human studies and 62 animal studies were retained for this opinion.

For the exposure, background dietary isoflavone exposure was estimated using levels of isoflavones in soy and soy-based products reported in the literature in combination with food consumption data from the EFSA Comprehensive European Food Consumption Database for the group of women older than 40 years of age. Exposure to isoflavones from food supplements in the population of peri- and post-menopausal women was taken from the range of doses used in the intervention studies in the target population included in the systematic review. In addition, information from the relevant industry associations was obtained on the labelled amount of isoflavones in the supplements and the recommended range of daily doses. This information was compared with published data reporting on the measured isoflavone content in the food supplements.

For humans, no data on absolute bioavailability can be given. In mice, absolute bioavailability amounted to 9–14 % for genistein and 29–34 % for daidzein. In rats, absolute bioavailability of genistein was 7 % in males and 15 % in females. In humans, the maximum concentration (Cmax) of

genistein aglucone and that of daidzein aglucone varied between 0.4 % and 3.9 % and between 1.4 % and 4.2%, respectively, expressed as percentage of the total isoflavone concentration. Hence, it can be deduced that the absolute bioavailability of the two main isoflavones must be low in humans. At least some data on the metabolism of the soy isoflavones daidzein and genistein are available for humans, monkeys, rats, and mice. The main metabolites are the sulphate and glucuronide conjugates of the substances. Monkeys, rats, and mice are described as 100 % equol producers, meaning that the microbiotas of these animals are uniformly able to transform daidzein to a considerable extent to *S*-equol. In humans only a part of the population is able to produce *S*-equol. Oxidative phase I metabolites of daidzein and genistein, mainly 6-hydroxy-, 8-hydroxy- and 3′-hydroxy-daidzein as well as 6-hydroxy-, and 3′-hydroxy-genistein, are found in humans, rats and mice, although as minor metabolites.

For the endpoint breast, four epidemiological studies investigating breast cancer incidence (involving, in total, 2 216 isoflavone users), eight interventional controlled studies, measuring mammographic density (741 participants), and two interventional controlled studies, investigating histopathological changes (75 participants), did not suggest an association between exposure to isoflavones-containing food supplements and adverse effects in the mammary gland. Ten studies in ovariectomised animals were found, investigating breast cell proliferation, and 11 animal studies were identified, investigating histopathological changes in the mammary gland of animals treated with isoflavones. Although in the majority of the studies no effect was noted, a stimulating effect on the mammary gland was observed in two rat studies (genistein 5.4 and 54 mg/kg/day and 221 mg/kg body weight (bw)/day, both studies carried out for 90 days). The findings are consistent with the results from the US National Toxicology Program study conducted in non-ovariectomised animals administered genistein at doses ranging 0.3–44 mg/kg bw/day, in which there was some evidence of carcinogenic activity of genistein in female Sprague–Dawley rats based on an increased incidence of mammary gland adenoma or adenocarcinoma (combined).

For the endpoint uterus, no study was found that investigated the association between intake of isoflavones from food supplements and risk of uterine cancer in the target population. Endometrial thickness was measured in 25 interventional controlled studies (1 484 participants) and histopathological investigations of endometrium were carried out in nine interventional controlled studies (677 participants). None of the studies reported statistically significant changes in endometrial thickness compared with control. In only two studies were some histopathological effects noted. One study was not properly controlled and had further methodological flaws. In the other study, there were no findings after 2.5 years of intervention, whereas after 5 years of intervention only five cases of simple hyperplasia and one case of complex hyperplasia of the endometrium were observed, but no cases of endometrial carcinoma. The findings could indicate an oestrogenic effect. Thirteen studies in animals investigated uterus cell proliferation, among them three in monkeys, and 22 animal studies studied uterus histopathological changes, among them four studies in monkeys. An effect of isoflavones was not seen in most of the studies. However, a daidzein-rich soy extract containing daidzein at doses above 40 mg/kg bw/day, caused an increase in cell proliferation of the epithelium and the stroma of the uterus as well as the vaginal epithelium of rats. Racemic equol (36 mg/kg/day), administered to rats induced an increase in proliferation of stromal cells of the uterus, whereas an equivocal effect was observed on proliferation of the epithelial cells of the uterus. In monkeys and rats, isoflavones obtained from soy extracts or soy protein isolates did not result in significant changes in endometrial thickness, endometrial hyperplasia, epithelial area or endometrial gland area. Daidzein-rich soy extract and red clover extracts at high doses (≥ 125 mg/kg bw/day) caused a significant increase in endometrial area, endometrial thickness, number of glands and myometrial area in rats. Genistein and daidzein did not induce histopathological changes such as hypertrophy, hyperplasia or squamous metaplasia in the uterus. Racemic equol at a dose of 10 mg/kg bw/day or higher resulted in a significant increase in uterine wall thickness when administered for 90 days, but no such changes were observed after 35 days' administration of the same dose.

Eleven human controlled randomised studies that reported effects of isoflavones administration on some thyroid-related endpoints were identified. In total, 925 subjects were allocated to isoflavones. In none of the studies was a clinically relevant effect on the thyroid detected. Although the studies have some flaws (thyroid function not the primary endpoint, sample size calculation not given, low power to detect changes) the Panel's conclusion is that administration of food supplements containing

isoflavones is not associated with clinically relevant changes in thyroid function (hypo- or hyperthyroidism) in the population of interest.

As regards the genotoxicity of genistein and the two catecholic oxidative metabolites of daidzein, 3′,4′,7-trihydroxyisoflavone and 4′,6,7-trihydroxyisoflavone, genotoxic effects found *in vitro* in mammalian cells, for which a thresholded mechanism of action has been demonstrated, have not been reproduced in valid *in vivo* micronucleus tests in rats and mice or in the comet assay and micronucleus test in human studies. Based on these findings, it can be concluded that isoflavones are not genotoxic.

In the human intervention studies included in this assessment, doses of isoflavones ranging 30–900 mg/day were used. On the basis of the published studies included in this assessment and measuring the actual content of isoflavones in a number of food supplements available on the market, it can be estimated that intake of isoflavones from food supplements is extremely variable, ranging approximately 0.1–100 mg/day for soy isoflavones, 30–160 mg/day for isoflavones from red clover and 20–50 mg/day for kudzu root isoflavones (all the values above are expressed as aglycones). The values above are in line with information provided by the relevant food sector business operators, with respect to the recommended daily doses of isoflavones in products mainly targeted at menopausal women. The overall intake of isoflavones from the diet in women (0.27–1.43 mg/day) is lower than the lowest recommended daily intake of isoflavones from food supplements (20–35 mg/day), although in women with special dietary habits (e.g. soy consumers and vegetarians) the intake of isoflavones from the diet could be within the range of exposure from food supplements. Consumption of certain soy-based food products (e.g. soy drink, soy yoghurt, tofu) alone in a single day might contribute to the intake of isoflavones of the same order of magnitude as estimated for food supplements.

In this opinion, data from human and animal studies have been assessed; however, the Panel identified a number of limitations which could introduce uncertainties in the evaluation. Owing to the nature of the uncertainties identified, it was not always possible to state in which direction they might have influenced the conclusions.

The Panel noted that the conclusions drawn in this opinion are based on the assumption that intake of isoflavones from the use of food supplements represents the major contribution to the intake of isoflavones. In line with the Terms of Reference, this risk assessment was focused on three target organs, mammary gland, uterus and thyroid, in a sub-group of the general European population: peri- and post-menopausal women. The assessment was limited to isoflavones ingested as food supplements at doses used in the human studies available in the published scientific literature and following a request for information from relevant interested parties.

The Panel considered that results obtained from the human studies and the studies in ovariectomised animals were most relevant for the target population. Furthermore, the Panel noted that, in addition to all the limitations described for the relevant studies, studies in other human populations (e.g. males) or animal models (e.g. juvenile animals, transgenic models) would not provide additional relevant information on the specific risks being assessed in peri- and post-menopausal women. Therefore, the result from this assessment cannot be extrapolated to other groups and other situations in the general population.

Based on the data reviewed and presented in the current opinion, and taking into account the uncertainties described above, the Panel reached the following conclusions:

1) An assessment could be provided only for human studies in the relevant population of peri- and post-menopausal women or from animal studies in OVX animals investigating the relevant pre-defined endpoints in mammary gland, uterus and thyroid. Details of the isoflavone composition of the preparations tested in the human and animal studies are given in the opinion.

2) In assessing the effects of isoflavones on the three target organs, the Panel decided that differences in functions, receptor density, proportions of ERα and ERβ and effects of receptor activation meant that it was not possible to directly extrapolate observations from any one organ to the others. The Panel noted differences in biological effects and activity between isoflavones from different sources and in different organs and, therefore, concluded that currently it is not generally feasible to apply a read-across approach either between different

preparations or between similar preparations in different organs. Hence, a full evaluation is possible only if study results are available for all three target organs.

3) There is overlap between peri- and post-menopause. The World Health Organization defines 'perimenopause' as the period immediately prior to the menopause (when the endocrinological, biological and clinical features of approaching menopause commence) and the first year after menopause and 'postmenopause' as the period dating from the final menstrual period. Although women progress through peri-menopause to post-menopause it may not be possible to definitively categorise them as peri- or post-menopausal. Only a small proportion of the participants included in the interventional studies would be classified as peri-menopausal women according to the definition above. Despite the uncertainties and limitations described, and given the overlap in the definitions, the Panel considered that the data on mammary gland and thyroid allow conclusions that are applicable to post- and peri-menopausal women.

   With respect to the data on uterus, the Panel considered that the database is not sufficient to draw conclusions on peri-menopausal women.

   Because not all three target organs were covered by the intervention studies in the peri-menopausal population, the overall conclusions of this opinion apply only to post-menopausal women.

4) For the target organ mammary gland, three case–control studies and one prospective cohort study did not support the hypothesis of an increased risk of breast cancer associated with the intake of isoflavones from food supplements. The Panel acknowledged that the central tendency was around 1, consistently across all the studies included in the review, and the upper limit of the confidence interval for the estimated odds ratio was always below 1.67.

   Based on interventional trials encompassing 816 women, the Panel concluded that neither enhanced breast density (741 women) nor histopathological changes (75 women) were observed for soy isoflavones/soy extracts, soy protein, daidzein-rich isoflavones, genistein and red clover extract. The Panel concluded that, on the basis of the evidence reviewed, there is no indication for adverse effects on the mammary gland in post-menopausal women from isoflavones when taken in doses and for durations as described above.

   The information on women with breast cancer obtained from this systematic review is limited; therefore, the opinion cannot conclude on the risk of oestrogenic isoflavones-based food-supplements in postmenopausal women with a current diagnosis or history of oestrogen-dependent cancer.

5) For the target organ uterus, neither changes in endometrial thickness (studies involving, in total, 1 484 participants) nor remarkable histo(patho)logical findings (studies encompassing 677 participants) were observed in any of the human interventional studies, with the highest isoflavone dose being 150 mg/day administered for a period of 2.5 years. An effect on uterine weight was found in rats with doses of various isoflavones of between 10 mg/kg body weight (bw)/day and 100 mg/kg bw/day. The Panel considered that this was not an adverse effect.

   On the basis of the evidence from human studies and the considerations on the findings from the animal studies, the Panel concluded that no adverse effects on the uterus were noted for soy isoflavones/soy extract, soy protein, daidzein-rich isoflavones, glycitein-rich isoflavones, genistein and red clover extract in post-menopausal women when taken in doses and for durations as described above.

   No information on women with uterine cancer was obtained from this systematic review; therefore, the Panel cannot conclude on the risk of oestrogenic isoflavones-based food-supplements in post-menopausal women with current diagnosis or a history of oestrogen-dependent cancer.

6) The assessment of effects on the thyroid function was exclusively based on the results from human interventional studies. Based on these studies (involving 925 participants taking isoflavones and 576 serving as controls), the Panel concluded that there are no statistically significant changes to indicate that food supplements containing soy isoflavones/soy extract,

soy protein, daidzein-rich isoflavones, genistein or red clover extract exert a hypothyroid effect in post-menopausal women with normal thyroid function.

7) Human studies investigating effects on the three target organs of intake of food supplements containing extracts from kudzu root and the isoflavones genistin, daidzin, daidzein, glycitin, glycitein, puerarin, biochanin A and formononetin were not retrieved. No animal studies other than those evaluating the effect of isoflavones on uterine weight were available. Taken together, these findings preclude an assessment of these substances and mixtures.

8) For genotoxicity, positive findings expressed *in vitro* in mammalian cells by the two catecholic oxidative metabolites of daidzein 3′,4′,7-trihydroxyisoflavone and 4′,6,7-trihydroxyisoflavone and by genistein through the stabilisation of the 'cleavable complex' and generation of DNA double-strand breaks at topoisomerase II–DNA binding sites, for which a thresholded mechanism of action has been demonstrated, have not been reproduced in valid *in vivo* micronucleus tests in rats and mice and in comet assay and micronucleus test in human studies. On these bases, the use of isoflavones in food supplements is not of genotoxic concern.

9) A comparison of the estimated intake of isoflavones from food supplements with estimates of intake based on food consumption data showed that the levels of daily intake of soy isoflavones from food supplements may be achieved by consumers of specific soy foods, such as tofu, soy yoghurt, soy milk and drinks.

Overall, the Panel concluded that it was not possible to derive a single health-based guidance value or a safe intake level for food supplements containing isoflavones. The doses and duration of treatment with the individual preparations used in the interventional studies may serve as guidance for a dose and duration of use at which no effect has been observed in all three target organs in the evidence considered for this opinion. The Panel noted that recommended daily doses of the marketed food supplements, with the exception of one product containing soy isoflavones and one product containing red clover, seem to fall within these ranges, albeit the food supplements do not bear any clear indication with respect to the recommended duration of use. For products containing kudzu root, no data were available which could guide their use in post-menopausal women.

The proposed values are applicable only to post-menopausal women without a current diagnosis or history of oestrogen-dependent breast or uterine cancer.

The Panel noted that the conclusions drawn in this opinion are based on the assumption that intake of isoflavones from the use of food supplements represents the major contribution to the intake of isoflavones, as has been the situation in the human interventional studies.

The Panel considered that more data on the doses and duration of consumption should be generated as this would improve the available database on the safety of prolonged use of food supplements containing isoflavones.

This assessment identified the need for a harmonised way of reporting the isoflavone content of food supplements.

Future studies should use a standardised description of the isoflavones and should explicitly state whether or not the content of isoflavones is expressed as aglycones and should report the ratio of the individual isoflavones.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background

Isoflavones are a class of naturally occurring substances, present in a number of plants, especially in soybeans, red clover and kudzu root.

The term isoflavones refers to a group of compounds, sharing a common diphenolic structure. Daidzein and genistein are most commonly found in soybeans together with glycitein. Red clover mostly contains formononetin and biochanin A, whereas puerarin and daidzein are the main isoflavones found in kudzu root. Typically, isoflavones occur naturally in the glucoside form, rather than the respective free form (aglycones).

Isoflavones are, in their chemical structure, related to the human hormone 17β-oestradiol, and for this reason are also known under the generic term of 'phytoestrogens'. Isoflavones have shown capacity to bind to oestrogen receptors (ERs) and to elicit oestrogen-like effects. For this reason their use in food supplements for the alleviation of menopausal symptoms in peri- and post-menopausal women has become increasingly popular and has been promoted as an alternative to hormone replacement therapy.

In 2007 the BfR issued an expert opinion[1] on the health assessment of isoflavones-containing food supplements in which it was concluded that, on the basis of the available evidence, it was not possible to establish a dose which could be considered safe for the use by post-menopausal women who are taking food supplements containing isolated isoflavones over a long period of time. The main safety concerns expressed in this opinion were the potential for isolated isoflavones to interfere with hormone-regulated processes such as the promotion of tumour growth in the breast, hyperplasia of the endometrium and possible effects on thyroid gland functioning.

This matter was referred to EFSA in 2008, which consequently started an activity aimed at the identification and characterisation of potential hazards and benefits associated with the dietary intake of isoflavones from food, food supplements and soy infant formulae. The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) has considered the beneficial effects of isoflavones in several scientific opinions on the substantiation of a number of health claims. However, the question of the possible health risks associated with the use of food supplements containing isolated isoflavones by peri- and post-menopausal women still needs to be assessed.

1.1.2. Terms of Reference

In accordance with Article 29 (1) of Regulation (EC) No 178/2002[2] the Bundesinstitut für Risikobewertung (BfR) asks the European Food Safety Authority to provide a scientific opinion on the possible health risks associated with the intake of isolated isoflavones in food supplements by peri- and post-menopausal women.

Isolated isoflavones used in dietary supplements are defined as extracts from soybeans containing a mixture of predominantly genistein, daidzein and glycitein and isolated forms of these soy-based compounds and extracts from red clover containing a mixture of predominantly formononetin and biochanin A.

In particular the scientific opinion requested by the BfR:

- Should address the relevant available scientific evidence on the potential adverse effects associated with intake of isolated isoflavones (as defined above) in food supplements by peri- and post-menopausal women. This should include data from both human and animal studies and should focus on possible harmful effects on mammary gland, uterus and thyroid;

[1] Updated BfR Expert Opinion No 039/2007, 3 April 2007. Available from: http://www.bfr.bund.de/cm/349/isolated_isoflavones_are_not_without_risk.pdf

[2] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

- If adverse effects are identified, should provide indication of the underlying potential modes of action;
- Should provide an estimate of exposure of the target populations to the isolated isoflavones from food supplements and, if possible, give advice on a safe intake level of isolated isoflavones from dietary supplements.

1.2. Interpretation of the Terms of Reference

The objective of this assessment is limited to the investigation of possible harmful effects on three target organs, namely the breast, the uterus and the thyroid, associated with the intake of food supplements containing isoflavones, in the specific population of peri- and post-menopausal women.

To this end, only studies conducted in this specific population or in the most appropriate animal model available which would be considered representative of this population (i.e. ovariectomised animals) have been considered for this risk assessment.

Specific criteria have been developed for selecting studies to address the objective of the assessment (see Annex A, section 3).

Human and animal studies were considered for this risk assessment if the botanical sources of the supplements were soy, red clover and kudzu root. Studies with supplements containing isoflavones from the botanical sources mentioned above combined with other phytoestrogens (e.g. coumestans, lignans) or other botanical extracts (e.g. flax, black cohosh) have not been considered for this assessment.

Since consumption of foods containing isoflavones, e.g. soy and soy-derived foods (e.g. miso, tempeh, tofu), is much higher among Asian populations than in those consuming Western diets, the ANS Panel agreed to minimise possible uncertainty linked to the baseline exposure to isoflavones via the diet, by limiting the inclusion of human studies to those conducted outside Asia. The ANS Panel is, however, aware that soy-based food products are increasingly available on the market in Western countries as dairy and meat alternatives, and that soy is increasingly incorporated in processed food for nutritional or technological purposes, e.g. soy protein used as meat extender (Belloque et al., 2002; Singh et al., 2008; Weiss et al., 2010).

It should be mentioned that the use of botanical supplements, including those containing isoflavones, is reported to occur among breast cancer patients or women with a history of breast cancer, including those who are being treated with anti-oestrogen medications or aromatase inhibitors (Velentzis et al., 2011). On the basis of the anticipated oestrogenic effects of isoflavones (see section 1.5.2), data on this sub-population were specifically looked for during the current risk assessment.

1.3. Disclaimer

The ANS Panel is aware that the current risk assessment is focused and, therefore, a number of aspects have deliberately not been considered as they were deemed to fall outside the scope of the current mandate.

In particular, the purported beneficial effects associated with consumption of isoflavones either via the diet or from intake of food supplements are clearly outside the remit of the ANS Panel and the scope of the current mandate.

The current risk assessment was performed to address questions on specific adverse effects in a pre-defined population and not as an overall evaluation of isoflavones in food supplements.

Isoflavones are often referred to as phytoestrogens; however, it should be noted that the two terms cannot be considered synonyms because the latter has a wider meaning and encompasses a number of other classes of substances, such as lignans and coumestans.

The ANS Panel is aware that a number of substances of botanical origin (e.g. from black cohosh, flaxseed, etc.) are used in food supplements targeted at peri- and post-menopausal women and that isoflavones are not the only type of phytoestrogens used in these supplements.

The ANS Panel noted that there are other botanical sources of isoflavones that are used in food supplements (i.e. alfalfa, black beans, *Pueraria mirifica*); however, for the purpose of this assessment

only data related to the isoflavones typically found in soy (*Glycine max*), red clover (*Trifolium pratense*) and kudzu root (*Pueraria montana*) have been considered relevant.

A detailed list of the isoflavones which were considered relevant for the risk assessment is presented in the protocol in Annex A to this scientific opinion and in section 1.4 a list of those substances for which human and animal studies in the relevant models have been found is presented.

The ANS Panel is aware that the current risk assessment excludes other populations which may be exposed to food supplements containing isoflavones, such as men or younger women; however, these populations were deliberately excluded from the assessment.

Moreover, exposure to isoflavones at levels which are higher than those typically found in food may happen at other life stages, such as during infancy in the case of babies fed soy formula, and this exposure may lead to effects that are substantially different from those observed in the population of peri- and post-menopausal women. For this reason, data from studies in infants or young children or in juvenile animals or deriving from peri-natal exposure were not considered of relevance for the current risk assessment.

When addressing peri-menopausal women, it is acknowledged that no commonly agreed definition exists (more detailed explanations are provided in the protocol in Annex A, section 1.1). The approach taken by the ANS Panel in selecting human studies has been to include those specifically reporting enrolment of peri-menopausal and post-menopausal women as defined by the authors of the study. In the absence of this information, studies conducted in women above the age of 40 years were included, possibly being representative of the peri-menopausal population. For details on the inclusion and exclusion criteria applied, see the protocol in Annex A. It is acknowledged, however, that the vast majority of the interventional studies were conducted in post-menopausal women because their main objectives were to investigate the effects of isoflavones on bone density and menopausal vasomotor symptoms.

In the majority of the human interventional studies included in this review, a current diagnosis or history of breast cancer was usually listed among the exclusion criteria. The exclusion in many interventional studies of these sub-populations means that they are likely to be under-represented in the current assessment.

In order to exclude transient effects associated with short-term supplementation with isoflavones, the Panel agreed that a minimum duration of 12 weeks or 3 months should be used for inclusion of human intervention studies in the current assessment. For similar reasons, a minimum duration of 5 days was used for animal studies, which was an intermediate cut-off point between the minimum recommended duration of 3 days for the test in ovariectomised (OVX) animals and the minimum recommended duration of 7 days for weak oestrogens in accordance with the uterotrophic bioassay in rodents (OECD TG 440, 2007, online).

With respect to the animal studies included in this risk assessment, the ANS Panel agreed to concentrate on studies conducted in OVX animals, which were considered of relevance to the menopausal condition in women. Typically in this surgical model of menopause, bilateral removal of the ovaries is performed in young reproductively competent animals, both rodents and non-human primates (Diaz Brinton, 2012). Although this animal model is widely used as a model of human menopause, it is acknowledged that it does not fully reflect the hormonal status of peri-menopausal women, in whom 17β-oestradiol levels decline over time rather than fall abruptly (within 1–2 weeks) in OVX animals. In the light of the above, the ANS Panel is aware that the current opinion suffers from limitations in its applicability to the specific sub-population of peri-menopausal women.

Human studies and animal studies in OVX animals were considered most appropriate for hazard characterisation in the target population of all peri- and post-menopausal women.

Animal studies conducted on special models such as athymic OVX mice implanted with cells from breast cancer cell lines, or studies on mammary gland carcinogenesis induced in rodents with well-known carcinogens were considered by the Panel as potentially useful in hazard identification and for developing a mode of action analysis. Some of these studies are briefly described in section 1.5.3 but were not used as basis for hazard characterisation.

For assessment of genotoxicity, to evaluate the risk of non-threshold effects *in vitro* studies and *in vivo* studies in non-OVX animals were considered relevant and were evaluated.

The Panel acknowledges that these restraints resulted in the limitation that not all the available studies on isoflavones in every population and every animal model have been considered in this opinion, and therefore this assessment is limited to the pre-defined target population and endpoints.

1.4. Definition and identification of isoflavones

In the context of this opinion, the term 'isoflavones', unless otherwise specified, indicates the total isoflavones including glycosides and aglycones from the following botanical sources: soybean, red clover and kudzu root.

For the purpose of this risk assessment the term 'isolated isoflavones' indicates the substances identified in the Table 1 of the protocol in Annex A when used in food supplements either individually (e.g. genistein alone) or as mixtures (e.g. as found in extracts from soy, red clover or kudzu root), or in combination with other compounds (e.g. formulations containing extracts from soy in combination with *Lactobacillus*).

1.4.1. Identity and nature of the source material

A description of the identity of the botanicals is provided in Table 1.

Table 1: Description of the identity of the botanical sources of isoflavones considered in this opinion

	Soybean	**Red clover**	**Kudzu**
Botanical family[(a)]	Fabaceae	Fabaceae/Leguminosae	Fabaceae
Genus[(a)]	*Glycine* Willd.	*Trifolium* L.	*Pueraria* DC.
Species[(a)]	*Glycine max* (L.) Merr.	*Trifolium pratense* L.	*Pueraria montana* (Lour.) Merr.
Variety[(a)]	–	–	*Pueraria montana* (Lour.) Merr. var. *lobata* (Willd.) Maesen & S. Almeida
Synonyms[(a)]	*Dolichos soja* L. *Glycine gracilis* Skvortzov *Glycine hispida* (Moench) Maxim. *Glycine soja* (L.) Merr., nom. illeg., non Glycine soja Siebold & Zucc. *Glycine ussuriensis* Regel & Maack *Phaseolus max* L. *Soja hispida* Moench *Soja max* (L.) Piper	*Trifolium pratense* L. var. *frigidum* Gaudin *Trifolium pratense* L. var. *sativum* (Mill.) Schreb.	*Dolichos lobatus* Willd. *Pueraria hirsuta* (Thunb.) C.K. Schneid.; *Pueraria lobata* (Willd.) Ohwi *Pueraria lobata* (Willd.) Ohwi var. *thomsonii* (Benth.) Maesen *Pueraria thunbergiana* (Siebold & Zucc.) Benth.
Part used	Germ, beans, seed[(b)]	Aerial herb with flowers[(b)]	Root
Geographical origin	Worldwide[(b)]	Europe, western Asia, northwest Africa, widespread[(b)]	NA
Growth and harvesting conditions	Cultivated[(b)(c)]	Cultivated[(b)(d)]	NA

NA: no information available further to request to food sector operators.
(a): From United States Department of Agriculture (USDA), Plants database (online). Available online: http://plants.usda.gov
(b): Information provided by food sector operators in response to request from EFSA (May–November 2014).
(c): Some of the members of the food sector business operators contacted stated that the plants are cultivated in China and harvested in the month of October.
(d): One of the food sector business operators contacted stated that the plants used for the production of the extract used in food supplements produced are cultivated in Europe (Poland, France, Germany), western Asia and northwest Africa and harvested when they reach a height of 25–30 cm, just before the flowering time (from July to end to October), when the isoflavones content is at the maximum level.

1.4.2. Constituents to characterise the biological activity

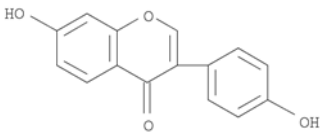
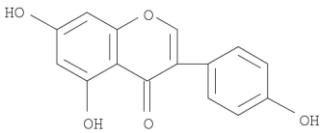
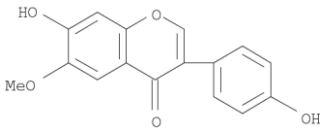
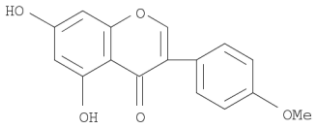
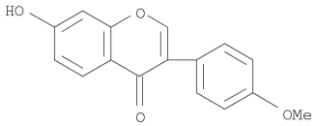
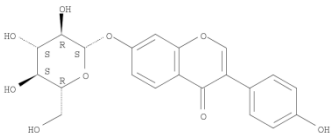
Soy isoflavones comprise three aglycone isoflavones molecules: daidzein, genistein and glycitein (isoflavone aglycones). These also exist as their corresponding glucosides (e.g. daidzin, genistin, glycitin). Most of the isoflavones found in foods exist as glycoside conjugates. Extracts obtained from soy typically maintain the naturally occurring glycosylated and derivated forms.

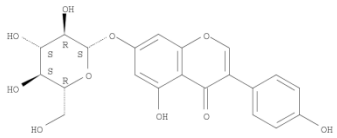
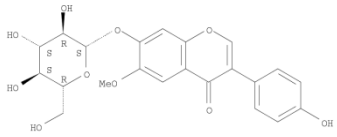
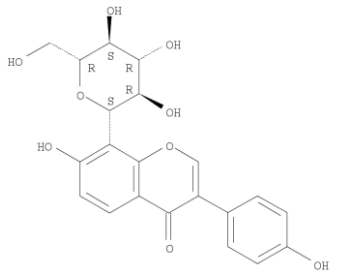
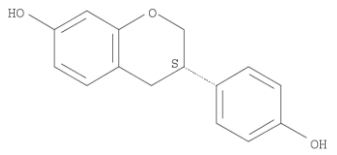
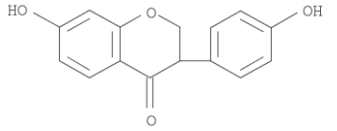
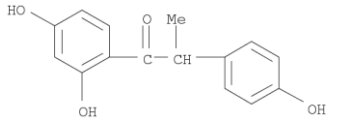
In red clover, the main isoflavones are formononetin and biochanin A (Spagnuolo et al., 2014), the latter being present essentially in the aglycone form. The glycosylated form of formononetin is named ononin.

In kudzu (*Pueraria lobata*), the main isoflavone is puerarin (Zeng et al., 2012). Besides puerarin, the main isoflavones are daidzein and then genistein.

The isoflavones (and some of their unconjugated metabolites) presented in Table 2 are those for which data have been retrieved and considered relevant for this risk assessment. The full list of isoflavones and their metabolites agreed with the requestor of the mandate and which were included in the search strings used in the literature search carried out for this risk assessment is presented in the protocol in Annex A to this opinion.

Table 2: Isoflavones (and some of their unconjugated metabolites) included in the risk assessment

Isoflavone	Chemical structure[(a)]	CAS Registry number[(a)]	Chemical formula[(a)]	Chemical name (*synonyms*)[(a)]
Aglycones				
Daidzein		486-66-8	$C_{15}H_{10}O_4$	7-Hydroxy-3-(4-hydroxyphenyl)-4-benzopyrone
Genistein		446-72-0	$C_{15}H_{10}O_5$	5,7-Dihydroxy-3-(4-hydroxyphenyl)-4-benzopyrone
Glycitein		40957-83-3	$C_{16}H_{12}O_5$	7,4'-Dihydroxy-6-methoxyisoflavone *(glycitin aglycone)*
Biochanin A		491-80-5	$C_{16}H_{12}O_5$	5,7-Dihydroxy-3-p-methoxyphenyl-4H-chromen-4-one
Formononetin		485-72-3	$C_{16}H_{12}O_4$	7-hydroxy-3-(4-methoxyphenyl)–4-benzopyrone *(biochanin B)*
Glycosides				
Daidzin		552-66-9	$C_{21}H_{20}O_9$	4H-1-Benzopyran-4-one, 7-(β-D-glucopyranosyloxy)-3-(4-hydroxyphenyl)- *(daidzein 7-O-glucoside)*

Isoflavone	Chemical structure[(a)]	CAS Registry number[(a)]	Chemical formula[(a)]	Chemical name (*synonyms*)[(a)]
Genistin		529-59-9	$C_{21}H_{20}O_{10}$	4H-1-Benzopyran-4-one, 7-(β-D-glucopyranosyloxy)-5-hydroxy-3-(4-hydroxyphenyl)- *(genistein 7-O-glucoside)*
Glycitin		40246-10-4	$C_{22}H_{22}O_{10}$	4H-1-Benzopyran-4-one, 7-(β-D-glucopyranosyloxy)-3-(4-hydroxyphenyl)–6-methoxy- *(glycitein 7-O-glucoside)*
Puerarin		3681-99-0	$C_{21}H_{20}O_9$	4H-1-Benzopyran-4-one, 8-β-D-glucopyranosyloxy-7-hydroxy-3-(4-hydroxyphenyl)- *(daidzein 8-C-glucoside)*
Metabolites				
S-equol		531-95-3	$C_{15}H_{14}O_3$	(S)-3,4-Dihydro-3-(4-hydroxyphenyl)–2H-1-benzopyran-7-ol
Dihydrodaidzein		17238-05-0	$C_{15}H_{12}O_4$	2H-1-Benzopyran-7-ol, 3,4-dihydro-3-(4-hydroxyphenyl)-, (3S)-
O-desmethylangolensin		21255-69-6	$C_{15}H_{14}O_4$	2,4-Dihydroxyphenyl p-hydroxyphenethyl ketone *(O-DMA)*

(a): SciFinder® database, online.

In addition to the substances listed above and in Table 1 in the protocol in Annex A, the Panel is aware that biochanin A and formononetin can be present in red clover in their glycosylated form, namely sissotrin and ononin. However, no data for these two specific compounds were retrieved.

1.5. Identification of potential hazards of concern: molecular effects on cells, tissues and organs

The potential hazards of concern for this assessment are those expected from the interaction of isoflavones with endocrine pathways.

The roles played by oestrogen receptors in enhancing cell proliferation in the mammary gland or in the uterus and the interaction of isoflavones with thyroid functioning led to endocrine-responsive cancers and endocrine dysfunction in these tissues being identified as the most relevant endpoints for hazard assessment. Together with the final endpoints, intermediate outcomes or related measures have been identified and considered for the assessment.

For the purpose of the current risk assessment, final endpoints are considered to be the most direct, or applicable, to the assessment, e.g. incidence of breast cancer. Surrogate endpoints are relevant, but less direct, and can include upstream indicators, intermediate outcomes or related measures to the final endpoints, i.e. pre-neoplastic lesions.

1.5.1. Hormone-independent effects

There are also hormone-independent actions of isoflavones, including inhibition of tyrosine kinase activity (Akiyama et al., 1987), inhibition of protein kinase C (Osada et al., 1988), inhibition of DNA topoisomerase II (Abe, 1999), antioxidant activity (György et al., 1964), anti-angiogenic effects (Fotsis et al., 1993) and inhibition of breast cancer resistance protein (BCRP), a cellular efflux protein (Imai et al., 2004). These effects are obtained with isolated compounds *in vitro* at doses typically exceeding 10 μM, whereas the ER-mediated effects occur at concentrations of 0.1–1 μM.

Genotoxicity

Evaluation of the genotoxic potential of any chemical agent (including those of anthropic origin but also compounds or contaminants of natural origin) is a key step in risk assessment, in view of the adverse consequences of genetic damage to human health.

In the present risk assessment, the issue of the genotoxicity of isoflavones has been raised mainly for genistein and daidzein (Kulling and Metzler, 1997; Kulling et al., 1999).

More specifically, genistein has been reported to be clastogenic in numerous studies conducted *in vitro*, but this effect has not been confirmed in studies *in vivo*. However, it has also been demonstrated to be mutagenic in a limited number of *in vitro* studies, through genotoxic mechanisms attributable to clastogenic events. The mechanisms of genistein genotoxicity appears to be associated with an inhibition of DNA topoisomerase II (Markovits et al., 1989; Yamashita et al., 1990; Austin et al., 1992; Kaufmann, 1998), an enzyme which catalyses the breakage and rejoining of both strands of DNA with requirement for ATP, thus relaxing the superhelical twist or decatenating intertwined DNA molecules. DNA topoisomerase II is necessary for separation of daughter DNA molecules after replication, but also for transcription, replication, chromosome condensation/decondensation, mitotic segregation and DNA recombination. Genistein binds to DNA topoisomerase II by stabilising the 'cleavable complex' with DNA, thus generating 'protein-concealed' DNA double-strand breaks (DSBs). This process is known to lead to the induction of chromosomal aberrations via a thresholded mechanism. This endpoint will be further elaborated in section 3.5 of this opinion.

1.5.2. Oestrogenic effects

The oestrogenic effects of isoflavones were first demonstrated in the 1940s when breeding problems were observed in sheep grazing on subterranean clover (*Trifolium subterraneum)* pastures (Bennetts et al., 1946; Braden et al., 1964). Later, the biological activities of isoflavones, and especially the soy isoflavones genistein and daidzein, were extensively investigated *in vitro*, in experimental animals and in humans (for reviews see Limer and Speirs, 2004; Rietjens et al., 2013; Kwon, 2014; Leclercq and Jacquot, 2014; Yoon et al., 2014).

The ability of isoflavones to interact with oestrogen receptors is ascribed to their structural analogy with 17β-oestradiol. The two types of oestrogen receptors have different biological actions. Oestradiol receptor alpha (ERα) is associated with cell proliferation while oestradiol receptor beta (ERβ) has pro-apoptotic and pro-differentiating effects.

In addition to ERα·and ERβ (Kuiper et al., 1998; Pike et al., 1999), GPR-30 was more recently identified as a further oestrogen receptor involved in breast cancer proliferation (Maggiolini et al., 2004). The ratio between ERα and ERβ varies between tissues, and their biological actions on normal cell proliferation and cell differentiation can counteract each other (Morani et al., 2008). Isoflavones can bind to both oestradiol receptors, but have a higher affinity for ERβ. The binding affinities of genistein to Erα and ERβ are 10 000 and 30 times lower, respectively, than that of 17β-oestradiol (Gutendorf and Westendorf, 2001). The ultimate biological activity is more complex since it requires consideration of other factors than receptor binding, including the recruitment of co-regulator proteins in the nuclear pathway as well as non-classical membrane oestrogen receptors (Carreau et al., 2009;

Yoon et al., 2014). Limited information is available on the interaction between isoflavones and these processes.

In addition to effects of isoflavones on ERα and ERβ, interactions are also known to occur with the oestrogen-related receptors ERRα, ERRβ or ERRγ (Suetsugi et al., 2003) and with GRP 30, identified as a further oestrogen receptor involved in proliferation of breast cancer cell lines *in vitro* (Maggiolini et al., 2004).

Some isoflavones have been shown to influence steroid synthesis and metabolism via modulating expression and activity of enzymes involved in steroidogenesis and steroid metabolism. Genistein increases the activity of aromatase *in vitro* (a 2.5-fold induction at 10 μM) (Sanderson et al., 2004); genistein and daidzein increase the synthesis of oestradiol *in vitro* (Taxvig et al., 2010); daidzein, genistein, biochanin A and formononetin inhibit 17β-hydroxysteroid synthetase *in vitro* (IC_{50} values of 2, 1, 0,5 and 2.7 μM respectively (Ohno et al., 2002); genistein decreases plasma corticosterone levels *in vivo* in rats by 50 % at plasma concentrations of 0.02 and 0.14 μM for genistein as parent compound and for parent plus phase II metabolite, respectively, that is within the range observed in humans following soy consumption and/or isoflavones supplements (Ohno et al., 2003); genistein inhibits glucuronosyltransferase whereas daidzein markedly stimulates it (Pfeiffer et al., 2005); and genistein and *S*-equol have also shown to be potent inhibitors of hepatic sulphotransferase (SULT) *in vitro* (Harris et al., 2004).

Results obtained with racemic equol (synthetic mixture of *S*-equol and *R*-equol) are of only limited relevance for this risk assessment, because *S*-equol and *R*-equol differ in their biological activity; for example, *R*-and *S*-equol have been shown to induce oestrogen receptor transactivation with opposite effects (Shinkaruk et al., 2010). *S*-equol has a high affinity for ERβ, whereas *R*-equol is relatively inactive (Setchell et al., 2005). Racemic equol is associated with strong antigenotoxic activity, in contrast to the purified *S*-equol, implicating the R-, rather than the S-enantiomer as responsible for the antioxidant effects of the racemic equol (Magee et al., 2006).

1.5.3. Effects on the different tissues

Mammary gland

Several *in vitro* and *in vivo* models have shown that some isoflavones stimulate proliferation of ER + human breast cancer cell lines, such as MCF-7 cells.

In oestrogen-dependent MCF-7 human breast cancer cells, genistein, at low concentrations (0.01 to 10 μM), was found to enhance cell proliferation, while higher concentrations inhibited cell growth (Martin et al., 1978; Hsieh et al., 1998). Genistein also induces the expression of the progesterone receptor and of the oestrogen-responsive pS2 gene (Wang et al., 1996). In a co-culture of MCF-7 and H295R cell lines (a cell line producing oestradiol), van Duursen et al. (2013) found that genistein and four menopausal food supplements containing isoflavones stimulated cell proliferation in the MCF-7 cells. The proliferation could not be prevented by a clinically used aromatase inhibitor (letrozole) or oestrogen receptor antagonist (hydroxytamoxifen). Glycitein was shown to be a pure ERβ agonist and did not induce MCF-7 proliferation *in vitro* (Kinjo et al., 2004).

A number of studies have investigated tumour growth in ovariectomised athymic mice implanted with MCF-7 cells and treated orally with isoflavones from soy providing total plasma isoflavones (parent compound plus phase II metabolites and/or different isoflavones) concentrations within the range observed in humans following soy consumption and/or isoflavones supplements. These studies are briefly summarised below.

A dose-dependent stimulation of tumour growth was observed after treatment with genistein or genistin (the glycoside form of genistein) in the diet (Hsieh et al., 1998; Ju et al., 2001).

Soy isolates containing genistein also stimulated tumour growth and pS2 gene expression in a dose-dependent manner (Allred et al., 2001a). The effect of genistin was found to be equivalent to that of genistein (Allred et al., 2001b).

An additive effect on stimulation of tumour growth by genistein was found in the presence of low levels of circulating oestradiol, similar to the levels observed in post-menopausal women (Ju et al., 2006).

The inhibitory effect on tumour growth of the oestrogen receptor antagonist tamoxifen and the aromatase inhibitor letrozol were negated by dietary genistein (Ju et al., 2002, 2008).

Dietary daidzein had a modest stimulatory effect on tumour growth, and its metabolite, *S*-equol, did not stimulate tumour growth (Ju et al., 2006).

In a study conducted within the US National Toxicology Program (NTP, 2008), female rats were exposed to doses of genistein ranging 0.3 to 44 mg/kg bw/day for 2 years. The authors reported '*some evidence of carcinogenic activity of genistein in female Sprague–Dawley rats, based on increased incidences of mammary gland adenoma or adenocarcinoma (combined)'.* An early onset of aberrant oestrus cycles was reported at the highest dose. These effects were interpreted to be consistent with an oestrogenic mechanism of toxicity.

Short-term (14 days) daily dietary intakes of 45 to 60 mg of isoflavones, within the range of those available in food supplements, were associated with an increased proliferation rate of breast lobular epithelium in biopsies of normal breast tissue from premenopausal women (McMichael-Phillips et al., 1998). A stimulatory effect on the breast tissue was also shown at the same doses by the increase of levels of pS2 in the nipple aspirate from premenopausal women (Hargreaves et al., 1999).

In short, some findings from *in vitro* and *in vivo* studies suggest that isoflavones may have an oestrogenic activity at concentrations within the range observed in humans following consumption of soy and/or isoflavones supplements.

Uterus

Like the mammary gland, uterus is a well-known target of oestrogens. Under normal conditions oestradiol secreted from the developing follicle increases the thickness of the endometrium by stimulating the growth of the epithelial cells. This biological response of the uterus to exogenous oestrogens is the basis of the uterotrophic bioassay in rodents, an OECD-validated guideline for screening of oestrogenic properties of chemicals (OECD TG 440, 2007, online). Either immature female or OVX rats or mice are administered test substances and uterine weight gain is assessed. Isoflavones are known to cause uterotrophic effects in rodents and the guideline includes specifications on maximum levels of genistein in the rodent feed, in order not to reduce the sensitivity of the test.

In vitro, cell proliferation was induced by genistein and daidzein at concentrations from 10 nM to 10 µM in uterine cancer cell lines in the absence of oestradiol (Schwartz et al., 1998; Kayisli et al., 2002). At concentrations over 18 µM, genistein induced HeLa cell apoptosis (Xiao et al., 2011). However, isoflavones, when present at nanomolar concentrations, can reduce the proliferative effect of oestradiol, while causing a slight induction at 1 µM (Sampey et al., 2011). This may be due to involvement of different pathways, including those triggered by GPR30 and the two oestrogen receptors (Gründker et al., 2008; Petrie et al., 2013). Furthermore, effects of genistein on glucocorticoid receptor signalling have been demonstrated in human uterine endometrial Ishikawa cells (Whirledge et al., 2015).

Genistein may also act via other than oestrogen-dependent pathways in the uterus.

Santell et al. (1997) showed that oral administration of genistein, resulting in plasma total genistein concentrations of 2.2 µM (genistein aglycone 0.4 µM), induced uterine growth in OVX female rats in a dose-dependent manner. These plasma levels can be reached in consumers of soy-based food or food supplements (Xu et al., 1994; Gardner et al., 2009). A dose-dependent increase in fluid secretion rate, fluid volume and uterine weight as well as evidence of glandular hyperplasia and an increase in proliferative cell nuclear antigen expression were found in genistein-treated rats (Salleh et al., 2013). It should be noted that Bennetts et al. (1946), who first reported on the fertility problems in sheep fed subterranean clover, also described the frequent occurrence of cystic endometrium in affected sheep. They also reported cystic endometrium in clover-fed guinea pigs.

Few data are available on adverse effect of isoflavones on uterus in women. Chandrareddy et al. (2008) reported three cases of adverse effects on endometrium (e.g. abnormal uterine bleeding and endometriosis) in women possibly due to high soy-food consumption. The symptoms disappeared or decreased after withdrawal of soy in their diet.

Thyroid

In vitro, isoflavones inhibit thyroid peroxidase (TPO), the enzyme that catalyses iodination and coupling of tyrosine residues in thyroglobulin in the synthesis of thyroxine and triiodothyronine (T_4 and T_3). In the absence of iodine, genistein and daidzein bind covalently to TPO, causing an irreversible inhibition of enzyme activity (as reviewed by Doerge and Sheehan, 2002; Marini et al., 2012). Surprisingly, the inhibition of thyroid peroxidase in rats by genistein did not result in any effects on T_3/T_4 or thyroid-stimulating hormone (TSH) levels.

Several mechanisms reported from *in vitro* and animal experiments, other than the inhibition of TPO, suggest that isoflavones have an effect on thyroid hormone metabolism. Among these mechanisms are the interaction of isoflavones with thyroid hormone receptors *in vitro* (Hofmann et al., 2009).

In addition to the well-known effect of oestrogen of increasing thyroxine-binding globulin (TBG), there is some evidence of a direct effect of oestrogen on thyroid cells mediated by the interaction with oestrogen receptors. This mechanism has been hypothesised to play a role in cell proliferation and ultimately in thyroid cancer (see review by Santin and Furlanetto, 2011).

Soy products can interfere with thyroid hormone absorption. Among infants with congenital hypothyroidism, treatment with levothyroxine resulted in a longer-lasting increase in TSH in those fed soy formula than in those fed non-soy formula (Conrad et al., 2004; Fruzza et al., 2012). A case of suspected interaction between the use of soy-based food supplements and decreased absorption of levothyroxine has been reported by Bell and Ovalle (2001).

1.6. Route of exposure

In the light of the above, only exposure via the oral route was considered relevant for hazard characterisation.

1.7. Additional information

The use of substances that can be used in the manufacturing of food supplements is harmonised at EU level by means of Directive 2002/46/EC[3] only with respect to vitamins and minerals and sources of vitamins and minerals.

Isoflavones would fall within the definition of substances with a nutritional and physiological effect other than vitamins and minerals used in food supplements, and as such would be regulated at EU level only in the event of their inclusion in the list of substances whose use in food supplements is prohibited or restricted, as foreseen by Regulation (EC) 1925/2006[4], which is not currently the case.

1.7.1. Isoflavones as ingredients of food supplements marketed in Europe

Regulatory status and maximum limits in the EU

On the basis of the information gathered from the relevant food sector operators, the ANS Panel was made aware of the maximal limits set by the relevant authorities of Belgium, France and Italy for the use of soy isoflavones, which are shown in Table 3.

[3] Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. OJ L 183, 12.7.2002, p. 51–57.

[4] Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods. OJ L 404, 30.12.2006, p. 26–38.

Table 3: Maximum limits set by EU national authorities with respect to the use of isoflavones in food supplements

Country	Responsible authority, year	Maximum limit	Additional warning
France	Ministry of the Economy and Finance arrêté plante, 2014[5]	1 mg/kg bw/day of isoflavone (as aglycone), equivalent to 60 mg/day for a 60-kg person[(a)]	Mandatory warning on the labelling: 'not suitable for women who have a personal or family history of breast cancer'
Belgium	Belgian Health and Social affairs Ministry, 2012[6]	40 mg/day isoflavones (expressed as glycosides of the main component)[(b)]	–
Italy	Italian Ministry of Health, 2012[7]	80 mg/day total isoflavones[(c)]	–

(a): This limit applies to *Glycine max*. (L.) Merr., *Trifolium pratense* L. and *Pueraria montana* var. *lobata* (Willd.) Sanjappa & Pradeep.
(b): This limit applies to isoflavones from *Glycine Max* (L.) Merr. (seed, germ), *Pueraria lobata* (Willd.) Ohwi (root, leaves, flowers) and *Trifolium pratense* L. (aerial parts).
(c): This limit applies only to isoflavones from *Glycine max* (L.) Merr. (semen, semen germinates).

The Panel noted that the information presented above may not be exhaustive and that other maximum limits may have been set in other EU Member States.

The Panel also noted that the limits set above are not directly comparable with each other.

Regulatory status and maximum limits in non-EU countries

In response to a request sent to relevant food business operators, asking for information on food supplements containing isoflavones and targeted at the peri- and post-menopausal population, it emerged that some of these products do not fall under food law in some of non-EU countries. This is the case, for example, for some standardised extracts of red clover containing 40–80 mg isoflavones, which are classified as complementary medicines (Australia), phyto-medicines (Brazil), natural health products (Canada), traditional medicines (e.g. in Indonesia and Malaysia) or over-the-counter medicines (South Korea).

In 2007, maximum limits for food supplements containing soy isoflavones were set by the Swiss Federal Food Safety and Veterinary Office (Bundesamt für Lebensmittelsicherheit und Veterinärwesen, BLV) at 50 mg/day.[8]

Two monographs have been prepared by the Natural and Non-prescription Health Products Directorate (NNHPD) of Health Canada for Soybean Extracts and Isolates, also covering genistein and soy protein isolate (SPI) (Health Canada, 2009, online), and red clover isoflavone extracts (Health Canada, 2015a, online).

No monograph for kudzu root was found on the Health Canada website; however, *Pueraria montana* var. *lobata* (Ge gen) is included in the list of Traditional Chinese Medicine Ingredients, and is under consultation at the time of writing (Health Canada, 2015b, online).

The monographs prepared by the Health Canada for red clover and soybean isoflavone extracts include a number of cautions and warnings aimed at post-menopausal women and contraindications for post-menopausal women with a current diagnosis or history of breast cancer. The recommendations and warnings are not based on a risk assessment but are simply accompanied by a list of references. In the two monographs, the doses of isoflavones are indicated as Aglycone

[5] Ministère de l'Économie, du Redressement Productif et du Numérique, Arrêté du 24 juin 2014 établissant la liste des plantes, autres que les champignons, autorisées dans les compléments alimentaires et les conditions de leur emploi. Journal Officiel de la République Française, 17.07.2014, Texte 26 sur 119.
[6] Arrete Royal du 29 Aout 1997 relatif à la fabrication et au commerce de denrées alimentaires composées ou contenant des plantes ou préparations de plantes (consolidated version 24.04.2014, M.B. 28.V.2014)
[7] Ministero della Salute, Decreto 09.07. 2012, Disciplina dell'impiego negli integratori alimentari di sostanze e preparati vegetali (G.U. 21-7-2012 serie generale n. 169)
[8] Eidgenössische Departement des Innern (EDI), Verordnung des EDI über Speziallebensmittel 817.022.104 vom 23. November 2005 (Stand am 4. Februar 2014)

Isoflavone Equivalents (AIEs), defined as the maximum amount of bioavailable isoflavone upon ingestion, calculated using the conversion factors presented in Table 4.

Table 4: Conversion of specific isoflavones quantities into AIEs (Health Canada, 2009, 2015a, online)

Isoflavone (1 mg)	Aglycone Isoflavone Equivalents (AIE) (mg)
Biochanin A	1
Biochanin A-7-O-glucoside	0.64
Formononetin	1
Formononetin-7-O-glucoside	0.62
Genistein	1
Genistin	0.625
Malonylgenistin	0.521
Daidzein	1
Daidzin	0.611
Malonyldaidzin	0.506
Glycitein	1

The Panel noted that the conversion factors indicated in the Health Canada monographs for soy and red clover isoflavones are exclusively based on the molecular weight of the substances and do not take into account actual bioavailability and bioactivity. The Panel also noted that food supplements containing isoflavones are not regulated as foods according to Canadian legislation; rather they are classified as 'non prescription health products'.

Overview of composition from industry

A request was sent to relevant food business operators, asking for information on the composition and recommended daily doses of food supplements containing isoflavones targeted at the peri- and post-menopausal population. An overview of the information received is reported in Table 5.

The percentage of total isoflavones in the extracts is extremely variable, ranging 10–43 % in soy extract and 8–40 % in red clover extracts. For kudzu root, isoflavones concentrations in the extracts used ranged 2–40%.

Table 5: Overview of information received from relevant food sector business operators.

Product identifier Data provider	Description	Recommended daily dose/number of serving
Soy isoflavones/soy extract (*Glycine max* (L.) Merr.)		
Product #1 AESGP	An extract from soy germ (cultivated, worldwide) manufactured by extraction/evaporation/spray drying. The extract contains 40–43 % isoflavones (daidzin, glycitin, genistin) and 13–17 % saponins	35 mg/day 1 tablet/day
Product #2 AESGP	Isoflavones extracted from soybean seeds (cultivated, Eastern Asia) and manufactured by aqueous extraction. The extract is standardised to contain 10 % total isoflavones. The concentration (mg/100 g) of the individual isoflavones in the extract is as follows: genistein, 80; genistin, 700; acetylgenistin,50; malonylgenistin, 7 000; daidzein, 50; daidzin, 50; acetyldaidzin, 40; malonyldaidzin, 1 900; glycitein, 5; glycitin, 25; acetylglycitin, 150; malonylglycitin, 0	75 mg/day 1 tablet twice a day
Product #3 EHPM	A preparation from soy sprout (cultivated, China) and manufactured by water–ethanol (70:30) extraction. The preparation contains > 10.0 % total isoflavones	Information not provided 2 capsules/day

Product identifier Data provider	Description	Recommended daily dose/number of serving
Product #4 EHPM	Soy isoflavones extracted from soy seeds (cultivated, China, harvested in October) and manufactured by ethanol (80 %) extraction and subsequent concentration and purification. The final product is standardised to > 40 % of total isoflavones (genistein, 0.5–1.5 %; genistin, 17–19 %; daidzein, 0.5–1.5 %, daidzin, 17–19 %; glycitein, 0.5–1.5 %; glycitin, 1.5–3.5 %)	60 mg/day single dose
Product #5 EHPM	Soy isoflavones extracted from soy germ (cultivated, worldwide) and manufactured by extraction/evaporation/spray drying. The final product is standardised to 40–43 % total isoflavones (genistein/genistin; daidzein/daidzin; glycitein/glycitin) and 13–17 % saponins	Information not provided
Product #6 FSE	Extract from soy germ (hypocotyl) manufactured by ethanol (60 %) extraction (ratio 15–25:1) and containing 10 % isoflavones (expressed as glycosides: genistin, daidzin, glycitin)	35 to 70 mg/day (glycosides) equivalent to 44 mg/day as aglycones 2–4 capsules/day
Product #7 FSE	Extract from soy germ (hypocotyl) manufactured by ethanol (60 %) extraction (ratio 60:1) and containing 30 % isoflavones (expressed as glycosides: genistin, daidzin, glycitin)	70 mg/day (glycosides) equivalent to 4 mg/day as aglycones 2 capsules/day
Product #8 FSE	Information not provided	300 mg/day
Product #9 FSE	Information not provided	150 mg/day
Products #10 FSE	Information not provided	100 mg/day
Products #11#12 FSE	Information not provided	60 mg/day
Product #13 FSE	Information not provided	40 mg/day
Product #14 FSE	Information not provided	24 mg/day
Products #15#16 FSE	Information not provided	20 mg/day
Product #17 Intertek	Isoflavones extracted from soya bean (wild or cultivated). The soya isoflavone extract (250 mg) provides 50 mg isoflavones	50–100 mg/day 1–2 tablets
Product #18 Intertek	Isoflavones extracted from soya bean (wild or cultivated)	50–100 mg/day 1–2 tablets
Product #19 Intertek	Isoflavones extracted from soy germ (wild or cultivated, the Netherlands). Extract obtained by ethanolic extraction/spray drying and containing isoflavones (genist(e)in, daidz(e)in, glycit(e)in) at a minimum concentration of 100 mg/g	20 mg/day 1 tablet
Red clover (*Trifolium pratense*)		
Product #20 AESGP	An extract from the aerial parts with flowers of red clover (cultivated, Europe, Western Asia, northwest Africa, widespread) and manufactured by ethanol–water (70:30) extraction. The extract is standardised to contain 8 % total isoflavones (formononetin, biochanin A, ononin, sissotrin, daidzein, genistein)	Information not provided 1–2 tablets/day
Product #21 FSE	Information not provided	40–80 mg/day
Products #22 #23 FSE	Information not provided	50 mg/day

Product identifier Data provider	Description	Recommended daily dose/number of serving
Products #24 Intertek	An extract from the aerial parts of red clover (cultivated, Switzerland, harvested from July to end to October) and manufactured by continuous extraction with ethanol. The extract is standardised to contain 36.0–44.0 % of the isoflavones genistein, biochanin A, daidzein and formononetin in the form of free aglycones	80 mg/day (peri-menopausal women) 40 mg/day (post-menopausal)
Products #25 Linnea SA	An extract from the aerial parts of red clover (cultivated, Poland, France, Switzerland, harvested end of July, end of August, beginning of October) and manufactured by continuous extraction with ethanol (60–80 % v/v). The extract is standardised to contain 36.0–44.0 % of the isoflavones genistein, biochanin A, daidzein and formononetin in the form of free aglycones. The ratio of (G + B)/(D + F) is 0.9–1.7:1	80 mg/day 1 tablet/day 40 mg/day) 1–2 tablets/day
Soy and red clover		
Product #26 FSE	Information not provided	50 mg/day
Kudzu root		
Product #27 EHPM	*Pueraria montana* var. *lobata* (Willd.) Sanjappa & Pradeep. Average isoflavone content 9.8 %	Information not provided
Product #28 EHPM	Extract from the root of *Pueraria lobata* standardised to contain 8 % isoflavones	1 tablet/day containing 175 mg of dry extract (14 mg/day isoflavones)
Product #29 EHPM	Product containing 500 mg of an extract from the root of *Pueraria lobata* standardised to contain 8 % isoflavones	1 capsule/day containing 500 mg extract (40 mg/day isoflavones)
Product #30 EHPM	*Pueraria montana* var. *lobata*	Information not provided
Product #31 EHPM	Dry water extract from *Pueraria lobata* standardised to contain 20 % isoflavones. Ratio plant/extract: 5–6:1 The recommended dose expressed in aglycone content is below the daily dose of 1 mg/kg bw/day	1 dose = 2 capsules; 2 doses/day 56 mg/day isoflavones
Product #32 EHPM	Dry extract from the root of *Pueraria lobata* standardised to contain 40 % isoflavones. Each tablet contains 100 mg extract, corresponding to 40 mg isoflavones	1–2 tablets/day (40–80 mg/day isoflavones)
Product #33 EHPM	Dry extract from the root of *Pueraria lobata* standardised to contain 40 % isoflavones. Each tablet contains 100 mg extract, corresponding to 40 mg isoflavones	1–2 tablets/day (40–80 mg/day isoflavones)
Product #34 EHPM	Dry extract from the root of *Pueraria lobata* standardised to contain 2 % isoflavones. Each tablet contains 150 mg extract corresponding to 3 mg isoflavones	1–2 tablets/day (3–6 mg/day isoflavones)
Product #35 EHPM	Powder from *Pueraria montana* var. *lobata* (Willd.) Sanjapa & Pradeep containing 9.79 % isoflavones	6 capsules/day
Product #36 EHPM	Water–ethanol extract from the root of *Pueraria montana* var. *lobata* standardised to contain 6 % isoflavones. Each tablet contains 600 mg extract, corresponding to 36 mg isoflavones	Information not provided
Product #37 EHPM	*Pueraria lobata*, 24.8 isoflavones/capsules	1–2 capsules/day (24.8–49.6 mg/day isoflavones)
Product #38 EHPM	Powder from the root of *Pueraria montana* var. *lobata* containing < 10 % isoflavones	3 capsules/day (600 mg powder, equivalent to a maximum dose of 60 mg/day isoflavones)
Product #39 EHPM	Powder from the root of *Pueraria montana* var. *lobata* containing < 3 % isoflavones. Extract from the root of kudzu (water–ethanol, 30 % ethanol; ratio plant/extract: 4–6:1) with an isoflavone concentration < 40 %	3 capsules/day (411 mg powder, equivalent to a maximum dose of 12 mg/day isoflavones and 68 mg/day extract equivalent to 27.4 mg/day isoflavones)

Product identifier Data provider	Description	Recommended daily dose/number of serving
Product #40 FSE	Information not provided	56–75 mg/day

AESGP: Association of the European Self-Medication Industry; EHPM: the European Federation of Associations of Health Product Manufacturers; FSE, Food Supplements Europe.

1.7.2. Evaluations performed by EFSA

Soy isoflavones

Glycine max (L.) Merr. is included in the EFSA 'Compendium of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements' (EFSA, 2012). The seed is the part identified as of possible concern and isoflavones are, together with soybean agglutinin (N-acetylgalactosamine-specific lectin), proteinase inhibitors and other toxic proteins, the chemical substances of concern mentioned in the compendium. The concentration range of total isoflavones reported in the compendium is 945–4 208 µg/g. The concentration of individual isoflavones is reported as follows: daidzin, 67–516 µg/g; genistin, 91–1 079 µg/g; glycitin, 12–177 µg/g; malonyldaidzin, 217–768 µg/g; malonylglycitin, 43–158 µg/g, malonylgenistin, 64–2 446 µg/g; genistein, 4.3–265 µg/g.

The compendium lists other botanical sources of isoflavones (i.e. *B. chinensis* (L.) DC. and *Pueraria mirifica* Airy Shaw & Suvat.), but not the other two botanical sources considered for this risk assessment (i.e. *Trifolium pratense* and *Pueraria lobata*).

The EFSA Panel on the Panel on Dietetic Products, Nutrition and Allergies (NDA) was requested on a number of occasions to assess the substantiation of health claims related to soy isoflavones (EFSA NDA Panel, 2009, 2011a, 2012a). The outcome of these evaluations is summarised in Table 6.

Table 6: Summary of EFSA NDA Panel opinions on health claims related to soy isoflavones

Claimed effect	NDA Panel opinion	Reference
Maintenance of bone mineral density	Insufficient evidence to establish a cause and effect relationship	EFSA NDA Panel, 2009, 2012a
Protection of DNA, proteins and lipids from oxidative damage	A cause and effect relationship has not been established	EFSA NDA Panel, 2011a
Maintenance of normal blood low-density lipoprotein (LDL) cholesterol concentrations	A cause and effect relationship has not been established	EFSA NDA Panel, 2011a
Reduction of vasomotor symptoms associated with menopause	Insufficient evidence to establish a cause and effect relationship	EFSA NDA Panel, 2011a EFSA NDA Panel, 2012a
Contribution to normal hair growth	A cause and effect relationship has not been established	EFSA NDA Panel, 2011a

The Panel noted that the doses reported in the NDA opinions for reduction of vasomotor symptoms associated with menopause were in the range of 27–100 mg/day of soy isoflavones for 3–24 months. For the maintenance of bone mineral density, the doses of soy isoflavones used in the intervention studies considered in the opinions were up to 200 mg/day.

Soy protein and soy protein isolate (SPI)

The NDA Panel also issued scientific opinions on the substantiation of a number of health claims related to soy protein (EFSA NDA Panel, 2010a, b) and SPI (EFSA NDA Panel, 2012b). The outcome of these evaluations is summarised in Table 7.

Table 7: Summary of EFSA NDA Panel opinions on health claims related to soy protein and isolated soy protein

Claimed effect	NDA Panel opinion	Reference
Reduction of blood cholesterol concentrations	A cause and effect relationship has not been established	EFSA NDA Panel, 2010a,b
Reduction of blood LDL-cholesterol concentrations	A cause and effect relationship has not been established	EFSA NDA Panel, 2012b
Contribution to the maintenance or achievement of a normal body weight	A cause and effect relationship has not been established	EFSA NDA Panel, 2010b
Protection of DNA, proteins and lipids from oxidative damage	A cause and effect relationship has not been established	EFSA NDA Panel, 2010b

Safety evaluation of fermented black bean extract as a novel food ingredient

In 2011, the EFSA NDA Panel adopted an opinion on the safety of Touchi extract, a protein-rich powder obtained by water extraction of small soybeans (*Glycine max*) fermented with *Aspergillus oryzae* to be used as a novel food ingredient in food supplements. The Panel concluded that the fermented black bean extract (Touchi extract) is safe at the proposed conditions of use (EFSA NDA Panel, 2011b).

Although the actual levels of isoflavones in the Touchi extract are not provided in the scientific opinion, in the light of the fact that the preparation process does not concentrate isoflavones, the NDA Panel concluded that the content of isoflavones ingested with the consumption of the extract at the maximum intake of 4.5 g/day was not of concern (EFSA NDA Panel, 2011b). Given that in the opinion it is reported that the extract should contain 300 mg of isoflavones per 100 g, it can be assumed that intake of isoflavones from the extract at the proposed maximum intake level would correspond to 13.5 mg/day.

1.7.3. Other opinions from other EU bodies

A number of concerns were raised by the German Federal Institute for Risk Assessment (BfR) in 2007 with respect to the potential harmful effects of isoflavones when consumed at high doses, such as in the case of menopausal women taking food supplements, on mammary gland and the thyroid. In its opinion the BfR advised against long-term consumption of isoflavone-containing products made from soya (BfR, 2007, online).

Previously, in 2005, the French Food Safety Agency (Agence française de sécurité sanitaire des aliments, AFFSA) had published an opinion on the safety and the beneficial effects of phytoestrogens from dietary sources (AFFSA, 2005, online). On the basis of the evidence reviewed for this opinion, recommendations on the consumption of soy-based products by menopausal women were made, limiting the intake of soy-based products in this population and suggesting a maximum intake for all isoflavones aglycones and coumestan not exceeding 1 mg/kg bw/day. This reference value had been derived from studies examining the effect of genistein alone (AFFSA, 2005, online).

In addition, the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) completed an extensive review on phytoestrogens and health in 2003, which included consumers of dietary supplements containing isoflavones (COT, 2003, online). In this report, some concerns regarding the potential interference between phytoestrogens and thyroxine replacement therapy were noted and further research was recommended to monitor the plasma thyroxine levels of children and adults with hypothyroidism who consume large quantities of dietary phytoestrogens. With respect to the potential effects exerted by phytoestrogens on breast cell proliferation, although no quantitative recommendation was given in the report, the UK COT suggested that, until further research is carried out, women with oestrogen-dependent breast disease should be cautious in supplementing their diet with phytoestrogen-rich foods or dietary supplements. Further research was commissioned that included three relevant projects, as follows: (i) a dietary and biomarker prospective study of phytoestrogens in breast and prostate cancer; (ii) an investigation of the phytoestrogen intake of a group of postmenopausal women previously diagnosed with breast cancer; and (iii) a double-blind placebo-controlled parallel trial of soy isoflavones on markers of bone

turnover in females in the early menopause. The first two of these were reviewed later (COT, 2012, online) and the final one was completed only in the final quarter of 2014. COT noted that these three studies were based on the recommendation that the programme concentrate on human studies. There was some lack of clarity in the scientific objectives of the study on phytoestrogen exposure in women diagnosed with breast cancer, and COT was disappointed that the results of this study had not been published in the peer-reviewed literature. COT noted that the analysis of phytoestrogens in a wide range of foods was useful and had allowed robust estimation of short-term dietary exposures to phytoestrogens. While the findings indicated no association between phytoestrogen intake and risk of breast cancer, the data on prostate cancer were inconclusive. COT had considered unpublished results from the final study. The minutes of COT's discussion have been temporarily withheld from publication while a report of the study is submitted for publication in a peer-reviewed scientific journal. COT judged that this delay was acceptable, since the results presented did not indicate any need for action to protect the health of the public.

2. Data and Methodologies

The current risk assessment was considered a candidate for applying a structured approach derived from systematic reviews methodology, which implies (i) developing a priori the protocol of the full risk assessment; (ii) performing each step of the risk assessment in line with the protocol; and (iii) thoroughly documenting the process. The protocol in Annex A to this opinion contains the method that has been applied in all steps of the risk assessment process including any decisions taken by the Working Group.

The systematic review method and principles have been applied to the sub-questions investigating a possible association between supplementation with isoflavones and adverse effects on the breast, uterus and the thyroid. To this end, the search strategy to be used, the criteria for screening the studies for relevance, the forms for data extraction from the included studies and the elements considered for appraising the studies have been defined beforehand in a protocol and applied throughout the assessment process.

2.1. Data

The steps followed for the acquisition of data, their selection and appraisal are documented in the protocol in Annex A to this scientific opinion.

The search strategies adopted for retrieving the literature used for this assessment were elaborated by an information specialist in consultation with the experts from the Working Group and are included in the protocol in Annex A to this scientific opinion.

2.1.1. Data on associations between intake of isoflavones from food supplements and outcomes of interest

The steps followed for the acquisition of data, their selection and appraisal are documented in the protocol in Annex A to this scientific opinion. A detailed analysis of the numbers of studies screened, and of the reasons for exclusion is reported in Annex B.

At the end of the systematic review process, a total of 7 841 studies were screened, which led to the identification of 43 studies in humans (4 observational and 39 intervention) and 62 studies in animals reporting on the three target organs of interest and fulfilling the criteria for inclusion pre-defined in the protocol in Annex A to this scientific opinion (see Figure 1 for PRISMA flow chart).

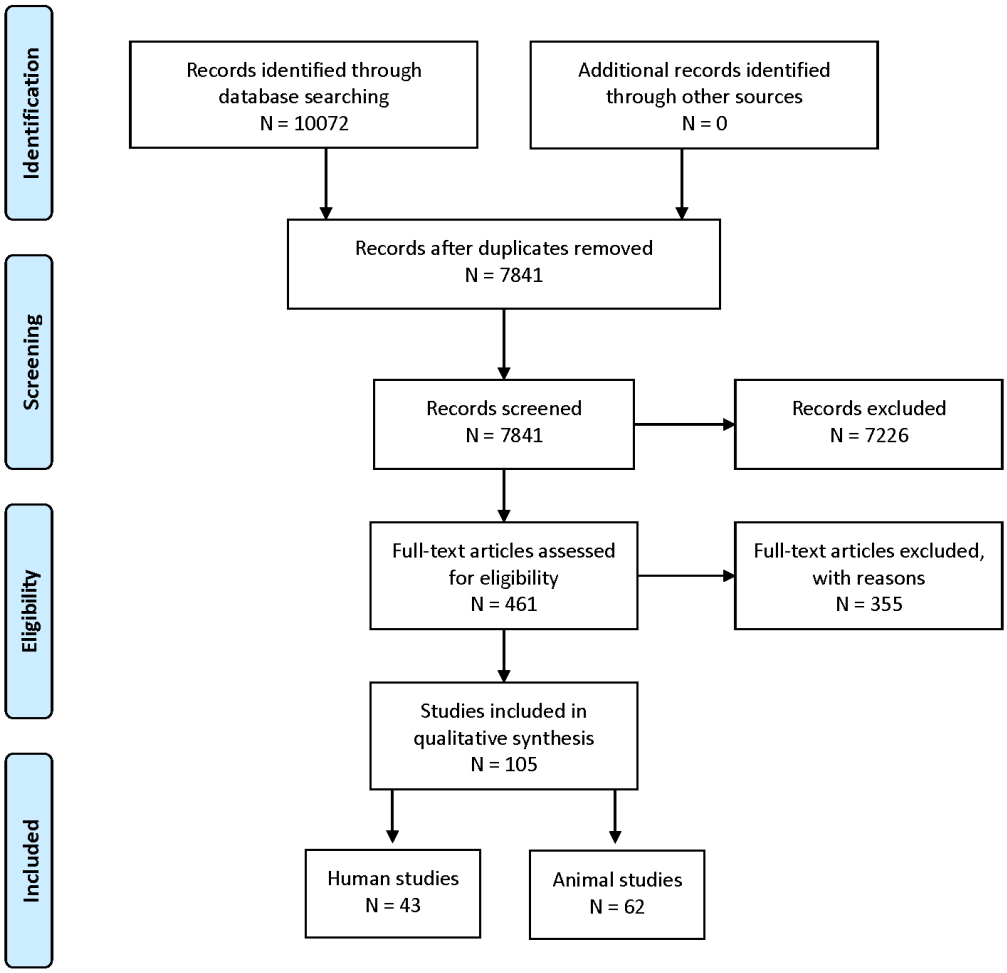


Figure 1: PRISMA flow chart (adapted from Moher et al., 2009)

The number of human interventional studies and animal studies included in the review for each target organ and endpoint of interest is presented in Table 8.

The studies included in the assessment are summarised in Tables 9, 10, 11, 12, 15, 16, 17, 18 and 19 and reported in more detail in Appendices A and B.

The studies included in Tables 9, 10, 11, 12, 15, 16, 17, 18 and 19 have been grouped by endpoint and within each endpoint by type of isoflavones tested, tier of reliability (for description see protocol in Annex A) (highest on top) and by duration (in decreasing order).

Table 8: Overview of number of human intervention studies and animal studies included in the systematic review

Intervention	Mammary gland			Uterus				Thyroid
	Mammographic density	Cell proliferation markers	Histo(patho)logical changes	Endometrial thickness	Histo(patho) logical changes	Cell proliferation markers	Uterine weight	Changes in thyroid hormone levels
Soy isoflavones/soy extract								
Human studies (n=15)	✓ (2)	✓ (2)	NA	✓ (8)	✓ (3)	×	NA	✓ (5)
Animal studies (n=15)	NA	✓ (1 monkeys, 1 rats)	✓ (1 monkeys, 1 rats)	NA	✓ (1 monkeys, 3 rats)	✓ (1 monkeys, 1 rats; 1 mice)	✓ (1, monkeys, 10 rats; 2 mice)	×
Soy protein (SPI)								
Human studies (n=6)	✓ (1)	×	NA	✓ (3)	✓ (3)	×	NA	✓ (2)
Animal studies (n=6)	NA	✓ (3 monkeys)	✓ (3 monkeys)	NA	✓ (3 monkeys, 2, rats)	✓ (2 monkeys, 2 rats)	✓ (1 monkeys, 3 rats)	×
Daidzein-rich isoflavones								
Human studies (n=4)	✓ (1)	×	NA	✓ (2)	✓ (1)	×	NA	✓ (2)
Animal studies (n=9)	NA	×	×	NA	✓ (3, rats)	✓ (2, rats)	✓ (8 rats)	×
Glycitein-rich isoflavones								
Human studies (n=1)	×	×	NA	✓ (1)	✓ (1)	×	NA	×
Animal studies	NA	×	×	NA	×	×	×	×
Genistein								
Human studies (n=8)	✓ (2)	×	NA	✓ (7)	×	×	NA	✓ (1)
Animal studies (n=0)	NA	✓ (2 rats)	✓ (3 rats)	NA	✓ (3, rats)	✓ (1 rat)	✓ (12 rats, 3 mice)	×
Red clover extract (RCE)								
Human studies (n=5)	✓ (2)	×	NA	✓ (4)	✓ (1)	×	NA	✓ (1)
Animal studies (n = 6)	NA	×	✓ (1 rats)	NA	✓ (1, rats)	✓ (3, rats)	✓ (1 rabbits, 3 rats)	×
Kudzu root								
Human studies	×	×	NA	×	×	×	NA	×
Animal studies (n=3)	NA	×	×	NA	×	×	✓ (1 rats, 2, mice)	×

Intervention	Mammary gland			Uterus				Thyroid
	Mammographic density	Cell proliferation markers	Histo(patho)logical changes	Endometrial thickness	Histo(patho) logical changes	Cell proliferation markers	Uterine weight	Changes in thyroid hormone levels
Equol (racemic)								
Human studies	×	×	NA	×	×	×	NA	×
Animal studies (n=8)	NA	✓ (2 monkeys, 2 rats)	✓ (2 monkeys, 1 rats)	NA	✓ (1 monkeys, 3 rats)	✓ (1 monkeys, 2 rats)	✓ (1 monkeys, 5 rats)	×
Genistin								
Human studies	×	×	NA	×	×	×	NA	×
Animal studies (n=1)	NA	×	×	NA	×	×	✓ (1 rats)	×
Daidzein								
Human studies	×	×	NA	×	×	×	NA	×
Animal studies (n=4)	NA	×	×	NA	✓ (1 rats)	×	✓ (1 rabbits, 3 rats)	×
Daidzin								
Human studies	×	×	NA	×	×	×	NA	×
Animal studies (n=1)	NA	×	×	NA	×	×	✓ (1 rats)	×
Glycitin								
Human studies	×	×	NA	×	×	×	NA	×
Animal studies (n=1)	NA	×	×	×	×	×	✓ (1 rats)	×
Puerarin								
Human studies	×	×	NA	×	×	×	NA	×
Animal studies (n=1)	NA	×	×	×	×	×	✓ (1 rats)	×
Biochanin A								
Human studies	×	×	NA	×	×	×	NA	×
Animal studies (n=1)	NA	×	×	×	×	×	✓ (1 rats)	×
Formononetin								
Human studies	×	×	NA	×	×	×	NA	×
Animal studies (n=1)	NA	×	×	×	×	×	✓ (1 mice)	×

NA: not applicable.
(n): number of studies included and, in the case of animal studies, animal species in which the studies were conducted.

2.1.2. Other supporting evidence

Additional literature searches were conducted to retrieve relevant information to support the current risk assessment, as detailed in the protocol in Annex A to this scientific opinion.

2.1.3. Exposure level

Levels of use of isoflavones in food supplements marketed in the EU and targeted at the population of interest in this risk assessment were gathered by EFSA through a request targeted at the relevant European industry associations, and from the range of recommended number of daily servings of the food supplements, as indicated on the label of the product. The data gathered are described in section 3.6.2.

Information gathered was compared with the range of doses used in the intervention studies in humans included in this risk assessment in the population of interest.

2.2. Methodologies

The method followed to perform this risk assessment is detailed in the protocol in Annex A to this scientific opinion.

2.2.1. Systematic review on the association between isoflavones intake from food supplements and possible effects on mammary gland, uterus and thyroid

All the studies included in the systematic reviews have been appraised for validity and risk of bias using pre-defined criteria agreed before the start of the assessment. These are described in the detail in the protocol in Annex A to this scientific opinion. The Tier of Reliability of the studies is based on the described appraisal. Tier 1 denotes studies with the lowest risk of bias whereas studies with some deficiencies are ranked to a higher tier (e.g. Tier 2 or Tier 3).

In the case of human intervention studies, the primary determinants of the study reliability were randomisation of intervention groups, blinding throughout the conduct of the study, allowance for confounding, limited attrition/exclusion of study subjects, quality control of analytical or diagnostic method and absence of selective reporting. No studies which fully met all these criteria were identified. The lack of long-term isoflavone supplementation to groups of sufficient size was the most frequent deficiency. The majority of studies were not registered in a clinical trial register and did not follow CONSORT criteria for reporting (Schulz et al., 2010).

In the case of animal data, results obtained in studies of ovariectomised monkeys would be considered to be of greater relevance than those obtained from studies in rats and mice or rabbits. However, only studies in which soy extracts and SPI were investigated have been conducted in monkeys.

The Panel considered that the most relevant data were those obtained from studies in the specific human sub-population of interest. Thus, the Panel decided that, in the risk assessment of isoflavones from food supplements used by peri- and post-menopausal women, the evidence from various sources should be ranked in the following order: studies in the specific population, studies in relevant animal models and *in vitro* studies (for genotoxicity studies).

2.2.2. Narrative approach for other evidence

The literature on kinetics and metabolism of isoflavones was retrieved using a two-step approach:

1) a literature search that searched only reviews, followed by
2) a hand search of the reviews to find primary literature sources.

The searches were intended to cover the topics on kinetics and metabolism which are of relevance in the context of the focused risk assessment of isoflavones.

Out of 168 articles for which title and abstract screening was performed, 29 remained to serve as starting point for primary literature search.

Data which describe the kinetics and the metabolism of substances are relevant for risk assessment if they allow calculation of internal exposure (absorption and bioavailability, area under the curve, concentration–time profile) in humans which can then be compared with the results from the animal species in which the toxicological studies were performed. Knowledge of metabolism is important to compare the profile in humans with the profile in the animal species in which the toxicological studies were performed, in particular if metabolites are active. This information might be used.

With respect to the approach followed for retrieving published literature on genotoxicity, a focused literature search was conducted using the strings detailed in the protocol in Annex A. A time limit was applied in order to limit the search to those studies conducted after the establishment of international guidelines. A total of 1 684 articles were screened for relevance by expert judgement using their title and abstract, which resulted in 161 articles undergoing full text screening.

3. Assessment

3.1. Kinetics and metabolism of isoflavones

General remarks

The absorption is the percentage of the dose which after oral intake is delivered from the lumen of the gastrointestinal tract into the cells lining the gastrointestinal tract. Absorption is different from bioavailability, which is the percentage of the dose delivered in the systemic circulation; in other words, this is the percentage of the dose which after absorption is escaping pre-systemic elimination in the cells of the gastrointestinal wall and in the liver. The concentration–time course in plasma/blood can be used to derive the percentage of absorption and bioavailability by comparing the area under the concentration–time profile (AUC) after oral administration with the dose-adjusted AUC after intravenous administration provided the kinetics of the substance is linear in the investigated dosage range. If data after intravenous administration are not available, the dose-adjusted AUCs of two oral preparations with the identical active ingredient can be compared, which provides information on the relative absorption/bioavailability. Data on the excretion in the urine of the substance and its metabolites may also be used to calculate the absolute or relative absorption. With the aim of obtaining data on the absorption and systemic availability (bioavailability) the papers were screened for reports of the concentrations of parent compounds. For metabolism, the goal was to identify the metabolic profile in the species relevant for risk assessment and to compare the profile in these species with the profile in humans.

3.1.1. Kinetics and metabolism in humans

Absorption and bioavailability

Mechanism of absorption

Setchell et al. (2002) showed that the glycosides daidzin and genistin are not found intact in the peripheral blood of healthy adults. According to Rowland et al. (2003), the glycoside forms are not found either in faeces or in the blood, indicating that all glycosidic forms are hydrolysed before glucuronidation or sulphation in the gut. They all concluded that isoflavone glycosides are not absorbed intact across the enterocyte but that hydrolysis by intestinal β-glucosidases is necessary for absorption. In addition, recent *in vitro* data (Kobayashi et al., 2013) may indicate that genistein is transported through the intestinal barrier by passive diffusion and is a substrate for the efflux protein BCRP after conjugation by phase II enzymes. Daidzein is a substrate for BCRP, multidrug resistance-associated proteins and P-glycoprotein without being conjugated.

Extracts from soy, red clover and kudzu root

Data on the absorption/bioavailability of extracts from red clover in humans are very limited (Maul and Kulling, 2010). Data on the isoflavones from kudzu roots have not been found, although data on concentration–time course are reported below. An extensive review on *Radix puerariae* (Zhang et al., 2013) cites several publications on the kinetics, but most of them are in Chinese.

Howes et al. (2002) reported on plasma concentrations after long-term administration of two tablets daily containing isoflavones from red clover (24.5 mg biochanin; 1.5 mg genistein; 16 mg formononetin; 1.5 mg daidzein) to eight female and six male volunteers. However, as they treated the plasma with β-glucuronidase before measuring the isoflavone aglucones, the study is not entirely helpful in the context of this risk assessment since it is not possible to determine the levels of conjugated and unconjugated forms present in plasma. The plasma concentration–time profile of isoflavones was measured in one male volunteer after oral administration of 25 mg glycitin and after 40 mg total isoflavone from red clover containing mainly formononetin and biochanin A (Setchell et al., 2001). The results were demonstrated in a figure showing concentrations of glycitin (C_{max} roughly 200 ng/ml) and daidzein (C_{max} roughly 10 ng/ml).

Peak concentrations after 40 mg total isoflavone from red clover containing mainly formononetin and biochanin A were 36 ng/ml genistein, 16 ng/ml formononetin, 7 ng/ml biochanin and 3 ng/ml daidzein. A single plasma sample measured 6.5 hours after intake of red clover isoflavones indicated that isoflavones are present in plasma (Maul and Kulling, 2010).

The concentration–time profile of puerarin was measured after oral administration of kudzu root extract containing 19 % puerarin, 4 % daidzin and 2 % daidzein. Peak plasma concentrations of puerarin were 36.9 ng/ml and 49.2 ng/ml and AUC 166.2 ng/ml × h and 208.9 ng/ml × h after 47.5 mg (n = 5 participants) and 95 mg of puerarin (n = 5 participants), respectively, indicating non-linear bioavailability, as also described for daidzein and genistein (see below) (Penetar et al., 2006).

For puerarin the mechanism of absorption includes also inhibition of efflux by P-glycoprotein.

Soy protein isolate

Although specific and sensitive methods are available to measure isoflavones in plasma, the information in the published literature does not allow estimation of isoflavone absorption and/or bioavailability (Soukup et al., 2014).

Individual isoflavones, alone and in combination

In a study by Busby et al. (2002), two preparations (Formulation A and Formulation B) containing genistein, daidzein and glycitein in different proportions were studied at five dose strengths, with doses of genistein varying between 1 mg and 16 mg and doses of daidzein varying between 0.49 mg and 7.8 mg daidzein. Formulation A contained 100 % total unconjugated isoflavones consisting of 90 ± 5 % genistein, 10 % daidzein and 1 % glycitein, whereas Formulation B contained 70 % unconjugated isoflavones consisting of 43 % genistein, 21 % daidzein and 2 % glycitein. Based on the AUC of unconjugated genistein, the relative bioavailability of genistein formulation A versus genistein formulation B was measured. Formulation A had a relative bioavailability of 60 % for a dose strength of 8 mg genistein (A/B) and of 15 % for a dose strength of 16 mg genistein (A/B), indicating a better bioavailability after intake of formulation B than intake of formulation A. When absorption was calculated based on total isoflavones, formulation B was also found to be better absorbed than formulation A; however, the values are not identical to the values for the bioavailability based on the AUC of unconjugated genistein. In the case of daidzein, data were not sufficient to calculate the AUC of unconjugated daidzein. In urine, total daidzein excretion was lower after ingestion of formulation A than after ingestion of formulation B. No further details are given in the publication. From this study, the concentration at C_{max} was used to calculate genistein aglucone as a percentage of total genistein. The value varied between 0.4 % and 3.9 % for the seven studied dose strengths for which data were available. Likewise, the concentration at C_{max} was used to calculate daidzein aglucone as percentage of total daidzein. The value varied between 1.4 % and 4.2 % for the five studied dose strengths for which data were available.

In a study by Shelnutt et al. (2002) six men and six women consumed a soy protein drink providing a dose of 1.0 mg genistein (aglycone) equivalents/kg bw and 0.6 mg daidzein (aglycone) equivalents/kg bw. Isoflavones were measured after treating the samples with β-glucuronidase and sulphatase, thus not enabling any statement about the presence or concentration of the conjugated or unconjugated aglycones in plasma. From the amount excreted in urine after 48 hours, a rough estimate can be made about the amount (but not the form) absorbed. If the data in Fig. 3 of the publication are summed up, in this study the absorption was 61.3 % of the dose for daidzin, 60.4 % for glycitin and

35.4 % for genistin. However, it should be noted that the sampling period could be too short and hence the percentage absorbed could be higher.

In a further study by Setchell et al. (2003a) three different doses of soy nuts were given to five premenopausal and five post-menopausal women on different occasions, 1 month apart. The isoflavone doses were conjugated forms of 6.6 mg, 13.2 mg and 26.4 mg of daidzein and of 9.8 mg, 19.6 mg and 39.2 mg of genistein. The absorption, calculated from urinary excretion, declined with increasing dose (daidzein: 63.2 %, 54.4 % and 44.0 %; genistein: 25.2 %, 13.4 % and 15.8 %). There was no difference between pre- and post-menopausal women.

A similar high absorption could be calculated from the data in Vergne et al. (2008): after intake of a soy supplement providing roughly 30 mg isoflavone equivalents of daidzein and intake of soy-based cheese providing approximately 7 mg isoflavone equivalents of genistein, roughly 25 mg and 3 mg, respectively (data extrapolated from figure 3 of the publication), was excreted in the urine over 48 hours. The resulting absorption would be 88.5 % for daidzein and 44.3 % for genistein.

In conclusion, wide variability in absorption is observed. van der Velpen et al. (2014) stated recently that the dose of isoflavones is a poor indicator of internal exposure. They found that plasma levels do not increase in proportion to the dose and that interindividual variability was 30–96 %.

Distribution

No data are available for the human situation. In one genistein and daidzein were measured in prostate tissue after 2 weeks of intervention with an isoflavone-containing supplement (Rannikko et al., 2006). As the analytical method for isoflavones included hydrolysis of samples, the results are not representative of the distribution of the individual substances.

Other studies also measured total genistein and the glucuronyl conjugates of genistein and daidzein in breast tissue. For example, Bolca et al. (2010) randomly allocated healthy women to a soy milk (containing 16.98 mg genistein and 5.40 mg daidzein aglycone equivalents per dose, three doses per day) group, a soy supplement (containing 5.27 mg genistein and 17.56 mg daidzein equivalents per dose, 3 doses per day) group or a control group for 5 days before surgery for aesthetic breast reduction. In some samples, Bolca et al. (2010) also measured the aglucones. In breast tissue, genistein concentration was 12 ± 2 (n =4) pmol/g tissue and daidzein 8 ± 1 (n = 6) pmol/g after soy milk whereas after soy supplement genistein concentration was 8 ± 2 (n = 3) pmol/g tissue and daidzein 22 ± 4 (n=6) pmol/g tissue.

Metabolism

The metabolism of the soy isoflavones daidzein and genistein is summarised in various review papers (Yuan et al., 2007; Larkin et al., 2008; Mortensen et al., 2009; Yang et al., 2012; Rafii, 2015). Based on these reviews there are no indications that the isoflavone source (soy food, soy supplement and individual isoflavones) has a fundamental impact on the general routes of metabolism.

Isoflavones are metabolised by endogenous phase I and phase II enzymes, mainly in the gut and the liver, as well as by the intestinal microbiota. Conjugation as well as microbial transformation reactions are major pathways. Minor metabolites are hydroxylated derivatives formed by the action of cytochrome P450 enzymes.

It is known that in humans, as in other species, biochanin A is mainly demethylated to genistein and formononetin is demethylated to daidzein by phase I hepatic enzymes (Setchell et al., 2001; Howes et al., 2002).

Hosoda et al. (2011) found that the 7-glucuronide-4′-sulphates were the major metabolites of daidzein (53.3 %) and genistein (54.0 %) in the plasma of 10 healthy Japanese men and women who ingested kinako (baked soybean flour). In contrast, in the 48-hour urine, daidzein-7-glucuronide constituted, on average, 48.1 % of the total daidzein metabolites. In the case of genistein, the excreted amounts of genistein-7-glucuronide and genistein-4′-glucuronide accounted for 28.5 % and 27.0 %, respectively, of total genistein metabolites.

Soukup et al. (2014) investigated the phase II metabolite profile in plasma and urine of 11 German post-menopausal women after ingestion of a bolus dose of a commercial soy extract. The result confirmed the findings of Hosoda et al. (2011) that sulphoglucuronides are the major metabolites of

daidzein and genistein in the plasma and that the 7-O-glucuronides are the predominant metabolites in the urine. Interindividual variation regarding the phase II metabolite pattern in humans appears to be low (Hosoda et al., 2011; Soukup et al., 2014). However, studies with a larger number of individuals are needed to confirm this.

Besides glucuronidation and sulphation, transformation reactions catalysed by the intestinal microbiota play a crucial role in the metabolism of isoflavones. Genistein can be reduced to dihydrogenistein and then via ring cleavage to 6′-hydroxy-O-desmethylangolensin. A further degradation to 2-(4-hydroxyphenyl)propionic acid is shown by incubation of genistein with faecal samples (Coldham et al., 2002; Braune et al., 2010). 4-ethylphenol is also mentioned in the literature as a genistein metabolite in humans (Setchell, 1998). However, the relevance of this degradation reaction *in vivo* has so far not been investigated. Daidzein can be converted to dihydrodaidzein and subsequently to O-desmethylangolensin and/or *S*-equol. A further degradation of these metabolites is not described. The extent of microbial metabolism of genistein and daidzein as well as the resulting microbial metabolite profile varies greatly among individuals. The prevalence of equol producers ranges from 20–30 % in the population of Western countries to 50–60 % in Asian populations consuming soy-containing diets.

The biotransformation pathways of daidzein, summarising the metabolites found so far in the plasma or urine of human or experimental animals (rats, mice, monkeys) is shown in Figure 2.

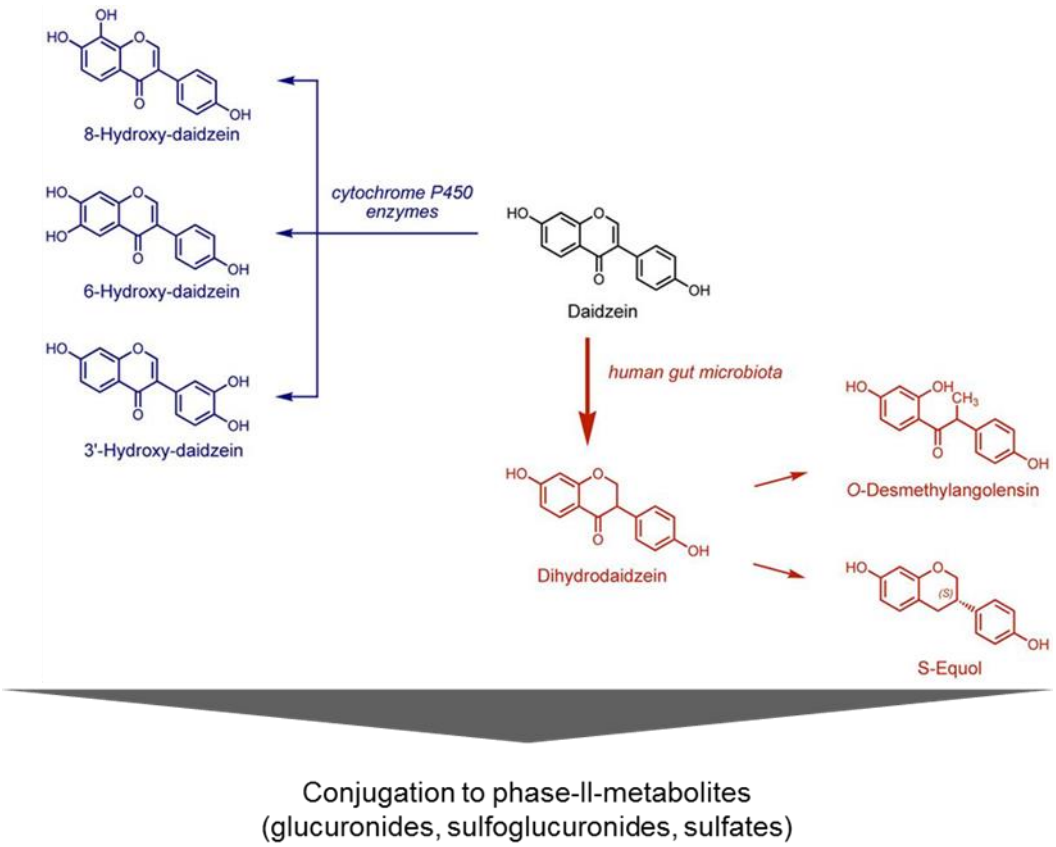


Figure 2: Metabolism of daidzein

Elimination

Most ingested genistein and daidzein is excreted as phase II conjugates and as phase II conjugates of microbial-derived metabolites in the urine. Faecal elimination has been found to be a minor route. Larkin et al. (2008), in their review, reported that total faecal excretion of isoflavones in humans accounts for less than 5 %, and is predominantly in the unconjugated form, with less than 10 % being conjugated.

Urine excretion was investigated in a study conducted by Xu et al. (1994). In this study 12 young adult women ingested defined doses of soy isoflavones as soy milk powder in three meals at defined time points within a 10-hour window. Urine was collected in 12-hour fractions for 48 hours after the

first dosing. Depending on the soy isoflavone dose ingested, 85 to 93.5 % of the total isoflavone amount excreted in the urine was excreted in the first 24 hours after first dosing (= 14 hours after last dosing); 6 to 14 % was excreted in the 24–48 hour urine fraction after first dosing, and after 48 hours (38 hours after last dosing) isoflavone excretion had reached baseline level. This result is consistent with other studies summarised by Larkin et al. (2008), who stated that the majority of urinary excretion of daidzein and genistein occurs within the first 24 hours after ingestion.

The urinary recovery rate was determined in several studies. Shelnutt et al. (2 000) conducted a study in 12 volunteers (six males and six females) who ingested a soy beverage prepared to provide a dose of 1.0 mg/kg genistein aglycone equivalents and 0.6 mg/kg daidzein aglycone equivalents; 7.2 % of the dose of genistein and 27.4 % of the dose of daidzein (aglycone plus phase II conjugates) was recovered in the 24-hour urine. Based on urinary dihydrogenistein, dihydrodaidzein and O-desmethylangolensin, total recovery was 9.3 % for genistein and 50.9 % for daidzein. *S*-equol was not measured in this study and therefore not included in the excretion rates determined over time. The apparent terminal half-lives for genistein and daidzein glucuronides, the main metabolites found in urine, were 6.0 ± 0.4 and 3.8 ± 0.4 hours, respectively, including the microbial metabolites in the calculation made for daidzein. In other studies the percentages of a genistein and daidzein dose excreted in the urine were reported to be, respectively, 14.6 % and 46.9 % (Lu et al., 1995), 17.6 % and 35.8 % (Watanabe et al., 1998), 22 % and 62 % (King and Bursill, 1998) and 16 % (range 5–42%) and 50 % (range 18–95 %) (Setchell et al., 2003a). At least part of the variability is certainly based on the fact that not all known microbial metabolites were included in each calculation and that enzymatic hydrolysis, which was used in all studies to quantify the resulting aglycones, might be not complete in every single case. However, the studies consistently showed that the urinary recovery rate is much higher for daidzein than for genistein.

The half-life ($t_{1/2}$) reported in most human studies is the half-life of total genistein and total daidzein. This means that the values were calculated on the basis of a genistein and daidzein measurement after enzymatic hydrolysis of the different phase II conjugates which accounted for > 95 % of the total genistein and daidzein concentration found in plasma. Reported values for the elimination $t_{1/2}$ of total genistein are in the range of 5.7–10.8 hours and were determined in intervention studies using different soy foods (soy flour, soy beverage, soy nuts, soybean powder) as isoflavone source. The values are summarised in the review of Yang et al. (2012). In one study, ^{13}C-labelled genistein and daidzein were used. The $t_{1/2}$ values of total genistein and total daidzein were determined to be 7.41 ± 0.39 hours and 7.18 ± 0.49 hours, respectively, for a dose of 0.8 mg genistein and 0.8 mg daidzein/kg bw (Setchell et al., 2003b).

3.1.2. Kinetics and metabolism in animals

Absorption and bioavailability

Extracts from soy, red clover and kudzu root

Monkey

No data were available.

Rats

A recent study in rats (Zhang et al., 2015) reported plasma concentration–time profiles of three isoflavones after administration of an extract of red clover. The dose of 3.5 mg/kg bw ononin (O), 7.6 mg/kg bw formononetin (F) and 6.1 mg/kg bw biochanin (B) resulted in peak concentrations of 36.9 ± 7.48 ng/ml (O), 94.0 ± 22.9 ng/ml (F) and 102 ± 17.9 ng/ml (B) and in AUCs of 301 ± 107 ng/ml × h (O), 568 ± 203 ng/ml × h (F) and 414 ± 163 ng/ml × h (B).

Mice

No data were available.

Soy protein isolate

Monkey

No data were available.

Rats

No data were available.

Mice

The absorption and absolute bioavailability of genistein and daidzein were measured in female BALB/c mice after intravenous (i.v.) and oral administration of pure substances in doses of 1.2 mg/kg genistein and 0.55 mg daidzein. In addition, both isoflavones were orally administered in food containing SPIs (Andrade et al., 2010). Absorption, estimated by the comparison of AUCs of the total isoflavones, was complete. The absolute bioavailability amounted to 9–14 % for genistein and 29–34 % for daidzein.

Genistein

Monkey

No data were available.

Rats

Coldham et al. (2002) determined absorption after oral and intravenous dosing with 4 mg/kg bw [^{14}C]genistein in rats. Measured by total radioactivity. the absorption from the gut was 56 % in males and 111 % in females. The absolute oral bioavailability of the parent compound genistein was 7 % in male rats and 15 % in female rats.

Higher oral genistein doses of 6.25 mg/kg bw, 12.5 mg/kg bw and 50 mg/kg bw and an i.v. dose of 12.5 mg/kg bw were given to rats in the study of Zhou et al. (2008). In this study the bioavailability at these three doses (estimated from AUCs after oral and after intravenous dosing) was 21.9 %, 33.5 % and 19.0%, respectively.

Mice

Yang et al. (2010) investigated the systemic availability after i.v. and oral dosing of 20 mg/kg genistein in FVB mice. The absolute bioavailability as calculated from the AUCs after oral and i.v. administration was 23.4 %.

Puerarin

Monkey

No data were available.

Rats

After i.v. administration of a medicinal preparation containing safflor yellow A, puerarin, 3′-methoxyl-puerarin and puerarinapioside to rats, the concentration–time profiles of the components were determined in plasma (Yu et al., 2007). The volume of distribution, expressed in ml/kg, was 91.0 ± 22.0 for safflor yellow A, 156 ± 24 for puerarin, 824 ± 114 for 3′-methoxyl-puerarin and 1096 ± 124 for puerarinapioside; the total clearance, expressed in ml/kg/h, was 144 ± 37 for safflor yellow A, 433 ± 62 for puerarin, 1 667 ± 417 for 3-methoxyl-puerarin and 2 401 ± 420 for puerarinapioside; the area under the plasma concentration–time profile, expressed in h µg/ml, was 7.39 ± 2.10 for safflor yellow A, 23.54 ± 3.70 for puerarin, 6.99 ± 1.80 for 3-methoxyl-puerarin and 7.10 ± 1.1 for puerarinapioside.

Prasain et al. (2007) measured the concentration–time profile after oral dosing of 50 mg/kg bw puerarin and derived pharmacokinetic parameters. However, no data are given for the

absorption/bioavailability. However, if the dose corrected AUC from this study is compared with the dose-corrected AUC from the study of Yu et al. (2007), the bioavailability of puerarin is about 1 %.

Mice

No data were available.

Distribution

Extracts from soy, red clover and kudzu root

Monkey, rats, mice

No data were available.

Soy protein isolate

Monkey, rats, mice

No data were available.

Genistein

Monkey

No data were available.

Rats

Three studies providing data on the distribution of genistein have been published. In the study of Coldham and Sauer (2000) radioactivity was found in every organ. After 2 and 7 hours, the highest levels of radioactivity (> 1 000 ng genistein-equ/g tissue) were in the gastrointestinal tract and in the excretory organs, liver and kidney. Intermediary concentrations (< 1 000 ng genistein-equ/g tissue to 250 ng genistein-equ/g tissue) were observed in the reproductive organs testis/ovary, uterus, prostate and vagina where most of the radioactivity was claimed to be the parent compound. In brain, fat, thymus, spleen, skeletal muscle and bone the radioactivity concentrations were low (< 100 ng genistein-equ/g tissue). Using a specific method Chang et al. (2 000) observed high proportions (up to 90%) of genistein aglucone in several tissues, in particular the reproductive tissues. This observation was confirmed by Zhou et al. (2008) which also observed the highest genistein concentrations in the gastrointestinal tract and in the excretory organs, liver and kidney. The concentrations in the reproductive organs were equal to the concentrations in the skeletal muscle and in fat.

Mice

No data were available.

Metabolism

Extracts from soy, red clover and kudzu root

Monkey

No data were available.

Rats

No data were available.

Mice

Allred et al. (2005) investigated the metabolism of genistein and daidzein in OVX Balb/c mice using soy products of different degree of processing including soy flour, soy molasses, soy extract and

purified isoflavone fractions. They found that the average fraction of aglycone genistein and daidzein in the blood plasma ranged from 5 % to 12 % and from 11 % to 18 %, respectively. The proportion of *S*-equol aglycone was < 1 % for all feeding groups. In addition, the group investigated the distribution of the conjugated forms. The glucuronides were the main metabolites in the case of genistein and daidzein (53 % of total genistein, 44 % of total daidzein). In contrast, *S*-equol was present to 78 % as sulphate.

Soy protein isolate

Monkey

Gu et al. (2006) analysed the metabolite profile of daidzein and genistein in female cynomolgus monkeys (n = 15) after feeding a diet formulated with SPI for 5 weeks. The isoflavones genistein and daidzein as well as the microbial-derived daidzein metabolite *S*-equol were present in the serum of the monkeys predominantly as sulphates (64.9 % of total daidzein, 72.8 % of total genistein, 64.2 % of total *S*-equol), and to a lower extent as glucuronides (34.5 % of total daidzein, 23.8 % of total genistein, 29.6 % of total *S*-equol). In the blood serum the proportion of the aglycone was 0.6 %, 3.5 % and 6.1 % for daidzein, genistein and *S*-equol, respectively. *S*-equol represented 52 % of the total isoflavones (isoflavones plus metabolites) in the serum of the monkeys. Based on these results, monkeys were classified as 100 % equol producers. Dihydrogenistein, dihydrodaidzein and O-desmethylangolensin were found in the plasma at concentrations considerably lower than *S*-equol. The microbial genistein metabolites 6-hydroxy-desmethylangolensin and 4-ethylphenol were not measured.

Rats

Gu et al. (2006) determined the metabolite profile in the serum of adult female Sprague–Dawley rats (n = 9) after feeding a diet enriched with SPI for 3 days. The intake of genistein and daidzein was estimated to be 13.0 and 9.9 mg/kg bw/day, respectively. In the blood serum the proportion of the aglycone was 7.3, 3.6 and 0.7 % for daidzein, genistein and *S*-equol, respectively. *S*-equol was the dominant metabolite, accounting for 77 % of total isoflavones (isoflavones plus metabolites) in the serum of the rats. The serum daidzein:*S*-equol ratio was 1:19. Based on these results, rats were classified as 100 % equol producers. Dihydrogenistein, dihydrodaidzein and O-desmethylangolensin were found in the plasma at concentrations very much lower than *S*-equol. The microbial genistein metabolites 6-hydroxy-desmethylangolensin and 4-ethylphenol were not measured.

Mice

No data were available.

Individual isoflavones, alone and in combination

Monkeys

No data were available.

Rats

No data were available.

Mice

Yang et al. (2010) investigated the systemic availability after i.v. and oral dosing of 20 mg/kg genistein in male FVB mice (8–10 weeks old). In addition to genistein, genistein-7-glucuronide, genistein-4′-glucuronide, genistein-7-sulphate and genistein-4′-sulphate were identified, with average maximum plasma concentration (C_{max}) values of 0.71 µM, 0.98 µM, 0.53 µM, 0.25 µM and 0.65 µM, respectively, after oral dosing.

Elimination

Extracts from soy, red clover and kudzu root

Monkeys

No data were available.

Rats

In a study comparing the bioavailability of conjugates of genistein and daidzein, rats were given a single oral dose of a soy extract providing 74 µmol genistein and 77 µmol daidzein/kg bw as conjugates (King, 1998). Urinary excretion of daidzein was 17 % of the dose ingested and 11.9 % in the case of genistein over a 48-hour post-dose period. *S*-equol excretion was 5 % of the daidzein dose, but 41.9 % of the genistein dose was excreted as 4-ethyl phenol. Faecal daidzein accounted for 2.3 ± 0.5 % and faecal genistein for 3.4 ± 0.4 % of the respective doses.

Mice

No data were available.

Soy protein isolate

Monkeys

Gu et al. (2006) reported that female monkeys excreted a very high percentage of daidzein, genistein and *S*-equol in the urine as aglycones (89–96 %). No information is given on the excretion kinetics of isoflavones.

Rats

Gu et al. (2006) found in their study that female Sprague–Dawley rats excreted a high percentage of daidzein, genistein and *S*-equol in the urine as aglycones (33–47 %). Of the original dose, 2.6 % of genistein and 3.3 % of daidzein was recovered in the 24-hour urine as aglycones plus phase II conjugates. In addition, 17.3 % of the daidzein dose was excreted as *S*-equol, compared with only 0.3 % and 0.2 % which were excreted as O-desmethylangolensin and dihydrodaidzein, respectively. Total daidzein (aglycone plus phase II conjugates plus microbial metabolites) recovery in the 24-hour urine was 21.2 % of the dose ingested. No information is given on the excretion kinetics of isoflavones in this study.

Mice

No data were available.

Individual isoflavones, alone and in combination

Monkeys

No data were available.

Rats

The excretion of daidzein was measured in male and female Fischer F344 rats after administration of daidzein (100 mg/kg bw, dissolved in corn oil) by gavage. For both sexes, 86 % of the dose was excreted as unchanged daidzein in the faeces within 36 hours after administration, and 8–9 % of the dose was excreted in the urine within 24 hours after administration (Bayer et al., 2001).

Mice

No data were available.

3.1.3. Discussion on absorption, distribution, metabolism and excretion

The term 'isoflavones' refers to a group of compounds; however, the kinetics can be described only for defined substances. Hence, the literature was searched for data on the kinetics of the most common isoflavones, daidzein, genistein, glycetin, formononetin, biochanin and puerarin. In addition, the kinetics may be species specific and, hence, the search included all the species in which studies on the effects have been performed (monkey, rat, mice).

Data on the absorption and absolute bioavailability of isoflavones were available for mice and rats. In mice, the absorption of genistein and daidzein, estimated by the comparison of AUCs of the total isoflavones, was complete. Absolute bioavailability amounted to 9–14 % for genistein and to 29–34 % for daidzein. In rats, genistein absorption was 56 % in males and 111 % in females. The absolute oral bioavailability of the parent compound genistein was 7 % in male rats and 15 % in female rats. In another study, oral genistein doses of 6.25 mg/kg bw, 12.5 mg/kg bw and 50 mg/kg bw were given and the bioavailability was 21.9 %, 33.5 % and 19.0 %, respectively. If the AUC, corrected for the oral dose, from the study of Prasain et al. (2007) is compared with the AUC, corrected for the i.v. dose, from the study of Yu et al. (2007) the bioavailability of puerarin is about 1 %.

In rats, doses of 3.5 mg/kg bw ononin (O), 7.6 mg/kg bw formononetin (F) and 6.1 mg/kg bw biochanin (B) resulted in peak concentrations of 36.9 ± 7.48 ng/ml (O), 94.0 ± 22.9 ng/ml (F) and 102 ± 17.9 ng/ml (B).

From three studies on genistein, it can be deduced that genistein is found in every organ, mainly in the form of the unconjugated substance.

In humans, calculated from urinary data, the absorption was estimated to be 61.3 % of the dose for daidzin, 60.4 % for glycitin and 35.4 % for genistin. After 6.6 mg, 13.2 mg and 26.4 mg of daidzein and of 9.8 mg, 19.6 mg and 39.2 mg of genistein in the glycosylated form, the absorption, calculated from urinary excretion, was found to decline with increasing dose (daidzein: 63.2 %, 54.4 % and 44.0 %; genistein: 25.2 %, 13.4 % and 15.8 %). In another study, the absorption, calculated from urinary excretion data, was 88.5 % for daidzein and 44.3 % for genistein.

From the study of Busby et al. (2002), who investigated several administrations of genistein and daidzein in healthy volunteers, C_{max} for genistein aglucone, as percentage of the total genistein, varied between 0.4 % and 3.9 % and the C_{max} for daidzein aglucone, as percentage of the total daidzein varied, between 1.4 % and 4.2 %.

A study by Setchell et al. (2011) provided relevant information by comparing the proportion of unconjugated genistein and daidzein in plasma in rat and humans. For daidzein, the percentage unconjugated substance was 8.1 % in rats and 0.98 % in female humans; for genistein, the percentage unconjugated was 4 % in rats and 0.26 % in female humans. This information could be used to derive a chemical specific adjustment factor to enable interspecies extrapolation.

At least some data on the metabolism of the soy isoflavones daidzein and genistein are available for humans, monkeys, rats and mice. Monkeys, rats, and mice are described as 100 % equol producers, meaning that the microbiotas of these animals are uniformly able to transform daidzein to a considerable extent to *S*-equol (Gu et al., 2006). In contrast, the microbial metabolism of daidzein in humans is characterised by a large interindividual variability, and only some of the population are able to produce *S*-equol. As a consequence of this heterogeneity, microbial metabolites other than equol, e.g. dihydrodaidzein or O-demethylangolensin, can be present in human plasma at higher concentrations than *S*-equol.

The unconjugated isoflavones are discussed as they are the biologically most active forms and are therefore of particular importance (Setchell et al., 2011). According to several intervention studies (Gu et al., 2006; Hosoda et al., 2011; Setchell et al., 2011; Soukup et al., 2014), the mean portion of unconjugated daidzein and genistein in plasma of adults ranges 1.4–2.1 % and 0.8–1.7 %, respectively, after the intake of various soy foods (soy beverage, kinako, soy nuts, tempeh) or soy extract.

Large species differences are found, as the plasma percentages of unconjugated genistein concentrations in Sprague–Dawley rats and C57BL/6, nude and transgenic AngptL4B6 mice were

4.0 %, 4.6 %, 11.6 %, and 30.1 %, respectively, which are 20, 23, 58, and 150 higher than the values in humans (Setchell et al., 2011).

Conjugated isoflavones may be deconjugated, as a recent study using U2OS and T47D cells showed, but the degree of deconjugation observed was low (0.2–1.6 %) (Islam et al., 2015). Using human breast tissue S9 fraction, the average total deconjugation after 24 hours was 2.4 % and 2.0 %, respectively, whereas the rat breast tissue S9 fraction was about 30 times more potent in deconjugating the 7-O-glucuronides (Islam et al., 2015). This is in line with the finding that only low concentrations of unconjugated daidzein and genistein (< 50 pmol/g breast tissue) were found in breast tissue homogenate from healthy women after the intake of soy milk or a soy supplement (Bolca et al., 2010). The authors estimated overall total glucuronidation of 98 % in breast tissue, although not all phase II metabolites were determined.

Compared with daidzein, the microbial degradation of genistein *in vivo* is less well investigated. There is evidence from one study that degradation to 4-ethyl phenol is the major pathway in the rat. However, the quantitative importance of this metabolite in humans is currently not known. The same applies for 6-hydroxy-O-methylangolensin, a metabolite which is considered more rarely than the analogous daidzein degradation product O-desmethylangolensin.

Oxidative phase I metabolites of daidzein and genistein, mainly 6-hydroxy-, 8-hydroxy and 3′-hydroxy-daidzein as well as 6-hydroxy- and 3′-hydroxy-genistein, are found in humans, rats and mice (Breinholt et al., 2000; Kulling et al., 2001; Rüfer et al., 2008). Although the extent of their formation is described as low, all these minor metabolites bear a catechol structure and might be easily oxidised to form reactive o-quinones. Moreover, o-quinones are described as reactive metabolites towards nucleophiles. No quantitative data were available on phase I metabolites that would allow a comparison between species.

The main phase II metabolites of daidzein and genistein in human plasma are the 7-glucuronide-4′-sulphates, whereas in female rats and mice the monoglucuronides are the predominant conjugates (Hosoda et al., 2011; Soukup et al., 2014). Data on the whole biotransformation, including phase I and phase II metabolism, of formononetin, biochanin A and puerarin are too incomplete to conclude on differences among the species.

3.2. Effects on the mammary gland

3.2.1. Results from human studies

At the end of the systematic review process the intervention studies shown in Table 9 and Table 10 were identified as relevant for the risk assessment of isoflavone supplements in mammary gland. The four observational studies included in this review are not included in the tables but only summarised narratively below.

Additionally, information on reported adverse events occurring in the clinical trials included in this review has been collected and assessed and is reported in Table C1 in Appendix C.

Endpoint: breast cancer incidence

A large number of epidemiological studies have investigated the association between breast cancer and isoflavones intake from diet, in most cases investigating the hypothesis of a possible protective effect of diets rich in phytoestrogens. Since the focus of this assessment is the intake of isoflavones from food supplements, only those studies in which the use of food supplements was analysed for association with incidence of breast cancer have been included in this review.

The association between consumption of food supplements containing isoflavones and incidence of breast cancer has been assessed in only four observational studies which fulfilled the pre-defined criteria specified in the protocol.

Studies with Tier 1 reliability

The association between use of 28 different isoflavone supplements and breast cancer risk was evaluated in a case–control study including 3 101 cases of breast cancer and 3 471 matched controls from Ontario, Canada (Boucher et al., 2013). Out of the total number of cases and controls, 2 140

cases and 2 228 controls were post-menopausal women. In the group of premenopausal women, 203 of the cases and 236 of the controls had an intake of isoflavone-containing food supplements. Of these, 78 cases and 85 controls reported the use of food supplements containing isoflavones for a period of 1 to 5 years and 37 cases and 43 controls reported using supplements for more than 5 years. Hence, premenopausal women were also included in this study. There was no indication that the intake of isoflavones in this group was associated with an increased risk of breast cancer (odds ratio (OR) 1.09; 95 % confidence interval (CI) 0.89–1.35). This study showed, in addition, that several individual supplements were associated with reduced breast cancer risk (e.g. OR 0.39; 95 % CI 0.22–0.69 for natural hormone replacement therapy (HRT)). There were no significant associations between breast cancer and ever use of isoflavone supplement in post-menopausal women, but there were inverse associations for cumulative use of two (OR 0.72; 95 % CI 0.55–0.93) or more than three supplements (OR 0.61; 95 % CI 0.46–0.82) and use for more than 5 years (OR 0.73; 95 % CI 0.54–0.98). These associations were more pronounced in consumers of high-isoflavone-containing supplements (≥ 0.676 mg/day).

Within the VITamins And Lifestyle (VITAL) Cohort, 35 016 post-menopausal women, aged 50 to 76 years, who were residents of western Washington State, completed a 24-page baseline questionnaire between 2000 and 2002 and were prospectively followed up for a mean of 6 years (Brasky et al., 2010). Incident invasive breast cancers (n = 880) from 2000 to 2007 were obtained from the Surveillance, Epidemiology, and End Results registry. Among the cohort population there were 1 589 users of soy-based supplements, 36 of whom developed incident breast cancer. The use of isoflavones supplements taken for menopausal symptoms in this cohort of women was not associated with breast cancer risk (OR 1.04, 95 % CI 0.74–1.48).

In a German case–control study, the possible association between usage patterns of herbal preparations (including phytoestrogens from soy isoflavones and red clover) and incident breast cancer was investigated in 10 121 post-menopausal women (3 464 women with breast cancer, 6 657 controls) (Obi et al., 2009). Information on the herbal preparations used was collected in face-to-face interviews supported by a list of brand names. Ever use of phytoestrogens, as reported by 86 (1.3 %) women in the control group and by 20 (0.6 %) cases, was not associated with invasive breast cancer (OR 0.64, 95 % CI 0.39–1.05).

Studies with Tier 2 reliability

A population-based case–control study conducted in the Philadelphia metropolitan area, USA, evaluated whether use of hormone-related supplements is associated with breast cancer risk (Rebbeck et al., 2007). The study included 949 breast cancer cases and 1 524 controls. Use of any phytoestrogen was not associated with breast cancer incidence (adjusted OR 0.76, 95 % CI 0.48–1.21). Similar results were found for any isoflavone or genistein (isoflavones: 30 cases and 52 controls; OR 0.74, 95 % CI 0.32–1.67), red clover (OR 0.78, 95 % CI 0.38–1.61) or soy medications (OR=0.81, 95 % CI 0.39–1.67).

From the limited number of observational studies, and the group sizes within the available studies (2 216 users of isoflavones across the four studies), no evidence of an association between use of isoflavone-containing supplements and breast cancer risk was found. The Panel noted that the results of the four observational studies were consistent, with low heterogeneity among the studies.

Endpoint: mammographic density

The effect of intake of isoflavones from food supplements and changes in the mammographic density of peri- and post-menopausal women has been investigated in several controlled trials.

Mammographic density is a method to estimate the radiographic dense part of the breast. This part is expressed as percentage of the breast tissue area on the mammogram (Ursin and Qureshi, 2009). It is commonly used as a measure of the changes in the tissue composition of the breast. Increasing age and menopause are associated with decreasing breast density, attributable to the reduction of epithelial and stromal tissues and increase in adipose tissues (Martin and Boyd, 2008). Boyd (2013) described 10 epidemiological studies in which breast density was demonstrated to be related to the risk of breast cancer, the results of which demonstrate the usefulness of this method. Two recent studies have confirmed that per cent mammographic density is the strongest risk factor for breast cancer besides genetic variants and family history (Vachon et al., 2015; Maskarinec et al., 2015). In

several interventional studies increased mammographic density has been shown to be related to the intervention if the intervention was a combined hormonal replacement therapy in postmenopausal women (reviewed in Ursin and Qureshi, 2009). On the other hand, treatment with tamoxifen, a selective oestrogen receptor modulator working as an oestrogen antagonist in breast tissue, reduced breast density in women (reviewed in Ursin and Qureshi, 2009). Hence, increased breast density might be seen as a surrogate endpoint related to an increased breast cancer risk in interventional trials with substances which are agonistic or antagonistic at the oestrogen receptor.

A total of eight studies fulfilling the pre-defined criteria for inclusion in the review were considered for the assessment of isoflavone supplements effect on mammographic density and are presented in Table 9.

None of the studies considered reported significant changes in mammographic density associated with the use of soy isoflavones/soy extract- (60–100 mg/day, 10–12 months), soy protein- (99 mg/day, 12 months), daidzein-rich isoflavones- (80–120 mg/day, 24 months), genistein- (54 mg/day, 12–36 months) and red clover extract- (40–43.5 mg/day, 12–36 months) containing food supplements.

Table 9: Human studies included reporting effects on breast: changes in mammographic density

Ref. ID[a] Author, year	Design	Duration (months)	Isoflavone dose (mg/day)[b]	Description of intervention	Active group (N)/ control group (N)[c]	Age[d]	Time since menopause (years) AND/OR age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
Soy isoflavones/soy extract										
3071 Colacurci et al., 2013	RCT, DB	12	60	Authors described the intervention as soy isoflavones, *Lactobacillus sporogenes* 1 billion spores, calcium 240 mg, vitamin D_3 5 µg and glucosamine 250 mg. The isoflavones dose is expressed as mg/day genistin and daidzin. Placebo was calcium and vitamin D_3 alone	ISO: 65/62; placebo: 65/62	ISO: 55.3 ± 7.6; placebo: 55.7 ± 7.7[f]	ISO: 3.2 ± 2.8; placebo: 2.8 ± 2.6	Yes	No statistically significant difference between isoflavones and placebo group in the mammographic density score.	1
16401 Delmanto et al., 2013	RCT, DB	10	100	Authors described the intervention as standardised soy extract 250 mg corresponding to 100 mg isoflavones/day administered as capsules twice a day. The authors stated that each capsule contained 125 mg soy extract plus 50 mg isoflavones. The Panel assumes that the total daily dose is still 100 mg isoflavones/day	ISO: 40/32; placebo: 40/34	ISO: 55.1 ± 6.0; placebo 56.2 ± 7.7	ISO: 6.6 (4.8), 48.4 (3.7); placebo: 7.1 (4.2), 47.7 (3.5)	Yes	No statistically significant differences in mammographic density between isoflavone and placebo group No differences in breast parenchyma evaluated by ultrasound	1
Soy protein										
3127 Verheus et al., 2008	RCT, DB	12	99	Authors described the intervention as soy powder. Dose expressed as aglycones weight	ISO: 100/70; placebo: 102/56	ISO: 66.3 (4.3); placebo: 65.3 (4.0)	ISO: 18.1 (7.0), 48.3 (5.5); placebo: 16.1 (5.8), 49.3 (3.6)	Subcohort of larger trial	No statistically significant differences between the two groups. Absolute density and per cent density decreased in both study groups in both the modified ITT and per protocol analyses. The analysis was repeated after stratification for equol-producing status (32.9 % and 21.1 % of the women in the isoflavones and placebo groups, respectively). Equol-producing status did not affect the results	1

Ref. ID[a] Author, year	Design	Duration (months)	Isoflavone dose (mg/day)[b]	Description of intervention	Active group (N)/ control group (N)[c]	Age[d]	Time since menopause (years) AND/OR age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
Daidzein-rich isoflavones										
1199 Maskarinec et al., 2009	RCT, DB	24	80; 120	Authors stated the isoflavone doses used in the study were equivalent to 300–500 g tofu or 500–1 000 ml soy milk	Low ISO: 120; high ISO: 115; placebo: 123	Low ISO: 54.8 ± 3.6; high ISO: 55.2 ± 4.0; placebo: 54.7 ± 3.8	Low ISO: 6.7 ± 5.4, 48.5 ± 5.7; high ISO: 6.9 ± 6.7, 47.9 ± 6.2; placebo: 6.5 ± 5.2, 48.3 ± 5.2	Sub-cohort of larger trial	No statistically significant differences between either treatment groups compared with placebo. Per cent densities decreased during the study in all groups.	1
Genistein										
3138 Marini et al., 2008	RCT, DB	36	54	Dose expressed as mg genistein/day. Authors report that purity of genistein used was approximately 98 %	GEN: 198/150/71; placebo: 191/154/67[h]	GEN: 53.8 ± 2.9; placebo: 53.5 ± 2.0	GEN: 3.6 ± 3.0; placebo: 3.6 ± 2.2[i]	Sub-cohort of larger trial	No statistically significant differences between groups at any time point (baseline, 2 years, 3 years) observed with digitised assessment. No statistically significant difference using visual classification.	1
2282 Morabito- et al., 2002	RCT, DB	12	54	Authors report that purity of genistein used was approximately 98 %. The HRT arm was given 1 mg/day of 17β-oestradiol combined with 0.44 mg/day norethisterone acetate, Activelle®	GEN:30; HRT: 30; placebo: 30	GEN: 52 ± 3; HRT: 52 ± 5; placebo: 51 ± 4	GEN: 7 ± 6; HRT: 7 ± 3; placebo: 6 ± 5	No	No statistically significant changes in mammography exams at 1 year in any of the groups	2
Red clover extract (RCE)										
3168 Atkinson et al., 2004	RCT, DB	12	43.5	Authors report that commercial name of isoflavone tablet was Promensil®, each tablet containing 26 mg biochanin A, 16 mg formononetin, 1 mg genistein and 0.5 mg daidzein derived from red clover	RCE: 102/86; Placebo: 103/91[j]	RCE: 55.1 ± 4.7; placebo: 55.2 ± 4.9	–	Yes	No statistically significant differences between treatment and placebo. Per cent densities decreased during the study in all groups	1

Ref. ID[(a)] Author, year	Design	Duration (months)	Isoflavone dose (mg/day)[(b)]	Description of intervention	Active group (N)/ control group (N)[(c)]	Age[(d)]	Time since menopause (years) AND/OR age at menopause (years)[(d)]	Primary endpoint?	Results	Tier of reliability [(e)]
16435 Powles et al., 2008	RCT, DB	36	40	Authors report that commercial name of isoflavone tablet was Promensil®	RCE total: 199; RCE post-menopausal: 39/22/22/8; RCE pre-menopausal: 160/133/123/111; placebo total: 202; Placebo post-menopausal: 38/25/22/11; Placebo pre-menopausal: 164/127/120/111[(k)]	RCE: 45 (35–69); placebo: 45 (35–69)	–	No	No statistically significant difference between treatment and control at any time point (1 year, 2 years, 3 years) in either the pre-menopausal or post-menopausal women	2

RCT: randomised controlled trial; DB: double-blind; –: information not available
(a): Refers to the Ref. ID number in Distiller
(b): Unless otherwise specified, doses are expressed as mg isoflavones/day.
(c): Allocated to treatment/completed.
(d): Mean ± SD or SEM, as reported in the original publications.
(e): See protocol in Annex A for appraisal of risk of bias.
(f): In the publication it is reported that mean age in the placebo group was '5.7 ± 7.7' years. Clarification has been received from the corresponding author.
(g): This study was embedded in another trial. Sample size was not specifically calculated to address the endpoint of interest.
(h): The third year of this study was an additional follow-up conducted on a subset of the initial patients. In the placebo group, 67 out of the 154 women who completed the second year of follow-up decided to continue for a third year; in the genistein arm, 71 women out of the 150 who completed the second year of follow-up decided to continue the trial for the additional year. No subjects were lost to follow-up in the third and last year.
(i): Values calculated by the Panel. The authors report time since menopause to be 42.9 ± 35.9 months and 42.8 ± 26.3 months for genistein and placebo, respectively.
(j): The population included in the study comprised premenopausal women (16 % and 17 % in the RCE and in the placebo group, respectively, peri-menopausal women (14 % and 16 % in the RCE and in the placebo group, respectively) and post-menopausal women (67 % and 68 % in the RCE and in the placebo group, respectively).
(k): The authors reported that women included in this study were at higher risk of breast cancer because of their family history.

Endpoint: histo(patho)logical markers/changes

The induction of histo(patho)logical changes in the mammary gland of peri- and post-menopausal women resulting from the intake of isoflavones from food supplements has been investigated in several controlled trials. Studies were included if they reported information on proliferation marker Ki-67 and atypical cytology.

The Ki-67 antigen was identified as a non-histone protein possibly playing an important role in cell proliferation. The expression of Ki-67 varies throughout the different cell cycle phases and it is typically low in healthy breast tissues (< 3%). Several investigators have reported that steroid receptor expression and Ki-67 antigen are detected in separate cell populations in healthy human breast epithelium, with Ki-67 expressed exclusively in ER-negative cells. Ki-67 antibody does not bind to ERα-positive cells in healthy human breast tissue. This separation between steroid receptor expression and proliferation markers does not exist in malignant tissue. A correlation has been described between expression of Ki-67 and breast density as well as with incidence of precancerous lesions (Yerushalmi et al., 2010).

Only two studies fulfilling the pre-defined criteria for inclusion in the review were considered for the assessment and are presented in Table 10.

No effects were reported in the two studies investigating soy isoflavones/soy extract supplementation for 3 to 6 months at doses ranging from 60 to 235 mg/day.

Table 10: Human studies included reporting effects on breast: proliferation marker Ki-67 and atypical cytology

Ref. ID[a] Author, year	Design	Duration (months)	Dose (mg/day)[b]	Description of intervention	Active group (N)/control group (N)[c]	Age[d]	Time since menopause (years) and/or age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
Soy isoflavones (ISO)/soy extract										
16409 Khan et al., 2012	RCT, DB	6	235 as aglycones	Authors described the intervention (PTIG-2535) as containing 150 mg genistein (GEN), 74 mg daidzein (DAI) and 11 mg glycitein (GLY) (ratio GEN:DAI:GLY was 2:1:0.15)	ISO: 49 total (21 post-menopausal); placebo: 49 total (24 post-menopausal)[f]	ISO: 48 (43–53)[g]; placebo: 50 (46–55)	–	Yes	Ki-67 labelling: No statistically significant differences in the post-menopausal women (45/98) within groups or between treated and placebo groups when post-intervention values were compared with those at baseline. In the group of pre-menopausal women (53/98) there was a relative increase in the Ki-67 index post intervention in the women treated with soy isoflavones; however, this was not statistically significant when compared with placebo. Atypical cytology: No significant changes between groups in morphological features of the epithelial cells[h]	1
3158 Cheng et al., 2007	RCT, DB	3	60	Authors described the intervention as fruit drink containing isoflavones from soya beans	ISO: 26/26; placebo: 25/24	ISO: 58.4 ± 5.0; placebo 56.9 ± 4.2	ISO: 8.4 ± 5.3; placebo: 7.0 ± 3.8	Not specified	Ki-67 labelling: The proliferation marker Ki-67 was seen in 0 % to 0.5 % of samples. No significant change was induced by isoflavones treatment. The analysis was performed in 102 samples (pre and post treatment)	2

(a): Refers to the Ref. ID number in Distiller.
(b): Participants included pre- and post-menopausal women at high risk of breast cancer, including women with a history of breast cancer (15 subjects in the treatment group, 10 in the control) group. Among the post-menopausal women included, 33.3 % in the soy group and 37.5 % in the placebo group had a history of unilateral breast cancer with all systemic therapy completed at least 1 year before the study.
(c): Unless otherwise specified doses are expressed as mg isoflavones/day.
(d): Allocated to treatment/completed.
(e): Mean ± SD or SEM, as reported in the original publications.
(f): Data reported as interquartile range.
(g): See protocol in Annex A for appraisal of risk of bias.
(h): Results were presented by the authors according to the menopausal status and also stratified by equol production status.

Discussion on results from human studies on the mammary gland

The association between intake of food supplements containing isoflavones and breast cancer risk in peri- and post-menopausal women was investigated in four observational studies fulfilling the criteria for this review. The studies have the obvious limitations that are intrinsic to observational design, which lead to uncertainty around the actual exposure of the subjects to the isoflavones from food supplements, e.g. in terms of dose and duration. The study population in prospective observational research is intended to be representative of the general population of interest, sub-groups with any particular exposure (e.g. in this context, food supplements containing isoflavones) will be only a limited proportion of this population.

On the other hand, the advantage of these studies is that they allow observation over prolonged time periods and may therefore provide information over those endpoints, such as the risk of developing cancer, which may occur as a late effect.

In total, three case–control studies were considered for this review, involving, in total, 7 514 women with breast cancer and 11 652 controls.

One prospective cohort study recruited a total of 35 016 subjects, observed for a mean of 6 years, and included a total of 880 breast cancer cases, 36 of which were reported in women taking isoflavones. Adding the numbers of isoflavones users in all epidemiological studies would result in a total of 2 216 peri- and post-menopausal women.

One of the studies (Boucher et al., 2013) found a significantly reduced risk of breast cancer in post-menopausal women associated with the use of any isoflavones supplement (ever use of three or more supplements or use for more than 5 years). However, the Panel noted that the number of subjects with the highest level of intake was very limited (82 cases, 134 controls); similarly, only 97 cases and 149 controls used supplements for a longer duration.

With respect to mammographic density, 741 subjects treated with isoflavones were followed up in eight intervention studies lasting between 10 and 36 months, in which isoflavones were supplemented at doses ranging from 40 to 100 mg/day. No significant changes were observed in any of the included studies.

Only two studies (Cheng et al., 2007; Khan et al., 2012), including 75 participants in total, were conducted to investigate the effect of soy isoflavones on changes in the proliferation marker Ki-67. Neither of the two studies reported significant changes. In one of these studies, a high dose of isoflavones was tested (235 mg/day, for 6 months) and women with previous history of breast cancer were also included. No significant effects were reported for this small sub-group of participants (12 women in the soy group, 10 in the control). In all the intervention trials that reported other adverse events related to the mammary gland, the events were distributed proportionately between treatment and control groups (see Appendix C). In the study of Chilibeck et al. (2013), all participants in the trial underwent mammographic assessment after 2 years of treatment with isoflavones (105 mg/day expressed as aglycones) with no difference between the groups (approximately 150 women/group).

During the process of screening of the literature for inclusion in the systematic review for the current risk assessment, the Panel became aware of a randomised placebo-controlled study (Shike et al., 2014) conducted in women with diagnosed breast cancer confirmed by biopsy. This study did not meet the inclusion criteria specified in the systematic review protocol (minimum 3 months' treatment) because duration of treatment from the date of diagnosis to the date of breast surgery varied between 7 and 30 days. No statistically significant difference between the groups was noted for the primary endpoints, Ki-67 proliferation marker and Cas3 as an apoptosis marker. The Panel noted that the results in the primary endpoints are consistent with the lack of an observed effect in all the other intervention studies included in the current systematic review.

In the same study, additional investigations were conducted in a subset of patients to investigate gene expressions revealing some changes for which the authors stated that *'the clinical impact of the subtle changes in gene expression has not been examined'*. For these changes in gene expression, the relevance of these findings for breast cancer risk is unclear. The Panel therefore agreed that these findings cannot be used for the current risk assessment.

Overall, the Panel considered that the available evidence from human studies does not suggest an association between exposure to isoflavones-containing food supplements and adverse effects in the mammary gland on the endpoints evaluated. It is however noted that the preparations tested in the intervention studies included are a limited representation of those on the market.

These conclusions are consistent with the results of a recent systematic review by Fritz et al. (2013) investigating the influence of soy intake from food, which also found lack of evidence for harmful effects of soy, red clover and isoflavones from food and food supplements with respect to risk of breast cancer or recurrence, based on long-term observational data at intake levels of 25–50 mg/day.

The evidence from this limited number of observational studies and the limited number of women taking isoflavone supplements does not support the hypothesis of an increased risk associated with the intake of isoflavones from food supplements. These findings are consistent with the results from the Women's Health Initiative study, which showed no increased risk of breast cancer associated to oestrogen-only HRT (Prentice, 2014).

Available data on the effect of HRT on mammographic density in menopausal women showed that treatment with oestrogen alone is associated with an increase in the frequency of high dense mammography, albeit to a much less extent than the combined regimen with oestrogens and progestin (Carmona-Sánchez et al., 2013). Similarly, the effect of HRT on the expression of Ki-67 antigen in post-menopausal women has been examined in a small study by Hofseth et al. (1999) in which combined treatment with oestrogen and progestin induced a significant increase in the percentage of cells expressing the Ki-67 antigen. The increase was not observed in women not taking HRT or taking oestrogens alone. Thus, the lack of effect on these two surrogate endpoints observed for isoflavone supplements was consistent with the results reported for oestrogen.

3.2.2. Results from animal studies

At the end of the systematic review process, the studies shown in Table 11 and Table 12 were identified as relevant for the risk assessment question. All the studies included in the assessment were assessed independently by two experts, using the appraisal tools described in the protocol in Annex A and allocated to different tiers of reliability.

Endpoint: cell proliferation in mammary gland

The effect of administration of isoflavones on cell proliferation in the mammary gland has been investigated in several experimental studies in animals. Studies were included if they reported information on the proliferation marker Ki-67 (a protein strictly associated with cell proliferation), PCNA (proliferating cell nuclear antigen, an essential DNA replication accessory protein) or atypical cytology. A total of 10 studies fulfilling the pre-defined criteria for inclusion in the review were considered for the assessment and are presented in Table 11.

A total of five studies in OVX monkeys (*Macaca fascicularis*) were identified as relevant. No significant changes were observed after administration of a soy extract providing 35.7 mg isoflavones/kg bw/day for approximately 1 month (Wood et al., 2006). In three of the five monkey studies, the source of isoflavones administered to the animals was a SPI providing doses of isoflavones ranging from 8.6 to 9.9 mg/kg bw/day for approximately 3 years and 6 months, respectively (Foth and Cline, 1998; Wood et al., 2004; Scott et al., 2008). According to the authors, the doses used in these studies were equivalent to human doses of 129–148 mg/day for women. In these three studies positive control groups were also present, in which animals were treated with oestradiol at doses equivalent to 0.625–1 mg/day for women. Administration of the SPI did not result in an increase in cell proliferation markers, which was instead observed in all the animals treated with oestradiol. On the contrary, a significant reduction of mammary expression of Ki-67 mRNA was observed in one of the studies (Scott et al., 2008). Equol (racemic) was administered at doses of 7.2 mg/kg bw/day and 68 mg/kg bw/day for 4 months and approximately 30 days, respectively (Wood et al., 2006, 2008). Also in these two studies, no significant changes were observed with respect to cell proliferation in the mammary gland.

Cell proliferation in the mammary gland was also measured in five studies in rats (Sprague–Dawley) identified as relevant for the assessment. In one of the studies, isoflavones from a soy extract were administered at doses of 15 mg/kg bw/day for 42 days with no observed effect on cell proliferation in the mammary gland (Gallo et al., 2006). In the other four studies either genistein or racemic equol

alone was administered to the animals. For genistein the doses tested ranged 5.4–221 mg/kg bw/day for 90 days (Wuttke et al., 2006; Rimoldi et al., 2007). In both studies an increase in PCNA immunostaining was observed in the mammary glands of the animal treated with genistein, although to a lesser extent than observed in the positive control groups treated with oestradiol at doses of 0.17–0.7 mg/kg bw/day. PCNA immunostaining was also measured in the two studies in rats in which animals were administered racemic equol at doses of 4.5 and 36 mg/kg bw/day for 90 days (Rachoń et al., 2008) and at doses of 4.5, 9 and 18 mg/kg bw/day for 56 days (Legette et al., 2009). A significantly higher percentage of PCNA-positive cells was observed in one of the two studies (Rachoń et al., 2008), but only for the highest dose tested (36 mg/kg bw/day), in terminal ducts and type II lobules. In the same study, animals in the positive control groups administered oestradiol-3-benzoate at doses of 0.4 and 1.6 mg/kg bw/day also showed a significant increase in all the mammary structures (terminal ducts, type I and type II lobules) and this was of a greater extent than in the high-dose equol (racemic) group.

Table 11: Animal studies included reporting effects on mammary gland: cell proliferation

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Soy isoflavones/soy extract								
1714 Wood et al., 2006	**Monkeys,** (*Macaca fascicularis*)	OVX control; ISO: 35.7; EQ: 68	The isoflavone supplement contained 65.5 % genistein, 29.2 % daidzein and 5.3 % glycitein (as expressed in aglycone equivalents), 96.0 % pure racemic mixture of *S*- and *R*-equol enantiomers. Actual dose administered 537 mg/1 800 kcal. Conversion reported by the authors	10	28–33	Breast epithelial expression MKI67, PGR, ESR1	No statistically significant difference among treatment groups for either lobules or ducts	1
15147 Gallo et al., 2006	**Rats** (Sprague–Dawley)	Sham control; OVX control; SSE: 15; E2	Authors report dose administered was 100 mg/kg bw/day of a standardised soy extract (SSE) Soyselect®. Dose conversion calculated on the basis of the percentage reported on the company website (13–17 % isoflavones). Animals in the E2 group were administered 17β-oestradiol, 0.5 mg/kg bw/day, by gavage	6–10	42	Immunohistochemical ERα, ERβ, PR, Ki-67 (epithelium and stroma)	No statistically significant changes in the number of cells expressing ERα in the epithelium. In the stroma, the final receptor score was similar in the sham, OVX and SSE groups. In animals treated with SSE, a downregulation of ERβ expression was observed in both epithelium and stroma. In the latter, the difference reached statistical significance compared with both sham and OVX control. Immunoreactivity to progesterone receptor (PR) was observed only in the epithelium. The final score of PR declined after ovariectomy. The SSE group showed a similar pattern to the OVX control group. Administration of SSE did not change the final score for the proliferative index Ki-67 both in the epithelium and in the stroma	1

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Soy protein isolate (SPI)								
16271 Wood et al., 2004	**Monkeys** (*Macaca fascicularis*)[c]	OVX control; SPI: 8.6; E2	Authors stated that the SPI treatment group received SPI containing isoflavones at a dose approximately equivalent to 129 mg/day for women (91 mg genistein, 31 mg daidzein and 7 mg glycitein). The doses were converted by the authors on the basis of the fact that the doses administered were scaled to 1 800 kcal of diet (estimated daily intake for US women) and that monkeys were fed 120 kcal/kg bw/day. Animals in the E2 group were given a dose corresponding to a human-equivalent dose of 0.625 mg/day on an energy basis. This dose was converted by the authors to 0.042 mg/kg bw/day	57–62	1080	Ki-67 labelling (lobules and ducts) PR and ER	No statistically significant difference among treatment groups for any of the parameters. In the E2-positive group, Ki-67 and PR and ERα expression were significantly increased compared with control and the SPI group	1
4730 Scott et al., 2008	Monkeys (*Macaca fascicularis*)[c]	OVX control; SPI: 8.6; E2	Authors stated that the dose administered corresponded to a human equivalent dose of 129 mg/day, expressed as aglycone units. The Panel assumed that the same conversion factor applied by the same research group in the publication by Wood et al. (2004) applies also to this case. Animals in the E2 group were fed an isoflavone-depleted diet supplemented with conjugated equine oestrogens (Premarin, Wyeth Pharmaceuticals) at a human-equivalent dose of 0.625 mg/day. The Panel assumed that the same conversion factor applied by the same research group in the publication Wood et al. (2004) (see above) applies also to this case	52–55	1080	Mammary mRNA expression of Ki-67, pS2 and PGR	Treatment with SPI produced a significant reduction (–48 %) in mammary expression of Ki-67 mRNA compared with control. No statistically significant differences in any other tissue biomarker were observed. Compared with monkeys on the control diet, animals treated with E2 experienced a 2-fold increase in Ki-67 mRNA expression, a 6-fold increase in progesterone receptor (PGR) and a 12-fold induction of pS2 mRNA	1
15120 Foth and Cline, 1998	Monkeys (*Macaca fascicularis*)	OVX control; SPI: 9.9; E2; E2 + SPI	Authors stated that the dose administered to the animals corresponded to a human-equivalent dose of 148 mg/day per woman, on an energy basis. Animals in the E2 groups were administered oestradiol in the diet. Authors stated that the dose administered to the animals corresponded to a human-equivalent dose of 1 mg/day per woman, on an energy basis. The Panel	15	180	Ki-67 labelling (lobules and large duct)	The SPI group was not different from control. Significant increase only in E2 group, in lobules; the effect was reduced in the E2 + SPI group	1

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
			assumed that the same conversion factor applied by the same research group in the publication by Wood et al. (2004) applies also to this case.					
Genistein								
4863 Rimoldi et al., 2007	Rats (Sprague–Dawley)	OVX control; low-GEN: 5.4; high-GEN: 54; low E2; high E2	In the publication it is reported that the doses of genistein were estimated by the authors on the basis of the daily food intake. Animals in the E2 groups were administered oestradiolbenzoate at 0.17 mg/kg bw/day (low E2) or 0.7 mg/kg bw/day (high E2)	11–12	90	PCNA and PR immunostaining	Genistein at both doses stimulated expression of PCNA compared with OVX control. PR-expressing nuclei were found in mammary glands of animals treated with high genistein and in both E2 groups but not in low genistein and OVX controls	1
16299 Wuttke et al., 2006	Rats (Sprague–Dawley)	OVX control; GEN: 221; E2[d]	In the publication it is reported that the dose administered to the animals was 53 mg/day. The authors were contacted and clarified that animal weight was 230–250 g. Dose conversion calculated by the Panel on the basis of the information provided by the authors. Animals in the E2 group administered 0.19 mg/day of oestrogen (free base)	10–12	90	PCNA immunostaining	Genistein produced a significant increase in PCNA-positive cells compared with OVX control but to a lesser extent than E2	1
Equol (racemic)								
16269 Wood et al., 2008	Monkeys (*Macaca fascicularis*)	OVX control; EQ: 7.2; E2	Authors stated that the dose administered corresponded to a human-equivalent dose of 105 mg/day of a 96.0 % pure racemic mixture of *S*- and *R*-equol enantiomers. Dose conversion reported in the publication. Animals in the E2 group were administered a dose corresponding to a human-equivalent dose of 1 mg/day micronised E2. Dose conversion to 0.066 mg/kg bw/day reported in the publication	20–22	240	Ki-67	No statistically significant difference between equol and control for either lobules or ducts. E2 significantly increased Ki-67 % of positive cells in lobules but not in ducts.	1
1714 Wood et al., 2006	Monkeys, *Macaca fascicularis*	OVX control; ISO: 35.7 EQ: 68	Conversion reported by the authors. Actual dose administered 1 020 mg/1 800 kcal	10	28–33	Breast epithelial expression of MKI67, PGR, ESR1	No statistically significant difference among treatment groups for either lobules or ducts	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
643 Rachoń et al., 2008	**Rats**, Sprague–Dawley	OVX control; EQ-50: 4.5; EQ-400: 36; low E2; high E2	Actual doses administered were 50 and 400 mg/kg chow of pure racemic mixture of equol (EQ-50 and EQ-400, respectively). On the basis of food intake, average consumption was 6.54 mg/day. Dose calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012). In the E2 groups, actual doses administered were 4.3 mg/kg and 17.3 mg/kg chow of oestradiol-3 benzoate for low E2 and high E2, respectively. On the basis of food intake, average consumption was 0.07 mg/day and 0.20 mg/day. Dose conversion to 0.4 mg/kg bw/day (low E2) and 1.6 mg/kg bw/day (high E2) calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012)	12	90	PCNA immunostaining (terminal ducts, type I lobules and type II lobules) PR	No statistically significant difference between EQ-50 and OVX control in either PCNA or PR. EQ-400 had significantly higher percentages of PCNA-positive cells in terminal ducts and type II lobules. EQ-400 resulted in a significantly higher percentage of PR-positive cells in type II lobules only. E2, at both doses, resulted in a significant increase in all endpoints considered	1
1203 Legette et al., 2009	Rats (Sprague–Dawley)	SHAM control OVX control; EQ-50: 4.5; EQ-100: 9; EQ-200: 18	Actual doses administered 50 mg/kg diet (EQ-50), 100 mg/kg diet (EQ-100) and 200 mg/kg diet (EQ-200) of equol powder (50 % *R*-equol, 50 % *S*-equol). Dose calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012). According to the authors the chosen doses correspond to human serum levels of equol of 3–39, and 5–49 µmol/l in response to dietary soy isoflavones of 56 and 90 mg/day (see Persky et al., 2002)	15–16	56	PCNA immunostaining	No statistically significant difference between OVX control and treated groups, at any dose	1

PR: progesterone receptor.
Text in bold indicates different animal species in which the studies were conducted. Text in grey indicates differents isoflavones being tested in the same study and refers to other rows in the table.
(a): Refers to the Ref. ID number in Distiller
(b): See protocol in Annex A for appraisal of risk of bias.
(c): Half of the monkeys had been previously treated with oral contraceptives over a 26-month period before ovariectomy.
(d): Additional group treated with *Cimicifuga racemosa* extract 133 mg/day. Data not presented in this table.

Endpoint: histo(patho)logy of the mammary gland

The effect of administration of isoflavones on the histopathology of the mammary gland has been investigated in several experimental studies in animals.

A total of 11 studies fulfilling the pre-defined criteria for inclusion in the review were considered for the assessment and are presented in Table 12.

A total of five studies in OVX monkeys (*Macaca fascicularis*) were identified as relevant. No proliferative lesions were observed in breast biopsies after administration of a soy extract providing 35.7 mg isoflavones/kg bw/day for approximately 1 month (Wood et al., 2006). In three of the five monkey studies, the source of isoflavones administered to the animals was a SPI providing doses of isoflavones ranging from 8.6 to 9.9 mg/kg bw/day for approximately 3 years and 6 months, respectively (Foth and Cline, 1998; Wood et al., 2004; Scott et al., 2008). According to the authors, the doses used in these studies were equivalent to human doses of 129–148 mg/day for women. In these three studies, positive control groups were also present, in which animals were treated with oestradiol at doses equivalent to 0.625–1 mg/day for women. Administration of the SPI did not result in histopathological changes, but some changes, such as an increase in the percentage of lobuloalveolar area and hyperplasia of the mammary epithelial area, were observed in animals in the positive control groups. On the contrary, a significant reduction in mammary thickness was observed in one of the studies (Wood et al., 2004). Equol (racemic) was administered at doses of 7.2 and 68 mg/kg bw/day for 4 months and approximately 30 days, respectively (Wood et al., 2006, 2008). In addition, in these two studies no significant changes were observed, with all treated animals exhibiting diffuse glandular atrophy.

Histo(patho)logy of the mammary gland was also measured in six studies in rats (Sprague–Dawley) identified as relevant for the assessment.

In one of the studies, isoflavones from a soy extract were administered at a dose of 15 mg/kg bw/day for 42 days. The animals showed a partial regression of glandular atrophy with a limited increase in the number of small lobuloalveolar units (Gallo et al., 2006).

In another study, a red clover extract was used as a source of isoflavones (Burdette et al., 2002) providing doses of isoflavones (expressed as aglycones) of 37.5, 75 and 112.5 mg/kg bw/day. Examination of duct branching and alveolar section did not reveal any treatment effect; on the contrary, the animals in the positive control groups showed extensive ductal branching and defined buds.

In the other four studies either genistein or equol (racemic) alone was administered to the animals.

For genistein the doses tested ranged 5.4–221 mg/kg bw/day for 90 days (Wuttke et al., 2006; Rimoldi et al., 2007). In another study of shorter duration (21 days), a dose of 90 mg/kg bw/day was administered to the animals alone or in combination with oestradiol (Santell et al., 1997). In the same study, lower doses of genistein were also tested (18 and 42 mg/kg bw/day) but only in combination with oestradiol. In both 90-day studies, animals treated with genistein were only slightly different from the OVX control and secretion was present only in the animals treated with oestradiol. The results from the 21-day study indicated that genistein 90 mg/kg bw/day prevented mammary gland regression, primarily in the lobuloalveolar structure (not in the ductal structure). Remarkably, mammary development in the oestradiol-treated rats was not different from that seen controls, whereas lobulo-alveolar development of the mammary gland was significantly greater in the group treated with genistein and oestradiol than in the controls. The authors stated that the reason dietary oestradiol exerts oestrogenic effects in the uterus but not in the mammary gland is unclear.

In one study rats were administered equol (racemic) at doses of 4.5 or 36 mg/kg bw/day for 90 days (Rachoń et al., 2008). Equol (racemic), at the highest dose tested, induced lumen formation but no secretion and increased the number of terminal ducts and type II lobules. No difference was observed in the low-dose group.

Table 12: Animal studies included reporting effects on mammary gland: histo(patho)logy

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Soy isoflavones/soy extract								
1714 Wood et al., 2006	Monkeys, (*Macaca fascicularis*)	OVX control; ISO: 35.7 EQ: 68	Conversion reported by the authors. Actual isoflavone dose administered 537 mg/1 800 kcal	10	28–33	Breast biopsies	All exhibiting diffuse glandular atrophy. No proliferative lesions.	1
15147 Gallo et al., 2006	Rats (Sprague–Dawley)	Sham control; OVX control; SSE: 15; E2	The authors reported dose administered was 100 mg/kg bw/day of standardised soy extract (SSE) Soyselect® by gavage. Dose calculated on the basis of the percentage reported 0n the company website (13–17 % isoflavones). Animals in the E2 group were administered 17β-oestradiol, 0.5 mg/kg bw/day, by gavage	6–10	42	Lobuloalveolar and ductal structures	Animals treated with soy extract showed partial regression of glandular atrophy with a limited increase in the number of small lobuloalveolar units. Histological appearance different between the sham and OVX control	1
Soy protein isolate								
4730 Scott et al., 2008	Monkeys (*Macaca fascicularis*)[c]	OVX control; SPI: 8.6; E2	Authors stated that the isoflavone dose administered corresponded to a human equivalent dose of 129 mg/day, expressed as aglycone units. The Panel assumed that the same conversion factor applied by the same research group in the publication Wood et al. (2004) applies also to this case. Animals in the E2 group were administered 17β-oestradiol 0.042 mg/kg bw/day	52–55	1080	Lobular area (%)	No statistically significant changes compared with OVX control. Significantly higher only in positive controls	1
16271 Wood et al., 2004	Monkeys (*Macaca fascicularis*)[c]	OVX control; SPI: 8.6; E2	Authors stated the isoflavone dose administered corresponded to a human-equivalent dose of 129 mg/day, expressed as aglycone units. Authors stated the dose administered in the E2 group (0.042 mg/kg bw/day) corresponded to a human equivalent dose of 0.625 mg/day of CEE	57–62	1080	Lobuloalveolar area (%) Mammary pad thickness Histological changes	Epithelial area not altered after 3 years of treatment. Mammary thickness was significantly lower in the SPI group than in controls. Mild to moderate lobular enlargement was observed in 2/57, 0/60 and 31/63 of the OVX control, SPI and E2 groups. Small focal papillary ductal hyperplasias were observed in 3/57, 0/60 and 1/63 of the OVX control, SPI and E2 groups	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
15120 Foth and Cline, 1998	Monkeys (*Macaca fascicularis*)	OVX control; SPI: 9.9; E2; E2 + SPI	Authors stated that the dose administered to the animals corresponded to a human-equivalent dose of 148 mg/day per woman, on an energy basis. Animals in the E2 groups were administered oestradiol in the diet. Authors stated that the dose administered to the animals corresponded to a human-equivalent dose of 1 mg/day per woman, on an energy basis. The Panel assumed that the same conversion factor applied by the same research group in the publication Wood et al. (2004) applies also to this case	15	180	Mammary lobuloalveolar hyperplasia Mammary gland thickness	Hyperplasia more present in E2-treated animals than in OVX control and SPI animals. Mammary gland thickness did not change in the SPI vs. OVX control. In the E2 and E2 + SPI groups mammary epithelial area was significantly increased compared with control	1
Red clover extract								
11684 Burdette et al., 2002	Rats (Sprague–Dawley)	OVX control; RC-250: 37.5 (aglycones); RC-500: 75; (aglycones); RC-750: 112.5 (aglycones); E2; RC-750 + E2	Authors report that animals were administered 250, 500 and 750 mg/kg/day of red clover extract standardised to a minimum 15 % isoflavone content by weight of four isoflavone—genistein, 0.850%; daidzein, 0.349 %; biochanin A, 6.57 %; and—hydrolysed aglycones. Dose of isoflavones calculated on the basis of the percentage (15 %) reported by the authors. According to the authors, the choice of the doses of red clover was based on a clinical dose of 40 mg isoflavones/day. Animals in the E2 groups were administered 17β-oestradiol 50 µg/kg bw/day	5–6	21	Examination of duct branching and alveolar structure	Administration of the red clover extract did not stimulate the mammary glands. Rats in the high-RC + E2 were comparable to those in E2 group, showing extensive ductal branching and defined buds	2
Genistein								
4863 Rimoldi et al., 2007	Rats (Sprague–Dawley)	OVX control; low-GEN: 5.4; high-GEN: 54; low-E2; high-E2	In the publication it is reported that the doses of genistein were estimated by the authors on the basis of the daily food intake. Animals in the positive E2 groups were administered oestradiolbenzoate. Daily doses were estimated on the basis of daily food intake at 0.17 mg/kg bw/day (low E2) and 0.7 mg/kg bw/day (high E2)	11–12	90	Degree of luminal formation; presence/ absence of secretion	Both low-GEN and high-GEN were only slightly different from OVX control. No secretion was induced and incipient luminal formation was present in half of the animals in the OVX control and in both GEN groups.	1

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
16299 Wuttke et al., 2006	Rats (Sprague–Dawley)	OVX control; GEN: 221; E2[d]	In the publication it is reported that the dose administered to the animals was 53 mg/day. The authors were contacted and clarified that animal weight was 230–250 g. Dose conversion calculated by the Panel on the basis of the information provided by the authors. Animals in the E2 group were administered 0.19 mg/day of oestrogen (free base)	10–12	90	Degree of luminal formation; presence/ absence of secretion	Slight increase in luminal and alveolar structures compared with OVX control Presence of secretion only in E2 animals	1
15958 Santell et al., 1997. Expt.2	Rats (Sprague–Dawley)	OVX control GEN 750: 90; E2; GEN 150+ E2: 18 + E2; GEN 375+ E2: 45 + E2; GEN 750+ E2: 90 + E2; Baseline animals	The authors stated the animals were administered genistein 750 µg/g diet (alone) or genistein 150 µg/g, 375 µg/g and 750 µg/g in combination with oestradiol. Animals in the E2 groups were administered oestradiol 1 µg/g diet corresponding to 0.12 mg/kg bw/day. Dose conversion calculated using a default value of 0.12 for sub-acute studies in rats (EFSA Scientific Committee, 2012)	6 (10 baseline animals)	21	Lobuloalveolar and ductal structure (extent of side branching)	Animals in the GEN 750 group were different from OVX control and similar to the baseline group, primarily in the lobuloalveolar score. Average ductal development did not differ in the GEN or E2 groups compared with OVX control. Lobuloalveolar development was significantly higher in the *GEN 750* and GEN 750 +E2 groups than in the control. Co-administration of genistein and E2 did not result in lower mammary scores than administration of E2 alone	1
Equol (racemic)								
16269 Wood et al., 2008	Monkeys, (*Macaca fascicularis*)	OVX control; EQ: 7.2; E2	Authors stated that the dose administered corresponded to a human equivalent dose of 105 mg/day, of a 96.0 % pure racemic mixture of *S*- and *R*-equol enantiomers. Dose conversion reported in the publication. The dose administered to E2 group corresponded to a human equivalent dose of 1 mg/day, micronized E2. Dose conversion to 0.066 mg/kg bw/day reported in the publication.	20–22	240	Mammary gland lesions Epithelial area	OVX control and equol-treated animals had diffusely atrophic mammary glands with scattered lobular units. Overall prevalence of mammary lesions on routine histology was 41 % (26 cases/63 animals). Cases were distributed as follows: 8 OVX control, 12 E2, 6 equol	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
1714 Wood et al., 2006	Monkeys, (Macaca fascicularis)	OVX control; ISO: 35.7; EQ: 68	Actual dose administered 1 020 mg/1 800 kcal racemic equol. Dose conversion reported in the publication	10	28–33	Mammary gland biopsies	All exhibiting diffuse glandular atrophy. No proliferative lesions	1
643 Rachoń et al., 2008	Rats (Sprague–Dawley)	OVX control; EQ-50: 4.5; EQ-400: 36; low E2; high E2	Actual doses administered 50 and 400 mg/kg chow of equol pure racemic mixture of equol (EQ-50 and EQ-400, respectively). On the basis of food intake, average consumption was 6.54 mg/day. In the E2 groups, actual doses administered were 4.3 mg/kg and 17.3 mg of oestradiol-3 benzoate per kg chow for low E2 and high E2, respectively. On the basis of food intake, average consumption was 0.07 mg/day (low E2: 0.4 mg/kg bw/day) and 0.20 mg/day (high E2: 1.6 mg/kg bw/day). Doses calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012)	12	90	Terminal ducts and type I and II lobules. Presence/absence of secretion	Animal in the low-equol group were similar to OVX control with scarce clusters of densely packed terminal structures, many of which did not show clear luminal formation. In the high-equol group, lumen formation was seen but no secretion. Animals in the high-equol group had significantly more terminal ducts and type II lobules. Animals in the low-equol group did not differ from OVX control in terms of the number of terminal ducts and type II lobules	1

CEE: conjugated equine (o)estroegen
Text in bold indicates different animal species in which the studies were conducted. Text in grey indicates differents isoflavones being tested in the same study and refers to other rows in the table.
(a): Refers to the Ref. ID number in Distiller.
(b): See protocol in Annex A for appraisal of risk of bias.
(c): Half of the monkeys had been previously treated with oral contraceptives over a 26-month period before ovariectomy.
(d): Additional group treated with *Cimicifuga racemosa* extract 133 mg/day. Data not presented in this table.

Discussion on results from animal studies reporting effects on the mammary gland

Overall, the Panel noted that in OVX monkeys there was no enhancing effect of isoflavones on cell proliferation in the mammary gland at doses up to 35.7 mg/kg bw/day for a period of approximately 30 days or at lower doses over periods ranging from 6 months to 3 years. Similarly, racemic equol did not produce a significant effect in the monkeys when tested at doses up to 68 mg/kg bw/day for 1 month or at the lower dose of 7.2 mg/kg bw/day for 6 months.

In the rat, treatment with equol (racemic) at 36 mg/kg bw/day induced lumen formation, but no secretion, and increased the number of terminal ducts and type II lobules in a 90-day study. At the same dose, equol (racemic) increased the percentage of PCNA-positive cells in terminal ducts and type II lobules. No effects were observed at the lower dose tested of 4.5 mg/kg bw/day (Rachoń et al., 2008).

A stimulating effect on cell proliferation in the mammary gland was observed in OVX rats treated with genistein at the doses of 5.4 and 54 mg/kg bw/day in a 90-day study (Rimoldi et al., 2007) and in OVX rats treated with genistein at a dose of 221 mg/kg bw/day for 90 days (Wuttke et al., 2006).

During the process of screening the literature for articles for inclusion in the systematic review for the current risk assessment, the Panel became aware of a carcinogenicity study on genistein performed by the US NTP (NTP, 2008) in non-OVX rats. The animals were treated with genistein at doses ranging from 0.3 to 44 mg/kg bw/day. The authors concluded that there was some evidence of carcinogenic activity of genistein in female Sprague–Dawley rats based on increased incidence of mammary gland adenoma or adenocarcinoma (combined). The effect was observed in females treated from conception to 2-years of age by comparison to females treated from conception to 20 weeks of age or from conception to weaning. This study did not meet the inclusion criteria specified in the systematic review protocol because the animals were not ovariectomised.

The Panel noted that, although sub-chronic exposure to genistein in OVX rats resulted in increased cell proliferation in mammary gland, the long-term genistein exposure of intact rats led to only equivocal evidence of carcinogenic activity (NTP, 2008).

3.2.3. Weight of evidence for effects on mammary gland

In this section an overview is given on the studies, their reliability (assessed according to the protocol), their relevance for the risk assessment in humans and the direction of the outcome in order to provide a formalised basis for a weight of evidence assessment.

All the studies included in the systematic reviews have been appraised for validity and risk of bias using pre-defined criteria agreed before the start of the assessment. These are described in the detail in the protocol in Annex A to this scientific opinion. The Tier of Reliability of the studies is based on the described appraisal. Tier 1 denotes studies with the lowest risk of bias whereas studies with some deficiencies are ranked to a lower tier.

Relevance for the risk assessment in humans was high (+++) if the studies were performed in humans and moderately high (++) if performed a relevant animal model (according to the protocol), e.g. in monkeys, and less high (+) if performed in rodents. What constitutes an endpoint relevant for the question was already defined in the protocol. For each outcome the symbol ⇔ means not statistically significant different from control, ⇧ means statistically significant increase in effect compared to control and ⇩ means statistically significant decrease of effect.

For the endpoint mammary gland, all human studies had a reliability score of 1 and all did not indicate a difference from control, irrespective of the tested isoflavone.

Among the animal studies with the endpoint cell proliferation, in one study in monkeys treated with SPI 8.6 mg/kg/day there was a reduction of Ki-67 mRNA expression. In two studies from the same group and in which rats were administered genistein in doses of 5.4 and 54 mg/kg bw/day (Rimoldi et al., 2007) or 221 mg/kg bw/day (Wuttke et al., 2006), an increase in cell proliferation was seen.

An increase in the percentage of PCNA-positive cells was noted for racemic equol (Rachoń et al., 2008) in rats.

No effects were seen in the animal studies for the endpoint histopathology irrespective of species and the isoflavone studied.

In the animal studies reporting results from histopathological analysis, an increase in the lobulo-alveolar score was reported in rats treated with genistein at 90 mg/kg bw/day. Such effect was not observed for ductal development (Santell et al., 1997). Some effects were also reported in rats treated with equol (racemic) at the dose of 36 mg/kg bw/day, but not at the lower dose of 4.5 mg/kg bw/day (Rachoń et al., 2008).

In a weight of evidence approach it can be concluded that adverse effects have not been seen neither in humans nor in animals. In animals at doses of genistein (ranging from 5.4 to 221 mg/kg bw/day) cellular proliferation but no histopathological changes were observed.

It should, however, be noted that this evaluation is restricted to the tested doses of the tested isoflavones.

The outcome of this analysis is summarised in Tables 13 and 14.

Table 13: Carcinogenicity/proliferation in the mammary gland: summary of human data

Endpoint/intervention	Reference	Reliability	Relevance	outcome
Breast cancer				
Use of food supplements containing isoflavones	Boucher et al., 2013	1	+++	⇔
	Brasky et al., 2010	1	+++	⇔
	Obi et al., 2009	1	+++	⇔
	Rebbeck et al., 2007	2	+++	⇔
Mammographic density				
Soy isoflavones/soy extract	Colacurci et al., 2013	1	+++	⇔
	Delmanto et al., 2013	1	+++	⇔
Soy protein	Verheus et al., 2008	1	+++	⇔
Daidzein-rich isoflavones	Maskarinec et al., 2009	1	+++	⇔
Genistein	Marini et al., 2008	1	+++	⇔
	Morabito et al., 2002	2	+++	⇔
Red clover extract (RCE)	Atkinson et al., 2004	1	+++	⇔
	Powles et al., 2008	2	+++	⇔
Proliferation marker Ki-67 and atypical cytology				
Soy isoflavones/soy extract	Khan et al., 2012	1	+++	⇔
	Cheng et al., 2007	2	+++	⇔

Table 14: Carcinogenicity/proliferation in the mammary gland: summary of animal data

Endpoint/ intervention	Reference	Animal species	Reliability	Relevance	Outcome
Cell proliferation/proliferation marker					
Soy isoflavones/ soy extract	Wood et al., 2006	Monkeys (*Macaca fascicularis*)	1	++	⇔
	Gallo et al., 2006	Rats (Sprague–Dawley)	1	+	⇔
Soy protein isolates	Wood et al., 2004	Monkeys (*Macaca fascicularis*)[(a)]	1	++	⇔
	Scott et al., 2008	Monkeys (*Macaca fascicularis*)[(a)]	1	++	⇔ For all endpoints ⇩ For Ki-67 mRNA
	Foth and Cline, 1998	Monkeys (*Macaca fascicularis*)	1	++	⇔
Genistein	Rimoldi et al., 2007	Rats (Sprague–Dawley)	1	+	⇧ 5.4 and 54 mg/kg bw/day
	Wuttke et al., 2006	Rats (Sprague–Dawley)	1	+	⇧ 221 mg/kg bw/day

Endpoint/ intervention	Reference	Animal species	Reliability	Relevance	Outcome
Equol (racemic)	Wood et al., 2008	Monkeys (*Macaca fascicularis*)	1	++	⇔
	Wood et al., 2006	Monkeys (*Macaca fascicularis*)	1	++	⇔
	Rachoń et al., 2008	Rats (Sprague–Dawley)	1	+	⇔ 4.5 mg/kg bw/day ⇧ 36 mg/kg bw/day
	Legette et al., 2009	Rats (Sprague–Dawley)	1	++	⇔
Histopathology					
Soy isoflavones/ soy extract	Wood et al., 2006	Monkeys (*Macaca fascicularis*)	1	++	⇔
	Gallo et al., 2006	Rats (Sprague–Dawley)	1	+	⇔
Soy protein isolates	Wood et al., 2004	Monkeys (*Macaca fascicularis*[(a)]	1	++	⇔
	Scott et al., 2008	Monkeys (*Macaca fascicularis*)[(a)]	1	++	⇔
	Foth and Cline, 1998	Monkeys (*Macaca fascicularis*)	1	++	⇔
Red clover extract	Burdette et al., 2002	Rats (Sprague–Dawley)	2	+	⇔
Genistein	Rimoldi et al., 2007	Rats (Sprague–Dawley)	1	+	⇔
	16299 Wuttke et al., 2006	Rats (Sprague–Dawley)	1	+	⇔
	15958 Santell et al., 1997. Expt.2	Rats (Sprague–Dawley)	1	+	⇧ Lobuloalveolar score ⇔ Duct score
Equol (racemic)	16269 Wood et al., 2008	Monkeys (*Macaca fascicularis*)	1	++	⇔
	1714 Wood et al., 2006	Monkeys (*Macaca fascicularis*)	1	++	⇔
	643 Rachoń et al., 2008	Rats (Sprague–Dawley)	1	+ +	⇔ 4.5 mg/kg bw/day ⇧ 36 mg/kg bw/day

(a): Half of the monkeys had been previously treated with oral contraceptives over a 26-month period before ovariectomy.

3.3. Effects on uterus

3.3.1. Results from human studies

At the end of the systematic review process the intervention studies shown in Table 15 and Table 16 were identified as relevant for the risk assessment of isoflavones from food supplements on the uterus of peri- and post-menopausal women.

No observational study was found that investigated the association between intake of isoflavones from food supplements and risk of uterine cancer in the target population.

Additionally, information on reported adverse events occurring in the clinical trials included in this review has been collected and assessed and is reported in Table C2 in Appendix C.

Endpoint: endometrial thickness

The effect of intake of isoflavones from food supplements and changes in the endometrial thickness of peri- and post-menopausal women have been investigated in several controlled trials.

After menopause, increased endometrial thickness may indicate proliferative endometrium (Wolfman et al., 2010). Hence, given the reproducibility and non-invasiveness of its assessment, endometrial thickness allows for serial surveillance in intervention and other epidemiological studies (Warming et al., 2002). The method has been used to evaluate the safety of HRT in post-menopausal women.

According to the review by Wolfman et al. (2010), values below 5 to 8 mm thickness have been considered as normal and in a study in which transvaginal ultrasound examination of the uterus was compared with biopsies of the endometrium the sensitivity of transvaginal ultrasound examination was given as 90 % if a cut-off of 5 mm was used (Langer et al., 1997).

A total of 25 studies, encompassing 1 484 participants in the active treatment groups and fulfilling the pre-defined criteria for inclusion in the review, were considered for the assessment and are presented in Table 15.

None of the studies considered reported significant changes in endometrial thickness associated with the use of food supplements containing soy isoflavones/soy extract (60–120 mg/day isoflavones, 3–36 months), soy protein (90–120 mg/day isoflavones, 4–36 months), daidzein-rich isoflavones (80–120 mg/day isoflavones, 24 months), glycitein-rich isoflavones (114 mg/day isoflavones, 3 months), genistein (30–54 mg/day, 3–36 months) and red clover extract (40–120 mg/day isoflavones, 12–36 months).

Table 15: Controlled studies measuring changes in endometrial thickness in peri- and post-menopausal women

Ref. ID[a] Author, year	Design	Duration (months)	Dose (mg/day)[b]	Description of intervention	Active group (N)/control group (N)[c]	Age[d]	Time since menopause (years) age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
Soy isoflavones (ISO)/soy extract										
14960 Chilibeck et al., 2013	RCT, DB	24	105 aglycones (165 total)	The authors reported that the intervention was an isoflavone supplement (Novasoy®)	ISO: 90/76; exercise (Ex): 86/77; Ex + ISO: 87/72; placebo: 88/73	ISO: 56.7 (6.5); Ex: 55.3 (6.3); Ex + ISO: 55.8 (5.0); Placebo: 56.4 (7.1)	At least 1 year[f]	No	No statistically significant difference between groups. In all the groups there was a decline in the thickness	1
3071 Colacurci et al., 2013	RCT, DB	12	60	Authors described the intervention as soy isoflavones, *Lactobacillus sporogenes* 1 billion spores, calcium 240 mg, vitamin D_3 5 µg and glucosamine 250 mg. The isoflavones dose is expressed as mg genistin and daidzin/day. Placebo was calcium and vitamin D_3 alone	ISO: 65/62; control: 65/62	ISO: 55.3 ± 7.6; placebo: 55.7 ± 7.7[g]	ISO: 3.2 ± 2.8; placebo: 2.8 ± 2.6	Yes	No statistically significant difference between isoflavones and placebo group	1
10231 Nahas et al., 2007	RCT, DB	9	100	The authors reported the intervention was a standardised soy extract (250 mg) corresponding to 100 mg isoflavones/day administered as capsules twice a day. Each capsule contained 125 mg soy extract plus 50 mg isoflavones each; the ratio between glycoside and aglycone form is 0.61 (38 % and 62 % in each capsule)	ISO: 40/38; placebo: 40/38	ISO: 55.1 ± 6.0; placebo 56.2 ± 7.7	ISO: 6.6 ± 4.8, 48.4 ± 3.7; placebo: 7.1 ± 4.2, 47.7 ± 3.5	No	After 9 months, no statistically significant changes in either the isoflavones or placebo group	1

Ref. ID[a] Author, year	Design	Duration (months)	Dose (mg/day)[b]	Description of intervention	Active group (N)/control group (N)[c]	Age[d]	Time since menopause (years) age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
14945 Alekel et al., 2015	RCT, DB	36	80; 120	Authors report that formulated versus mean (SD) actual doses in tablets were control, 0 vs. 0.3 mg ± 0.4 mg; 80 mg, 89.5 ± 5.0 mg vs. 84.3 ± 4.5 mg; 120 mg, 124.0 ± 7,7 vs. 122.5 ± 3.4 mg. Ratio of genistein to daidzin to glycitein (GEN:DAI:GLY) was 1.3:1:0.3	Low ISO: 87/77; high ISO: 85/73; placebo: 83/74	Low ISO: 54.3 (45.8–61.4); high ISO: 54.7 (46.5–62.0); placebo: 54.2 (45.8–65.0)	Low ISO: 3.0 (0.9–10.0), 50 (44.0–60.0); high ISO: 2.8 (1.0–8.0), 51.0 (41.0–59.0) placebo: 2.7 (0.8–7.9), 51.0 (42.0–57.0)	No	Median values declined from baseline through the duration of the study. The data from the two investigation sites were analysed separately owing to the differences in the ultrasound equipment. No statistically significant differences between treatments across time	2
1640 Kaari et al., 2006	RCT, DB	6	120	The authors reported isoflavone intervention contained daidzein congeners 20 %, genistein congeners 75 % and glycitein congeners 5%. The ratio between glycoside and aglycone forms was 0.61. Women in the E2 group were given 0.625 CEE	ISO: 40/33; E2: 40/35	ISO: 53.9 ± 0.9; E2: 53.7 ± 0.9	ISO: 5.3 ± 0.5, 48.6 ± 0.8; E2: 6.7 ± 0.5, 46.8 ± 0.9	Not specified	After 6 months, no statistically significant changes in the isoflavone group compared with baseline. In the E2 group there was a statistically significant increase after 3 and 6 months, compared with baseline and with isoflavone treatment	2
2414 Han et al., 2002	RCT, DB	4	100	Authors report that the intervention was a capsule containing soy protein 50.3 mg (60%) and isoflavone 33.3 mg (40%). The specific amount of the individual isoflavones, in aglycone form, was genistein, 23.3 mg; daidzein, 6.2 mg; glycitein, 3.8 mg	ISO: 40/40; Placebo: 40/40	ISO: 48 ± 1.1, Placebo: 49 ± 1.3	ISO: 1.8 ± 0.2 placebo: 2 ± 0.3	No	The authors reported no statistically significant difference between isoflavones and the placebo group	2
3158 Cheng et al., 2007	RCT, DB	3	60		ISO: 26/26; placebo: 25/24	ISO: 58.4 ± 5.0; placebo 56.9 ± 4.2	ISO: 8.4 ± 5.3; placebo: 7.0 ± 3.8	Not specified	No statistically significant difference at baseline and after treatment between the two groups.	2

Ref. ID[a] Author, year	Design	Duration (months)	Dose (mg/day)[b]	Description of intervention	Active group (N)/control group (N)[c]	Age[d]	Time since menopause (years) age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
16165 Upmalis et al., 2000	RCT, DB	3	50	The authors reported isoflavone intervention contained daidzein congeners 20 %, genistein congeners 75 % and glycitein congeners 5 % The ratio between glycoside and aglycone forms was 0.61	ISO: 89/59; placebo: 86/63	ISO: 54.8; placebo: 54.4	Not reported	Not specified	After 3 months, NSS difference in either the isoflavones or the placebo group compared with baseline or between the two groups.	2
Soy protein										
16436 Quaas et al., 2013	RCT, DB	36	91	Dose expressed as aglycones equivalent. The authors reported intervention to contain 154 mg of total isoflavones (conjugates plus aglycones). Genistein: 52 mg of aglycone equivalents (88 mg in total); daidzein: 36 mg aglycone (61 mg total): glycitein 3 mg aglycone (5 mg total)	ISO: 121; Placebo: 103	ISO: 60.9 (7.0); placebo: 60.1 (6.6)	ISO: 11.0 (8.0); placebo: 9.8 (6.8)	No	No statistically significant difference in the endometrial thickness between treatment groups either at baseline or at the end of the study period	1
1103 Carmignani et al., 2010	RCT, DB	4	90	Authors report the intervention as two portions of a food powder (Previna®) each containing 12 g of soy protein and a total of 45 mg of isoflavones (26.5 mg aglycones)	ISO: 20; HRT:20; placebo: 20	ISO: 52.9 ± 3.5; HRT: 53.3 ± 4.5; placebo: 50.9 ± 3.4	ISO: 2.5 (1.5–4.0)[h]; HRT: 5.6 (1.5–10), placebo: 2.5 (1.0–5.5)	No	No statistically significant difference between the three groups at baseline and after treatment. A slight, albeit not significant, increase was observed only in the HRT group	1
11323 Murray et al., 2003	RCT, DB	6	120 (aglycones)		Low E2: 7; high E2: 7; low E2 + ISO: 8; high-E2 + ISO: 8	Low E2: 53.0 ± 3.4; high-E2: 53.4 ± 4.1; low E2 + ISO: 56.3 ± 7.4; high E2 + ISO: 56.6 ± 9,1	Not reported	Yes	No statistically significant difference between groups, albeit the increase in thickness observed in the E2 + ISO groups was less prominent	3

Ref. ID[a] Author, year	Design	Duration (months)	Dose (mg/day)[b]	Description of intervention	Active group (N)/control group (N)[c]	Age[d]	Time since menopause (years) age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
Daidzein-rich isoflavones										
3110 Penotti et al., 2003	RCT, DB	6	72	The authors reported that the active substance in each tablet contained a total of 36 mg of soy-derived isoflavones (5.5 mg of genistein/genistin, 18 mg of daidzeine/daidzine, and 12.5 mg of glyciteine/ glycitine) and 48 mg of soy saponin.	ISO: 28/22; placebo: 34/27	ISO: 52.5 (2.5), range 49–58; placebo: 52.5 (2.3), range 49–57	ISO: 2.4 ± 1.2 (0.5–4.3); placebo: 2.35 ± 1.5 (0.5–5.8)	Yes	No stastistically significant changes between baseline and end of treatment for either the treated and or the placebo group, nor significant changes between groups.	1
4366 Steinberg et al., 2011	RCT, DB	24	80; 120 (aglycone equivalents)	The authors reported that the tablets used in this 2-year study had an isoflavone content ranging from 34.10 mg to 40.51 mg aglycone equivalent of total isoflavones (daidzein, 17.87–22.01; genistein, 4.96–5.00; glycitein, 11.22–13.54; ratio GEN:DAI:GLY= 0.4:1:0.2), with the majority (> 95 %) in the form of glycosides	Low ISO: 135; high ISO: 134; placebo: 134	Low ISO: 54.9 ± 4.0; high ISO: 54.5 ± 4.1; placebo: 55.0 ± 3.7	Low ISO: 48.5 ± 5.5; high ISO: 47.6 ± 6.3; placebo: 48.2 ± 5.1	No	This measurement was conducted in a sub-cohort of women (n = 116) from one of the two investigation sites. The values declined over time, with no statistically significant difference between groups at any time point (baseline, 2 years)	2
Glycitein-rich isoflavones										
1639 Nikander et al., 2005	C-O, RCT, DB	3	114	Authors report that the supplement used (Bonette®) contained glycitein (58 %), daidzein (36 %) and genistein (6 %)	ISO: 32/28; placebo: 30/28	54 ± 6[i]	5.3 ± 5.5 (0.6–27.0)	Yes	No statistically significant changes between baseline and end of treatment for either the treated and or the placebo group	1
Genistein										
3138 Marini et al., 2008	RCT, DB	24 + 12[j]	54		GEN: 71; placebo: 67 [k]	GEN: 53.8 ± 2.9; placebo: 53.5 ± 2.0	GEN: 3.6 ± 3.0; placebo: 3.6 ± 2.2 [l]	Sub-cohort of parent trial.	No statistically significant difference between groups at any time point (baseline, 2 years, 3	1

Ref. ID[a] Author, year	Design	Duration (months)	Dose (mg/day)[b]	Description of intervention	Active group (N)/control group (N)[c]	Age[d]	Time since menopause (years) age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
									years). A significant reduction from baseline values was observed for both groups at the end of the study.	
4922 Marini et al., 2007	RCT, DB	24	54		GEN: 198/178/150; placebo: 191/172/154	GEN: 54.7 ± 3.5; placebo: 54.2 ± 2.7	GEN: 5.6 (3.8) placebo: 4.9 (3.2)	No	No statistically significant difference between groups at any time point (baseline, 1 year, 2 years)	1
15431 Lappe et al., 2013	RCT, DB	6	30		GEN: 35/30; calcium: 35/28	GEN: 54.8 ± 2.5; placebo: 54.7 ± 2.3	GEN: 2.2 ± 0.8; placebo: 2.1 ± 0.8	No	No statistically significant difference between treatment and control either at baseline or at the end of the study period. In the genistein group, at the end of the study, endometrial thickness was significantly decreased compared with baseline, whereas in the placebo group no change was observed	1
2282 Morabito et al., 2002	RCT, DB	12	54		GEN: 30; HRT:30; placebo: 30	GEN: 52 ± 3; HRT: 52 ± 5; placebo: 51 ± 4	GEN: 7 ± 6 HRT: 7 ± 3 Placebo: 6 ± 5	No	No statistically significant changes in the endometrial thickness measured at 1 year. Values > 5 mm were reported in 3/30 women in the genistein and placebo groups and in 2/30 women in the HRT group	2
256 Irace et al., 2013	RCT, DB	6	54		GEN: 20; placebo: 15	GEN: 60.1 ± 5.9; placebo: 57.5 ± 8.6	Not reported	No	No statistically significant difference compared with control	2
15095 Evans et al., 2011	RCT, DB	3	30		GEN:41/32; placebo: 42/36	GEN: 53.39 ± 5.05; placebo: 53.50 ± 4.44	See footnote (m)	No	No statistically significant difference compared with control after 12 weeks of treatment	2

Ref. ID[a] Author, year	Design	Duration (months)	Dose (mg/day)[b]	Description of intervention	Active group (N)/control group (N)[c]	Age[d]	Time since menopause (years) age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
15955 Sammartino et al., 2003	RCT, open	12	36		GEN: 35; calcium: 35	GEN: 51.9 ± 1.85; calcium: 51.6 ± 1.75	GEN: 1.5 ± 0.3; calcium: 1.4 ± 0.3[n]	No	No statistically significant difference between treatment and control at any time point (baseline, 6 months, 12 months). After 12 months, 1 woman in the GEN group and 2 women in the calcium supplement group were above the 5 mm cut-off values and therefore underwent biopsy. In all the three cases the histological examination report was of proliferative endometrium	3
Red clover extract (RCE)										
19610 Geller et al., 2009	RCT, DB	12	120 [o]	Ethanolic extract of aerial parts of red clover providing 57.5 mg biochanin A, 56.6 mg formononetin, 1.6 mg genistein and 0.9 mg daidzein. Women in the E2 arm were given conjugated equine oestrogen and medroxyprogesterone acetate	RCE: 22; placebo: 22; E2: 23	RCE: 52.4 (4.6); placebo: 52.0 (4.2); E2: 53.3 (4.0)	RCE: 4.1 (2.8); placebo: 2.8 (2.9); E2: 3.6 (2.9)	No	No stastistically significant difference compared with placebo	2
16435 Powles et al., 2008	RCT, DB	36	40		RCE: 39/18/17/17; placebo: 38/18/18/17[p]	RCE: 45 (35–69); placebo: 45 (35–69)	–	No	No stastistically significant difference between treatment and control at any time point (1 year, 2 years, 3 years	2
15280 Imhof et al., 2006	C-O, RCT, DB	3	80		Group A: 50; Group B: 59	53.5 ± 7.1	Not reported [q]	Unclear	Significant reduction in endometrial thickness in the RCE group compared with placebo	2

Ref. ID[(a)] Author, year	Design	Duration (months)	Dose (mg/day)[(b)]	Description of intervention	Active group (N)/control group (N)[(c)]	Age[(d)]	Time since menopause (years) age at menopause (years)[(d)]	Primary endpoint?	Results	Tier of reliability [(e)]
16405 Hale et al., 2001	RCT, DB	3	50		RCE: 11; placebo: 13	RCE: 47.9 (3); placebo: 46.5 (1.8)	See table footnote [(r)]	Yes	Increase in endometrial thickness in the RCE group, but not significantly different from control	2

(a): Refers to the Ref. ID number in Distiller.
(b): Unless otherwise specified doses are expressed as mg isoflavones/day.
(c): Allocated to treatment/completed.
(d): Mean ± SD or SEM, as reported in the publication.
(e): See protocol in Annex A for appraisal of risk of bias.
(f): Time since menopause was used as a criterion for stratification of the randomisation (1–9 years; more than 9 years).
(g): In the publication it is reported that mean age in the placebo group was '5.7 ± 7.7' years. Clarification has been received from the corresponding author.
(h): Median (Q1, first quartile; Q3, third quartile).
(i): Participants were all post-menopausal women who had undergone treatment for breast cancer more than 6 months before the start of the trial. Study population included 10 women (18%) with a previous hysterectomy.
(j): For the last 12 months of this study, the participants were recruited among those completing the study described in Marini et al. (2007).
(k): Number of participants refers exclusively to those enrolled in the additional follow-up.
(l): Values calculated by the Panel. The authors report time since menopause to be 42.9 ± 35.9 months and 42.8 ± 26.3 months for genistein and placebo, respectively.
(m): The information is reported as follows: in the genistein group, 16/40 (40.0 %) women had been menopausal for 1–5 years, 13/40 (32.5 %) for 6–10 years, 11/40 (27.5 %) for more than 10 years and one subject was not able to recall number of years since natural menopause; in the placebo group 20/41 (48.8 %) had been in menopause for 1–5 years, 10/41 (24.4 %) for 6–10 years, 11/41 (26.8%) for more than 10 years and one subject was not able to recall number of years since natural menopause. The population included women with surgical menopause: these were 15/41 (36.6%) in the genistein group and 13/42 (31.0 %) in the placebo group.
(n): Values calculated by the Panel. The authors report time since menopause to be 17.6 ± 3.1 months and 17.0 ± 3.4 months for genistein and calcium supplements, respectively
(o): Data from an additional group treated with Black Cohosh extract are not presented in this table.
(p): The authors state that women included in this study were at higher risk of breast cancer because of their family history. Although pre-menopausal women were also included in this study, the assessment of endometrial thickness was limited to the post-menopausal women.
(q): The population included women with surgical menopause. Their distribution in the two groups was: 18 % in the RCE group and 13.6 % in the placebo group
(r): The subjects included in this study were described by the authors as healthy women of late reproductive age who had experienced at least two menstrual periods in the 6 moths before enrolment.

Endpoint histo(patho)logical changes

The effect of intake of isoflavones from food supplements and histo(patho)logical changes in the endometrium of peri- and post-menopausal women has been investigated in several controlled trials. Studies were included if they reported information on proliferation marker Ki-67 and atypical cytology.

Nine studies encompassing 677 participants in the active treatment groups and fulfilling the pre-defined criteria for inclusion in the review were considered for the assessment and are presented in Table 16.

Significant changes were reported in a controlled trial (Unfer et al., 2004) in which 376 subjects were randomised to receive either 150 mg isoflavones/day or placebo. No cases of endometrial hyperplasia or malignancy were detected in either group at 30 months. However, after 5 years of treatment, six cases of hyperplasia (five cases of simple hyperplasia and one case of complex hyperplasia) were detected in the isoflavones group compared with none in the placebo group. No cases of endometrial carcinoma occurred during the 5-year period of the study.

Endometrial hyperplasia was also reported in a small randomised controlled trial (Murray et al., 2003) in which 39 post-menopausal women were randomised to one of four treatment arms: 0.5 mg/day oestradiol (low E2), 1 mg/day oestradiol (high E2), low E2 and 120 mg/day isoflavones (expressed as aglycones) or high E2 and 120 mg/day isoflavones (expressed as aglycones). The study did not have an inactive control group. The number of subjects per group was limited (n = 7–8). The subjects underwent endometrial biopsy at baseline and at the end of the treatment period. After 6 months, endometrial hyperplasia occurred in women from all groups (low E2: 1/7; high E2: 4/7; low E2 + ISO: 4/8; high E2 + ISO: 4/8). All the cases were classified as simple hyperplasia with the exception of one complex hyperplasia without atypia in the low E2 + ISO group. The proliferation marker Ki-67 was significantly increased in the stroma of the high E2 + ISO and in the glands of the low E2 + ISO group and marginally in the high E2 + ISO group. Owing to the lack of an inactive control group it is not possible to interpret these findings. In addition, important methodological flaws were noted in the appraisal of the study (see Appendix A for the appraisal of the study).

None of the other studies considered reported significant histo(patho)logical changes in the uterus associated with the use of food supplements containing soy isoflavones/soy extract (60–120 mg/day, 3–6 months), soy protein (65–154 mg/day isoflavones, 3–36 months), daidzein-rich isoflavones (80–120 mg/day, 24 months), glycitein-rich isoflavones (114 mg/day isoflavones, 3 months) or red clover extract (50 mg/day isoflavones, 3 months).

Table 16: Controlled studies measuring histo(patho)logical changes in peri- and post-menopausal women

Ref. ID[a] Author, year	Design	Duration (months)	Dose (mg/day) [b]	Description of intervention	Active group (N)/control group (N)[c]	Age[d]	Time since menopause (years) and/or age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
Soy isoflavones/soy extract										
3106 Unfer et al., 2004	RCT, DB	60	150	Authors state the formulated percentage of isoflavones was genistein 40–45 %, daidzein 40–45 % and glycitein 10–20 %	ISO: 179/154; placebo: 197/165	ISO: 49 ± 4.3; placebo: 50 ± 3.9	ISO: 5.6 ± 4.3, 50.2 ± 6.5; Placebo: 5.8 ± 4.3, 49.8 ± 6.3	Yes	Endometrial biopsy: At 30 months, no cases of endometrial hyperplasia or malignancy were detected in either the isoflavones group or the placebo group. After 5 years of treatment, six cases (five cases of simple hyperplasia and one case of complex hyperplasia) were detected in the isoflavones group, compared with none in the placebo group. No cases of endometrial carcinoma occurred during the 5-year period of the study	1
1640 Kaari et al., 2006	RCT, DB	6	120	The authors reported isoflavone intervention contained daidzein congeners 20 %, genistein congeners 75 % and glycitein congeners 5 %. The ratio between glycoside and aglycone forms was 0.61. Women in the E2 group were given 0.625 CEE	ISO: 40/33; CEE: 40/35	ISO: 53.9 ± 0.9; CEE: 53.7 ± 0.9	ISO: 5.3 ± 0.5, 48.6 ± 0.8; CEE: 6.7 ± 0.5, 46.8 ± 0.9	Unclear	Endometrial biopsy: Significantly higher prevalence of endometrial proliferation in the CEE group (10 proliferative, 1 hyperplasia) than in the isoflavone group (1 proliferative, 0 hyperplasia) after 6 months of treatment Papanicolaou smears: No statistically significant changes in the percentage of cells in the parabasal, intermediate and superficial layers between baseline and end of treatment in the isoflavones group. A significant increase in the percentage of superficial cells was observed in the CEE group at 3 and 6 months, compared with baseline and isoflavones group	2

Ref. ID[a] Author, year	Design	Duration (months)	Dose (mg/day) [b]	Description of intervention	Active group (N)/control group (N)[c]	Age[d]	Time since menopause (years) and/or age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
3158 Cheng et al., 2007	RCT, DB	3	60		ISO: 26/26; placebo: 25/24	ISO: 58.4 ± 5.0; placebo 56.9 ± 4.2	ISO: 8.4 ± 5.3; placebo: 7.0 ± 3.8	Not specified	Ki-67 (endometrium): The proliferation marker Ki-67 was seen in 0 % to 3 % of samples. No significant change was induced by isoflavone treatment. The analysis was performed in only 38 samples	2
Soy protein										
16436 Quaas et al., 2013	RCT, DB	36	154		ISO: 121; placebo: 103	isoflavones: 60.9 ± 7.0; placebo: 60.1 ± 6.6	ISO: 11.0 (8.0); placebo: 9.8 (6.8)	No	Endometrial biopsy: Biopsy was performed only in those women in whom there was an indication for it: 9/121 (7.4 %) participants in the isoflavones group and 7/103 (6.8 %) in the control group. The indication for biopsy was post-menopausal bleeding in 3/9 cases in the isoflavones group and in 3/7 cases and in the control group. In all the other cases it was asymptomatic endometrial thickening. The results were benign in all nine cases in the isoflavones group, whereas one subject in the placebo group was diagnosed with stage IB endometrial cancer	1
3179 Duncan et al., 1999	C-O, RCT, DB	3	65 ± 11; 132 ± 22	Daily doses expressed as unconjugated phytoestrogen units. Placebo contained a minimal (10-fold less than low-ISO group) amount of isoflavones, estimated to be 7.1 ± 1.1 mg/day	Placebo: 18; low ISO: 17; high ISO: 18	56.9 ± 5.8	7.6 ± 4.7	Yes	Endometrial biopsy: Not significantly different between baseline and high-ISO diet (8/18 subjects were inactive at both time points; 4/18 were proliferative at both time points; two were proliferative at baseline and inactive on the high-ISO diet).	2

Ref. ID[a] Author, year	Design	Duration (months)	Dose (mg/day) [b]	Description of intervention	Active group (N)/control group (N)[c]	Age[d]	Time since menopause (years) and/or age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
11323 Murray et al., 2003	RCT, DB	6	120 (aglycones)		Low E2: 7; high E2: 7; lo E2 + ISO: 8; high E2 + ISO: 8	Low E2: 53.0 ± 3.4; high E2: 53.4 ± 4.1; low E2 + ISO: 56.3 ± 7.4; high E2 + ISO: 56.6 ± 9.1	Not reported	Yes	Endometrial biopsy: After 6 months endometrial hyperplasia occurred in all women from all groups (low E2: 1/7; high E2: 4/7; low-E2 + ISO: 4/8; high E2 + ISO: 4/8). All the cases were classified as simple hyperplasia with the exception of one complex hyperplasia without atypia in the low-E2 + ISO group. Ki-67 (endometrium): The proliferation marker Ki-67 was significantly increased in the low E2 + ISO.	3
Daidzein-rich isoflavones										
4366 Steinberg et al., 2011	RCT, DB	24	80; 120 (aglycone equivalents)	The authors reported that the tablets used had an isoflavone content ranging from 34.10 to 40.51 mg aglycone equivalent of total isoflavones (daidzein, 17.87–22.01; genistein, 4.96–5.00; glycitein, 11.22–13.54; ratio GEN:DAI:GLY= 0.4:1:0.2), with the majority (> 95 %) in the form of glycosides	Low ISO: 135; high ISO: 134; placebo: 134	Low ISO: 54.9 ± 4.0; high ISO: 54.5 ± 4.1; placebo: 55.0 ± 3.7	Low ISO: 48.5 ± 5.5; high ISO: 47.6 ± 6.3; placebo: 48.2 ± 5.1	Sub-cohort of patients	Uterine fibroids: This measurement was conducted in a sub-cohort of women (n = 116) from one of the two investigation sites. In total. 13 women had detectable uterine fibroids. No growth of any uterine fibroids and no statistical differences between the treatment groups over the 2-year duration of the study were observed	2

Ref. ID[(a)] Author, year	Design	Duration (months)	Dose (mg/day) [(b)]	Description of intervention	Active group (N)/control group (N)[(c)]	Age[(d)]	Time since menopause (years) and/or age at menopause (years)[(d)]	Primary endpoint?	Results	Tier of reliability [(e)]
Glycitein-rich isoflavones										
1639 Nikander et al., 2005	C-O, RCT, DB	3	114		ISO: 32/28; placebo: 30/28	54 ± 6	5.3 ± 5.5 (0.6–27.0)[(f)]	Yes	Ki-67 (epithelial and stromal cells of the endometrium): At screening Ki-67 was detected in only one endometrial sample. The isoflavones regimen was associated with the detection of Ki-67 in epithelial endometrial cells of three women, whereas Ki-67 remained undetectable in all cases during the placebo regimen Papanicolaou smears: No statistically significant changes in the percentage of cells in the parabasal, intermediate and superficial layers between baseline and end of treatment in the treated group. A trend towards an increase in the percentage of parabasal cells was observed during the placebo regimen (not statistically significant)	2
Red clover extract (RCE)										
16405 Hale et al., 2001	RCT, DB	3	50		RCE: 11; placebo: 13	RCE: 47.9 (3); placebo: 46.5 (1.8)	See footnote (g)	Yes	No statistically significant changes in Ki-67 index (epithelial and stromal) in the RCE group compared with control	2

(a): Refers to the Ref. ID number in Distiller.
(b): Unless otherwise specified, doses are expressed as mg isoflavones/day.
(c): Allocated to treatment/completed.
(d): Mean ± SD or SEM, as reported in the publication.
(e): See protocol in Annex A for appraisal of risk of bias.
(f): Study population included 10 women (18 %) with a history of hysterectomy.
(g): The subjects included in this study were described by the authors to be healthy women of late reproductive age who had experienced at least two menstrual periods in the 6 months before enrolment.

Discussion on results from human studies on uterus

A total of 25 trials of isoflavones supplementation assessed changes in endometrial thickness in peri-/post-menopausal women. None of the studies included reported statistically significant changes in endometrial thickness compared with control.

With respect to histo(patho)logical changes, the majority of the studies included in the review did not demonstrate any effect of isoflavones in the target population. In only two of the studies included in the review (Murray et al., 2003; Unfer et al., 2004) were histopathological effects noted. The Panel noted that in both of these studies the isoflavones doses used were at the higher end of the dose range used in other studies. Moreover, with respect to the study by Murray et al. (2003), the lack of an inactive control group and other important methodological flaws make it not possible to interpret these findings

In the study by Unfer et al. (2004), there were no findings after 2.5 years of intervention, but after 5 years five cases of simple hyperplasia and one case of complex hyperplasia were observed in the soy isoflavones group, compared with none in the placebo group. No cases of endometrial carcinoma were reported after 5 years. Some methodological weaknesses of the study need to be mentioned. For example, the Panel is aware of the correspondence regarding this publication by Foth and Nawroth (2005), which noted that a considerable number (up to 25 %) of specimens of endometrium were neither obtained nor assessable at each time point and that these samples were not consistently obtained from the same participants at each time point. In addition, based on the explanation given in Hofmeister et al. (1966), the histopathological reference given in Unfer et al. (2004), the Panel considered that the effects observed were indicative of a possible oestrogenic but not a carcinogenic effect.

All the other 23 studies reported no statistically significant findings for any of the endpoints considered.

Additional information on adverse events related to endometrial/uterine effects from the studies included in this review is reported in Appendix C. No disproportionate reporting was noted for any of the effects observed.

3.3.2. Results from animal studies

At the end of the systematic review process, the studies shown in Table 17; Table 18 and Table 19 were identified as relevant for the risk assessment question. All the studies included in the assessment were assessed independently by two experts, using the appraisal tools described in the protocol in Annex A and allocated to different tiers of reliability.

Endpoint: cell proliferation endometrium

The effect of administration of isoflavones on cell proliferation in the endometrium has been investigated in several experimental studies in animals. Studies were included if they reported information on proliferation marker Ki-67 (a protein strictly associated with cell proliferation), PCNA (an essential DNA replication accessory protein) or atypical cytology.

A total of 13 studies fulfilling the pre-defined criteria for inclusion in the review were considered for the assessment and are presented in Table 17.

A total of three studies in OVX monkeys (*Macaca fascicularis*) were identified as relevant.

No significant changes in either the superficial or the basal epithelium were observed after administration of a soy extract providing 35.7 mg isoflavones/kg bw/day for approximately 1 month (Wood et al., 2006).

In the other two studies, the source of isoflavones administered to the animals was a SPI providing doses of isoflavones ranging 8.6–9.9 mg/kg bw/day for approximately 3 years and 6 months, respectively (Foth and Cline, 1998; Wood et al., 2004). According to the authors, the doses used in these studies are equivalent to human doses of 129–148 mg/day for women. These two studies also included positive control groups, in which animals were treated with oestradiol at doses equivalent to

0.625–1 mg/day for women. Administration of the SPI did not result in an increase in cell proliferation markers, which was instead observed in all the animals treated with oestradiol.

Equol (racemic) was administered at a dose of 68 mg/kg bw/day for approximately 30 days (Wood et al., 2006). Again, in this study, no significant changes were observed with respect to cell proliferation in either superficial or basal epithelium.

Cell proliferation in the endometrium was also measured in nine studies in rats (Sprague–Dawley and Wistar) identified as relevant for the assessment.

Isoflavones from a soy extract were administered in one study at a dose of 15 mg/kg bw/day for 42 days with no observed effect on cell proliferation in the endometrium (Gallo et al., 2006).

In another study, isoflavones from a soy bean isolate and from an alcoholic extract of SPI were administered at doses of approximately 10 and 20 mg/kg bw/day for 7 weeks. Animals treated with isoflavones did not differ from OVX controls with respect to PCNA immunostaining (Bahr et al., 2005).

Isoflavones from a soy bean extract and from an alcohol-extracted isolate were also administered at doses of 15.7 and 1.5 mg/kg bw/day, respectively for 60 days, also in combination with oestradiol. The analysis of PCNA in endometrial surface cells and glands was performed only in the animals treated with the higher isoflavones doses; no proliferative effect compared with control was observed (Tansey et al., 1998).

A daidzein-rich soy extract providing different isoflavones doses, ranging from 4.3 up to 255.6 mg/kg bw/day, was administered to rats for 21 days. An additional group receiving 17β-oestradiol by means of subcutaneous implant served as positive control. An increase in PCNA in the epithelium and in the stroma was observed at the highest doses, starting from 42.6 mg/kg bw/day, and was comparable to the results observed in the positive control group (Mosquette et al., 2007). The same soy extract was also tested at doses of 19.6 and 53.25 mg/kg bw/day for 21 days for effects on the vaginal epithelium in rats. Increased PCNA and Ki-67 were observed at the higher dose tested, similarly to the effects observed in groups treated with oestradiol. No significant differences were observed in the 19.6 mg/kg bw/day group compared with control (Carbonel et al., 2011a).

In only one identified study, in which isoflavones from a red clover extract were administered at a dose of 1.15 mg/kg bw/day for 28 days, was Ki-67 protein expression in the endometrium significantly decreased compared with control (Alves et al., 2008).

In the remaining two studies, racemic equol alone was administered to the animals at doses of 4.5 and 36 mg/kg bw/day for 90 days (Rachoń et al., 2007a) and at doses of 4.5, 9 and 18 mg/kg bw/day for 56 days (Legette et al., 2009). The percentage of PCNA-positive cells in the stroma was significantly higher in the animals treated with equol 36 mg/kg bw/day than in control animals, whereas in the epithelium the percentage of PCNA-positive cells was significantly lower. No significant difference was observed for the lower dose of 4.5 mg/kg bw/day (Rachoń et al., 2007a). In the other study, a significantly higher proliferative index was observed in the epithelium of animals treated with the two higher doses (9 mg/kg bw/day and 18 mg/kg bw/day) but not in animals treated with the lower dose of 4.5 mg/kg bw/day. In the stroma, only the highest dose tested produced a significant positive response compared with control and the lower dose (Legette et al., 2009).

Cell proliferation in the endometrium was also measured in one study in mice (129/C57BL/6) in which isoflavones from a soy extract and from a formulation containing equal parts of genistein, daidzein and equol were administered at doses of 10 mg/kg bw/day for 9 months with no significant changes in either Ki-67 or PCNA in either of the two groups (Zhao et al., 2011).

Table 17: Overview of animal studies reporting effects on cell proliferation in the uterus

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Soy isoflavones (ISO)/soy extract								
1714 Wood et al., 2006	**Monkeys** (*Macaca fascicularis*)	OVX control; ISO: 35.7; EQ: 68	Conversion reported by the authors. Actual isoflavone dose administered 537 mg/1 800 kcal	10	28–33	Endometrial gland expression MKi-67, PGR	No statistically significant difference among treatment groups in either superficial or basal epithelium.	1
15146 Gallo et al., 2008	**Rats** (Sprague–Dawley)	Sham control; OVX control; SE-100: 16.8; E2	The authors reported that animals were administered soy extract (SE) 100 mg/kg bw/day. The extract used was a standardised extract containing 16.8 % isoflavones. Dose of total isoflavones calculated on the basis of the percentage reported by the authors. Animals in the positive E2 group were administered 17β-oestradiol, 0.5 mg/kg/day, by gavage	10	42	Ki-67, luminal epithelial (LE) and glandular epithelial (GE) cells	The percentage of Ki-67-positive nuclei was significantly reduced in LE cells in the SE-100 animals compared with OVX control. No statistically significant changes were observed in GE cells E2 significantly increased Ki-67 in both luminal and glandular compartment.	1
9236 Zhao et al., 2011	**Mice** (129/C57BL/6)	Sham control; OVX control; phyto-β-SERM: 10 SE: 10	The phyto-β-SERM formulation contained equal parts genistein, daidzein and equol providing a total of 100 mg/kg diet of isoflavones. According to the authors the dose was equivalent to a human dose of 50 mg/day. Animals in the other group (SE) were given a commercial soy extract product providing a total of 100 mg/kg diet of genistein/genistin, daidzein/daidzin and glycitein/glycitin. According to the authors the dose was equivalent to a human dose of 50 mg/day	5–7	270	Ki-67; PCNA	No statistically significant changes compared with OVX control in any of the treatment groups	1
Soy protein isolate (SPI)								
16271 Wood et al., 2004	**Monkeys**, (*Macaca fascicularis*) [c]	OVX control; SPI: 8.6; E2	Authors stated that the SPI treatment group received SPI containing isoflavones at a dose approximately equivalent to 129 mg/day for women (91 mg genistein, 31 mg daidzein and 7 mg glycitein). The doses were converted by the authors on the basis of the fact that the doses administered were scaled to 1 800 kcal of diet (estimated daily intake for US women) and that monkeys were fed 120 kcal/kg bw/day. Animals in the E2 group were given a dose corresponding to a human-equivalent dose of 0.625 mg/day on an energy basis. This dose was converted by the authors to 0.042 mg/kg bw/day	57–62	1080	Ki-67 labelling (superficial glands and basal glands), PR and ER	No statistically significant difference among treatment groups	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
15120 Foth and Cline, 1998	Monkeys (*Macaca fascicularis*)	OVX control; SPI: 9.9 E2; E2 + SPI	Authors stated that the dose administered to the animals corresponded to a human-equivalent dose of 148 mg/day per woman, on an energy basis. Animals in the E2 groups were administered oestradiol in the diet. Authors stated that the dose administered to the animals corresponded to a human-equivalent dose of 1 mg/day per woman, on an energy basis. The Panel assumed that the same conversion factor applied by the same research group in the publication Wood et al. (2004) applies also to this case	15	180	Ki-67 labelling (epithelium and stroma)	The SPI group was not different from control. Significant increase only in E2 group in both epithelium and stroma; however, the effect was reduced in the E2 + SPI group	1
10960 Bahr et al., 2005	**Rats** (Sprague–Dawley)	Intact control; OVX control; low SPI:10.5 (aglycones); high SPI: 21.6 (aglycones); low SPIEx: 9.4 (aglycones); high SPIEx: 19.3 (aglycones); E2	The authors reported that animals in the SPI groups were administered SPI at 100 and at 200 g/kg diet (low SPI and high SPI, respectively). The SPI used contained 2.14 mg/g aglycone from isoflavones (genistein, 1.10 mg/g; daidzein, 0.84 mg/g; and glycitein, 0.20 mg/g). Animals in the SPIEx groups were administered an alcoholic extract from SPI (SPIEx) at 17.2 and 34.4 g/kg diet (low-SPIEx and high-SPIEx group, respectively). The SPIEx contained 11.37 mg/g aglycone from isoflavones (genistein, 7.09 mg/g; daidzein, 3.81 mg/g; and glycitein, 0.47 mg/g). Daily doses estimated by the authors on the basis of food consumption. Animals in the positive E2 group were implanted subcutaneously (s.c.) with a silastic implant maintaining blood E2 concentrations of 30–40 pg/ml plasma (i.e. within normal cycling physiological range)	5–9	84	PCNA and C3 immunostaining	Any of the isoflavones-treated groups did not differ from OVX control group	1
2803 Tansey et al., 1998	Rats (Sprague–Dawley)	OVX control; Sham control; low ISO: 1.5; high ISO: 15.7; sham–low ISO: 1.5; sham–high ISO: 15.7; low E2; high E2; low ISO + low E2; low ISO + high E2; high ISO + low E2; high ISO + high E2	The authors reported the animals in the high-ISO groups were administered a soy bean extract (SBE) isolate (SUPRO 670) added at 15.40 g/100 g diet and providing 117.8 mg isoflavones/1 800 cal; animals in the low-ISO groups were administered an alcohol-extracted SPE isolate (SUPRO 670-IF)) added at 16.00 g/100 g diet and providing 11.6 mg isoflavones/1 800. The dose conversion has been calculated by the Panel assuming the daily caloric intake for a rat to be 60 cal/day and an average body weight of 250 g. Hence daily isoflavones intake would be 0.38 mg/day and 3.93 mg/day for the low-ISO and the high-ISO groups, respectively. These doses were converted assuming an animal body weight of 250 g into 0.04 mg/kg bw/day (low-E2) and 0.08 mg/kg	8–12	60	PCNA (endometrial surface cells and glands)	In both the OVX control and high-ISO groups the levels of proliferation were very low. The analysis was not performed in the low-ISO group. The addition of E2, at both doses significantly increased proliferation, with no additive effect observed for isoflavones	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
			bw/day (high-E2). Animals in the E2 groups were given daily doses of a pharmaceutical oestrogen preparation (conjugated equine oestrogens) equal to 0.313 mg/1 800 cal and 0.625 mg/1 800 cal. The conversion has been calculated assuming the daily caloric intake of a rat is 60 cal/day.					
Daidzein-rich isoflavones								
1618 Mosquette et al., 2007	Rats (Wistar)	OVX control; SE-10: 4.3; SE-50: 21.3; SE-100: 42.6; SE-300: 127.8; SE-600: 255.6 E2	Authors report that the soy extract used contained 42.6 % isoflavones. Approximately 36 % were genistein/genistin, 62 % were daidzein/daidzin and 2 % glycitein/glycitin. The authors stated that the animals received doses of the soy extract of 10 mg/kg bw/day, 50 mg/kg bw/day, 100 mg/kg bw/day, 300 mg/kg bw/day and 600 mg/kg bw/day. Animals in the E2 group were administered conjugated equine oestrogens at 200 µg/kg bw/day	8	21	PCNA (epithelium and stroma)	No statistically significant differences between SE-10 and SE-50 compared with OVX control. Increased PCNA expression in SE-100, SE-300 and SE-600 was observed, comparable with the results for the E2 group	1
14909 Carbonel et al., 2011a	Rats	OVX control; SE-46: 19.6; SE-120: 53.25; E2; SE 120 + E2	Authors report that the soy extract used contained 42.6 % isoflavones. Approximately 36 % were genistein, 62 % were daidzein and 2 % glycitein (including isoflavones isoforms). The authors stated that the animals received doses of the soy extract of 46 mg/kg bw/day (SE-46), 120 mg/kg bw/day (SE-120). The Panel calculated the dose of isoflavones provided by the extract on the basis of the percentage values given in the publication. Animals in the E2 groups were administered conjugated equine oestrogens at a dose of 50 µg/kg/day	10	21	PCNA, Ki-67 (vaginal epithelium)	No statistically significant changes for SE-46 compared with OVX control. Increased PCNA and Ki-67 expression in SE-120, comparable with the results for the E2 group	1
Red Clover extract								
14764 Alves et al., 2008	Rats (Wistar)	SHAM control; OVX control; RCE: 1.15; E2[(d)]	The authors reported that the red clover extract (RCE) used in this study contained 9.7 % of isoflavones. According to the authors, the dose chosen was equivalent to that for a 70-kg adult, i.e. 80 mg. Animals in the E2 group were administered oestradiol valerate 0.029 mg/kg	7–8	28	Endometrial Ki-67 expression	Ki-67 protein expression was significantly lower in RCE compared with OVX control.	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
Genistein								
19012 Carbonel et al., 2015	Rats	OVX control; OVX control-late; GEN: 50; OVX-GEN-late: 50; E2; E2-late	Administration of vehicle, genistein and 17β-oestradiol was initiated either immediately after OVX (OVX control, GEN, E2) or 30 days later (OVX control-late, GEN-late, E2-late). Animals in the E2 groups were administered 17β-oestradiol, 10 µg/kg bw/day, s.c.	10	30	Endometrial Ki-67 expression	No statistically significant difference between animals treated with GEN immediately after ovariectomy and both control groups. A significant increase was observed in animals in the GEN-late group compared with the other GEN group and both control groups. Ki-67 was significantly higher in both E2 groups. The effect was more pronounced in the E2-late group than in the OVX control group	1
Equol (racemic)								
1714 Wood et al., 2006	**Monkeys**, (*Macaca fascicularis*)	OVX control; ISO:35.7; EQ: 68	Actual dose administered 1 020 mg/1 800 kcal racemic equol. Dose conversion reported in the publication	10	28–33	Endometrial gland expression MKI67, PGR	No statistically significant difference among treatment groups in either superficial or basal epithelium.	1
15876 Rachoń et al., 2007a	**Rats** (Sprague–Dawley)	OVX control; EQ-50: 4.5; EQ-400: 36; low E2; high E2	Actual doses administered were 50 and 400 mg/kg chow of a pure racemic mixture of equol (EQ-50 and EQ-400, respectively). On the basis of food intake, average consumption was 6.54 mg/day. In the E2 groups, actual doses administered were 4.3 mg/kg and 17.3 mg/kg chow of oestradiol-3 benzoate for low E2 and high E2, respectively. On the basis of food intake, average consumption was 0.07 mg/day (low E2: 0.4 mg/kg bw/day) and 0.20 mg/day (high E2: 1.6 mg/kg bw/day). Doses calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012)	12	90	PCNA immunostaining (epithelium and stroma)	No statistically significant difference between low-EQ and OVX control. Significantly higher percentage of PCNA-positive cells in the stroma for high-EQ compared with control. Significantly lower percentage of positive PCNA-positive cells in the epithelium for high-EQ compared with control	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
1203 Legette et al., 2009	Rats (Sprague–Dawley)	Sham control; OVX control; EQ-50: 4.5; EQ-100: 9; EQ-200: 18	Actual doses administered w50, 100 and 200 mg/kg diet (EQ-50, EQ-100 and EQ-200, respectively) of equol powder (50 % *R*-equol, 50 % *S*-equol). Dose calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012). According to the authors the chosen doses correspond to human serum levels of equol of 3–39, and 5–49 µmol/l in response to dietary soy isoflavones of 56 and 90 mg/day (see Persky et al., 2002)	15–16	56	PCNA immunostaining (epithelium and stroma)	Significantly higher epithelial proliferative index in sham control, EQ-100 and EQ-200 groups than in the OVX control and EQ-50 groups. Significantly higher uterine stroma cell proliferative index in the sham control and EQ-200 groups than in the OVX control and EQ-50 groups, but not the EQ-100 group	1

phyto-β-SERM: phyto-β-Selective (o)estrogen receptor modulator
Text in bold indicates different animal species in which the studies were conducted. Text in grey indicates differents isoflavones being tested in the same study and refers to other rows in the table.
(a): Refers to the Ref. ID number in Distiller.
(b): See protocol in Annex A for appraisal of risk of bias.
(c): Half of the monkeys had been previously treated with oral contraceptives over a 26-month period before ovariectomy.
(d): An additional group was treated with an extract of *Cimicifuga racemosa*. Data are not presented in this table.

Endpoint: histo(patho)logy

The effect of administration of isoflavones on the histopathology of the endometrium has been investigated in several experimental studies in animals.

A total of 21 studies fulfilling the pre-defined criteria for inclusion in the review were considered for the assessment and are presented in Table 18.

A total of four studies in OVX monkeys (*Macaca fascicularis*) were identified as relevant.

No significant changes were observed after administration of a soy extract providing 35.7 mg isoflavones/kg bw/day for approximately 1 month. All treatment groups showed diffuse atrophy with no hyperplastic lesions reported (Wood et al., 2006).

In the other three studies, the source of isoflavones administered to the animals was a SPI providing doses of isoflavones of 8.6 mg/kg bw/day for 3 years and 9.9 mg/kg bw/day for 6 months. According to the authors, the doses used in these studies were equivalent to human doses of 129–148 mg/day for women. These three studies also included positive control groups, in which animals were treated with oestradiol at doses equivalent to 0.625–1 mg/day for women. Administration of the SPI alone did not result in any significant changes in endometrial thickness, epithelial area or endometrial gland area or in the number of hyperplastic lesions. Endometrial hyperplasia and/or increase in endometrial thickness were instead observed in the animals treated with oestrogen, alone or in combination with the SPI (Foth and Cline, 1998; Wood et al., 2004; Scott et al., 2008).

Equol (racemic) was administered at a dose of 68 mg/kg bw/day for approximately 30 days. Again, in this study, no significant changes were observed compared with control (Wood et al., 2006).

Histo(patho)logical changes in the uterus were also measured in 17 studies in rats (Sprague–Dawley and Wistar) identified as relevant for the assessment.

Soy extracts were used as a source of isoflavones in three studies at doses ranging from 16.8 to 50 mg/kg bw/day for a period of 30–42 days. All three of these studies also included positive control groups treated with 17β-oestradiol or diethylstilboestrol (Gallo et al., 2005, 2008; Teixeira et al., 2014). No significant changes were observed in any of the three studies compared with the control groups.

In another study, isoflavones from a SPI and from an alcoholic extract of SPI were administered at doses of approximately 10 and 20 mg/kg bw/day for 7 weeks. Animals treated with isoflavones did not differ from OVX controls with respect to the histological features examined (Bahr et al., 2005). Isoflavones from a soy bean extract and an alcohol-extracted isolate were also administered to rats at dose of 1.57 mg/kg bw/day and 1.5 mg/kg bw/day, respectively, for 60 days, also in combination with oestradiol. Again, in this case, there was no statistically significant difference between the two groups treated with isoflavones and the OVX controls, whereas concomitant oestrogenic treatment produced a significant increase in luminal epithelial height (Tansey et al., 1998).

Three studies were identified in which the source of isoflavones was probably the same daidzein-rich soy extract (Mosquette et al., 2007; Carbonel et al., 2011b; Francisco et al., 2013).

A daidzein-rich soy extract was administered to Wistar rats, providing doses of 42, 125 and 250 mg/kg bw/day genistein equivalents for 30 days. No significant changes were detected at morphological analysis in the animals treated with the lowest dose compared with OVX controls. Squamous metaplasia was observed in all the animals in the highest dose, compared with none in the OVX control and in the other two treatment groups. Endometrial area, number of glands/area and area of endometrial glands were significantly increased in the medium- and high-dose groups compared with controls (Carbonel et al., 2011b). In another study, the same extract was tested for 21 days, providing different isoflavones doses, ranging from 4.3 up to 255.6 mg/kg bw/day. An additional group treated with conjugated equine oestrogen served as positive control. No significant changes were detected at morphometric analysis in the animals treated with the two lower doses (4.3 and 21.3 mg/kg bw/day) compared with OVX controls. Animals in the three higher dose groups (42.6, 127.8 and 255.6 mg/kg bw/day) had significantly increased endometrial area, thickness, number of glands and myometrial area compared with animals in the lower isoflavones dose groups. In addition, the number of vessels was increased in these three groups compared with the lower doses.

Furthermore, with respect to the histological features analysed, there was no significant difference between OVX controls and the animals treated with the two lower doses. In animals treated with 42.6 mg/kg bw/day isoflavones, a slight proliferation of endometrium was observed, whereas in animals treated with the two higher doses proliferation was marked. The endometrial enlargement observed at a dose of 127.8 mg/kw bw/day was comparable to the results observed in the positive control group, whereas the myometrium of animals in the lower dose groups was not different from control (Mosquette et al., 2007). In the third study, the doses of isoflavones administered were, according to the authors, 21.3 and 42.6 mg/kg bw/day. Again in this case there were two additional groups, one treated with the oestrogen alone and serving as a positive control, the other being concomitantly treated with the high dose of isoflavones together with oestrogen. As in the other two studies, no significant effects were observed at the lower dose tested, whereas the dose of 42.6 mg/kg bw/day induced increases in endometrial area, in the number of glands and in the gland area. Myometrial area was also increased, as was the number of vessels. The endometrium was better developed among animals treated with high isoflavones doses, with increased size of the endometrial glands and myometrium (Francisco et al., 2013).

Three studies were identified in which red clover extracts were used as a source of isoflavones. In the first study, isoflavones administered at a dose of 1.15 mg/kg bw/day for 28 days did not produce any significant effect on uterine histology (Alves et al., 2008). In the second study, three different doses were tested (37.5, 75 and 112.5 mg/kg bw/day for 21 days. Two additional groups of animals were treated with oestradiol, alone or in combination with the higher dose of isoflavones. In this study all three doses produced a significant increase in endometrial thickness. The response observed was dose dependent, although significantly less than the positive control (Burdette et al., 2002). In the third study (Kang et al., 2015) the dose of 32 mg/kg/day was tested and demonstrated to be active.

In the remaining six studies, the effects of the individual isoflavones genistein, daidzein and equol were investigated.

Genistein was administered to the animals at doses of 5.4 and 54 mg/kg bw/day for 90 days. Two groups treated with two different doses of oestradiol served as positive controls. Lamina propria cells were found in half of the animals treated with the lower dose and in all animals treated with the high dose, as well as in all animals in both positive control groups. All animals in the higher dose group exhibited endometrial cell hypertrophy, but no pathological signs were detected (no squamous metaplasia, hyperplastic/hypertrophic glands, cystic glands and pyometra) (Rimoldi et al., 2007). In another 90-day study, no changes compared with controls were detected in animals administered genistein at 10 mg/kg bw/day (Phrakonkham et al., 2007). After treating animals for 84 days with genistein at a dose of 45 mg/kg bw/day, the epithelial cells of the treated animals appeared cubic and more similar to the sham control than to the OVX animals (Li and Yu, 2003). In a more recent study, oestrogenic effects were noted after treatment with 50 mg/kg/day genistein for 30 days. However, the effects were smaller than in E2-treated positive controls (10 µg/kg/day s.c.). Squamous metaplasia was seen in E2-treated animals but not in genistein-treated animals. In another study either genistein at a dose of 50 mg/kg bw/day or E2 was administered immediately after ovariectomy or 30 days later (Carbonel et al., 2015). Animals in the late genistein administration group had significantly higher values of all the parameters measured compared with the control groups, but the effect was smaller than in the E2 treated groups.

No changes compared with controls were detected in a study in which animals were treated with daidzein at a single dose of 10 mg/kg bw/day (Phrakonkham et al., 2007).

In three studies, equol, either racemic or in its S-form, was administered to rats at doses ranging 4.5–36 mg/kg bw/day. The duration of treatment ranged 35–90 days.

In a 90-day study, no significant difference between treated animals and OVX controls was observed at histological and morphometric analysis in animals treated with racemic equol at 4.5 mg/kg bw/day. At the higher dose tested of 36 mg/kg bw/day, animals showed some signs of oestrogenic stimulation (Rachoń et al., 2007a). Uterine wall thickness and section surface were significantly increased compared with controls after administration of racemic equol at a single dose of 10 mg/kg bw/day for 90 days (Phrakonkham et al., 2007).

No significant changes compared with control were instead observed in a 35-day study in which animals were treated with either *S*-equol or a soy germ extract, both providing a dose of 11.5 mg/kg bw/day of *S*-equol (Yoneda et al., 2011).

Table 18: Overview of animal studies measuring histo(patho)logical changes in the uterus

Ref. ID[a] Author, year	Animal species	Isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Soy isoflavones (ISO)/soy extract								
1714 Wood et al., 2006	**Monkeys** (*Macaca fascicularis*)	OVX control; ISO: 35.7; EQ: 68	Conversion reported by the authors. Actual isoflavone dose administered 537 mg/1 800 kcal	10	28–33	Endometrial thickness Epithelial area uterine area Histological evaluation	No statistically significant difference among treatment groups. Diffuse atrophy in all groups. No hyperplastic lesions	1
15146 Gallo et al., 2008	**Rats** (Sprague–Dawley)	Sham control; OVX control; SE-100: 16.8; E2	The authors reported animals were administered 100 mg soy extract (SE)/kg/day. The extract used was a standardised extract containing 16.8 % isoflavones. Dose of total isoflavones calculated on the basis of the percentage reported by the authors. Animals in the positive E2 group were administered 17β-oestradiol, 0.5 mg/kg/day via gavage	10	42	Histopatholo-gical analysis	No statistically significant changes compared with OVX control for animals in the SE-100 group. In SE-100 group sub-atrophic/atrophic epithelium was reported in 1/10 animals, endometrial hyperplasia in1/10 animals. Endometrial hyperplasia was reported in 9/10 animals in E2 group	1
15145 Gallo et al., 2005	Rats (Sprague–Dawley)	Sham control; OVX control; SE-50: 7.5; SE-100: 15; E2	The authors reported doses administered were 50 and 100 mg/kg bw/day of extract Soyselect® by gavage (SE-50 and SE-100, respectively). Isoflavone dose calculated on the basis of the percentage reported in the company website (13–17 % isoflavones). Animals in the positive E2 group were administered with 17β-oestradiol, 0.5 mg/kg/day via gavage	10–12	42	Histopatholo-gical analysis	The atrophy of the uterine epithelium induced by OVX was not reverted by treatment with SE. Stromal oedema was observed in the majority of SE animals at both doses tested	1
16112 Teixeira et al., 2014	Rats (Wistar)	SHAM control; OVX control; SE: 50 (genistein equivalents); E2	The authors state that the animals received 50 µg/g bw/day of genistein from a soy extract (SE), Novasoy®, containing 40 % isoflavones, in a ratio of 1.3: 1: 0.3 of genistein:daidzein:glycitein. Animals in the E2 group were treated with diethylstilboestrol (DES) at 10 µg/kg bw/day, s.c.	5	30	Endometrial thickness Myometrial thickness Glandular area	No statistically significant difference between animals treated with soy extract and OVX control	1

Ref. ID[a] Author, year	Animal species	Isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Soy protein isolate								
4730 Scott et al., 2008	**Monkeys** (*Macaca fascicularis*)[c]	OVX control; SPI: 8.6; E2	Authors stated that the isoflavone dose administered corresponded to a human equivalent dose of 129 mg/day, expressed as aglycone units. The Panel assumed that the same conversion factor applied by the same research group in the publication Wood et al., 2004 applies also to this case. Animals in the E2 group were administered 17β-oestradiol 0.042 mg/kg bw/day	52–55	1080	Endometrial thickness Epithelial area Glandular area	No statistically significant changes compared with OVX control. Significantly higher only in positive controls.	1
16271 Wood et al., 2004	Monkeys (*Macaca fascicularis*)[c]	OVX control; SPI: 8.6; E2	Authors stated the isoflavone dose administered corresponded to a human equivalent dose of 129 mg/day, expressed as aglycone units. Authors stated the dose administered in the E2 group (0.042 mg/kg bw/day) corresponded to a human equivalent dose of 0.625 mg/day of CEE	57–62	1080	Endometrial thickness Epithelial area Histological changes	Endometrial hyperplasia of moderate to marked degree was observed in 0/57, 0/60 and 26/62 for OVX control, SPI and E2 respectively. Minimal or mild proliferative changes were seen in 3/57, 1/60 and 32/60 for OVX control, SPI and E2, respectively	1
15120 Foth and Cline, 1998	Monkeys (*Macaca fascicularis*)	OVX control; SPI: 9.9; E2; E2 + SPI	Authors stated that the dose administered to the animals corresponded to a human equivalent dose of 148 mg/day per woman, on an energy basis. Animals in the E2 groups were administered oestradiol in the diet. Authors stated that the dose administered to the animals corresponded to a human equivalent dose of 1 mg/day per woman, on an energy basis. The Panel assumed that the same conversion factor applied by the same research group in the publication Wood et al. (2004) applies also to this case	15	180	Endometrial gland area Histopathology	Endometrial hyperplasia in all animals given E2 or E2 + SPI, degree and irregularity of hyperplasia less pronounced in the W2 + SPI group. Atypical hyperplasia not observed. Significant increase of endometrial thickness and gland area observed in E2 and E2 + SPI groups	1
10960 Bahr et al., 2005	**Rats** (Sprague–Dawley)	Intact-control; OVX control; low SPI: 10.5 (aglycones); high SPI: 21.6 (aglycones); low SPIEx: 9.4 (aglycones); high SPIEx: 19.3 (aglycones); E2	The authors reported that animals in the SPI groups were administered soy protein isolate (SPI) at 100 and at 200 g/kg diet (low SPI and high SPI, respectively). The SPI used contained 2.14 mg/g aglycone from isoflavones (genistein, 1.10 mg/g; daidzein, 0.84 mg/g and glycitein, 0.20 mg/g). Animals in the SPIEx groups were administered an alcoholic extract from SPI (SPIEx) at 17.2 and 34.4 g/kg diet (low -SPIEx and high SPIEx, respectively). The SPIEx contained:	5–9	84	Histology (uterus and vagina)	Histological features do not differ from OVX-group in any of the isoflavones-treated groups. Uterine epithelial cells were low columnar to cuboidal in shape in all rats	1

Ref. ID[(a)] Author, year	Animal species	Isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
			11.37 mg/g aglycone from isoflavones (genistein, 7.09 mg/g; daidzein, 3.81 mg/g and glycitein, 0.47 mg/g). Daily doses estimated by the authors on the basis of food consumption. Animals in the positive E2 group were implanted s.c. with a silastic implant maintaining blood E2 concentrations of 30–40 pg/ml plasma (i.e. within normal cycling physiological range)					
2803 Tansey et al., 1998	Rats (Sprague–Dawley)	OVX control; Sham control; low ISO: 1.5; high ISO: 15.7; sham-low-IF: 1.5; sham-high-IF: 15.7; low E2; high E2; low ISO + low E2; low ISO + high-E2; high ISO + low E2; high ISO + high E2	The authors reported the animals in the high-ISO groups were administered a soy bean extract (SBE) isolate (SUPRO 670) added at 15.40 g/100 g diet and providing 117.8 mg isoflavones/1 800 cal; animals in the low- ISO groups were administered an alcohol-extracted SPE isolate (SUPRO 670-IF)) added at 16.00 g/100 g diet and providing 11.6 mg isoflavones/1 800. The dose conversion has been calculated by the Panel assuming the daily caloric intake for a rat to be 60 cal/day and an average body weight of 250 g. Hence daily isoflavones intake would be 0.38 mg/day and 3.93 mg/day for the low- ISO and the high- ISO groups, respectively. These doses were converted assuming an animal body weight of 250 g into 0.04 mg/kg bw/day (low E2) and 0.08 mg/kg bw/day (high E2). Animals in the E2 groups were given daily doses of a pharmaceutical oestrogen preparation (conjugated equine oestrogens) equal to 0.313 mg/1 800 cal and 0.625 mg/1 800 cal. The conversion has been calculated assuming the daily caloric intake of a rat is 60 cal/day	8–12	60	Luminal epithelial height Vaginal cytology	No statistically significant difference between low-IF and high- ISO and OVX control. Addition of E2, at both doses, produced a significant increase, but no additive effect observed for isoflavones. No statistically significant difference in vaginal cytology between low- ISO or high- ISO groups and OVX control	1

Ref. ID[(a)] Author, year	Animal species	Isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
Daidzein-rich isoflavones								
12969 Carbonel et al., 2011b	Rats (Wistar) EPM-1	OVX control; low SE: 42 (genistein equivalent); medium SE: 125 (genistein equivalent); high SE: 250 (genistein equivalent)	The authors reported that the soy extract (SE) administered to the animals contained 42.6 % isoflavones, in a ratio of 0.6:1:0.03 of genistein:daidzein:glycitein	10	30	Morphological analysis Squamous metaplasia Endometrial area, number of endometrial glands/area; area of endometrial glands	On morphological analysis, there were no differences between the low-SE and OVX control group. Squamous metaplasia was not seen in any of the animals in the OVX control, low-SE and medium-SE groups, but was found in 10/10 animals in the high-SE group. Endometrial area and number of glands/area and area of endometrial glands were significantly increased in medium-SE and high-SE groups	1
1618 Mosquette et al., 2007	Rats (Wistar)	OVX control; SE-10: 4.3; SE-50: 21.3; SE-100: 42.6; SE-300: 127.8; SE-600: 255.6; E2	Authors report that the soy extract used contained 42.6 % isoflavones. Approximately 36 % were genistein, 62 % were daidzein and 2 % glycitein (including isoflavones isoforms). The authors stated that the animals received doses of the soy extract of 10 mg/kg bw/day, 50 mg/kg bw/day, 100 mg/kg bw/day, 300 mg/kg bw/day and 600 mg/kg bw/day. The Panel calculated the dose of isoflavones provided by the extract on the basis of the percentage values given in the publication. Animals in the E2 group were administered conjugated equine oestrogens 200 µg/kg bw/day	8	21	Endometrium: area, number and gland area, number of vessels Myometrium: area, number of vessels Histological analysis	The morphometric analysis did not show significant changes for SE-10 and SE-50 compared with OVX control. Animals in the SE-100, SE-300 and SE-600 groups had higher endometrial area, thickness index, number of glands and myometrial area than animals treated with the lower isoflavones doses. The number of vessels was also increased in the SE-100, SE-300 and SE-600 groups compared with the lower-dose groups. The endometrium of OVX control was dense and thin. Animals in the SE-50 and SE-100 groups did not have histological features very different from OVX control. In SE-100 a slightly proliferative endometrium was observed. Proliferation was marked in SE-300 and	1

Ref. ID[(a)] Author, year	Animal species	Isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
							SE-600. SE-100, SE-300 and SE-600 showed many endometrial glands. The endometrial enlargement observed in SE-300 was comparable with E2 control. The myometrium of SE-10 was comparable with that of OVX control. At higher doses an increase in thickness, in the number of myocytes was observed and a large number of collagen fibres	
454 Francisco et al., 2013	Rats	OVX control; SE-46: 21.3; SE 120: 42.6; E2; SE 120 + E2	The authors reported that the soy extract used contained 42.6 % isoflavones, in a ratio of 0.6:1:0.03 of genistein:daidzein:glycitein, and was administered at the doses of 46 and 120 mg/kg bw/day (SE-46 and SE-120, respectively). The authors reported that the doses administered correspond to 21.3 mg and 42.6 mg of isoflavones for SE-46 and SE-120, respectively. The Panel, however, noted that, according to the per cent of isoflavones reported, the calculated dose of isoflavones would be 19.6 mg and 51.1 mg for SE-46 and SE-120, respectively. Animals in the E2 groups were administered conjugated equine oestrogens at a dose of 50 µg/kg bw/day	10	21	Endometrium: area, number and gland area, number of vessels Myometrium: area, number of vessels Histological analysis	No statistically significant changes for SE46 compared with OVX control SE 120 and SE 120 + E2 had higher endometrial area, number and gland area. Myometrial area and number of vessels were increased in SE 120 and SE 120 + E2 compared with OVX control and SE 46. Endometrium and myometrium of OVX control and SE 46 were dense and thin. In SE 120 and SE 120 + E2 animals increased size of endometrial glands and myometrium were noted. Endometrium in groups E2, SE120 and SE 120 + E2 was better developed compared with SE 46. No additional effect was observed in SE 120 + E2 compared with SE 120 and E2	2

Ref. ID[a] Author, year	Animal species	Isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Red Clover extract								
19187 Kang et al., 2015	Rats (Sprague–Dawley)	Sham control; OVX control; RCE: 3.2[e]	The authors reported that animals were dosed with 40 mg/kg bw/day of a red clover extract (RCE) standardised to contain 8 % total isoflavones (genistein, 0.62%; biochanin A, 5.43%; formononetin, 3.66%; and daidzein, 0.47%). Dose of isoflavones calculated on the basis of the percentage (8%) reported by the authors	8	84	Total thickness, epithelium thickness, mucosa thickness. Uterine gland (%)	All the measured parameters were significantly increased compared with OVX control, albeit different from sham control	1
14764 Alves et al., 2008	Rats (Wistar)	Sham control; OVX control; RCE: 1.15; E2[d]	The authors reported that the red clover extract (RCE) used in this study contained 9.7 % isoflavones. According to the authors, the dose chosen was equivalent to that for a 70-kg adult, i.e. 80 mg. Animals in the E2 group were administered oestradiol valerate 0.029 mg/kg	7–8	28	Histology	Histological features did not differ from OVX group. The endometrium remained atrophic	1
11684 Burdette et al., 2002	Rats (Sprague–Dawley)	OVX control; RCE-250: 37.5 (aglycones); RCE-500: 75; (aglycones); RCE-750: 112.5 (aglycones); E2; RCE-750 + E2	Authors report that animals were administered 250, 500 and 750 mg/kg/day of red clover extract (RCE) standardised to a minimum 15 % isoflavone content by weight of four isoflavones genistein, 0,850%; daidzein, 0.349%; biochanin A, 6.57 %; and formononetin, 8.56 %, as hydrolysed aglycones. Dose of isoflavones calculated on the basis of the percentage (15%) reported by the authors. According to the authors the choice of the doses of red clover was based on a clinical dose of 40 mg isoflavones/day. Animals in the E2 group were administered 17β-oestradiol 50 µg/kg bw/day	5–6	21	Endometrial thickness Vaginal cytology	Dose-dependent increase in endometrial thickness; however, the response was significantly less than that observed in E2-treated rats	2

Ref. ID[a] Author, year	Animal species	Isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Genistein								
4863 Rimoldi et al., 2007	Rats (Sprague–Dawley)	OVX control; low-GEN: 5.4; high-GEN: 54; low-E2; high-E2	In the publication it is reported that the doses of genistein were estimated by the authors on the basis of the daily food intake. Animals in the positive E2 groups were administered oestradiolbenzoate. Daily doses were estimated on the basis of daily food intake at 0.17 mg/kg bw/day (low-E2) and 0.7 mg/kg bw/day (high-E2)	11–12	90	Endometrial thickness, myometrium thickness, morphological analysis, determination of hypertrophy and hyperplasia of glands and endometrial epithelium	Lamina propria cells were found in 6/12 of the animals in the low-GEN group and 12/12 in the high-GEN group, as well as in all animals in both low-E2 and high E-2 groups. In the high-GEN group endometrial cell hypertrophy was observed in 12/12 animals but no pathological signs were detected (no squamous metaplasia, hyperplastic/hypertrophic glands, cystic glands or pyometra)	1
16713 Phrakonkham et al., 2007	Rats (Wistar)	OVX control; GEN: 10; EQ: 10; DAI: 10		10	90	Wall thickness (uterine and vaginal), section surface (uterus); epithelium thickness (vaginal); per cent cornified cells	No statistically significant changes compared with OVX control	1
11367 Li and Yu, 2003	Rats (Sprague–Dawley)	Sham control; OVX control; GEN: 45; E2	Animals in the E2 group were administered oestradiol 10 µg/kg once every 2 days	10	84	Histological analysis	Epithelial cells from GEN animals appeared cubic as in SHAM-operated animals. E2 animals had squamous cells, whereas the epithelium in OVX control appeared thin and flat	1
19012 Carbonel et al., 2015	Rats	OVX control; OVX control-late; GEN: 50; OVX-GEN-late: 50; E2; E2-late	Administration of vehicle, genistein and 17β-oestradiol was initiated either immediately after OVX (OVX control, GEN, E2) or 30 days later (OVX control-late, GEN-late, E2-late). Animals in the E2 groups were administered 17β-oestradiol, 10 µg/kg bw/day, s.c.	10	30	Endometrium: area, thickness, epithelial thickness, number and gland area, number of vessels	No statistically significant changes for GEN compared with both groups of OVX control, with the exception of the number of vessels/area which was significantly higher in the GEN group. Animals in the GEN-late group had significantly higher	1

Ref. ID[(a)] Author, year	Animal species	Isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
						Histological analysis	values of all the parameters measured compared with both groups of OVX control and, with the exception of the number of vessels/area, also of the group treated with GEN immediately after OVX. All the morphological parameters measured had significantly higher values in both groups treated with E2 compared with OVX control and GEN treated animals. The effect was significantly higher in the group of animals treated 30 days after OVX. Histological analysis revealed that. in both GEN groups, the uteri was enlarged compared with OVX control, with thicker layers covered by simple prismatic epithelium. Treatment with E2 resulted in even larger uteri covered by simple, prismatic epithelium containing leucocyte infiltration. In the E2-late group, some areas were indicative of squamous metaplasia	
Daidzein								
16713 Phrakonkham et al., 2007	Rats (Wistar)	OVX control; GEN: 10; DAI: 10; EQ: 10		10	90	Wall thickness (uterine and vaginal), section surface (uterus); epithelium thickness (vaginal); per cent of cornified cells	No statistically significant changes compared with OVX control	1

Ref. ID[(a)] Author, year	Animal species	Isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
Equol								
1714 Wood et al., 2006	**Monkeys** (*Macaca fascicularis*)	OVX control; ISO: 35.7; EQ: 68	Actual dose administered 1020 mg/1 800 kcal racemic equol. Dose conversion reported in the publication	10	28–33	Endometrial thickness, epithelial area, uterine area. Histological evaluation	No statistically significant difference among treatment groups. Diffuse atrophy in all groups. No hyperplastic lesions.	1
15876 Rachoń et al., 2007a	**Rats** (Sprague–Dawley)	OVX control; EQ-50: 4.5; EQ-400: 36; low E2; high E2	Actual doses administered 50 and 400 mg/kg chow of pure racemic mixture of equol (EQ-50 and EQ-400, respectively). On the basis of food intake, average consumption was 6.54 mg/day. In the E2 groups, actual doses administered 4.3 mg/kg and 17.3 mg/kg chow of oestradiol-3 benzoate for low E2 and high E2, respectively. On the basis of food intake, average consumption was 0.07 mg/day (low E2: 0.4 mg/kg bw/day) and 0.20 mg/day (high E2: 1.6 mg/kg bw/day). Doses calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012)	12	90	Histological and morphometric analyses	Low-EQ did not show any features of oestrogenic stimulation, similar to OVX control. In high-EQ 6/12 animals the endometrial epithelium was composed of tall columnar cells with increased mitotic activity, some differentiation in the stroma. All animals in both E2 groups showed oestrogenic stimulation. High-EQ animals had significantly greater endometrial epithelium height, thickness of the stroma and myometrium. These features were no different between low-EQ and OVX control	1
16713 Phrakonkham et al., 2007	Rats (Wistar)	OVX control; GEN: 10; DAI: 10; EQ: 10		10	90	Wall thickness (uterine and vaginal), section surface (uterus); epithelium thickness (vaginal); per cent of cornified cells	Uterine wall thickness and section surface were significantly increased compared with OVX control	1

Ref. ID[(a)] Author, year	Animal species	Isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
16333 Yoneda et al., 2011	Rats (Sprague–Dawley)	Sham control; OVX control; S-EQ: 11.7; SE5-OH: 11.7; E2	SE5-OH is described as a fermented soy germ containing *S*-equol. The dose is expressed as *S*-equol equivalent. Animals in the E2 group were administered CEE 6 mg/kg/day	5–15	38	Histological analysis: epithelial height, endometrial stromal thickness, myometrial thickness	No statistically significant changes compared with OVX control in either of the *S*-equol treated groups	1

Text in bold indicates different animal species in which the studies were conducted. Text in grey indicates differents isoflavones being tested in the same study and refers to other rows in the table.
(a): Refers to the Ref. ID number in Distiller.
(b): See protocol in Annex A for appraisal of risk of bias.
(c): Half of the monkeys had been previously treated with oral contraceptives over a 26-month period before ovariectomy.
(d): An additional group was treated with an extract of *C. racemosa*. Data are not presented in this table.
(e): Other additional groups were treated with a pomegranate concentrate powder (PCP) alone and in combination with red clover extract (RC:PCP 2:1 mixture). Data for these additional groups not presented in this table.

Endpoint: uterine weight

The effect of administration of isoflavones on uterine weight has been investigated in several experimental studies in OVX animals.

Most of the studies included were primarily designed to investigate other parameters, e.g. effects on the bone or on the cardiovascular system: in these studies uterine weight was also determined as an ancillary endpoint or marker of oestrogenic activity. None of the studies was designed as a standard uterotrophic assay according to OECD TG 440 (2007).

A total of fifty-three studies fulfilling the pre-defined criteria for inclusion in the review were considered for the assessment and are presented in Table 19.

Two studies in OVX monkeys (*Macaca fascicularis*) were identified (Foth and Cline, 1998; Wood et al., 2006).

No significant changes were observed after administration of a soy extract providing 35.7 mg isoflavones/kg bw/day for approximately 1 month (Wood et al., 2006).

No significant changes were observed after administration of a SPI providing doses of isoflavones of 9.9 mg/kg bw/day for 6 months. In contrast, a significant increase in uterine weight was observed in the animals treated with oestrogen, alone or in combination with the SPI (Foth and Cline, 1998).

Most of the studies included in this review were conducted in rats. An overview of the findings from the forty-two studies conducted in this animal species is presented in Figure 3. Studies are reported more than once if different isoflavones were tested within the same experiment.

The results presented in Table 19 are reported as stated in the corresponding publications; hence it should be noted that there may be differences in the parameter measured (wet weight, dry weight, absolute, relative weight to body weight, or not further specified).

Figure 3 shows at which doses the compounds tested showed a significant (full red dot) effect on uterine weight compared with control for each of the studies included in the review. Empty dots represent no statistically significant difference from control. The size of the full dot is proportional to the relative effect on the uterine weight compared with the control within the same study. The use of relative weight means that differences in the parameter measured have no influence on the overall analysis.

The Panel encourages readers to exercise caution in interpreting Figure 3, since many assumptions were made and sometimes default values were applied to convert the doses reported in the publications to mg/kg bw/day (see footnotes in Table 19 and explanations in Table 28).

Moreover, it should be noted that the doses shown in Figure 3 do not differentiate between the doses expressed as total isoflavones and doses expressed as aglycones and are only indicative of the range of doses used in the studies and their relative effect.

In calculating the relative increase in uterine weight compared with control, several assumptions were made or numerical values had to be extrapolated from graphical representations of the results. Thus, although the data presented in Figure 3 provide an overview of the observations from a large number of animal studies reporting on the same endpoints at comparable doses, there could still be several uncertainties around the data shown.

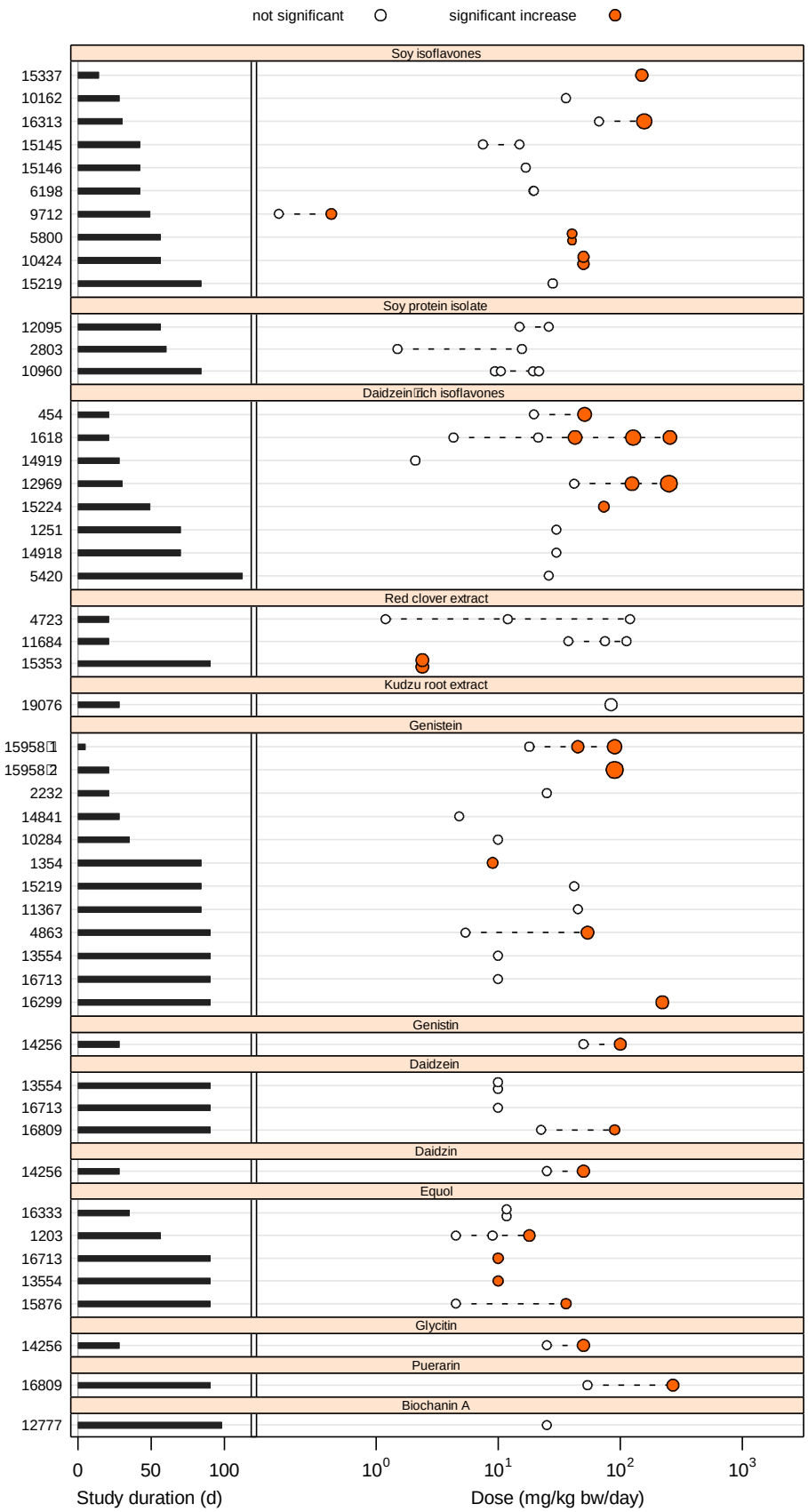


Figure 3: Graphical representation of the results of the studies measuring uterotrophic effects in OVX rats included in this systematic review

A total of eight studies in OVX mice (different strains) were identified.

Two studies investigated the uterotrophic effects of soy extracts in mice. In the first one, no significant changes were observed in 129/C57BL/6 mice after administration of isoflavones from a soy extract and from a formulation containing genistein, daidzein and equol in equal parts for 9 months, both providing an isoflavones dose of 10 mg/kg bw/day (Zhao et al., 2011). No significant difference in uterine weight was reported after administration of isoflavones from a soy extract providing an isoflavone dose of 200 mg/kg bw/day for 35 days in C57BL/6J mice (Zhang et al., 2009).

In two studies, kudzu root (*Pueraria lobata*) was used as a source of isoflavones. In the first study, a powder from kudzu root was administered to ddY mice for 28 days, providing isoflavones doses of 60 mg/kg bw/day, 120 mg/kg bw/day and 240 mg/kg bw/day. No statistically significant changes were observed compared with the animals in the control group (Wang et al., 2003). No significant difference with the uterine weight of the control animals was reported also in the other study in which ICR mice were administered an extract providing isoflavones doses of 27 mg/kg bw/day and 68 mg/kg bw/day for 28 days (Cho et al., 2012).

The uterotrophic effect of genistein alone was investigated in three studies in mice. In the first one, seven doses of genistein, ranging from 2 mg/kg bw/day to 2 000 mg/kg bw/day were administered to C57BL/6J mice for 90 days. There was no significant difference in the uterine weight of the animals in the five lower doses compared with control (i.e. up to 1 000 mg/kg bw/day). Uterine weight of the animals treated with genistein at 600 mg/kg bw/day and 2 000 mg/kg bw/day was significantly increased compared with the OVX controls. Uterine weight of the animals treated with the highest dose was significantly increased compared with a control group of intact animals (Nguyen et al., 2013). These findings were consistent with the results of another study in which a dose of genistein of 400 mg/kg bw/day administered for 84 days did not induce a significant change in the uterine weight of the treated animals compared with OVX control (Chen et al., 2009). No significant changes were also reported in a study in which a dose of 100 mg/kg bw/day was administered to C57BL/6J mice (Zhang et al., 2009).

The uterotrophic effect of formononetin was investigated in a study in which two doses of 50 mg/kg bw/day and 500 mg/kg bw/day were administered to Kunmig mice for 6 months. The lower dose produced a significant increase in the uterine weight compared with OVX control, whereas no difference was observed for the animals treated with the higher dose (Mu et al., 2009).

Only one study in rabbits was found, in which both isoflavones from a red clover extract and daidzein alone were tested at equivalent doses of 0.1 mg/kg bw/day for 84 days. There was no significant difference in the uterine weight of either treated groups and OVX controls.

Table 19: Overview of animal studies measuring effects uterus weight

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Soy isoflavones/soy extracts								
1714 Wood et al., 2006	**Monkeys** (*Macaca fascicularis*)	OVX control; ISO: 35.7; EQ: 68	Conversion reported by the authors. Actual isoflavone dose administered 537 mg/1 800 kcal	10	28–33	Uterine weight	No statistically significant difference among treatment groups.	1
15219 Hertrampf et al., 2009	**Rats** (Wistar)	Sham control; OVX control; IRD: 28; GEN: 42; E2[c]	Animals were fed an isoflavone-rich diet (IRD) containing genistein (242 µg/g diet) and daidzein (232 µg/g diet) corresponding to a daily intake of 14 mg/kg bw/day for both genistein and daidzein. Animals in the positive E2 group were administered oestradiol 2 µg/kg bw/day s.c.	7	84	Uterine weight (wet, absolute)	No statistically significant changes compared with OVX control	1
5800 Breitman et al., 2003	Rats (Sprague–Dawley)	Sham control; OVX control; ISO: 40.3 + 0.2 % calcium; ISO-Ca: 40.3 + 2.5 % calcium[d]	Authors report dose as 1.6 g/kg diet. The isoflavone extract used (1.6 g) contained: genistein, 217 mg; daidzein, 196 mg and glycitein, 35 mg. Dose calculated by applying the EFSA default value of 0.09 per sub-chronic studies in rats (EFSA Scientific Committee, 2012) to the sum of isoflavones reported in the publication (448 mg)	10	56	Weight (relative)	Uterine weight was slightly but significantly lower in animals in the ISO group than in OVX control sand slightly but significantly higher in the ISO-Ca group	1
10424 Liu et al., 2007	Rats (Wistar)	Sham control; OVX control; OVX-exercise; ISO-50: 50; ISO-50 + exercise	The authors reported that intervention consisted of soybean isoflavone with or without exercise	10	56	Weight (relative)	Relative uterine weight significantly increased in ISO-50 and ISO-50 + exercise compared with OVX controls	1
9712 Hong et al., 2009	Rats (Sprague–Dawley)	Sham control; OVX control; SP: 0.16 (aglycones); FSP: 0.43 (aglycones)	The authors reported that total isoflavone aglycone (daidzein and genistin) concentration was 3.45 mg/kg soy powder (SP). Authors report dose as 1.8 mg/kg feed expressed as aglycones. The intervention in the other group was fermented soy pulp (FSP). Total isoflavone aglycone (daidzein and genistin) concentration was 17.90 mg/kg FSSP powder. The authors reported the dose as 4.8 mg/kg feed expressed as aglycones. Doses calculated by the Panel by applying EFSA default value of 0.09 per sub-chronic studies in rats (EFSA Scientific Committee, 2012)	6	49	Weight (absolute)	No statistically significant changes for SP compared with OVX control. Slight but statistically significant increase in FSP compared with OVX control. The change was no longer significant when compared with SP	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
15146 Gallo et al., 2008	Rats (Sprague–Dawley)	Sham control; OVX control; SE-100: 16.8; E2	The authors reported that animals were administered 100 mg soy extract/kg/day. The extract used was a standardised extract containing 16.8 % isoflavones. Dose of total isoflavones calculated on the basis of the percentage reported by the authors. Animals in the positive E2 group were administered 17β-oestradiol, 0.5 mg/kg/day via gavage	10	42	Weight (relative to body weight)	No statistically significant changes compared with OVX controls	1
15145 Gallo et al., 2005	Rats (Sprague–Dawley)	Sham control; OVX control; SE-50: 7.5; SE-100: 15; E2	The authors reported that animals were administered 50 and 100 mg soy extract/kg/day (SE-50 and SE-100, respectively). The extract used was a standardised extract containing 16.8 % isoflavones. Dose calculated on the basis of the percentage reported in the company website (13–17 % isoflavones). Animals in the positive E2 group were administered 17β-oestradiol, 0.5 mg/kg/day via gavage.	11–12	42	Weight (dry, absolute)	No statistically significant changes for SE compared with OVX control	1
6198 Kikuchi-Hayakawa et al., 1998	Rats (Wistar)	Sham control; OVX control; SM: 19.6; FSM: 19.4	Authors report that total isoflavones concentration was 1 691 µg/g and 1 726 µg/g in the soy-milk (SM) powder and in the fermented soy milk (FSM) powder, respectively. The powders constituted 30 % of the diet of the animals. Dose estimated by the Panel on the basis of the information provided in the publication (i.e. mean food intake 13.1 g/day; mean initial weight of the animal, 339 g in the SM group and mean food intake 12.5 g/day; mean initial weight of the animal, 333 g in the FSM)	5	42	Weight	No statistically significant changes compared with OVX control in any of the treatment groups	1
16313 Yamaguchi et al., 2001	Rats (Wistar)	Intact control; OVX control; low ISO: 67 (aglycones); high ISO: 157 (aglycones); E2	Authors report that the extract used (SoyAct®) contained genistein, 52 %: daidzein, 42 %; and glycitein, 6 % as aglycones. Animals in the positive E2 group were administered 17β-oestradiol propionate 300 µg/kg s.c. once/week	5–10	30	Weight (wet, absolute)	No statistically significant changes compared with OVX control in the low-ISO group. Uterine weight in the high-ISO group was significantly increased	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
10162 Kishida et al., 2008. Expt. 4	Rats (Sprague–Dawley)	OVX control; ISO-300: 36; E2	The authors stated the animals were administered isoflavones 300 mg/kg diet. Authors report that the extract used contained genistein, 155 mg/g; daidzein, 127 mg/g; glycitein, 18 mg/g; genistin, daidzin and glycitin < 1 mg/g (ratio GEN:DAI:GLY = 1.2:1:0.1). Dose calculated using a default value of 0.12 for sub-acute studies in rats (EFSA Scientific Committee, 2012). Animals in the positive E2 groups were administered 17β-oestradiol, 4.2 µg/animal/day, s.c.	6	28	Weight (absolute)	No statistically significant changes compared with OVX control.	1
15337 Kakehashi et al., 2012. Expt. 1	Rats (Donryu)	OVX control; ISO: 150 (aglycones)	Rats were given 0.6 % IA-rich extract SoyAct®. The authors reported that the extract contained in total 30 % of soy phytoestrogens: genistein 52%; daidzein, 42%; and glycitein, 6 % (ratio GEN:DAI:GLY = 1.2:1:0.1)	5	14	Relative weight	Significantly increased compared with OVX control	1
9236 Zhao et al., 2011	**Mice** (129/C57BL/6)	Sham control; OVX control; phyto-β-SERM: 10; SE: 10	The phyto-β-SERM formulation contained equal part of genistein, daidzein and equol providing a total of 100 mg/kg diet of isoflavones. Animals in the other group were given a commercial soy extract (SE) product providing a total of 100 mg/kg diet of genistein/genistin, daidzein/daidzin and glycitein/glycitin. According to the authors the doses used were equivalent to a human dose of 50 mg/day	5–7	270	Wet weight (relative to % of phytoestrogen free diet)	No statistically significant changes compared with OVX control in any of the treatment groups.	1
4561 Zhang et al., 2009	Mice (C57BL/6J)	Sham control; OVX control; SE-2500: 200; GEN-500: 100; E2	The authors reported that animals were administered a commercial soy extract (Novasoy®, SE) at a dose of 2 500 mg/kg diet. The soy extract contained 40 % of isoflavones in the ratio of 1.3:1:03 of genistein: daidzein: glycitein. Animals in the E2 group were administered 17β-oestradiol 2 mg/kg diet corresponding to 0.4 mg/kg bw/day. Doses calculated using a default value of 0.2 for sub-chronic/sub-acute studies in mice (EFSA Scientific Committee, 2012)	10–12	35	Weight (relative)	No statistically significant changes compared with OVX control.	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
Soy protein isolate								
15120 Foth and Cline, 1998	**Monkeys** (*Macaca fascicularis*)	OVX control; SPI:9.9; E2; E2 + SPI	Authors stated that the dose administered to the animals corresponded to a human equivalent dose of 148 mg/day per woman, on an energy basis. Animals in the E2 groups were administered oestradiol in the diet. Authors stated that the dose administered to the animals corresponded to a human-equivalent dose of 1 mg/day per woman, on an energy basis. The Panel assumed that the same conversion factor applied by the same research group in the publication Wood et al., 2004 applies also to this case.	15	180	Weight	Uterine weight significantly increased only in E2 or E2 + SPI groups compared with OVX control or SPI control	1
10960 Bahr et al., 2005	**Rats** (Sprague–Dawley)	Intact control; OVX control; low SPI: 10.5 (aglycones); high SPI: 21.6 (aglycones); low SPIEx: 9.4 (aglycones); high SPIEx: 19.3 (aglycones); E2	The authors reported that animals in the SPI groups were administered SPI at 100 and at 200 g/kg diet (low SPI and high SPI, respectively). The SPI used contained 2.14 mg/g aglycone from isoflavones (genistein, 1.10 mg/g; daidzein, 0.84 mg/g and glycitein, 0.20 mg/g). Animals in the SPIEx groups were administered an alcoholic extract from SPI (SPIEx) at 17.2 and 34.4 g/kg diet (low SPIEx and high SPIEx, respectively). The SPIEx contained 11.37 mg/g aglycone from isoflavones (genistein, 7.09 mg/g; daidzein, 3.81 mg/g and glycitein, 0.47 mg/g). Daily doses estimated by the authors on the basis of food consumption. Animals in the positive E2 group were implanted s.c. with a silastic implant maintaining blood E2 concentrations of 30–40 pg/ml plasma (i.e. within normal cycling physiological range)	5–9	84	Weight (wet, relative)	No statistically significant changes compared with OVX control for any of the isoflavones-treated group	1
2803 Tansey et al., 1998	Rats (Sprague–Dawley)	OVX control; Sham control; low ISO: 1.5; high ISO: 15.7; sham-low-ISO: 1.5; sham-high-ISO: 15.7; low E2; high E2;	The authors reported the animals in the high-ISO groups were administered a soy bean extract (SBE) isolate (SUPRO 670) added at 15.40 g/100 g diet and providing 117.8 mg isoflavones/1 800 cal; animals in the low-ISO groups were administered an alcohol-extracted SPE isolate (SUPRO 670-IF)) added at 16.00 g/100 g diet and	8–12	60	Uterine weight	No statistically significant difference between animals in the soy-alone groups and controls	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
		low ISO + low E2; low ISO + high-E2; high ISO + low E2; high ISO + high E2	providing 11.6 mg isoflavones/1 800. The dose conversion has been calculated by the Panel assuming the daily caloric intake for a rat to be 60 cal/day and an average body weight of 250 g. Hence daily isoflavones intake would be 0.38 mg/day and 3.93 mg/day for the low-ISO and the high-ISO groups, respectively. These doses were converted assuming an animal body weight of 250 g into 0.04 mg/kg bw/day (low E2) and 0.08 mg/kg bw/day (high E2). Animals in the E2 groups were given daily doses of a pharmaceutical oestrogen preparation (conjugated equine oestrogens) equal to 0.313 mg/1 800 cal and 0.625 mg/1 800 cal. The conversion has been calculated assuming the daily caloric intake of a rat is 60 cal/day					
12095 Pan et al., 1999	Rats (Sprague–Dawley)	OVX control-young; OVX control-old; SE-young: 26; SE-old: 15; E2-young; E2-old	Old animals were retired breeders, 8–10 months at time of OVX and weighing 300–360 g; young animals were 2–3 months old at time of OVX and weighed 180–200 g. According to the authors, the isoflavone dose administered was equivalent to a woman's dose of 150 mg total isoflavones/day (150 mg/1 800 cal). Soy phytoestrogen concentrate contained 66 % isoflavones on a dry basis (genistein, 43 %; daidzein, 21 %; and glycitein, 2 %). Animals in the E2 group received an oestradiol dose which was considered by the authors to be equivalent to a woman's dose of 2 mg/day (2 mg/1 800 cal). Doses conversion calculated on the assumption that caloric intake of a rat is 60 cal/day	5	56	Absolute weight	No statistically significant changes compared with OVX control. No differences observed in relation with the age of the animals	1

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Daidzein-rich isoflavones								
5420 Lee et al., 2004	Rats (Sprague–Dawley)	Sham control; OVX control; SHE: 26; E2	The authors reported that animals were administered soy hypocotyl extract (SHE) providing isoflavones at 521.94 mg/kg diet. The authors report that 6.25 g of isoflavone extract contained: genistein 1.79 mg; daidzein 3.06 mg; glycitein, 8.36 mg; genistin, 57.63 mg; daidzin, 258.99 and glycitin 171.31 mg. Animals in the E2 group were administered 17β-oestradiol at a dose of 3.9 mg/kg diet (0.195 mg/kg bw/day). Dose calculated using the EFSA default value of 0.05 for chronic studies in rats (EFSA Scientific Committee, 2012).	6	112	Weight	No statistically significant changes compared with OVX control	1
1251, Baeza et al., 2009	Rats (Wistar)	OVX–control; SHE: 30; E2[e]	The authors reported that the animals were administered a commercial soy extract containing 9–11 % isoflavones (4–6 % daidzein, 2–4 % glycitein, 1–3 % genistein) at a dose of 300 mg/kg bw/day. Isoflavones dose calculated by the Panel assuming the extract contained 10 % of total isoflavones. Animals in the positive E2 group received oestradiol valerate 125 µg/week single dose s.c.	7–8	70	Absolute weight	No statistically significant changes compared with OVX control for animals treated with the soy extract	1
15224 Hidaka et al., 2003	Rats (Sprague–Dawley)	Sham control; OVX control; P40: 64–83; E2[f]	The product used was 'Fujiflavone P40' obtained from soybean seed and containing 46.63 % isoflavones (daidzin, 24.13 %, glycitin, 16.48 %; genistin, 5.93 %). On the basis of food consumption the Authors estimated a daily dose of isoflavones ranging from 64–83 mg/kg bw/day. Animals in the positive E2 group were administered 17β-oestradiol,10 µg/kg bw, 5 times/week dose s.c.	6	49	Absolute weight	Uterine weight in P40 group was significantly increased	1
12969 Carbonel et al., 2011b	Rats (Wistar EPM-1)	OVX control; low SE: 42 (genistein equivalent); medium SE: 125 (genistein equivalent); high SE: 250 (genistein equivalent)	The authors reported that the soy extract administered to the animals contained 42.6 % isoflavones, in a ratio of 0.6:1:0.03 of genistein: daidzein: glycitein.	10	30	Weight	No statistically significant changes in the low-SE group compared with OVX controls. Significantly increased in medium-SE 120 and high-SE compared with OVX control and SE 46	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
14919 Catania et al., 2002	Rats (Sprague–Dawley)	Sham control; OVX control; sham-SE: 2.1; OVX-SE: 2.1; sham-E2; OVX-E2	The authors reported that the soy extract used in the study was administered at a dose of 5 mg/kg/day. The extract was reported to contain 42.3 % isoflavones (genistein and genistin, 17 %; daidzein and daidzin, 22 %, glycitein and glycitin, 3.1 %). Dose calculated on the basis of the percentage given in the publication. Animals in the positive E2 groups were administered 17β-oestradiol 20 µg/kg, s.c.	6	28	Weight (% reduction vs. SHAM)	No statistically significant changes between SE treated animals vs. control	1
1618 Mosquette et al., 2007	Rats (Wistar)	OVX control; SE-10: 4.3; SE-50: 21.3; SE-100: 42.6; SE-300: 127.8; SE-600: 255.6; E2	Authors report that the soy extract used contained 42.6 % isoflavones. Approximately 36 % were genistein, 62 % were daidzein and 2 % glycitein (including isoflavones isoforms). The authors stated that the animals received doses of the soy extract of 10 mg/kg bw/day, 50 mg/kg bw/day, 100 mg/kg bw/day, 300 mg/kg bw/day and 600 mg/kg bw/day. The Panel calculated the dose of isoflavones provided by the extract on the basis of the percentage values given in the publication. Animals in the E2 group were administered conjugated equine oestrogens 200 µg/kg bw/day	8	21	Weight	No statistically significant differences between SE-10 and SE-50 compared with OVX control. Increased uterine weight in SE-100, SE-300 and SE-600 was observed. For the SE-300 (but not for SE-600) the observed effect was comparable to the result for the E2 group	1
14918 Castillo et al., 2006	Rats (Wistar	Young-sham control; old-OVX control; old-SE: 30; old-OVX-E2	The authors reported that the soy extract used in the study contained 9–11 % isoflavones (daidzin, 4–6 %; glycitin, 2–4 %; genistin 1–3 %) and was administered at a dose of 300 mg/kg bw/day. Isoflavones dose calculated on the basis of the percentage reported in the publication. Animals in the E2 group were administered oestradiol valerate 125 µg/week	6	70	Weight (relative)	No statistically significant difference between animals in soy extract and OVX control.	2

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
454 Francisco et al., 2013	Rats	OVX control; SE-46: 21.3; SE 120: 42.6; E2; SE 120 + E2	The authors reported that the soy extract used contained 42.6 % isoflavones, in a ratio of 0.6: 1: 0.03 of genistein: daidzein: glycitein, and was administered at the doses of 46 and 120 mg/kg bw/day (SE-46 and SE-120, respectively). The authors reported that the doses administered correspond to 21.3 mg and 42.6 mg of isoflavones for SE-46 and SE-120, respectively. The Panel however noted that, according to the% of isoflavones reported, the calculated dose of isoflavones would be 19.6 mg and 51.1 mg for SE-46 and SE-120, respectively. Animals in the E2 groups were administered conjugated equine oestrogens at a dose of 50 µg/kg bw/day	10	21	Weight	No statistically significant changes in SE 46 compared with control. Significantly increased in SE 120 and in SE 120 + E2 compared with control and SE 46	2
Red clover extract								
14737 Adaikan et al., 2009	**Rabbits** (New Zealand White)	Intact-control; OVX control; RCE: 0.1; DAI: 0.1 E2	Dose reported by the authors as daidzein equivalent. The authors reported that the animals were administered 6.68 mg/kg bw/day of a 0.6 % red clover extract. Animals in the E2 group were administered E2 valerate 0.1 mg/kg bw/day	6	84	Weight (absolute)	No statistically significant changes compared with OVX control	1
15353 Kawakita et al., 2009	**Rats** (Sprague–Dawley)	Sham control; OVX control; RCE: 2.4; RCE + BP: 2.4 + alkaline supplementation (16 mg)	The authors reported that animals were fed with 6 mg/kg/day food mixed with the red clover extract Menoflavon Forte® standardised to contain 40 % isoflavones by weight (genistein, daidzein, biochanin A and formononetin present as hydrolysed aglycones). Authors report that the dose chosen corresponds to a human dose of 240 mg/day. It is assumed that the dose is expressed as mg/kg bw/day. Isoflavone dose calculated on the basis of the percentage (40%) reported by the authors.	20	90	Weight	Animals in both RCE groups had increased uterine weight compared with OVX control. The weights were still 50 % less than sham control	1

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
4723 Overk et al., 2008	Rats (Sprague–Dawley)	OVX control; low RCE: 1.2; medium RCE: 12; high RCE: 120; E2; high RC + E2[g]	The authors reported that the extract used was standardised to a minimum of 30 % isoflavone content by weight of four isoflavones: genistein, 0.41%; daidzein, 0.23%; biochanin A, 14.47%; and formononetin, 14.26%, as hydrolysed aglycones. Animals were administered the extract at doses of 4, 40 and 400 mg/kg bw/day (low RCE, medium RCE and high RCE, respectively). The middle dose of 40 mg/kg bw/day was based on a clinical dose of 120 mg/day for a woman contained in 400 mg/day of *T. pratense* extract. Isoflavone dose calculated on the basis of the percentage (30%) reported by the authors. Animals in the positive E2 group were administered 17β-oestradiol 10 µg/rat/day	6–7[h]	21	Uterine weight	No statistically significant changes in any of the RCE-groups compared with OVX control	1
11684 Burdette et al., 2002	Rats (Sprague–Dawley)	OVX control; RCE-250: 37.5 (aglycones); RCE-500: 75; (aglycones); RCE-750: 112.5 (aglycones); E2; RCE-750 + E2	Authors report that animals were administered 250, 500 and 750 mg/kg/day of red clover extract standardised to a minimum 15 % isoflavone content by weight of four isoflavones genistein, 0.850%; daidzein, 0.349 %; biochanin A, 6.57%; and formononetin, 8.56 %, as hydrolysed aglycones. Dose of isoflavones calculated on the basis of the percentage (15%) reported by the authors. Animals in the E2 groups were administered 17β-oestradiol, 50 µg/kg bw/day	5–6	21	Uterine weight	Dose-dependent increase in uterine weight and thickness, however the response was significantly less than that observed in E2 treated rats. Concomitant administration of high-RCE and E2 neither stimulated nor antagonised the E2-induced changes in uterine weight	2

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Kudzu root (*Pueraria lobata*)								
19076 Dong et al., 2014	**Rats** (Sprague–Dawley)	Sham control; OVX control; PR-300: 56; E2[i]	The authors reported that animals were administered 300 mg/kg/day of a water extract of *Puerariae* radix (PR-300) containing 18.7 % of total isoflavones (the two main isoflavones puerarin and daidzin accounted for 9.7 % and 2.3 %, respectively). Isoflavones dose calculated on the basis of the percentage reported in the publication, assuming the dosing of the extract is expressed as mg/kg bw/day. Animals in the E2 group were administered 17β-oestradiol, injected i.p. at a weekly dose of 200 µg/kg in the diet	12	84	Uterine weight	Treatment with PR extract significantly increased (+ 75%) uterine weight compared with OVX control however to a lesser extent than E2 (+ 108%)	1
11449 Wang et al., 2003	**Mice** (ddY)	Sham control; OVX control; PR-5: 60; PR-10: 120; PR-20: 240; E2	Authors stated that the amounts of daidzein and genistein in Pueraria radix powder were quantified at 8.03 and 1.01 mg/g, respectively. They also speculated that overall daily intake of isoflavones in the PR-5, PR-10 and PR-20 groups was 1.8, 3.6 and 7.2 mg/day, respectively. Dose converted by dividing for the approximate mean weight of the animals (30 g), as reported in the publication. Animals in E2 group were administered oestradiolbenzoate 17β-oestradiol 0.03 µg/day s.c.	8	28	Uterine weight	No statistically significant changes compared with OVX control	1
4121 Cho et al., 2012	Mice (ICR)	Sham control; OVX control; IPL-200: 27; IPL-500: 68	The authors reported that animals were administered 200 and 500 mg/kg bw/day (IPL-200 and IPL-500, respectively) of an extract from *Pueraria lobata* containing 13.6 % of total isoflavones in the following proportion: puerarin, 7.5 %; daidzin, 4.2 %; genistin, 1.9 %. Isoflavones dose calculated on the basis of the percentage reported in the publication	5	28	Uterine weight	No statistically significant changes compared with OVX control	1

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
Genistein								
4863 Rimoldi et al., 2007	**Rats** (Sprague–Dawley)	OVX control; low GEN: 5.4; high GEN: 54; low E2; high E2	In the publication it is reported that the doses were estimated by the authors on the basis of the daily food intake. Animals in the E2 groups were administered oestradiolbenzoate. at 0.17 mg/kg bw/day (low-E2) and 0.7 mg/kg bw/day (high-E2)	11–12	90	Weight	High-GEN significantly increased uterine weight, but not low-GEN.	1
16299 Wuttke et al., 2006	Rats (Sprague–Dawley)	OVX control; GEN: 221; E2[j]	In the publication it is reported that the dose administered to the animals was 53 mg/day. The authors were contacted and clarified that animal weight was 230–250 g. Dose conversion calculated by the Panel on the basis of the information provided by the authors. Animals in the E2 group were administered 0.19 mg/day of oestrogen (free base)	10–12	90	Weight	Uterine weight significantly increased compared with OVX control, but to lesser extent than E2	1
13554 Mathey et al., 2007	Rats (Wistar)	Sham control; OVX control; GEN: 10; EQ: 10; DAI: 10; DAI-FOS: daidzein 10 + scFOS; DAI-L: daidzein 10 + *Lactobacillus casei*		10	90	Weight (relative to body weight)	No statistically significant changes compared with OVX control	1
16713 Phrakonkham et al., 2007	Rats (Wistar)	OVX control; GEN: 10; EQ: 10; DAI: 10		10	90	Weight (relative to 100 g BW)	No statistically significant changes compared with OVX control	1
11367 Li and Yu, 2003	Rats (Sprague–Dawley)	Sham control; OVX control; GEN: 45; E2	Animals in the E2 group were administered oestradiol 10 µg/kg once every 2 days	10	84	Weight (absolute and index)	No statistically significant changes compared with OVX control	1
1354 Wang et al., 2008	Rats (Sprague–Dawley)	Sham control; OVX control; GEN: 9; NO-GEN: 4.5; NO-GEN: 9; NO-GEN: 18	NO-releasing pro-drug of genistein. Doses are equimolar of genistein	8	84	Weight	Significant increase observed in the GEN group and in the NO-GEN 4.5 mg/kg/day (23.4 % and 26.6 % heavier than OVX control)	1

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
15219 Hertrampf et al., 2009	Rats (Wistar)	Sham control; OVX control; IRD: 28; GEN: 42; E2	Animals in the E2 group were administered oestradiol 2 µg/kg bw/day	7	84	Uterine weight (wet, absolute)	No statistically significant changes compared with OVX control	1
10284 Vera et al., 2007	Rats (SHR)	Sham control; OVX control; GEN: 10; E2	Animals in the E2 group were administered 17β-oestradiol 2 mg/kg bw/week s.c.	8	35	Weight (absolute)	No statistically significant changes in the genistein treated animals compared with OVX control	1
14841 Bitto et al., 2009	Rats (Wistar Kyoto)[k]	Intact control; OVX control; intact GEN: 4.8; OVX GEN: 4.8	Authors report that the dose administered was equivalent to a human-equivalent dose (HED) of 54 mg/day	10	28	Weight	No statistically significant changes in the genistein treated animals compared with OVX control in Wistar animals	1
2232 Ye et al., 2003. Expt.2	Rats (Wistar)	Intact control; OVX control; GEN: 25[l]		5	21	Weight	No statistically significant changes in the genistein treated animals compared with OVX control	1
15958 Santell et al., 1997. Expt.2	Rats (Sprague–Dawley)	OVX control; GEN 750: 90; E2; GEN 150 + E2: 18 + E2; GEN 375 + E2: 45 + E2; GEN 750 + E2: 90 + E2; baseline animals	The authors stated the animals were administered genistein 750 µg/g diet (alone) or genistein 150 µg/g, 375 µg/g and 750 µg/g in combination with oestradiol. Animals in the E2 groups were administered oestradiol 1 µg/g diet corresponding to 0.12 mg/kg bw/day. Dose conversion calculated using a default value of 0.12 for sub-acute studies in rats (EFSA Scientific Committee, 2012).	6 (10 baseline)	21	Weight	Significantly increased at all doses compared with OVX control and baseline animals. The effect of E2 was not affected by the concomitant treatment with GEN, at any dose.	1
15958 Santell et al., 1997. Expt.1	Rats (Sprague–Dawley)	OVX control; GEN-150: 18; GEN-375: 45; GEN-750: 90; E2-0.5; E2-1.0; E2-1.5; baseline animals	The authors stated the animals were administered genistein 150, 375 and 750 µg/g diet (GEN-150, GEN-375, GEN-750). Animals in the E2 groups were administered oestradiol at 0.5, 1.0 and 1.5 µg/g diet corresponding to 0.06, 0.12 and 0.18 mg/kg bw/day. Dose conversion calculated using a default value of 0.12 for sub-acute studies in rats (EFSA Scientific Committee, 2012).	6–8	5	Weight (wet, dry)	GEN produced a dose-dependent increase, significantly different from OVX control only for GEN-375 and GEN-750. GEN-750 animals were comparable with E2–1.0.	1

Ref. ID[a] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [b]
4038 Nguyen et al., 2013	**Mice** (C57BL/6J)	Intact-control; OVX control; GEN-0.01: 2; GEN-0.03: 6; GEN-0.1: 20; GEN-0.3: 60; GEN-1: 200; GEN-3: 600; GEN-10: 2000	The authors stated the animals were administered genistein at levels of 10 mg/kg diet, 30 mg/kg diet, 100 mg/kg diet, 300 mg/kg diet, 1 000 mg/kg diet, 3 000 mg/kg diet and 10 000 mg/kg diet. Dose conversion calculated by the Panel using a default value of 0.2 for sub-chronic/sub-acute studies in mice (EFSA Scientific Committee, 2012)	9–10	90	Weight (absolute, relative)	No statistically significant changes in uterus weight (both absolute and relative) compared with OVX control except for GEN-3 and GEN-10. Uterine weight in GEN-10 was significantly increased compared with intact-control.	1
4676 Chen et al., 2009	Mice (BALB/c)	Sham control; OVX control; GEN-2: 400; E2[m]	The authors stated the animals were administered genistein at 2 g/kg diet. Animals in the E2 group were administered 17β-oestradiol at 2 mg/kg diet (0.4 mg/kg bw/day) Dose conversion calculated by the Panel using a default value of 0.2 for sub-chronic/sub-acute studies in mice (EFSA Scientific Committee, 2012)	8–11	84	Weight	No statistically significant changes compared with OVX control.	1
4561 Zhang et al., 2009	Mice (C57BL/6J)	Sham control; OVX control; SE-2500: 200; GEN-500: 100; E2	The authors reported that animals were administered genistein at a dose of 500 mg/kg diet. Animals in the E2 group were administered 17β-oestradiol 2 mg/kg diet corresponding to 0.4 mg/kg bw/day. Doses calculated using a default value of 0.2 for sub-chronic/sub-acute studies in mice (EFSA Scientific Committee, 2012)	10–12	35	Weight (relative)	No statistically significant changes compared with OVX control	1
Genistin								
14256 Uesugi et al., 2001	**Rats** (Sprague–Dawley)	SHAM control; OVX control; low GEN: 50; high GEN: 100; low DAI: 25; high DAI: 50; low GLY: 25; high GLY: 50; E2	Animals in the E2 group were administered oestrone 7.5 µg/kg bw/day s.c.	6	28	Weight (relative to bw)	Significantly increased in high-GEN but to a much lesser extent than oestrone	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
Daidzein								
14737 Adaikan et al., 2009	**Rabbits** (New Zealand White)	Intact-control; OVX control; RCE: 0.1; DAI: 0.1 E2	Dose reported by the authors as daidzein equivalent. The authors reported that the animals were administered 6.68 mg/kg bw/day of a 0.6 % red clover extract. Animals in the E2 group were administered E2 valerate 0.1 mg/kg bw/day	6	84	Weight (absolute)	No statistically significant changes compared with OVX control	1
13554 Mathey et al., 2007	**Rats** (Wistar)	Sham control; OVX control; GEN: 10; EQ: 10; DAI: 10; DAI-FOS: daidzein 10 + scFOS; DAI-L: daidzein 10 + *Lactobacillus casei*		10	90	Weight (relative to body weight)	No statistically significant changes compared with OVX control in any of the daidzein group.	1
16713 Phrakonkham et al., 2007	Rats (Wistar)	OVX control; GEN: 10; EQ: 10; DAI:10		10	90	Weight (relative to 100 g BW)	No statistically significant changes compared with OVX control	1
16809 Rachoń et al., 2007b	Rats (Sprague–Dawley)	OVX control; low DAI: 22.5; high DAI: 90; low PUE: 54; high PUE: 270; low E2; high E2	The authors reported that the animals were administered daidzein at doses of 250 and 1 000 mg/kg diet (low DAI and high DAI, respectively). Dose calculated by the Panel using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012). Animals in the E2 groups were administered oestradiol-3-benzoate at doses of 4.3 and 17.3 mg/kg diet (low E2: 0.07 mg/day; high E2: 0.20 mg/day)	11–12	90	Weight (absolute, relative to body weight)	No statistically significant changes in low-DAI. Significantly increased in the high-DAI group but to a much lesser extent than E2 (~5-fold less than in the low-E2 group)	2
Daidzin								
14256 Uesugi et al., 2001	**Rats** (Sprague–Dawley)	SHAM control; OVX control; low GEN: 50; high GEN: 100; low DAI: 25; high DAI: 50; low GLY: 25; high GLY: 50; E2	Animals in the E2 group were administered oestrone 7.5 µg/kg bw/day s.c.	6	28	Weight (relative to body weight)	Significantly increased in high-DAI but to a much lesser extent than oestrone	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
Equol								
1714 Wood et al., 2006	**Monkeys** (*Macaca fascicularis*)	OVX control; ISO: 35.7; EQ: 68	Actual dose administered 1020 mg/1 800 kcal racemic equol. Dose conversion reported in the publication	10	28–33	Weight	No statistically significant difference among treatment groups	1
13554 Mathey et al., 2007	**Rats** (Wistar)	Sham control; OVX control; GEN: 10; EQ: 10; DAI: 10; DAI-FOS: daidzein 10 + scFOS; DAI-L: daidzein 10 + *Lactobacillus casei*		10	90	Weight (relative to body weight)	Slight but significant increase compared with OVX control	1
16713 Phrakonkham et al., 2007	Rats (Wistar)	OVX control; GEN: 10; EQ: 10; DAI: 10		10	90	Weight (relative to 100 g BW)	Significantly increased compared with OVX control	1
15876 Rachoń et al., 2007a	Rats (Sprague–Dawley)	OVX control; EQ-50: 4.5; EQ-400: 36; low E2; high E2	Actual doses administered 50 and 400 mg/kg chow of equol pure racemic mixture of equol (EQ-50 and EQ-400, respectively). On the basis of food intake, average consumption was 6.54 mg/day. In the E2 groups, actual doses administered 4.3 mg/kg and 17.3 mg/kg chow of oestradiol-3 benzoate for low E2 and high E2, respectively. On the basis of food intake, average consumption was 0.07 mg/day (low E2: 0.4 mg/kg bw/day) and 0.20 mg/day (high E2: 1.6 mg/kg bw/day). Doses calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012)	12	90	Absolute and relative (uterine/body) weight	No statistically significant difference between low-EQ and OVX control. Significantly increased in high-EQ compared with OVX control but to a much lesser extent than E2 (1.3-fold increase, high EQ; 6.3-fold, low E2; 6.8-fold, high-E2B). Comparable results for relative weight vs. OVX control (0.9-fold, low EQ; 1.5-fold, high EQ; 8.2-fold, low E2; 10.3-fold, high E2)	1

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
1203 Legette et al., 2009	Rats (Sprague–Dawley)	Sham control; OVX control; EQ-50: 4.5; EQ-100: 9; EQ-200: 18	Actual doses administered 50 mg/kg diet (EQ-50), 100 mg/kg diet (EQ-100) and 200 mg/kg diet (EQ-200) of equol powder (50 % *R*-equol, 50 % *S*-equol). Dose calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012). According to the authors the chosen doses correspond to human serum levels of equol of 3–39, and 5–49 µmol/l in response to dietary soy isoflavones of 56 and 90 mg/day (see Persky et al., 2002)	15–16	56 days	Weight	Significantly higher in OVX-200 than OVX control but still significantly less than in SHAM.	1
16333 Yoneda et al., 2011	Rats (Sprague–Dawley)	Sham control; OVX control; *S*-EQ: 11.7; SE5-OH: 11.7; E2	SE5-OH is described as a fermented soy germ containing *S*-equol. The dose is expressed as *S*-equol equivalent. Animals in the E2 group were administered CEE 6 mg/kg/day	5–15	38	Weight (absolute and relative to body weight)	No statistically significant changes compared with OVX control in either of the *S*-equol treated groups.	1
Glycitin								
14256 Uesugi et al., 2001	Rats (Sprague–Dawley)	SHAM control; OVX control; low GEN: 50; high GEN: 100; low DAI: 25; high DAI: 50; low GLY: 25; high GLY: 50; E2	Animals in the E2 group were administered oestrone 7.5 µg/kg bw/day s.c.	6	28	Weight (relative to bw)	Significantly increased in high-GLY but to a much lesser extent than oestrone	1
Puerarin								
16809 Rachoń et al., 2007b	Rats (Sprague–Dawley)	OVX control; low DAI: 22.5; high DAI: 90; low PUE: 54; high PUE: 270; low E2; high E2	The authors reported that the animals were administered puerarin (PUE) at doses of 600 mg/kg diet (low PUE) and 3 000 mg/kg diet (high PUE). Dose calculated by the Panel using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012). Animals in the E2 groups were administered oestradiol-3-benzoate at doses of 4.3 mg/kg diet (0.07 mg/day; low E2) and 17.3 mg/kg diet (0.20 mg/day, high E2)	11–12	90	Weight (absolute, relative to body weight)	No statistically significant changes in low PUE. Significantly increased in high-PUE but to a much lesser extent than E2 (~4-fold less than low-E2)	2

Ref. ID[(a)] Author, year	Animal species	Treatment groups and isoflavone dose(s) (mg/kg bw/day)	Description of intervention	No of animals/ group	Duration (days)	Measured endpoint	Results	Tier of reliability [(b)]
Biochanin A								
12777 Su et al., 2013	Rats (Sprague–Dawley)	SHAM control; OVX control; BCA: 25 E2	Animals in the E2 group were administered 17β-oestradiol 23 µg/kg bw/day i.p.	10	98	Weight	No statistically significant difference between animals treated with biochanin A and OVX control	1
Formononetin								
15689 Mu et al., 2009	Mice (Kunming)	Sham control; OVX control; low FOR: 50; high FOR: 500; E2	Animals in the E2 group were administered diethylstilboestrol (DES) at a dose of 0.20 mg/kg bw	10	180	Weight (relative to body weight)	Significantly increased in low-FOR compared with OVX control. No statistically significant difference between high-FOR and OVX control	1

Text in bold indicates different animal species in which the studies were conducted. Text in grey indicates differents isoflavones being tested in the same study and refers to other rows in the table.
(a): Refers to the Ref. ID number in Distiller.
(b): See protocol in Annex A for appraisal of risk of bias.
(c): An additional group was treated with genistein 10 mg/kg bw/day s.c. Data not presented in this table.
(d): An additional group of animals received calcium at 2.5 % (data not reported in this table).
(e): Additional groups of animals treated with recombinant growth hormone and melatonin. Data not presented in this table.
(f): An additional group of animals was sham treated with the vehicle used for s.c. administration of 17β-oestradiol. These data are not presented in this table.
(g): Additional groups of animals treated with *Humulus lupulus* extract, isoxanthohumol and 8-prenylnaringenin (data not presented in this table).
(h): Two animals in the high-RCE group and two animals in the high *Humulus lupulus* groups were euthanised owing to morbidity caused by gavage.
(i): Additional groups treated with a water extract of *Fructus Ligustri Lucidi* and with a combination of the two botanical extracts not shown in this table.
(j): Additional group treated with *Cimicifuga racemosa* extract 133 mg/day. Data not presented in this table.
(k): Additional groups of SHROB rats treated. Results not shown in this table.
(l): An additional group of animal was administered daidzein, 25 mg/kg bw/day. Data not included in this review because the number of animal in the group was less than 5.
(m): Additional group of animals treated with *Dioscorea alata* (Yam) extract 630 g/kg diet. Data not presented in this table.

Discussion on results from animal studies reporting effects on uterus

Because of the oestrogenic activity of isoflavones, changes in several uterine parameters have been evaluated in animal models treated with isoflavones. Most of the studies included were primarily designed to investigate other parameters, e.g. effects on the bone or on the cardiovascular system: in these studies uterine weight was also determined as an ancillary endpoint or marker of oestrogenic activity. None of the studies was designed as a standard uterotrophic assay according to OECD TG 440 (2007).

1) The effects of administration of isoflavones on uterine cell proliferation have been investigated in monkeys, rats and mice. Isoflavones obtained from soy extract or SPI did not modulate proliferation of epithelial cells in the uterus of monkeys, rats or mice. A daidzin-rich soy extract, however, at doses above 40 mg/kg bw/day, caused an increase in cell proliferation of the epithelium and the stroma of the uterus as well as the vaginal epithelium of rats. Interestingly, racemic equol administered to rats at high doses induced an increase in proliferation of stromal cells of the uterus, whereas an equivocal effect was observed on proliferation of the epithelial cells of the uterus.
2) In monkeys and rats, isoflavones obtained from soy extracts or SPIs did not result in significant changes in endometrial thickness, endometrial hyperplasia, epithelial area or endometrial gland area. In contrast, daidzein-rich soy extract and red clover extracts used as sources of isoflavones administered to rats at high doses (≥ 125 mg/kg bw/day) caused a significant increase in endometrial area, endometrial thickness, number of glands and myometrial area. Individual isoflavones such as genistein, daidzein and equol showed different effects. Genistein and daidzein did not induce histopathological changes such as hypertrophy, hyperplasia or squamous metaplasia in the uterus, whereas rats treated with racemic equol at doses of 10 mg/kg bw/day or higher for 90 days demonstrated a significant increase in uterine wall thickness and section surface. Rats treated with the same dose for 35 days did not show these histopathological changes.
3) In mice, isoflavones obtained from a soy extract or from a formulation containing equal parts genistein, daidzein and equol did not have a significant effect on uterine weight, whereas genistein alone at doses of 600 mg/kg bw/day and higher induced an increase in uterine weight of mice. In rabbits, isoflavones from a red clover extract and daidzein alone did have an effect on uterine weight.

In rats, isoflavones at doses between 10 and 100 mg/kg bw/day consistently caused an increase in uterine weight. This effect appeared to be dependent on the duration of the exposure. Although the results from isoflavone treatments are not completely consistent across all species, experimental designs and endpoints, the data show that sufficiently high doses of isoflavones and racemic equol can elicit uterotrophic effects in animals. Concerning the latter, it should be noted that *S*-equol does not act as does the racemic mixture of synthetic R- and *S*-equol (Shinkaruk et al., 2010).

3.3.3. Weight of evidence for effects on uterus

As previously described in section 3.2.3, an overview is given on the studies, their reliability (assessed according to the protocol), their relevance for the risk assessment in humans and the direction of the outcome in order to facilitate weight of evidence assessment.

For the endpoint uterus the reliability score of human studies varied between 1 and 3. Out of the 35 included studies, 34 did not indicate a difference from control, irrespective of the tested isoflavone. In one study (Unfer et al., 2004), no difference was seen compared with placebo after 2.5 years. After 5 years, in 6 out of 154 subjects hyperplastic lesions, but no cancerous lesions, were observed.

The effect of isoflavones on uterine weight in OVX animals was assessed in the current systematic review, but not included in the weight of evidence analysis. This is because the Panel considered that the effects observed are indicative of oestrogenic properties, as indicated in the OECD Guideline TG 440 (OECD, 2007).

In the 37 animal studies, there were no differences between treatments groups and placebo groups with the exception of two studies of racemic equol, in which in doses of 10 mg/kg bw/day and above

resulted in hyperplasia but no cancerous lesions were observed. There were indications that red clover extract (3.2 mg/kg bw/day) may also cause hyperplasia but no cancerous lesions.

From the weight of evidence approach, it can be concluded that isoflavones may have some hyperplastic effects, indicating oestrogenic activity, but no adverse effects were reported. It should, however, be noted that this evaluation is restricted to the tested doses of the tested isoflavones.

The outcome of this analysis is summarised in Table 20 and Table 21.

Table 20: Carcinogenicity/proliferation in the uterus: summary of human data

Endpoint/intervention	Reference	Reliability	Relevance	outcome
Endometrial thickness				
Soy isoflavones/soy extract	Chilibeck et al., 2013	1	+++	⇔
	Colacurci et al., 2013	1	+++	⇔
	Nahas et al., 2007	1	+++	⇔
	Alekel et al., 2015	2	+++	⇔
	Kaari et al., 2006	2	+++	⇔
	Han et al., 2002	2	+++	⇔
	Cheng et al., 2007	2	+++	⇔
	Upmalis et al., 2000	2	+++	⇔
Soy protein	Quaas et al., 2013	1	+++	⇔
	Carmignani et al., 2010	1	+++	⇔
	Murray et al., 2003	3	+++	⇔
Daidzein-rich isoflavones	Penotti et al., 2003	1	+++	⇔
	Steinberg et al., 2011	2	+++	⇔
Glycitein-rich isoflavones	Nikander et al., 2005	1	+++	⇔
Genistein	Marini et al., 2007	1	+++	⇔
	Marini et al., 2008	1	+++	⇔
	Lappe et al., 2013	1	+++	⇔
	Morabito et al., 2002	2	+++	⇔
	Irace et al., 2013	2	+++	⇔
	Evans et al., 2011	2	+++	⇔
	Sammartino et al., 2003	3	+++	⇔
Red clover extract	Geller et al., 2009		+++	⇔
	Powles et al., 2008	2	+++	⇔
	Imhof et al., 2006	2	+++	⇩
	Hale et al., 2001	2	+++	⇔
Histo(patho)logical changes				
Soy isoflavones/soy extract	Unfer et al., 2004	1	+++	⇔ after 2.5 years ⇧ hyperplasia after 5 years in 6/154 subjects. No cancerous lesions
	Kaari et al., 2006	2	+++	⇔
	Cheng et al., 2007	2	+++	⇔
Soy protein	Quaas et al., 2013	1	+++	⇔
	Duncan et al., 1999	2	+++	⇔
	Murray et al., 2003	2	+++	⇧ no inactive group
Daidzein-rich isoflavones	Steinberg et al., 2011	2	+++	⇔
Glycitein-rich isoflavones	Nikander et al., 2005	2	+++	⇔
Red clover extract (RCE)	Hale et al., 2001	2	+++	⇔

Table 21: Carcinogenicity/proliferation in the uterus: summary of animal data

Endpoint/ intervention	Reference	Animal species	Reliability	Relevance	outcome
Cell proliferation/Proliferation marker					
Soy isoflavones, soy extract	Wood et al., 2006	Monkeys (*Macaca fascicularis*)[(a)]	1	++	⇔
	Gallo et al., 2008	Rats (Sprague–Dawley)	1	+	⇔
	Zhao et al., 2011	Mice (129/C57BL/6)	1	+	⇔
Soy protein isolates	Wood et al., 2004	Monkeys (*Macaca fascicularis*)[(a)]	1	++	⇔
	Foth and Cline, 1998	Monkeys (*Macaca fascicularis*)	1	++	⇔
	Bahr et al., 2005	Rats (Sprague–Dawley)		+	⇔
	Tansey et al., 1998	Rats (Sprague–Dawley)	1	+	⇔
Daidzein-rich isoflavones	Mosquette et al., 2007	Rats (Wistar)	1	+	⇧ PCNA expression in SE-100, SE-300 and SE-600
	Carbonel et al., 2011a	Rats	1	+	⇔
Red Clover extract	Alves et al., 2008	Rats (Wistar)	1	+	⇔
Equol (racemic)	Wood et al., 2006	Monkeys (*Macaca fascicularis*)	1	++	⇔
	15876 Rachoń et al., 2007a	Rats (Sprague–Dawley)	1	+	⇔
	1203 Legette et al., 2009	Rats (Sprague–Dawley)	1	+	⇔
Histopathology					
Soy isoflavones/ soy extract	Wood et al., 2006	Monkeys (*Macaca fascicularis*)	1	++	⇔
	Gallo et al., 2008	Rats (Sprague–Dawley)	1	+	⇔
	15145 Gallo et al., 2005	Rats (Sprague–Dawley)	1	+	⇔
	16112 Teixeira et al., 2014	Rats (Wistar)	1	+	⇔
Soy protein isolates	4730 Scott et al., 2008	Monkeys (*Macaca fascicularis*)[(a)]	1	++	⇔
	16271 Wood et al., 2004	Monkeys (*Macaca fascicularis*)[(a)]	1	++	⇔
	15120 Foth and Cline, 1998	Monkeys (*Macaca fascicularis*)	1	++	⇔
	10960 Bahr et al., 2005	Rats (Sprague–Dawley)		+	⇔
	2803 Tansey et al., 1998	Rats (Sprague–Dawley)	1	+	⇔
Daidzein-rich isoflavones	12969 Carbonel et al., 2011b	Rats (Wistar) EPM-1	1	+	⇔
	1618 Mosquette et al., 2007	Rats (Wistar)	1	+	⇔
	454 Francisco et al., 2013	Rats	2	+	⇔

Endpoint/ intervention	Reference	Animal species	Reliability	Relevance	outcome
Red clover extract	19187 Kang et al., 2015	Rats (Sprague–Dawley)	1	+	⇧ Total thickness, epithelium thickness, mucosa thickness. Uterine gland (%) were significantly increased compared with OVX control, albeit different from SHAM control at the dose of 3.2 mg/kg bw/day of isoflavones.
	14764 Alves et al., 2008	Rats (Wistar)	1	+	⇔
	11684 Burdette et al., 2002	Rats (Sprague–Dawley)	2	+	⇔
Genistein	4863 Rimoldi et al., 2007	Rats (Sprague–Dawley)	1	+	⇔ Low GEN ⇧ High GEN: endometrial cells hypertrophy observed in 12/12 animals but no pathological signs detected (no squamous metaplasia, hyperplastic/hypertrophic glands, cystic glands and pyometra)
	16713 Phrakonkham et al., 2007	Rats (Wistar)	1	+	⇔
	11367 Li and Yu, 2003	Rats (Sprague–Dawley)	1	+	⇔
Daidzein	16713 Phrakonkham et al., 2007	Rats (Wistar)	1		⇔
Equol (racemic)	1714 Wood et al., 2006	Monkeys (*Macaca fascicularis*)	1	++	⇔
	15876 Rachoń et al., 2007a	Rats (Sprague–Dawley)	1	+	⇔ Low EQ (4.5 mg/kg bw/day) ⇧ High EQ (36 mg/kg bw/day): animals had significantly greater endometrial epithelium height, thickness of the stroma and myometrium
	16713 Phrakonkham et al., 2007	Rats (Wistar)	1	+	⇧ EQ (10 mg/kg bw/day) Uterine wall thickness and section surface were significantly increased compared with OVX control
	16333 Yoneda et al., 2011	Rats (Sprague–Dawley)	1	+	⇔

(a): Half of the monkeys had been previously treated with oral contraceptives over a 26-month period before OVX.

3.4. Effects on thyroid

3.4.1. Results from human studies

The intervention studies shown in Table 22 were identified as relevant for the risk assessment of isoflavones food supplements and their effects on the thyroid in peri- and post-menopausal women.

No observational study was found that investigated an association between intake of isoflavones from food supplements and risk of thyroid cancer in the target population.

Additionally, information on reported adverse events occurring in the clinical trials included in this review has been collected and assessed and is reported in Table C3 in Appendix C.

Endpoint: changes in thyroid hormone levels

The effect of the intake of isoflavones from food supplements on circulating thyroid hormone levels have been investigated in several controlled trials.

A total of 11 studies fulfilling the pre-defined criteria for inclusion in the review were considered for the assessment and are presented in Table 22.

Only serum TSH was measured in all 11 studies, T_4 and/or fT_4 was assayed in eight studies, T_3 and/or fT_3 was assayed in five studies, thyroid autoantibodies in two studies and thyroxine-binding globulin (TBG) or thyroglobulin in one study.

Nine studies did not detect significant differences in the endpoints measured.

Only in the two studies in which soy protein was administered (Duncan et al., 1999; Persky et al., 2002) were some changes in the thyroid endpoints considered for this assessment reported, although they were clinically insignificant.

In one study (Persky et al., 2002), T_4 concentrations in the low-ISO group (56 mg/day isoflavones aglycones) were significantly higher when averaged across time (102.1 nmol/l at 0 months, 100.9 nmol/l at 3 months, 104.3 nmol/l at 6 months) compared with the control (93.3 nmol/l at 0 months, 86.4 nmol/l at 3 months, 89.4 nmol/l at 6 months). Similar findings were reported for FTI in the low-ISO group (2.44 % at 0 months, 2.40 at 3 months, 2.52 at 6 months) compared with the control (2.29 % at 0 months, 2.13 at 3 months, 2.24 at 6 months); TSH was significantly increased (2.04 mU/l at 0 months, 2.39 mU/l at 3 months, 2.22 mU/l at 6 months) when averaged across time in the high-ISO group (90 mg/day isoflavones aglycones) compared with the control (2.25 mU/l at 0 months, 2.23 mU/l at 3 months, 2.00 mU/l at 6 months). In the high-ISO group, T_3 was also increased, although the group × time interaction reached statistical significance only at 6 months (1.75 nmol/l at 0 months, 1.66 nmol/l at 3 months, 1.80 nmol/l at 6 months in the high-ISO group; 1.72 nmol/l at 0 months, 1.65 at 3 months, 1.66 at 6 months in the control). The magnitude of these effects was not clinically relevant.

In the study by Duncan et al. (1999) a modest effect of isoflavones consumption on TBG was reported only in the low-dose group (65 ± 11 mg isoflavones/day) (TBG, 600.4 nmol/l versus 559.3 nmol/l in the control group). Non-significant changes in the other measured parameters (total T_4, fT_4, total T_3, fT_3 and TSH) were observed.

Table 22: Overview of human studies reporting effects on thyroid and considered for the risk assessment

Ref. ID[a] Author, year	Design	Duration (months)	Isoflavone dose (mg/day)[b]	Description of intervention	Active group (N)/control group (N)[c]	Age [d]	Time since menopause (years) and/or age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
Soy isoflavones/soy extract										
14945 Alekel et al., 2015	RCT, DB	36	80; 120	Authors report that formulated versus mean (SD) actual doses in tablets were: control, 0 vs. 0.3 mg ± 0.4 mg; 80 mg, 89.5 ± 5.0 mg vs. 84.3 ± 4.5 mg; 120 mg, 124.0 ± 7,7 vs. 122.5 ± 3.4 mg. Ratio of GEN:DAI:GLY was 1.3:1:0.3	Low-ISO: 87/77; high-ISO: 85/73; placebo: 83/74	Low ISO: 54.3 (45.8–61.4); high ISO: 54.7 (46.5–62.0); placebo: 54.2 (45.8–65.0)	Low ISO: 3.0 (0.9–10.0), 50 (44.0–60.0); high ISO: 2.8 (1.0–8.0), 51.0 (41.0–59.0); placebo: 2.7 (0.8–7.9), 51.0 (42.0–57.0)	No	No statistically significant changes at any time point between groups in TSH values and in free thyroxine levels. Free thyroxine was only measured in those samples that revealed abnormal TSH values at each time point	1
16411 Levis et al., 2011	RCT, DB	24	200	Authors report that each daily dose of the intervention (Novasoy®) contained 91 mg genistein and 103 mg daidzein (ratio GEN:DAI=0.9:1)	ISO: 122/96/93; Placebo: 126/81/75	ISO: 53 (3.3); placebo: 52 (3.3)	See footnote[f]	No	No statistically significant changes in TSH levels. No statistically significant changes in the number of women with positive thyroid peroxidase auto-antibodies.	2
14889 Bruce et al., 2003	RCT, DB	6	90 (aglycones)	The authors reported that the isoflavone supplement used (Novasoy®) contained 50 mg/serving (approximately 30 mg expressed as aglycone weight)	ISO: 22; placebo: 16	ISO: 68.9 (1.03); placebo: 69.9 (0.98)	Not reported	Yes	No statistically significant changes in TSH, T_4 and T_3 levels between groups and over time	2
3185 Ryan-Borchers et al., 2008	RCT, DB	4	70; 71.6 ± 3.1	Authors state that one of the treatment arm (ISO) was given an isoflavone supplement (70 mg/day) formulated and verified to match composition of 706 ml/day of soy milk administered to the other treatment arm (SOY)	ISO: 24; SOY:26; placebo: 27	ISO:54.8 ± 0.7; SOY: 55.8 ± 0.9; placebo: 55.7 ± 0.8	ISO: 6.0 ± 1.0; SOY: 7.7 ± 1.3; placebo: 10.1 ± 1.7	Yes	No statistically significant changes in TSH levels	2

Ref. ID[a] Author, year	Design	Duration (months)	Isoflavone dose (mg/day)[b]	Description of intervention	Active group (N)/control group (N)[c]	Age [d]	Time since menopause (years) and/or age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
3288 Pop et al., 2008	RCT, DB	3	900 (aglycones)	The authors described the intervention (PTI G-2535) as hard gelatin capsules that contained ≥ 70 % active substance as total unconjugated isoflavones unconjugated Isoflavones. Each capsule contained 150 mg genistein activity. The composition of PTI G-2535 capsules (Lot # UPM 9809–021) used in this clinical study was as follows: genistein 139.5 mg per capsule, daidzein 74 mg per capsule and glycitein 11 mg per capsule	ISO: 18; placebo: 12	ISO: 56.8; placebo: 53.5	Not reported	No	No statistically significant change in the mean difference from baseline for T_4, TSH, T_3 uptake, and FTI when comparing active and placebo groups	2
Soy protein										
3193 Persky et al., 2002	RCT	6	56; 90	The test protein was incorporated at 40 mg/day into baked products, ready-to-mix beverages and soups, providing moderate (56 mg/day) or higher (90 mg/day) doses of isoflavones	Low ISO: 24; high ISO: 23 placebo: 26	Low ISO: 59.3 high ISO: 61.9 placebo: 61.0	Not reported	Yes	When averaged across 3 and 6 months, T_4 concentrations and the FTI were significantly higher in the low-ISO group compared with control and TSH was significantly increased in the high-ISO group compared with control. In the high-ISO group T_3 was also increased, however the increase reached statistical significance only at 6 months. The magnitude of the effect was small and unlikely to be clinically relevant	1
3179 Duncan et al., 1999	C-O, RCT, DB	3	65 ± 11; 132 ± 22 (aglycones)	Placebo contained a minimal (10-fold less than low-ISO group) amount of isoflavones estimated to be 7,1 ± 1,1 mg/day	low ISO: 17; high ISO: 18; placebo: 18	56.9 ± 5.8	7.6 ± 4.7	Yes	Significant effect of isoflavones consumption was noted on TBG, albeit modest and with no apparent dose response. No statistically significant changes were observed for the other measured parameters (total T_4, free T_4 (fT_4), total T_3, fT_3 and TSH)	1

Ref. ID[a] Author, year	Design	Duration (months)	Isoflavone dose (mg/day)[b]	Description of intervention	Active group (N)/control group (N)[c]	Age [d]	Time since menopause (years) and/or age at menopause (years)[d]	Primary endpoint?	Results	Tier of reliability [e]
Daidzein-rich isoflavones										
4366 Steinberg et al., 2011	RCT, DB	24	80; 120 (aglycones)	The authors reported that the tablets used in the study had an isoflavone content ranging from 34.10 mg to 40.51 mg aglycone equivalent of total isoflavones (daidzein, 17.87–22.01; genistein, 4.96–5.00; glycitein, 11.22–13.54; ratio GEN:DAI:GLY= 0.4:1:0.2), with the majority (> 95 %) in the form of glycosides	Low ISO: 135/122/119; high ISO: 134/123/117; placebo: 134/128/126	Low ISO: 54.9 ± 4.0; high ISO: 54.5 ± 4.1; placebo: 55.0 ± 3.7	Low ISO: 48.5 ± 5.5; high ISO: 47.6 ± 6.3; placebo: 48.2 ± 5.1	No	No statistically significant changes between groups at any time point (baseline, 1 year, 2 years) in the values of TSH and fT_4	1
3195 Khaodhiar et al., 2008	RCT, DB	3	40; 60 (aglycones)	The authors described the intervention as a concentrated isoflavone product prepared from soybean germ fermentation with Koji fungus (*Aspergillus awamori*). The ratio GEN:DAI:GLY was 0.1:1:0.3	Low ISO: 48; high ISO: 49; placebo: 45	Low ISO: 52.2 ± 4.8; high ISO: 53.2 ± 5.6; placebo: 53.8 ± 5.1	Low ISO: 4.0 ± 4.9; high ISO: 5.8 ± 6.1; placebo: 5.6 ± 5.9	No	No statistically significant changes between groups in the levels of TSH, thyroglobulin, fT3, fT_4, total T_3 and total T_4 at any time point (4 weeks, 8 weeks, 12 weeks)	2
Genistein										
16393 Bitto et al., 2010	RCT, DB	36	54		GEN: 71/40; placebo: 67/37	See Marini et al., 2008[g]	Not reported	Sub-cohort of larger trial	No statistically significant difference between the two groups in TSH, fT_3 and fT_4 levels and the values did not significantly change over time in either of the two groups. No significant alteration of thyroid specific antibodies was observed during the 3-year period in either genistein or placebo groups	1

Ref. ID[(a)] Author, year	Design	Duration (months)	Isoflavone dose (mg/day)[(b)]	Description of intervention	Active group (N)/control group (N)[(c)]	Age [(d)]	Time since menopause (years) and/or age at menopause (years)[(d)]	Primary endpoint?	Results	Tier of reliability [(e)]
Red clover extract										
19610 Geller et al., 2009	RCT, DB	12	120[(h)]	Ethanolic extract of aerial parts of red clover providing 57.5 mg biochanin A, 56.6 mg formononetin, 1.6 mg genistein, 0.9 mg daidzein. Women in the E2 arm were given conjugated equine oestrogen and medroxyprogesterone acetate	RCE:22; placebo: 22; E2: 23	RCE: 52.4 (4.6); Placebo: 52.0 (4.2); E2: 53.3 (4.0)	RCE: 4.1 (2.8); Placebo: 2.8 (2.9); E2: 3.6 (2.9)	No	No statistically significant difference between any of the groups in TSH levels after 12 months of treatment	2

FTI: free thyroxine index
(a): Refers to the Ref. ID number in Distiller.
(b): Unless otherwise specified doses are expressed as mg isoflavones/day.
(c): Allocated to treatment/completed.
(d): Mean ± SD or SEM, as reported in the publication.
(e): See protocol in Annex for appraisal of risk of bias.
(f): According to the inclusion criteria, in order to qualify women had to be without menses for more than 12 months but less than 5 years or absence of menses for 6 to 12 months and FSH> 40 IU/l.
(g): The age of the study population is not reported in the full text. Authors state the women enrolled were a sub-cohort of post-menopausal women described in another publication (Marini et al., 2008) also included in this assessment.
(h): Data from an additional group treated with black cohosh extract not presented in this table.

Discussion on results from human studies reporting effects on thyroid

Based on the criteria applied throughout the assessment process, 11 randomised controlled studies in humans that reported effects of isoflavones administration on some thyroid-related endpoints were selected.

The duration of the studies ranged from 3 months (inclusion criterion for this review) to 3 years (one single study on genistein).

In total, 925 patients were allocated to the intervention with isoflavones and 576 served as controls.

The interventions administered in the studies were food supplements containing soy isoflavones, soy extract, soy protein, daidzein-rich isoflavones, genistein alone and red clover extract. No studies were retrieved in which the intervention was food supplements containing kudzu root.

The doses used in the studies ranged 40–200 mg/day, with one study using a significantly higher dose (900 mg isoflavones/day), albeit for 3 months and in a limited number of participants.

The Panel noted that the primary endpoints in most studies included in this review were not related to thyroid function. As a consequence, the sample sizes were mainly determined on the basis of expected changes in other endpoints, rather than thyroid function. Even studies in which thyroid parameters were the primary focus were not adequately powered to detect clinically significant changes, and sample size calculations are not always provided in methodological description of the studies.

The criteria used to select subjects for inclusion in the studies do not allow adequate characterisation of the participants in terms of the variables that properly define their thyroid status (thyroid volume, occurrence of partial functional autonomy, thyroid autoimmunity, concomitant use of drugs that interfere with thyroid function, timing of blood sampling). Additionally, in most of the studies included in the current review the presence of chronic conditions, such as thyroid diseases, was an exclusion criterion.

The Panel concluded on the basis of the studies included in this review that intake of food supplements containing isoflavones is not associated with clinically relevant changes in thyroid function (hypo- or hyperthyroidism) in the population of interest. However, the many confounding factors that increase the variability of results may have prevented the detection of minor effects. These effects (that could potentially become clinically relevant in the long term) might be unravelled if an adequate number of subjects, properly characterised and adequately matched to controls, were to be studied.

At the end of the systematic review process no sub-populations with specific sensitivity could be identified. The Panel was aware of a study published by Sathyapalan et al. (2011) that does not meet the inclusion criteria for this systematic review (male and female subjects included in the study, but data not reported separately) but which suggested a possible effect of isoflavones supplementation on the risk of progression to overt hypothyroidism in a sub-group of the population with slightly elevated TSH levels.

The Panel is aware that an updated statement from UK COT on possible effects of soy phytoestrogen intake on thyroid hormone levels was under discussion at the time of writing this opinion.

In the process of assessing information for this opinion, the Panel became aware of the possible interaction between isoflavone-containing food and food supplements and medicinal products. Specifically, in subjects treated with medicinal products containing levothyroxine as the active ingredient and indicated for the treatment of hypothyroidism, isoflavones could result in impaired absorption of levothyroxine. Since levothyroxine is a drug with a narrow therapeutic margin, impaired absorption could affect its effectiveness in the treatment of hypothyroidism.

3.4.2. Results from animal studies

At the end of the systematic review process no study was found that fulfilled all the inclusion criteria set in the protocol.

One study identified reported the effects of isoflavones administration on some thyroid-related endpoints. In this study, OVX monkeys (*Macaca fascicularis*) were administered a SPI providing a dose of 9.3 mg isoflavones/kg bw/day, stated by the authors to be equivalent to a human dose of

140 mg/day of isoflavones aglycones. There were no statistically significant changes in the levels of T_3 or TSH in the treated animals compared with OVX controls. However, treatment with isoflavones preceded ovariectomy in the animals, so that these animals were intact when the treatment started (Silverstein et al., 2014).

Discussion on results from animal studies reporting effects on thyroid

The criteria applied for the selection of studies to be included in this systematic review limited inclusion to those studies conducted in OVX animals. However, the effect on the thyroid may not be mediated by oestrogen and, therefore, limiting inclusion to OVX animals could have excluded relevant studies on the thyroid. The Panel acknowledged that the potential effects of isoflavones on thyroid endpoints may have been investigated in other animal models, such as juvenile or non-OVX animals. However, the differences in thyroid physiology between the rat and the human make extrapolation from rodents to humans problematic (see, for example, explanation reported in the publication by Silverstein et al. (2014): '*Studies using animal models can control for many of the environmental variables thought to influence thyroid function. Controlled experiments with rodents demonstrate that isoflavones induce increases in serum T4 concentration. However, rodent thyroid function and regulation differ from those in primates. The main thyroid hormone carrier protein in primates, thyroid-binding globulin (TBG), is not present in rodents. Isoflavone metabolism also differs markedly between rodents and humans. Accordingly, we used nonhuman primates (NHPs) in a long-term, controlled, randomized study to determine the effects of dietary soy on thyroid function and the relationship between thyroid function and ovarian function.*').

Additionally, in the current assessment, data from human studies were available for this specific endpoint and, although limited, were considered more relevant for the population of interest.

3.4.3. Weight of evidence for effects on thyroid

As previously described in section 3.2.3, an overview is given of the studies, their reliability (assessed according to the protocol), their relevance for the risk assessment in humans and the direction of the outcome in order to provide a formalised basis for a weight of evidence assessment.

The outcome of this analysis is summarised in Table 23.

For the endpoint thyroid the reliability score of human studies varied between 1 and 2. In none of the 11 studies was a clinically significant change observed.

In a weight of evidence approach it can be concluded that no adverse effects of isoflavones on the thyroid were seen. It should, however, be noted that this evaluation is restricted to the tested doses of the tested isoflavones.

Table 23: Effects on thyroid function: summary of human data

Endpoint/ intervention	Reference	Reliability	Relevance	Outcome
Effects on thyroid function				
Soy isoflavones/ soy extract	Alekel et al., 2015	1	+++	⇔
	Levis et al., 2011	2	+++	⇔
	Bruce et al., 2003	2	+++	⇔
	Ryan-Borchers et al., 2008	2	+++	⇔
	Pop et al., 2008	2	+++	⇔
Soy protein isolates	Persky et al., 2002	1	+++	⇔
	Duncan et al., 1999	1	+++	⇧ TBG (modest and with no apparent dose response) ⇔ other parameters
Daidzein-rich isoflavones	Steinberg et al., 2011	1	+++	⇔
	Khaodhiar et al., 2008	2	+++	⇔
Genistein	Bitto et al., 2010	1	+++	⇔
Red clover extract	Geller et al., 2009	2	+++	⇔

3.5. Genotoxicity data

A focused literature search did not retrieve any *in vitro* or *in vivo* studies of SPI, or of extracts from soy, red clover, kudzu root with a defined composition which would have allowed an extrapolation to the preparations used in the human and animal studies included in this review, the only exception being the test compound PTI G-2535 used in two of the human studies included in this review (Pop et al., 2008; Khan et al., 2012) for which genotoxicity data were identified.

In the other genotoxicity studies presented in this assessment, the individual isoflavones from soy, red clover and kudzu root which are of interest for the whole risk assessment, alone or in combination, have been tested.

3.5.1. *In vitro* data

The study by Nakayama et al. (2014) shows that genistein at 100 μM in HeLa S3 cells *in vitro* causes abnormal cell division and cleavage furrow regression, resulting in the generation of binucleated cells and hence polyploidy. Moreover, it affects the formation of the central spindle, which is essential for completion of cytokinesis. Its impairment is accompanied by aberrant chromosome segregation, such as a chromosome bridge and lagging chromosomes.

In the study by Baechler et al. (2014), daidzein and possible human hydroxylated metabolites, 6-hydroxydaidzein (6-HO-DAI) and 8-hydroxydaidzein (8-HO-DAI), were assessed for their genotoxicity in the human colon carcinoma (HT29) cell line by means of a comet assay. Potential catalytic inhibition and poisoning of DNA topoisomerase was also investigated. In the comet assay, cells were treated for either 1 or 24 hours at concentrations ranging from 1 to 50 μM and the results obtained indicated that, in contrast to daidzein, 6-HO-DAI and 8-HO-DAI significantly increased the rate of DNA strand breaks in HT29 cells after 24 hours' treatment and caused a cell cycle delay in S-phase. In addition, the hydroxylated metabolites also suppressed the catalytic activity of topoisomerase II in the decatenation assay but not the level of covalent topoisomerase II–DNA intermediates within HT29 cells by the 'isolating *in vivo* complexes of enzyme to DNA (ICE) bioassay', thus arguing for a catalytic inhibition of topoisomerase II rather than poisoning activity.

Mizushina et al. (2013) investigated the potential inhibitory activity of three soy isoflavones, daidzein, genistein and glycitein, and their glycosides, daidzin, genistin and glycitin, on purified DNA topoisomerases I and II (topo I and II) from human placenta which generate DNA single- and double-strand breaks respectively. The catalytic activity of both topo I and II was evaluated by detecting supercoiled plasmid DNA (form I) in its nicked state (form II). The results obtained indicated that none of the six soy isoflavones influenced the topo I nicking activity at concentrations of 100 μM and greater. In contrast, genistein at 100 μM completely inhibited the nicking activity of topo II, while the other compounds were not active, indicating that genistein is a true inhibitor of human DNA topo II. It interacts directly with the enzyme and not through the stabilisation of the 'cleavable complex' as indicated by the interaction of genistein with DNA double-strand breaks through its thermal transition. This specificity determines that genistein does not induce DNA double-strand breaks in the same way classical topo II inhibitors, which stabilise the 'cleavable complex'. Such inhibitors are adriamycin, amsacrine, ellipticine, saintopin, streptonigrin, terpentecin, etoposide, etc. Instead, genistein induces arrest of cell proliferation. The Panel noted that the authors' conclusion that genistein is not a topo II poison which stabilises the 'cleavable' complex is rather speculative. Indeed, this opinion is mostly based on thermal profiles of the transition of double-stranded DNA to single-stranded DNA with or without genistein. However, the evidence that genistein suppresses growth of HCT116 human colon carcinoma cells in a dose-related way indicate a true cell cycle-inhibitory effect.

The study by Lepri et al. (2013), which aimed to evaluate the ability of soy isoflavones (genistein and daidzein) to prevent micronuclei formation in HTC hepatoma cells, found that genistein and daidzein, when added alone to cell cultures at a single dose level of 10 μM, did not induce significant increases in micronuclei. The Panel noted that the results obtained are of limited validity since no change in the number of binucleated cells was observed in cultures treated with the test compound or in solvent-treated cultures owing to selection of an inadequate, i.e. not sufficiently high, dose level.

The study by Masuda et al. (2012) investigated the genotoxic properties of genistein after a nitrite treatment under acidic conditions in the Ames test (*Salmonella typhimurium* strains TA98 and TA100). When genistein was applied alone, no mutagenicity was observed in the Ames test at 10 μM per plate

either in the absence or in the presence of S9 metabolic activation. However, the Panel noted the limited value of results obtained for genistein alone, which is attributable to a number of shortcomings, including the use of a single dose level, and one that is not sufficiently high (10 μM/plate), and the use of only two *S. typhimurium* tester strains (TA98 and TA100).

The study by Zou et al. (2012) aimed to investigate mutagenic effects of genistein in the mouse lymphoma assay. Dose levels of 2.5–20 μg/ml genistein were used to treat cells for 3 or 24 hours in the absence of S9 metabolic activation. The results obtained showed statistically significant and dose-related increases in mutation frequencies at both treatment times. The authors concluded that the induced mutations were mainly of hemizygous type caused by loss of heterozygosity at the TK locus. The Panel noted that the relative total growth in the solvent control was rather low, thus limiting the strength of the test.

In the study by Schwen et al. (2010), *S*-equol, a metabolite of daidzein, was assessed for its mutagenicity in the reverse mutation assay using *S. typhimurium* strains TA1535, TA1537 and TA98 TA100 and *Escherichia coli* WP2 uvrA, in accordance with the method of Ames, and for its clastogenicity in a chromosomal aberration assay in human lymphocytes both in the absence and in the presence of S9 metabolism. In the reverse mutation assay, the pre-incubation and plate incorporation methods both in the absence and in the presence of rat liver S9 metabolism were used. A range of half-$\log_{10}$ concentrations of *S*-equol up to the standard limit dose of 5 000 μg/plate was assayed. In the *in vitro* chromosome aberration assay, cultures were treated with appropriate 2-fold dose intervals up to the dose limit of 10 mM (2 422.7 μg/ml) for 4 hours in the absence or presence of S9 mix. In addition, one set of cultures was treated with *S*-equol in the absence of S9 mix for 21 hours without medium change. The results obtained indicate that *S*-equol did not show genotoxic activity in any of the tests employed. The Panel noted that the studies were adequately performed and met the requirements of the relevant OECD guidelines (TG 471 and TG 473, respectively).

In the study by Chung et al. (2009), puerarin and its glycoside, glucosyl-α-(1,6)-puerarin, were assessed for their mutagenicity in the reverse mutation assay using *S. typhimurium* strains TA98 TA100 in accordance the method of Ames. The pre-incubation method both in the absence and in the presence of rat liver S9 metabolism was used and a four concentrations ranging from 50 to 200 μg/plate were assayed. The results obtained did not show any genotoxic potential. However, the Panel noted significant shortcomings, which include the use of two *S. typhimurium* tester strains (TA98 and TA100) only and the use of very low dose levels (50–200 μg/plate) in the absence of adequate cytotoxicity, thus limiting the strength of the study.

In the study by Ullah et al. (2009), the genotoxicity of genistein and biochanin A was assessed using the alkaline comet assay in purified human lymphocytes at dose levels ranging from 10 to 50 μM. The test compounds were applied directly onto gelatinised slides. The results obtained indicated that both compounds induced dose-related and statistically significant increases in DNA breakage, as evinced by increased tail length in the comets. The authors concluded that the clastogenic activity of both compounds was caused by their pro-oxidant activity, mediated by copper and not by iron and zinc, as supported by the action of copper chelators. In addition, the authors asserted that these compounds have antioxidant activity. However, the Panel noted that the pro-and antioxidant activities of genistein and biochanin A claimed by the authors do not appear to be fully supported by data.

In the study by López-Lazaro et al. (2007), the dietary flavonoids genistein and luteolin were evaluated as topo I and topo II poisons and catalytic inhibitors in human K562 leukaemia cells using the cell-based assay TARDIS (trapped in agarose DNA immunostaining) at dose levels of 100–300 μM. Both flavonoids induced topo II–DNA complexes with both topo IIα and topo IIβ. Genistein, in the same range of concentrations, also decreased the topo II–DNA complexes induced by the topo II poison etoposide, suggestive of a catalytic inhibition of topo II, and luteolin decreased the topo I–DNA complexes induced by the topo I poison camptothecin, indicative of a catalytic inhibition of topo I. However, when using murine transgenic cells lacking topo IIβ, the authors found resistance to genistein-induced cell growth inhibition and cytotoxicity, indicating a key role for genistein in catalytic inhibition of topo IIβ. Since slow-growing tumours contain significant levels of this isoenzyme, genistein might display anticancer effects in these types of tumours. The authors also report that catalytic inhibition of topo II and topo I occurs at relatively high concentrations *in vitro* (175 and 146 μM genistein and luteolin, respectively). They suggest that it is unlikely that genistein or luteolin inhibits the catalytic activity of topo II and topo I when ingested via the diet or supplements,

considering the pharmacokinetics of dietary flavonoids in humans. The authors further point out that the *in vivo* plasma concentrations of genistein after supplementation have been reported to be 0.1–8 μM, a concentration range that encompasses the threshold for genistein induction of topo II–DNA-mediated clastogenicity. This indicates that some flavonoids may exert topo II-mediated toxic and carcinogenic effects when ingested at relatively high concentrations, such as those present in some dietary supplements. On the other hand, accumulating evidence suggests that consumption of flavonoid-containing foods is associated with a reduced cancer risk, indicating that low concentrations of some flavonoids through a diet rich in plant-derived foods, may exert anticancer effects via topoisomerase-independent mechanisms, e.g. oestrogen receptor β-mediated effects or other effects. To sum up, the authors conclude that some flavonoids such as genistein or luteolin may produce anticancer effects at concentrations achievable through a diet rich in plant-derived foods (submicromolar) through topoisomerase-independent mechanisms (e.g. antioxidant, anti-oestrogenic). However, at higher concentrations (micromolar, non-cytotoxic), these agents induce topo II-mediated DNA damage that may produce carcinogenic effects. At high concentrations (micromolar, cytotoxic), these dietary agents may produce cancer chemotherapeutic effects through a catalytic inhibition of topo II in the cells, mainly involving topo IIβ.

In the study by McClain et al. (2006), the mutagenicity of genistein was evaluated in the reverse mutation assay using *S. typhimurium* strains TA1535, TA1537, TA98 TA100 and *Escherichia coli* WP2 uvrA in accordance with the method of Ames and in mouse lymphoma L5178Y $tk^{+/-}$ cells *in vitro*. In the reverse mutation assay, triplicate plates were prepared for each experimental point and treated with a range of concentrations up to 3 300 μg/plate in the first experiment using the plate test methods and with up to 1 000 μg/plate in the second experiment using the pre-incubation method owing to excessive precipitation of genistein at the higher dose levels. Treatments were performed both in the absence and in the presence of rat liver S9 metabolism. In the mouse lymphoma assay, four independent experiments were conducted: two in the absence of S9 (exposure times of 3 and 24 hours) and two in presence of S9 (exposure time 3 hours in both cases). In the absence of S9, cells were treated with up to 60 and 7.5 μg/ml for 3 and 24 hours, respectively, based on the relative cell survival. In the presence of S9-mix, the experiments were carried out up to a maximum concentration of 6.5 and 7.5 μg/ml. Genistein did not show any genotoxic activity in the reverse mutation assay (Ames test) using either the standard plate incorporation method or the pre-incubation method and either in the presence of in the absence of metabolic activation. In contrast, in the *in vitro* mouse lymphoma assay, genistein significantly increased resistant mutant colonies both in the absence and in the presence of metabolic activation (S9). These were predominantly small colonies, indicating that genistein acts as a clastogen. This observation is in agreement with published data on the inhibitory action of genistein on topoisomerase II, which is known to lead to chromosomal damage with a threshold dose–response. The Panel noted that the studies performed were conducted in accordance with the relevant OECD guidelines, TG 471 and TG 476 for the bacterial mutation assay and the *in vitro* mammalian cell gene mutation assay, respectively.

The study by Lehmann et al. (2005) aimed to investigate the genotoxic potential of equol, 3-hydroxy-daidzein (3-HO-DAI) and 6-hydroxy-daidzein (6-HO-DAI), three human metabolites of daidzein, by assessing the induction micronuclei in cultured human endometrial carcinoma cells (Ishikawa cells). Cells were exposed at concentrations of up to 50 μM for 48 hours, after which time the number of cells with micronuclei was determined. Using fluorescence-labelled anti-kinetochore antibodies, micronuclei containing whole chromosomes (kinetochore-positive micronuclei) could be discriminated from micronuclei containing acentric chromosome fragments (kinetochore-negative micronuclei). The results obtained indicated that at 4 hours sampling time only equol, at concentrations between 5 and 20 μM, induced statistically significant increases in micronuclei (mainly kinetochore negative, thus reflecting a clastogenic activity) compared with the untreated control. However, after an additional 24 hours of compound-free incubation, the number of micronuclei in cells exposed to 3-HO-DAI at concentrations between 5 and 50 μM showed a two-fold increase relative to controls in a dose-dependent way. Again the induced micronuclei were predominantly kinetochore negative, thus reflecting a clastogenic activity. For 6-HO-DAI, the induction of micronuclei was observed neither immediately after treatment for 48 hours nor after the subsequent compound-free incubation period of 24 hours. However, the Panel noted that the reliability of this study is limited since the frequency of micronucleated cells in the untreated control is unusually high, possibly reflecting an elevated genomic instability of the cell line employed

In the study by Di Virgilio et al. (2004), an *in vitro* micronucleus test in Chinese hamster V79 cells was used to assess the genotoxicity of genistein, daidzein and equol. Cells were treated for 18 hours (1.5 cell cycles) with 5, 10, 18, 25, 50 or 75 μM genistein, 25, 50, 75 or 100 μM daidzein or 5, 10, 18, 25, 50, 75 μM equol. One solvent (dimethylsulphoxide)-treated control and two positive controls, treated with methylmethanesulphonate at 50 μg/ml or vincristine at 10 nM, were also employed. In addition, the alkaline version of the comet assay was also used to detect DNA strand breaks for genistein only. The results showed that genistein at concentrations up to 25 μM induced a statistically significant and dose-related increase in micronucleated cells, with values being more than triple the control values. At higher dose levels the incidence of micronucleated cells decreased, probably as a result of concomitant cytotoxic effects. In the comet assay, positive effects in terms of DNA breakage were observed only at dose levels higher than 100 μM, which were confirmed to be cytotoxic. This outcome can be explained by the fact that repair of DNA single- and double-strand breaks proceeds during processing of cells for comet assay, unless blocked by ice-cold treatment. Consequently, repair of DNA strand breaks, though illegitimate, cannot be seen by the comet assay. For daidzein slight, dose-related increases in micronucleated cells were also observed and were statistical significant at concentrations of 50 μM and higher. At 75 μM the frequency of micronucleated cells was double the control values. Similarly, for equol, slight increases were observed and were statistically significant at concentrations of 50 μM and above. At 75 μM, a maximum 2.5-fold increase compared with the control values was observed. In all cases, the tested compounds induced mostly CREST (–) micronuclei, indicating a clastogenic mechanism of action compatible with the poisoning of topo II. Overall, genistein proved to be genotoxic in the *in vitro* micronucleus test while the outcome for daidzein and equol should be considered as equivocal.

Snyder and Gillies (2003) showed that some flavonoids, such as galangin, fisetin, biochanin, hesperitin naringenin, biochanin, galangin and, particularly, daidzein, which is a major constituent of marketed soy products, are catalytic topo II inhibitors (not poisons). In their study, Snyder and Gillies (2003) assessed the topo II catalytic inhibitory activity of these compounds by their ability to antagonise the induction of micronuclei by genistein in V79 cells at 75 μM. The results obtained indicated that selected compounds were all able to reduce genistein-induced micronuclei by at least 80 % when genistein was added to cultures first, thus confirming the topo II catalytic inhibition activity of these compounds.

In the study by Misra et al. (2002), the purified soy isoflavone product PTI G-2535, containing predominantly genistein (40 % to 50 %) but also daidzein (18 % to 25 %), glycitein (1 % to 4 %) and small quantities of residual lipids and carbohydrates, was evaluated for its mutagenicity in the reverse mutation assay using *S. typhimurium* strains LT2, TA1535, TA1537, TA98 TA100 and *Escherichia coli* WP2 uvrA in accordance with the method of Ames and in mouse lymphoma L5178Y $tk^{+/-}$ cells *in vitro*. In the reverse mutation assay, a range of concentrations up to 625 μg/plate were used in the first experiment based on the toxicity observed in the preliminary toxicity test and up to 1 250 μg/plate was used in the second experiment in an attempt to achieve cytotoxicity with *S. typhimurium* TA98 and *E. coli* WP2*uvrA*, based on the results obtained in experiment 1. Treatments were performed both in the absence and in the presence of rat liver S9 metabolism. In the mouse lymphoma assay, four independent experiments were conducted: two in the absence and two in the presence of S9 metabolic activation with 4 hours' exposure in all case. In the absence of S9, cells were treated with up to 300 μg/ml in the first experiment and up to 50 μg/ml in the second experiment because of the toxicity observed in initial experiments. In the presence of S9-mix, the maximum concentration used was 5 μg/ml in the first experiment and 2.5 μg/ml in the second because of the toxicity observed in initial experiments.

PTI G-2535 did not show genotoxic activity in the reverse mutation assay (Ames test) either in the absence or in presence of metabolic activation although some increases in the presence of S9 were observed at doses of 156.2 and 312.5 μg/plate in the TA100 strain. However, such increases, which were dose related and achieved statistical significance, were less than two-fold the control values. In contrast, in the mouse lymphoma assay, statistically significant and dose-related increases in mutation frequency were induced by PTI G-2535 both in the absence and in the presence of S9 metabolic activation in all the experiments performed and small and large colonies were observed.

The Panel noted that the studies performed were conducted in accordance with the relevant OECD Guidelines, TG 471 and TG 476 for the bacterial mutation assay and the *in vitro* mammalian cell gene mutation assay, respectively.

In the study by Salti et al. (2000), genistein was assessed for its capability to induce DNA breakage by means of the alkaline comet assay in HT-29 colon cancer cells. Cultures were treated with dose levels ranging from 2 to 200 μM for 1 or 48 hours. The results obtained indicated marked and dose-related increases in DNA breakage from 10 up to 100 μM following 1 hour's treatment. After 48 hours' treatment, marked DNA breakage was only observed at 100–200 μM. The authors concluded that DNA breakage induced by genistein in HT-29 colon cancer cells was the result of poisoning of topo II through the stabilisation of the cleavable complex since aclarubicin, a known catalytic inhibitor of topo II, markedly antagonised the DNA damage induced by both genistein and VP-16 in HT-29 cells in a similar manner, as determined by the comet assay.

Morris et al. (1998) assessed the ability of genistein to induce micronuclei and gene mutations in the human lymphoblastoid cells AHH-1 $tk^{+/-}$ ($p53^{+/-}$) and L3 ($p53^{+/-}$), which differ in the functional status of the tumour suppressor gene, *p53*. The mutant fraction at the thymidine kinase (*tk*) locus was determined by resistance to trifluorothymidine and at the hypoxanthine phosphoribosyl transferase (*hprt*) locus by resistance to 6-thioguanine. The results obtained indicated that genistein induced micronuclei and gene mutation in both cell lines.

Summary of *in vitro* data

Bacterial gene mutation tests (Ames tests) yielded no evidence for the mutagenicity the isoflavones. In contrast, in mammalian cells *in vitro*, genistein proved to be markedly mutagenic (although with a clastogenic mechanism of action) and clastogenic through the induction of micronuclei or DNA breakage as measured by the comet assay. These effects are attributable to the poisoning of the DNA topoisomerase II through the stabilisation of the 'cleavable complex' and generation of DNA double strand breaks (DSBs) at topoisomerase II–DNA binding sites. Such an indirect effect on DNA is the concept of a threshold for clastogenicity (Müller and Kasper, 2000; Sofuni et al., 2000).

Daidzein, *S*-equol, glycitein and puerarin did not show significant genotoxic activity. On the other hand, the two oxidative daidzein metabolites (3′,4′,7- and 4′,6,7-trihydroxyisoflavone) proved to induce micronuclei. This clastogenic effect was attributed to their oxidation to o-quinones, which are known to be direct clastogens.

3.5.2. *In vivo* data

The study by Masuda et al. (2012) investigated the genotoxic properties of genistein after a nitrite treatment under acidic conditions in an *in vivo* micronucleus test in peripheral blood cells of ICR male mice. When genistein was applied alone to male mice at a single dose of 2 mmol/kg bw by oral gavage, for 24 or 48 hours, no genotoxicity was observed. However, the Panel noted the use of a single dose level, not sufficiently high to cause any reduction in the ratio of mature to immature erythrocytes. Furthermore, the number of cells scored was low.

In the study by Schwen et al. (2010) *S*-equol, a metabolite of daidzein, was assessed for its genotoxicity in an *in vivo* micronucleus test. Groups of five male and female Sprague–Dawley rats were treated by oral gavage with 500, 1 000 or 2 000 mg/kg bw *S*-equol, the last dose level being the limit dose for this test. Animals were sacrificed at 24 hours (all dose levels) and at 48 hours (2 000 mg/kg only). Bone marrow smears were prepared and stained with acridine orange and 2 000 PCEs per animal were analysed for the presence of micronuclei. *S*-equol did not show any genotoxic activity. The Panel noted that the study was adequately performed and met the requirements of the relevant OECD guideline, TG 474, with the exception that at the 48-hour sampling time only three animals rather than five were analysed. The study by Pop et al. (2008) was a phase I double-blinded clinical trial that aimed to evaluate changes indicative of oestrogenic stimulation and genotoxicity, following administration of a high oral dose of soy isoflavones (genistein, daidzein and glycitein) in 30 healthy (18 in the active group and 12 in the placebo group), post-menopausal women for 84 days. The genotoxicity was evaluated by means of the alkaline comet assay and analysis of apurinic/apyrimidinic (AP) sites in peripheral blood lymphocytes on day 1 and day 84 following administration of purified isoflavones. The active group (n = 18) received four capsules of the dietary

supplement PTI G-2535 (containing approximately 600 mg genistein/day and 30 mg/day daidzein) divided into two equal daily doses. The placebo group (n = 12) received the same number of placebo capsules. The unconjugated isoflavones (PTI G-2535) were formulated in capsules, each containing 139.5 mg genistein, 74 mg daidzein and 11 mg glycitein. The mean values obtained in the comet assay were similar for the active and placebo groups at baseline (day 1) and at day 84 and the mean for number of AP sites at day 84 was higher in the placebo group than in the active group, but not significantly so. The authors concluded that unconjugated soy isoflavones are safe and well tolerated in healthy post-menopausal women at doses of up to 900 mg per day. The Panel noted that the analysis of AP sites is not a standard test and that, in the case of comet assay, the elapsed time between the last administration and processing of cells was not reported, which partly reduces the strength of the study.

In the study by Chung et al. (2009), puerarin and its glycoside, glucosyl-α-(1,6)-puerarin, were assessed for their genotoxicity in an *in vivo* mouse bone marrow micronucleus test. Groups of five male ICR mice were treated once by oral gavage with 500, 1 000, 2 000 or 5 000 mg/kg bw and sacrificed at 36 hours after treatment. Bone marrow smears were prepared and stained with 5 % Giemsa, and 1 000 PCEs per animal were analysed for the presence of micronuclei. The results obtained did not provide any evidence of genotoxic activity. The Panel noted minor shortcomings, which include the use of a single sampling time at 36 hours (instead of the recommended two, comprised between 24 and 48 hours) and scoring of 1 000 rather than 2 000 PCEs per animal to identify the induction of micronuclei. However, the result obtained was considered reliable also in the light of the high dose-levels employed. The highest dose-level employed (5 000 mg/kg bw) fairly exceeds the limit dose of 2 000 mg/kg bw recommended by the OECD Guideline No 474.

In the study by McClain et al. (2005), genistein was evaluated for its clastogenicity/aneugenicity in three independent micronucleus tests (one test in Moro albino mice, one in RAIf rats and one in Wistar rats). In the in mouse micronucleus test, five male and five female animals were treated by oral gavage with dose levels of 0, 0.2, 2 and 20 mg/kg bw genistein for 14 days and were sacrificed 30 hours after the last administration. Peripheral blood smear slides were prepared and stained with May–Gruenwald–Giemsa. The slides were scored for the presence of micronuclei and 2 000 PCEs per animal were analysed. In the rat micronucleus assays, five male and five female rats of each strain (RAIf and Wistar) were treated by oral gavage once at dose levels of 0, 500, 1 000 and 2 000 mg/kg bw, the last dose level being the limit dose level for this assay. Animals were sacrificed at 24 hours (all dose levels) and at 48 hours (2 000 mg/kg only) and bone marrow was collected from femurs. Smears were prepared and stained with May–Gruenwald–Giemsa and 2 000 PCEs per animal were scored for the presence of micronuclei. The results obtained indicated that genistein has no genotoxic activity. The Panel noted that the studies were conducted in accordance with the relevant OECD guideline, TG 474, with the exception of the micronucleus test in mice. This study showed major shortcomings, including the use of 20 mg/kg bw as the highest dose, selected based on the upper range of human exposure to genistein. Since no signs of toxicity were observed, this dosing regime is considered inadequate for an *in vivo* genotoxicity test. In addition, the sampling time of peripheral blood, 30 hours rather than the 36 hours recommended by the relevant OECD guideline, TG 474, is considered inappropriate. On this basis, the Panel considered the mouse peripheral blood micronucleus test unreliable.

The study by Manjanatha et al. (2006) aimed to assess ability of genistein to potentially protect against dimethylbenz(a)anthracene (DMBA)-induced *lacI* mutation in the heart of OVX Big Blue rats. The study was not designed as a standard genotoxicity test. However, when genistein was fed in the diet to female Big Blue rats homozygous for *lacI* transgene at 250 or 1 000 μg genistein/g diet, no increases in the mutation frequencies were observed compared with the untreated control values. The Panel acknowledged a limited value of this study for risk assessment since it was not specifically designed as a standard *in vivo* mutagenicity test.

The study by Miltyk et al. (2003) aimed to assess the genotoxicity of purified soy isoflavones (genistein, daidzein and glycitein) in 20 patients with prostate cancer and six healthy volunteers, by means of the alkaline comet assay and the cytokinesis-block micronucleus assay in peripheral blood lymphocytes. Chromosome translocation of the myeloid/lymphoid leukaemia (MLL) gene (11q23) was also assessed by using fluorescence *in situ* hybridisation. The ingredients were formulated in capsules which contained each 139.5 mg genistein, 74 mg daidzein and 11 mg glycitein. The patients with

prostate cancer were initially administered 300 mg (approximately 4 mg/kg bw) for 28 days. The dose was then escalated to 600 mg (approximately 8 mg/kg bw) given as two divided doses in the morning and evening for an additional 56 days. At the end of the dosing period, fresh blood was collected by venepuncture from each patient, centrifuged to separate mononuclear cells and subsequently processed for comet assay and cytokinesis-block micronucleus test in accordance with standard procedures. The plasma profiles of genistein, daidzein, and glycitein in the patients were measured at 24 hours post dose on days 5, 9, 14, 21 and 28 and 12 hours post dose on days 31, 35, 42 and 56, and the highest concentration of plasma genistein ranged 4.1–27.1 μmol/l (ng/ml). No genotoxic activity was detected in any of the assays performed. The Panel noted that the studies were accurately and correctly performed. The only limitation observed in the comet assay was that the elapsed time between last administration and processing of cells was not reported. Chromosome translocation of the MLL gene (11q23) is not a standard assay for chromosomal aberrations

In the study by Misra et al. (2002), the purified soy isoflavone product PTI G-2535, containing predominantly genistein (40 % to 50%) but also daidzein (18 % to 25%), glycitein (1 % to 4%) and small quantities of residual lipids and carbohydrates, was evaluated for potential clastogenicity/aneugenicity in the micronucleus test in mice. The dose levels used in the experiments performed were based on genistein concentration. In the mouse micronucleus test, five male and five female animals were treated once by oral gavage at dose levels of 0, 500, 1 000 and 2 000 mg/kg bw, the last dose level being the limit dose level for this assay. Animals were sacrificed at 24 and 48 hours after test compound administration and bone marrow was collected from femurs. Bone marrow smear slides were prepared and stained with May–Gruenwald–Giemsa and 2 000 PCEs per animal were scored for the presence of micronuclei.

The results obtained indicated that PTI G-2535 has no genotoxic activity although statistically significant increases in the frequency of micronucleated PCEs were seen in only male mice treated with 500 and 1 000 mg/kg bw at 24 hours after treatment. Such increases were small, were not dose related, and fell within the historical range of micronucleus frequencies for control animals in the laboratory. The Panel noted that the studies performed were conducted in accordance with the relevant OECD guideline, TG 474.

Summary of *in vivo* data

No genotoxic effects were observed for genistein, daidzein, *S*-equol, glycitein and puerarin when assayed in pivotal mouse and rat micronucleus tests, comet assays or in humans in phase I double-blinded clinical trials

3.5.3. Discussion on genotoxicity

In this review, study results from genotoxicity of isoflavones has been assessed which were conducted in numerous *in vitro* and *in vivo* studies. The studies were identified in a focussed literature review. The available studies focused mainly on genistein and daidzein and at lesser extent on soy extracts. A limited number studies on daidzein metabolites (*S*-equol 3′,4′,7-trihydroxyisoflavone, 4′,6,7-trihydroxyisoflavone, o-desmethylangolesin) and glycitein were also available.

Genistein yielded no evidences for mutagenicity in the bacterial gene mutation test (Ames tests) (Misra et al., 2002; McClain et al., 2005; Masuda et al., 2012). In contrast, in mammalian cells *in vitro*, genistein proved to be markedly mutagenic (Morris et al., 1998; Misra et al., 2002; McClain et al., 2005; Zou et al., 2012), compatible with a clastogenic mechanism of action as indicated by the partial or complete deletion of the $tk^{+/-}$ locus following DNA sequencing. It also proved to be clastogenic through the induction of micronuclei (Morris et al., 1998; Snyder and Gillies, 2003; Di Virgilio et al., 2004) or DNA breakage as measured by the comet assay (Salti et al., 2000; Di Virgilio et al., 2004; Ullah et al., 2009). It is widely recognised that this genotoxicity arises from poisoning of DNA topoisomerase II through the stabilisation of the 'cleavable complex', thus resulting in protein-concealed DNA double DSBs at topoisomerase II–DNA binding sites (Markovits et al., 1989; Yamashita et al., 1990; López-Lazaro et al., 2007). However, genistein has also been shown to be a catalytic inhibitor of topo II with the capability to disrupt the enzyme physiology, as reported by Mizushina et al. (2013) and López-Lazaro et al. (2007), who reported that genistein completely inhibited the nicking activity of topo II. An important implication of an indirect (topo II-mediated) effect on DNA is the concept of a threshold for clastogenicity, as demonstrated by Lynch et al. (2003). In this study, a

number of topoisomerase type II inhibitors with different clastogenic potencies were investigated and 'pragmatic thresholds' for clastogenicity in mouse lymphoma L5178Y cells were defined. For genistein the 'pragmatic threshold' was defined at 1 μg/ml. No evidence of *in vivo* genotoxicity was observed for genistein, as shown by negative results obtained in two mouse micronucleus tests (Misra et al., 2002; Masuda et al., 2012) and in two pivotal, limit bone marrow micronucleus tests in RAIf and Wistar rats, at 2 000 mg/kg bw (McClain et al., 2005). Negative results were obtained in a phase I double-blinded clinical trial in 30 healthy post-menopausal women, in which genotoxicity was evaluated by means of the alkaline comet test and analysis of AP sites in peripheral blood lymphocytes (Pop et al., 2008). Nor were any genotoxic effects observed in an alkaline comet assay and cytokinesis-block micronucleus assay in peripheral blood lymphocytes in 20 patients with prostate cancer in whom genistein 300 mg/day (approximately 4 mg/kg bw/day) was administered for 28 days, increasing to 600 mg/day (approximately 8 mg/kg bw/day) for a further 56 days (Miltyk et al., 2003).

Daidzein was not mutagenic in the bacterial reverse mutation assay (Misra et al., 2002), and no relevant genotoxic effects were generally observed in mammalian cells *in vitro* (Schmitt et al., 2003; Di Virgilio et al., 2004; Lehmann et al., 2005). Similarly, negative results were observed *in vivo* in a pivotal limit mouse bone marrow micronucleus test (Misra et al., 2002). As for genistein, the analysis of peripheral blood lymphocytes from 30 healthy post-menopausal women (Pop et al., 2008) and from 20 men with prostate cancer (Miltyk et al., 2003) yielded no evidence of genotoxicity of yielded no evidences of genotoxicity.

In contrast, the two oxidative daidzein metabolites (3',4',7-trihydroxyisoflavone and 4',6,7-trihydroxyisoflavone) found after incubation with human hepatic microsomes, and also identified in the urine of volunteers after ingestion of soy food, proved to be clastogenic through the induction of micronuclei in mammalian cells (Schmitt et al., 2003). The clastogenic effect of these two catecholic metabolites may be attributed to oxidation to o-quinones, which are known to be clastogens and could represent a potential hazard *in vivo.* However, as soy isoflavones and their metabolites are rapidly conjugated with glucuronic acid and sulphate *in vivo,* it is unlikely that the high concentrations of the free metabolites required for adverse effects are reached in humans even after ingestion of high levels of isoflavones. Furthermore, the negative outcome observed for daidzein *in vivo* (Misra et al., 2002; Miltyk et al., 2003; Pop et al., 2008) is reassuring about the genotoxicity of these catecholic metabolites.

For *S*-equol, which is produced by intestinal bacteria in the human gut after ingestion of daidzein, no genotoxicity was reported in the bacterial reverse mutation assay (Schwen et al., 2010). Equivocal findings in terms of induction of micronuclei were observed in mammalian cells *in vitro* (Schmitt et al., 2003; Di Virgilio et al., 2004; Lehmann et al., 2005) though a negative finding for induction of chromosomal aberrations was observed in a valid study in human lymphocytes *in vitro* (Schwen et al., 2010). *In vivo*, *S*-equol proved to be devoid of any genotoxic activity in a 'limit' bone marrow micronucleus test performed in accordance with the relevant OECD guideline, TG 474 (Schwen et al., 2010).

Glycitein (Misra et al., 2002) and puerarin (Chung et al., 2009) were not mutagenic in the bacterial reverse mutation assay. Negative results were also observed *in vivo.* Puerarin showed no induction of micronuclei in a mouse bone marrow micronucleus test while glycitein was negative when peripheral blood lymphocytes from 30 healthy post-menopausal women (Pop et al., 2008) and 20 patients with prostate cancer (Miltyk et al., 2003) were analysed for different genotoxicity endpoints.

Overall, it can be concluded that the genotoxicity expressed *in vitro* in mammalian cells by the two catecholic oxidative metabolites of daidzein 3',4',7-trihydroxyisoflavone and 4',6,7-trihydroxyisoflavone and by genistein, for which a thresholded mechanism of action has been demonstrated, has not been reproduced in valid *in vivo* micronucleus tests in rats and mice or in comet assay and micronucleus test in human studies. On these bases, the use of isoflavones as food supplement is not of genotoxic concern.

3.6. Exposure assessment

3.6.1. Background exposure from the diet

Levels of isoflavones in food

The highest levels of isoflavones can be found in soy and soy-based foods, but lower levels can also be found in other foods of vegetable and animal origin. In particular, soy milk can contain about 10 mg isoflavones/100 g and tofu and soy yoghurt can contain up to 48 mg and 84 isoflavones/100 g, respectively. Isoflavone levels in foods as reported in the literature and in compositional databases are reported in Table 24.

Variability in isoflavone content can be noticed within the same food group. For example, the Phenol-Explorer database (online) reports 18 mg isoflavones/100 g in soy milk, whereas the USDA Database for the Isoflavone Content of Selected Foods (USDA, 2008, online) reports levels ranging from 0.25 to 10.7 mg/100g. Soy milk from Foo jook or yuba has been reported contain up to 196.05 mg isoflavones/100 g in the USDA database. The reason for differences in isoflavones content reported in the literature may be that many studies measure only daidzein and genistein, but not the other isoflavones.

It is important to highlight that soy enzymes can be used in the processing of flour, which results in isoflavones being present in products made thereof, such as breakfast cereals, bread, cakes and confectionery. Among individuals who regularly consume soy-based products, such as vegetarians, these foods are the most important sources of isoflavones. However soy-based products are not commonly consumed in the general population, and the main sources of isoflavones in this case could be foods to which soy enzymes are added directly or which contain other soy-containing ingredients. The use of soy enzymes in the baking process is quite common in the USA and, within the European Union, at least in the UK (Clarke et al., 2004). In the USA the presence of isoflavones can therefore be identified in non-soy-based foods, such as 'meatballs' (6.0 mg/100 g), pizza, beef topping (0.5 mg/100 g) and frankfurter beef (1.9 mg/100 g). Among non-soy consumers in the UK, more than 80 % of the isoflavone intake comes from bread (Mulligan et al., 2007). The presence of 'hidden soy' in other foods and countries is poorly characterised, but it is expected to be significant (Clarke et al., 2004). In the general European population, legumes, cereals and confectionery are the most important sources (Zamora-Ros et al., 2012).

Table 24: Isoflavones content in soy and soy-based products

Food	Isoflavones mg/100 g	Reference
Soy milk	18.0	Phenol-Explorer, online
Soy milk, seven different products (min–max)	6–10	Chan et al., 2009
Soy milk, nine different products (min–max)	0.7–10.7	USDA database, online
Soy milk (Foo jook or yuba), two different products (min–max)	44.7–196	USDA database
Tofu, 16 different products (min–max)	9.4–48.5	USDA database
Soy cheese, seven different products (min–max)	6.0–25.7	USDA database
Soy flour, four different products (min–max)	151–178	USDA database
Soy bread, four different products (min–max)	0.3–14.7	USDA database
Bread, nine different products (min–max)	0.02–1.0	USDA database
Bread, average from 21 different breads	0.9	Umphress et al., 2005
Soy yoghurt	84.3	Phenol-Explorer
Soy yoghurt	33.2	USDA database
Soy sausage	26.8	Phenol-Explorer
Common bean, black, whole raw	1.4	Phenol-Explorer

Food	Isoflavones mg/100 g	Reference
Black bean sauce	10.3	USDA database, online
Soy or soy milk (tonyu) drink	15.0	ANSES, 2011, online
Soy-based vegetable cutlets	12.8	ANSES, 2011, online
Chocolate-flavoured soy dessert	5.9	ANSES, 2011, online
Soy dessert with fruit	6.8	ANSES, 2011, online
Plain soy dessert	4.6	ANSES, 2011, online
Soy sauce	0.9	ANSES, 2011, online
Tofu	53.3	ANSES, 2011, online
Soybean oil	0.8	ANSES, 2011, online

Intake

Eleven studies assessing the dietary intake of isoflavonoids in the European population have been identified and are presented in Table 25.

The intake of isoflavones in the general European population is relatively low, most studies showing intake estimates below 1 mg/day, with the exception of the UK, where intake levels are up to 3 mg/day. Higher intakes have been reported among men than among women. Among vegetarians and consumers of soy, intake estimates are much higher than those for the general population, up to 20 mg/day, but still lower than what can be expected in a supplement (see section 3.6.2).

It is expected that most of the reported studies underestimated the intake of isoflavones because many of the hidden sources of soy are unmeasured (Clarke et al., 2013).

Table 25: List of studies assessing average daily intake of isoflavones in the European population.

Population group	Number of subjects	Age group (years)	Average intake of isoflavones (mg/day)	Reference
General population, women				
Irish women	662	18–64	0.60	van Erp-Baart et al., 2003
Italian women	827	Up to 94	0.49	van Erp-Baart et al., 2003
Dutch women	2 206	Up to 97	0.83	van Erp-Baart et al., 2003
UK women, n = 168, age 40–64 years	168	40–64	0.66	van Erp-Baart et al., 2003
UK women, non-soy consumers, part of the Norfolk arm in EPIC	708	39–49	0.58	Mulligan et al., 2007
UK women, non-soy consumers, part of the Norfolk arm in EPIC	1 638	50–59	0.50	Mulligan et al., 2007
Spanish women	148	19–69	0.27	Hernandez-Elizondo et al., 2013
European women	23	35–74	1.43	Zamora-Ros et al., 2012
Dutch women, part of EPIC	17	50–69	0.88	Boker et al., 2002
Soy consumers, vegetarians, women and men				
UK women, soy consumers, part of the Norfolk arm in EPIC	41	39–49	11.00	Mulligan et al., 2007
UK women, soy consumers, part of the Norfolk arm in EPIC	83	50–59	8.40	Mulligan et al., 2007
UK vegetarians	10	21–56	7.40	Ritchie et al., 2006
UK adults, vegetarian duplicate diet study	35	39	10.50	Clarke et al., 2003
UK adults, 'health conscious' (including vegetarians)	309	'Adults'	19.40	Zamora-Ros et al., 2012
General population, men and women				
French adults, Total Diet Study TDS2/INCA2	2 624	18–79	0.50	ANSES, 2011
Scottish men, controls in PCANDIET cancer study	197	50–74	1.60	Heald et al., 2006
'Average adult', Total Diet Study, UK 1998	–	'Average adult'	3.00	Clarke and Lloyd, 2004
Finnish adults	2 862	'Adults'	0.79	Valsta et al., 2003
Mediterranean adults, EPIC study	11 285	'Adults'	0.47	Zamora-Ros et al., 2012
Non-Mediterranean, EPIC study	23 469	'Adults'	0.76	Zamora-Ros et al., 2012
UK adults, EPIC study	974	'Adults'	2.34	Zamora-Ros et al., 2012

Exposure to isoflavones from the EFSA database for the population of European women > 40 years old

The EFSA Comprehensive European Food Consumption Database (Comprehensive Database) has been used to roughly estimate the intake of isoflavones from soy and soy-based products in European women. The Comprehensive Database provides a compilation of existing national information on food consumption at individual level. It was first built in 2010 (EFSA, 2011a; Huybrechts et al., 2011; Merten et al., 2011) and then updated in 2015.[9] Details on how the Comprehensive Database is used are published in the Guidance of EFSA (EFSA, 2011b).

The database contains data from 41 surveys in 23 different European countries for a total of 78 990 participants. Data from six surveys were available for 'Infants' (< 12 months old), from 11 surveys for 'Toddlers' (≥ 12 months to < 36 months old), from 19 surveys for 'Other children' (≥ 36 months to < 10 years old), from 19 surveys for 'Adolescents' (≥ 10 years to < 18 years old), from 21 surveys for 'Adults' (≥ 18 years to < 65 years old), from 15 surveys for the 'Elderly' (≥ 65 years to < 75 years old) and from 13 surveys for the 'Very elderly' (≥ 75 years old). Two additional surveys provided information on specific population groups: 'Pregnant women' (Latvia) and 'Lactating women' (Greece).

In the surveys above, consumption data were collected using single or repeated 24- or 48-hour dietary recalls or dietary records covering from 3 to 7 days per subject. Owing to the differences in the methods used for data collection, direct country-to-country comparisons must be made with caution.

The subset of women above 40 years of age was used as a proxy for the target population group of peri- and post-menopausal women. The number of consumers of the different soy and soy-based products in women above 40 years of age is reported in Table 26. These results confirm that soy and soy-based products are rarely consumed in the European Union. The product with the highest percentage of consumers is soy milk, consumed by 12 % of Spanish women, with the percentage of consumers of all other foods being below 8 %. Average intake of isoflavones has been estimated by combining, for each soy and soy-based product, the average consumption among consumers only with the maximum levels of isoflavones derived from the Phenol-Explorer database (online) or, when this information was not available, from the USDA database (online) ('Soya cheese', 26 mg/100 g; 'Soya drink', 18 mg/100 g; 'Soya yoghurt', 81 mg/100 g; 'Tofu', 48 mg/100 g; 'Soya bread', 14.7 mg/100 g; 'Soya beans flour', 178 mg/100 g). Soy yoghurt and soy drink presented the highest intake estimates, ranging from 38 to 122 mg/day and from 5 to 34 mg/day, respectively.

The consumption of food supplements containing isoflavones was reported in only three surveys included in the Comprehensive Database. In the subset of women above 40 years of age, the consumption of 'Protein and amino acids supplements' based on soy and of 'Plant extract formula' containing isoflavones were reported in Finland (FINDIET2012, four consumers) and the UK (NDNS-RollingProgrammeYears1–3, one consumer) and in Finland (FINDIET2012, four consumers) and Ireland (NANS_2012, one consumer), respectively. As the consumption of food supplements has not been captured in all dietary surveys included in the Comprehensive Database, the consumption of food supplements containing isoflavones in other countries is possible even if it was not reported.

[9] http://www.efsa.europa.eu/en/press/news/150428

Table 26: Intake estimates of isoflavones from soy and soy-based products in women above 40 years of age

Country	Survey	Number of subjects	Food products	Number of consumers	% of consumers	Mean consumption (g/day)	Mean exposure to isoflavones (mg/day)
Austria	ASNS_Adults	133	Soya cheese	1	1 %	27.5	7.2
			Soya drink	2	2 %	187.5	33.8
			Soya yoghurt	1	1 %	150.0	121.5
			Tofu	1	1 %	50.0	24.3
Belgium	Diet_National_2004	916	Soya bread	1	<1 %	69.8	10.3
			Soya drink	10	1 %	147.3	26.5
			Soya yoghurt	4	<1 %	74.9	60.7
			Tofu	2	<1 %	17.3	8.4
Denmark	DANSDA 2005–08	686	Soya beans flour	7	1 %	31.0	55.1
			Soya drink	4	1 %	28.9	5.2
Finland	FINDIET2012	670	Soya beans flour	6	1 %	3.8	6.7
			Soya cheese	7	1 %	9.9	2.6
			Soya drink	11	2 %	188.3	33.9
			Soya yoghurt	10	1 %	127.8	103.5
			Tofu	5	1 %	11.6	5.6
France	INCA2	932	Soya beans flour	7	1 %	3.5	6.3
			Soya drink	28	3 %	115.6	20.8
			Soya yoghurt	57	6 %	56.7	45.9
			Tofu	3	<1 %	36.9	17.9
Germany	National_Nutrition_Survey_II	4946	Soya beans flour	1	<1 %	22.5	40.1
			Soya drink	57	1 %	190.3	34.3
			Soya yoghurt	9	<1 %	93.8	75.9
			Tofu	28	1 %	40.4	19.6
Ireland	NANS_2012	461	Soya cheese	1	<1 %	3.3	0.8
			Soya drink	6	1 %	106.3	19.1
			Soya yoghurt	5	1 %	96.2	77.9
			Tofu	5	1 %	9.4	4.5
Italy	INRAN_SCAI_2005_06	1034	Soya bread	3	<1 %	37.3	5.5
			Soya drink	2	<1 %	175.0	31.5

Country	Survey	Number of subjects	Food products	Number of consumers	% of consumers	Mean consumption (g/day)	Mean exposure to isoflavones (mg/day)
Netherlands	VCPBasis_AVL2007_2010	547	Soya drink	19	3 %	188.8	34.0
			Soya yoghurt	1	<1 %	75.0	60.8
			Tofu	2	<1 %	39.4	19.1
	VCP-Elderly	366	Soya drink	13	4 %	171.5	30.9
			Soya yoghurt	8	2 %	113.0	91.5
			Tofu	2	1 %	60.4	29.3
Romania	Dieta_Pilot_Adults	440	Soya cheese	2	<1 %	10.7	2.8
			Soya drink	3	1 %	152.4	27.4
Spain	AESAN	91	Soya drink	11	12 %	184.1	33.1
Sweden	Riksmaten 2010	647	Soya drink	9	1 %	163.2	29.4
			Soya yoghurt	6	1 %	95.8	77.6
			Tofu	3	<1 %	34.2	16.6
United Kingdom	NDNS-RollingProgrammeYears1–3	554	Soya cheese	2	<1 %	10.6	2.8
			Soya drink	18	3 %	157.0	28.3
			Soya yoghurt	2	<1 %	46.9	38.0
			Tofu	3	1 %	26.0	12.6

3.6.2. Exposure to isoflavones from food supplements in the population of peri- and post-menopausal women

Range of doses used in the intervention studies in the target population included in the systematic review

The range of doses of isoflavones from food supplements ingested by the target population was obtained from the information reported in the human intervention studies included in this review. The description of the intervention administered to the participants was extracted from the publications and, whenever possible, data have been expressed as (or converted to) the daily dose of isoflavones (mg/day) administered to the women participating in the studies.

Information on the composition of the interventions used was also extracted and whenever possible the ratio of the individual isoflavones genistein:daidzein:glycitein (GEN:DAI:GLY) was either extracted from the publications, as reported, or calculated using the information provided by the authors, thus allowing a better comparison on the type of the interventions used in the different studies. An overview of the data is presented in Table 27.

In eight studies included the intervention used was the isoflavone genistein alone, at daily doses ranging from 30 to 54 mg/day (Morabito et al., 2002; Sammartino et al., 2003; Marini et al., 2007, 2008; Bitto et al., 2010; Evans et al., 2011; Irace et al., 2013; Lappe et al., 2013).

In two studies (Upmalis et al., 2000; Colacurci et al., 2013) the daily dose administered to the participants was expressed as total daidzein and genistein and ranged 50–60 mg/day. In both studies the intervention was a soy extract.

The same commercial extract, Novasoy®, was used in four intervention studies at doses of 80–120 mg/day of total isoflavones and 90–200 mg/day when expressed as aglycones (Bruce et al., 2003; Levis et al., 2011; Chilibeck et al., 2013; Alekel et al., 2015)

In three studies (Kaari et al., 2006; Nahas et al., 2007; Delmanto et al., 2013) the intervention used was probably the same standardised soy extract, delivering a total daily dose of isoflavones ranging from 100 to 120 mg/day. In these three studies the interventions were characterised by a higher proportion of genistein than of the other isoflavone congeners, daidzein and glycitein, with a GEN:DAI:GLY ratio of 3.75:1:0.25. On the basis of the GEN:DAI:GLY ratio it can be assumed that interventions in these studies are likely to be similar to the one used in a previous study in which soy isoflavones at a dose of 100 mg/day were administered for 4 months (Han et al., 2002).

Soy protein was used in six of the included studies, with daily doses of isoflavones ranging from 43 to 132 mg/day as isoflavones aglycones. The GEN:DAI:GLY ratio was broadly consistent across these studies, with values ranging from 1.3 to 1.9:1:0.1–0.4 (Duncan et al., 1999; Persky et al., 2002; Murray et al., 2003; Verheus et al., 2008; Carmignani et al., 2010; Quaas et al., 2013).

Four studies reported the use of daidzein-rich soy extracts, such as those obtained from soy-germ. The interventions used in these studies were characterised by a GEN:DAI:GLY ratio of approximately 0.1–0.3:1:0.3–0.9. Daily doses of isoflavones in these studies ranged from 72 to 120 mg/day when expressed as total isoflavones and 40–120 mg/day as aglycones (Penotti et al., 2003; Khaodhiar et al., 2008; Maskarinec et al., 2009; Steinberg et al., 2011).

In a single study a glycitein-rich preparation was used, characterised by a GEN:DAI:GLY ratio of approximately 0.2:1:1.6 and providing a daily dose of total isoflavones of 114 mg/day (Nikander et al., 2005).

Red clover extracts were used in five studies: in three studies the intervention is likely to be the same commercial product (Promensil®) at doses ranging between 40 and 50 mg day, expressed as total isoflavones (Hale et al., 2001; Atkinson et al., 2004; Powles et al., 2008). In the remaining two studies the doses reported were 80–120 mg/day of isoflavones expressed as aglycones (Imhof et al., 2006; Geller et al., 2009).

One study (Pop et al., 2008) was conducted using a preparation (PTI G-2535) at a daily dose of 900 mg/day of isoflavone aglycones. The same preparation was used also in another study (Khan et al., 2012), although at the lower dose of 235 mg/day isoflavone aglycones. The Panel noted that

these preparations were not intended to be used as food supplements and, moreover, that the doses used in these studies are approximately up to 10-fold higher than those typically reported by food sector business operators for food supplements on the market (see Table 5). Furthermore, the preparations contain purified aglycones not reflecting the typical composition of commercial products. In the light of the above, the Panel considered that these doses could not be used to provide guidance for the intake of isoflavones from food supplements (see section 3.7).

In the remaining three studies, the interventions used were either poorly described or could not be compared with the other interventions (Unfer et al., 2004; Cheng et al., 2007; Ryan-Borchers et al., 2008). In these studies, the daily dose ranged 90–200 mg/day in the two studies that expressed doses as isoflavones aglycones per day and was 150 mg/day as total isoflavones in the study by Unfer et al. (2004).

Table 27: Overview of isoflavones composition, doses, duration used and target organs assessed in the human intervention studies included in the systematic review.

Ref. ID Authors, year	Brand name, producer	Description	Ratio GEN:DAI:GLY	Dose (mg/day)	As	Duration of treatment (months)	Organs assessed in the study
Soy isoflavones/soy extract							
3106 Unfer et al., 2004	–	Capsules containing 50 mg of soy isoflavones	–	150	Total isoflavones	60	U
16411 Levis et al., 2011	Novasoy®, Archer Daniels Midland Inc., Decatur, IL, USA	Each dose contained, on average, 91 mg of genistein and 103 mg of daidzein. The total isoflavones daily dose ranged 2.05–4.50 mg/kg bw	0,9:1:–	200	Aglycones	24	T
14945 Alekel et al., 2015	Novasoy®, Archer Daniels Midland Inc., Decatur, IL, USA	Tablets providing 120 mg aglycone equivalents per day. Formulated versus mean (SD) actual doses in tablets were 124.0 ± 7.7 mg vs. 122.5 ± 3.4 mg	1.3:1:0.3	120	Total isoflavones	36	U T
		Tablets providing 80 mg aglycone equivalents per day. Formulated versus mean (SD) actual doses in tablets were 89.5 ± 5.0 mg vs. 84.3 ± 4.5 mg	1.3:1:0.3	80	Total isoflavones	36	U T
3288 Pop et al., 2008	PTIG-2535	The composition of PTI G-2535 capsules (Lot # UPM 9809-021) used in this clinical study was as follows: genistein 139.5 mg per capsule, daidzein 74 mg per capsule and glycitein 11 mg per capsule	1.9:1:0.15	900	Aglycones	3	T
14960 Chilibeck et al., 2013	Novasoy®, Archer Daniels Midland Inc., Decatur, IL, USA	Tablets providing 105 mg aglycone equivalents per day	1:1:0.2	105	Aglycones	24	U
16409 Khan et al., 2012	PTIG-2535	The study agent contained 150 mg genistein, 74 mg daidzein and 11 mg glycitein	2:1:0.15	235	Aglycones	6	M
16401 Delmanto et al., 2013	Glycine Max AT	Capsules containing 125 mg standardised soy extract (50 % genistein and 35 % daidzein) plus 50 mg isoflavones	–	100	Total isoflavones	10	M
10231 Nahas et al., 2007	Glycine Max AT	Capsules containing 125 mg standardised soy extract (50 % genistein and 35 % daidzein) plus 50 mg isoflavones. The ratio between glycoside and aglycone form is 0.61 (38 % and 62 % in each capsule)	n.s.	100	Total isoflavones	9	U
3071 Colacurci et al., 2013	Rottapharm Madaus, Italy	SI 60 mg (30 mg of genistin, 30 mg of daidzin), *Lactobacillus sporogenes* 1 billion spores, calcium 240 mg, vitamin D_3 5 µg and glucosamine 250 mg	1:1:–	60	Genistin and daidzin	12	M U

Ref. ID Authors, year	Brand name, producer	Description	Ratio GEN:DAI:GLY	Dose (mg/day)	As	Duration of treatment (months)	Organs assessed in the study
1640 Kaari et al., 2006	ACHÉ Laboratório Ldta.	Capsule containing 150 mg standardised soy extract containing 60 mg isoflavones (as glycosides and aglycones). The extract contained daidzein congeners 20%, genistein congeners 75 % and glycitein congeners 5%. The ratio between glycoside and aglycone form is 0.61 (38 % and 62 % in each capsule)	3.75:1:0.25	120	Total isoflavones	6	U
14889 Bruce et al., 2003	Novasoy®, Archer Daniels Midland Inc., Decatur, IL, USA	The supplement consisted of 40 % isoflavones, 40 % natural soy components (mostly soy saponins) and represented approximately 2–3 times the typical Japanese adult intake	1.3:1:0.3	90	Aglycones	6	T
2414 Han et al., 2002	Eugenbio Co. Ltda, Seoul, South Korea	Capsule containing purified soy protein (50.3 mg, 60 %) and isoflavone 33.3 mg (40 %). The specific amount of genistein, daidzein and glycitein in aglycone form in each capsule was 23.3 mg, 6.2 mg and 3.8 mg respectively	3.75:1:0.6	100	Total isoflavones	4	U
3185 Ryan-Borchers et al., 2008	–	Soy milk containing providing a total daily dose of isoflavone s of 71.6 ± 3.1. Individual daily doses of isoflavones were: daidzein, 30.9 ± 1.5; genistein, 37.4 ± 1.3; glycitein, 3.6 ± 0.5	1.2:1:0.1	71.6	Total isoflavones	4	T
		The isoflavone content and composition of the supplements was formulated to match that of soy milk used in the study	1.1:1:0.2	70	Total isoflavones	4	T
3158 Cheng et al., 2007	Carlshamns Mejeri	Fruit drink containing 60 mg isoflavones	–	60	Total isoflavones	3	M
16165 Upmalis et al., 2 000	–	Soy extract standardised for total content of genistin and daidzin	–	50	Genistin and daidzin	3	U
Soy protein							
16436 Quaas et al., 2013	Solae™; The Solae Company	Soy protein (25 g) providing 91 mg of aglycone equivalent (154 mg of total isoflavones conjugates plus aglycones): genistein, 52 mg of aglycone equivalents (88 mg total); daidzein, 36 mg (61 mg total); glycitein, 3 mg (5 mg total)	1.4:1:0.1	91	Aglycones	36	U
3127 Verheus et al., 2008	Solae™; The Solae Company	Intervention consisted of 25.6 g of soy protein containing 52 mg genistein, 41 mg daidzein and 6 mg glycitein (aglycone weights) as a powder	1.3:1:0.15	99	Aglycones	12	M

Ref. ID Authors, year	Brand name, producer	Description	Ratio GEN:DAI:GLY	Dose (mg/day)	As	Duration of treatment (months)	Organs assessed in the study
3193 Persky et al., 2002	Supro 675, Protein Technologies International, St. Louis, MO, USA	Test protein (40 g) containing high concentrations of isoflavones (56 mg or 2.25 mg/g protein, expressed as aglycones)	1.5:1:0.3	90	Aglycones	6	T
		Test protein (40 g) containing moderate concentrations of isoflavones (56 mg or 1.39 mg/g protein, expressed as aglycones)	1.9:1:0.3	56	Aglycones	6	T
11323 Murray et al., 2003	Protein Technologies International, St Louis, MO, USA	Soy powder containing 25 g SPI and 120 mg aglycone isoflavones	1.5:1:0.2	120	Aglycones	6	U
3179 Duncan et al., 1999	Supro Brand Isolated Soy Protein, Protein Technologies International, St. Louis, MO, USA	Soy powder providing 2 mg/kg bw/day total isoflavones expressed as unconjugated phytoestrogen units. Proportion of genistein, daidzein and glycitein averaged 58%, 33 % and 9 % respectively, with 91 % of the genistein, 90 % of the daidzein and 82 % of the glycitein present as the glucoside conjugates	1.8:1:0.3	132	aglycones	3	T
		Soy powder providing 1 mg/kg bw/day total isoflavones expressed as unconjugated phytoestrogen units. Proportion of genistein, daidzein and glycitein averaged 58 %, 33 % and 9 % respectively, with 91 % of the genistein, 90 % of the daidzein and 82 % of the glycitein present as the glucoside conjugates	1.8:1:0.3	65	aglycones	3	T
1103 Carmignani et al., 2010	Previna®, Sanavita Functional Foods, Piracicaba, São Paulo, Brazil	20 g portions of a food powder containing 12 g of soy protein and a total of 45 mg of isoflavones (26.5 mg aglycones)	1.9:1:0.4	43	aglycones	4	U
Daidzein-rich isoflavones							
4366 Steinberg et al., 2011	Frutarom Netherlands BV	Each tablet contained 34.10–40.51 mg aglycone equivalent of total isoflavones with the majority (> 95 %) in the form of glycosides	0.4:1:0.2	120	Aglycones	24	T
				80	Aglycones	24	T
1199 Maskarinec et al., 2009	Acatris Holding	Soy-germ isoflavone tablets containing 42 % daidzin, 2 % daidzein, 13 % genistin, 1 % genistein, 39 % glycitin and 3 % glycitein	0.9:1:0.9	120	Total isoflavones	24	M
				80	Total isoflavones	24	M

Ref. ID Authors, year	Brand name, producer	Description	Ratio GEN:DAI:GLY	Dose (mg/day)	As	Duration of treatment (months)	Organs assessed in the study
3110 Penotti et al., 2003	Solgar Italia Multinutrient srl, Padua, Italy	Tablets containing a total of 36 mg of soy-derived isoflavones (5.5 mg of genistein/genistin; 18 mg of daidzein/daidzin; and 12.5 mg of glycitein/glycitin) and 48 mg of soy saponin. Aglycate:glycate ratio was 3:2	0.3:1:0.7	72	Total isoflavones	6	U
3195 Khaodiar et al., 2008	AglyMax, Nichimo Co, Ltd, Shinagawa, Tokyo, Japan	Concentrated isoflavone product prepared from soybean germ fermentation with Koji fungus (*Aspergillus awamori*). The concentration of the individual isoflavones was daidzein, 70 %; genistein, 10 %; and glycitein, 20 %	0.1:1:0.3	40	Aglycones	3	T
				60	Aglycones	3	T
Glycitein-rich isoflavones							
1639 Nikander et al., 2011	Bonette®, Novomed, Helsinki, Finland	Each tablet consisted of glycitein (11 mg, 58 %), daidzein (7 mg, 36 %) and genistein (1 mg, 6%), the total dose of isoflavonoids being 114 mg/day	0.2:1:1.6	114	Total isoflavones	3	U
Genistein							
3138 Marini et al., 2008; 4922 Marini et al., 2007; 16393 Bitto et al., 2010	Lab Plants, Messina, Italy	Each tablet contained 27 mg genistein, purity approx. 98 %	1:-:–	54	Genistein	12	M T U
2282 Morabito et al., 2002	Lab Plants, Messina, Italy	Each tablet contained 27 mg genistein, purity approx. 98 %	1:-:–	54	Genistein	12	M U
15955 Sammartino et al., 2003	Fitogen, Bergamo, Italy	–	1:-:–	36	Genistein	12	U
256 Irace et al., 2013	–	–	1:-:–	54	Genistein	6	U
15431 Lappe et al., 2013	Genivida®, DSM Nutritional products	Each capsule contained 15 mg genistein (geniVida 99.1 % genistein), 500 mg PUFAs (ROPUFA 75 n-3 ethyl ester), 75 µg vitamin K1 (99.7 % phylloquinone) and 400 IU vitamin D_3 (100 % cholecalciferol) together with maize oil and bees wax.	1:-:–	30	Genistein	6	U
15095 Evans et al., 2011	Genivida®, DSM Nutritional products	A patented, 99 % synthetic pure aglycone	1:-: –	30	Genistein	3	U

Ref. ID Authors, year	Brand name, producer	Description	Ratio GEN:DAI:GLY	Dose (mg/day)	As	Duration of treatment (months)	Organs assessed in the study
16435 Powles et al., 2008	Promensil®; Novogen Ltd		n.a.	40	Total isoflavones	36	M
19610 Geller et al., 2009		Each tablet contained 116.6 mg isoflavones (57.5 mg biochanin A, 56.6 mg formononetin, 1.6 mg genistein, 0.9 daidzein)	n.a.	120	Aglycones	12	T
3168 Atkinson et al., 2004	Promensil®; Novogen Ltd, Sidney, Australia	Each tablet provided 26 mg biochanin A, 16 mg formononetin, 1 mg genistein and 0.5 mg daidzein	n.a.	43,5	Total isoflavones	12	M
15280 Imhof et al., 2006	MF11RCE; Melbrosin International, Vienna, Austria	Standardised content of 40 mg aglyconic isoflavones per capsule	n.a.	80	Aglycones	3	U
16405 Hale et al., 2011	Novogen Ltd, North Ride, Australia	Purified red clover extract containing high proportion of methylated isoflavone biochanin A and a smaller amount of formononetin, genistein and daidzein	n.a.	50	Total isoflavones	3	U

M: mammary gland; U: uterus; T: thyroid.

Range of doses used in the animal studies included in the systematic review

Information on the composition of the test substances used in the animal studies included in the current systematic review was also extracted. As for the human intervention studies, whenever possible the ratio between the individual isoflavones, genistein:daidzein:glycitein (GEN:DAI:GLY), was either extracted from the publications, as reported, or calculated using the information provided by the authors, thus allowing a better comparison of the type of the interventions used in the different studies. An overview of the data is presented in Table 28. Data are presented grouped by type of intervention (e.g. soy isoflavones, red clover extract, genistein) and animal species in which the tests were performed. Within each group the data are presented with the highest dose tested on top and then in descending order.

Despite the heterogeneity of the reporting of the data available, all the doses tested in the studies have been converted to mg/kg bw/day to facilitate comparison. The assumptions made by the Panel to convert doses are clearly documented in the table, although it should be noted that in some cases there are still important uncertainties with respect to the exact dose tested.

In 15 studies, the animals were administered test products that could be classified as soy isoflavones or soy extracts (Kikuchi-Hayakawa et al., 1998; Yamaguchi et al., 2001; Breitman et al., 2003; Gallo et al., 2005, 2006, 2008; Wood et al., 2006; Liu et al., 2007; Kishida et al., 2008; Hertrampf et al., 2009; Hong et al., 2009; Zhang et al., 2009; Zhao et al., 2011; Kakehashi et al., 2012; Teixeira et al., 2014).

The doses used ranged from 7.5 to 200 mg/kg bw/day (doses expressed as total isoflavones) and from 0.16 to 157 mg/kg bw/day expressed as aglycones. In two studies (Gallo et al., 2006; Hertrampf et al., 2009) the dose of isoflavones administered to the animals was expressed as total daidzein and genistein and ranged from 16.8 to 28 mg/kg bw/day.

The commercial soy extract Novasoy®, which was used in four of the human studies included in the current assessment, was also tested in rats at doses of 50 mg/kg bw/day expressed as genistein equivalent (Teixeira et al., 2014) and at a dose of 200 mg/kg bw/day in mice (Zhang et al., 2009).

Soy protein or SPI was used in three studies in monkeys (Foth and Cline, 1998; Wood et al., 2004; Scott et al., 2008), providing isoflavones at doses ranging from 8.6 to 9.9 mg/kg bw/day. The doses of isoflavones from soy protein or SPI tested in the three studies in rats ranged from 1.5 to 26 mg/kg bw/day expressed as total isoflavones and from 9.4 to 28.5 mg/kg bw/day when expressed as aglycones (Tansey et al., 1998; Pan et al., 1999; Bahr et al., 2005). In all the studies above, genistein was always the main component of the total isoflavones.

Nine studies performed in rats reported the use of daidzein-rich soy extracts as the source of isoflavones (Catania et al., 2002; Hidaka et al., 2003; Lee et al., 2004; Castillo et al., 2006; Mosquette et al., 2007; Baeza et al., 2009; Carbonel et al., 2011a, b; Francisco et al., 2013). The interventions used in these studies were characterised by a GEN:DAI:GLY ratio of approximately 0.2–0.8:1:0.03–0.7. Doses of isoflavones in these studies ranged from 2.1 to 255 mg/kg bw/day.

Red clover extracts were used in five studies in rats (Burdette et al., 2002; Alves et al., 2008; Overk et al., 2008; Kawakita et al., 2009; Kang et al., 2015) and in one in rabbits (Adaikan et al., 2009). The doses used in the rats studies ranged from 1.15 (total isoflavones) to 112.5 (aglycones) mg/kg bw/day whereas, in the rabbit study, a single dose providing an equivalent dose of 0.1 mg/kg bw/day daidzein was tested.

Isoflavones from kudzu root were tested in one study in rat (Dong et al., 2014) at a single dose of 56 mg/kg bw/day and in two studies in mice (Wang et al., 2003; Cho et al., 2012) at doses ranging from 2 to 240 mg/kg bw/day.

In 13 studies included in the assessment, the isoflavone genistein alone, at doses ranging from 4.5 to 90 mg/kg bw/day, was investigated in rats (Santell et al., 1997; Li and Yu, 2003; Ye et al., 2003; Vera et al., 2006; Wuttke et al., 2006; Mathey et al., 2007; Phrakonkham et al., 2007; Rimoldi et al., 2007; Chen et al., 2009; Wang et al., 2008; Bitto et al., 2009; Hertrampf et al., 2009; Zhang et al., 2009; Carbonel et al., 2015). Additionally, one study investigated the glycosylated form, genistin, at doses of 50 to 100 mg/kg bw/day (Uesugi et al., 2001).

Genistein was also investigated in three studies in mice (Chen et al., 2009; Zhang et al., 2009; Nguyen et al., 2013) at doses ranging from 6 to 2 000 mg/kg bw/day.

The isoflavone daidzein alone was investigated in three studies in rats at doses ranging from 10 to 90 mg/kg bw/day (Mathey et al., 2007; Phrakonkham et al., 2007; Rachoń et al., 2007b). Its glycosylated form, genistin, was also tested in rats at doses of 25 and 50 mg/kg bw/day (Uesugi et al., 2001).

In the same study also the glycosylated form of glycitein, glycitin, was tested at the same doses (Uesugi et al., 2001).

Racemic equol was tested in two studies in monkeys at doses ranging from 7.2 to 68 mg/kg bw/day (Wood et al., 2006, 2008) and in five studies in rats at doses ranging from 4.5 to 36 mg/kg bw/day (Mathey et al., 2007; Phrakonkham et al., 2007; Rachoń et al., 2007a, 2008; Legette et al., 2009). The isomer *S*-equol and a fermented soy germ product containing *S*-equol were tested in one study in rats at a single dose of 11.7 mg/kg bw/day (Yoneda et al., 2011).

With respect to the other isoflavones puerarin and biochanin A, these were investigated in only one study each in rats; puerarin was tested at two doses of 54 and 270 mg/kg bw/day (Rachoń et al., 2007b) and biochanin A at a single dose of 25 mg/kg bw/day (Su et al., 2013).

Formononetin was tested at two doses of 50 to 500 mg/kg bw/day in only one study in mice (Mu et al., 2009).

Table 28: Overview of isoflavones composition, doses (and methods for dose conversion), and duration of treatment in the animal studies included in the systematic review.

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
Soy isoflavones/soy extract							
1714 Wood et al., 2006	The isoflavone supplement contained genistein (65.5 %), daidzein (29.2 %) and glycitein (5.3 %), expressed in aglycone equivalents. Actual dose administered 537 mg/1 800 kcal	Reported in the publication	2.2:1:0.2	35.7	Total isoflavones	30	Monkeys (*Macaca fascicularis*)
16313 Yamaguchi et al., 2001	Isoflavone aglycone (IA)-rich extract (Soy Act®, Kikkoman Corporation, Chiba) containing genistein (52 %), daidzein (42 %) and glycitein (6 %)	Reported in the publication	1.2:1:0.1	157 67	Aglycones	30	Rats (Wistar)
15337 Kakehashi et al., 2012. Expt.1	Isoflavone aglycone (IA)-rich extract (Soy Act®, Kikkoman, Noda, Japan) containing genistein (52 %), daidzein (42 %) and glycitein (6 %). Total content of isoflavones in the extract was 30 %	Reported in the publication	1.2:1:0.1	150	Aglycones	14	Rats, Donryu
10424 Liu et al., 2007	Soybean isoflavone (Harbin high-tech Corp., Heilongjiang, China)	Reported in the publication	–	50	Total isoflavones	56	Rats (Wistar)
16112 Teixeira et al., 2014	Soy isoflavone extract (Novasoy®, Archer Daniels Midland, Decatur, IL, USA) containing 40 % total isoflavones	Reported in the publication	1.3:1:0.3	50	Genistein	30	Rats (Wistar)
5800 Breitman et al., 2003	Isoflavone extract (1.6 g) containing: genistein, 217 mg; daidzein, 196 mg and glycitein, 35 mg. Authors report dose as 1.6 g/kg diet	Dose calculated by applying the EFSA default value of 0.09 per sub-chronic studies in rats (EFSA Scientific Committee, 2012) to the sum of isoflavones reported in the publication (448 mg/kg diet)	1.1:1:0.2	40.3	Aglycones	56	Rats (Sprague–Dawley)

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
10162 Kishida et al., 2008. Expt.4	Fermented soy bean extract (Kikkoman, Soy Act, Noda, Japan) containing genistein, 155 mg/g; daidzein, 127 mg/g; glycitein, 18 mg/g; genistin, daidzin and glycitin < 1 mg/g. Isoflavones were administered at 300 mg/kg diet	Dose calculated by the Panel by applying a default value of 0.12 for sub-acute studies in rats (EFSA Scientific Committee, 2012)	1.2:1:0.1	36	Aglycones	28	Rats (Sprague–Dawley)
15219 Hertrampf et al., 2009	Isoflavone-rich diet (Haraln Winkelmann, Borchen, Germany) containing genistein (240 ± 36 µg/g) and daidzein (232 ± 10 µg/g)	As reported in the publication	1:1:-	28	Genistein and daidzein	84	Rats (Wistar)
6198 Kikuchi-Hayakawa et al., 1998	Unfermented soy milk containing 1 691 µg/g isoflavones (expressed as aglycones). Most of the isoflavones (98.5 %) were present as glycosides: daidzin, 777.8 µg/g; genistin, 887.6 µg/g; daidzein, 14.5 µg/g; genistein, 11.1 µg/g). The soy milk powder was 30 % of the diet.	The Panel calculated the dose on the basis of the mean food intake reported (13.1 g/day) and mean animal initial animal weight (0.339 kg)		19.6	Total isoflavones	30	Rats (Wistar)
	Crude soy milk fermented with *Bifidobacterium breve* YIT4065 containing 1 725.7 µg/g isoflavones (expressed as aglycones). Most of the isoflavones were present as aglycones: daidzin, 521.9 µg/g; genistin, 184.5 µg/g; daidzein, 248.1 µg/g; genistein, 771.2 µg/g. The fermented soy milk powder was 30 % of the diet	The Panel calculated the dose on the basis of the mean food intake reported (12.5 g/day) and mean animal initial animal weight (0.333 kg)		19.4			
15146 Gallo et al., 2008	Standardised soy extract (Indena SpA, Milan, Italy) administered at 100 mg/kg bw/day. The extract was standardised to contain 13–17 % isoflavones glycosides genistin and daidzin and not less than 18 % of B-group saponins. The batch used in the study contained 16.8 % isoflavone glycosides	Dose calculated on the basis of the percentage reported by the authors	–	16.8	Genistin and daidzin	42	Rats (Sprague–Dawley)

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
15147 Gallo et al., 2006	Standardised soy extract Soyselect® administered at 100 mg/kg bw/day	Dose calculated on the basis of the percentage reported in the company website (13–17 % isoflavones, mean 15 %)	–	15	Total isoflavones	42	Rats (Sprague–Dawley)
15145 Gallo et al., 2005	Standardised soy extract Soyselect® (Indena SpA, Milan, Italy) administered at 50 and 100 mg/kg bw/day	Dose calculated on the basis of the percentage reported in the company website (13–17 % isoflavones, mean 15 %)	–	15	Total isoflavones	42	Rats (Sprague–Dawley)
				7.5			
9712 Hong et al., 2009	Fermented soy pulp (FSP) containing 17.9 mg/kg isoflavones, administered at 4.8 mg/kg diet	Dose calculated by applying EFSA default value of 0.09 per sub-chronic studies in rats (EFSA Scientific Committee, 2012)	–	0.43	Aglycones	49	Rats (Sprague–Dawley)
	Soy pulp (SP) supplement containing 3.4 mg/kg isoflavones, administered at 1.8 mg/kg diet			0.16			
4561 Zhang et al., 2009	Novasoy® (Archer Daniels Midland) containing 40 % isoflavones at a dose of 2.5 g/kg diet	Dose calculated using a default value of 0.2 for sub-chronic/sub-acute studies in mice (EFSA Scientific Committee, 2012)	1.3:1:0.3	200	Total isoflavones	35	Mice (C57BL/6J)
9236 Zhao et al., 2011. Expt. 2	Commercial soy extract product (Healthy Women Soy Extract Supplement, Amazon, Seattle, WA, USA) providing a total of 100 mg/kg diet of genistein/genistin, daidzein/daidzin and glycitein/glycitin	Reported in the publication. Dose calculated by the authors assuming a 25-g mouse eating a 2.5 g per day diet	1:1:–	10	Total isoflavones	270	Mice (129/C57BL/6)
	A phyto-β-SERM diet containing equal parts (33 mg/kg diet) of genistein, daidzein and equol and providing a total of 100 mg/kg diet			10	Aglycones		

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
Soy protein							
15120 Foth and Cline, 1998	Isoflavone-rich SPI fed at a dose equivalent, on an energy basis, to 148 mg/day per woman	The Panel assumed that the same conversion factor applied by the same research group in the publication Wood et al. (2004) applies also to this case	–	9.9		180	Monkeys (*Macaca fascicularis*)
16271 Wood et al., 2004	Isolated soy protein from The Solae Company. The unextracted soy protein (SUPRO 670-HG) contained isoflavones at a dose approximately equivalent to 129 mg/day for women (~91 mg genistein, 31 mg daidzein, and 7 mg glycitein)	Reported in the publication. Dose converted by the authors on the basis of the fact that the doses administered were scaled to 1 800 kcal of diet (estimated daily intake for US women) and that monkeys were fed 120 kcal/kg bw/day	2.9:1:0.2	8.6	Total isoflavones	1 080	Monkeys (*Macaca fascicularis*)
4730 Scott et al., 2008	Isolated soy protein from The Solae Company. Authors stated that the dose administered corresponded to a human equivalent dose of 129 mg/day, expressed as aglycone units.	The Panel assumed that the same conversion factor applied by the same research group in the publication Wood et al. (2004) applies also to this case	-	8.6	Total isoflavones	1080	Monkeys (*Macaca fascicularis*)
12095 Pan et al., 1999	Soy phytoestrogen concentrate (Protein Technologies International, St. Louis, MO, USA) containing 66 % on dry basis of isoflavones (genistein, 43%; daidzein, 21 %; and 2 % glycitein). The extract was administered at a dose of 0.0833 mg/cal. According to the authors the dose was equivalent to a human dose of 150 mg/day for a woman (150 mg/1 800 kcal)	Dose calculated on the assumption that caloric intake of a rat is 60 cal/day. Mean weight of the animals was 0.330 kg (old animals) and 0.190 kg (young animals)	2:1:0.1	26 (young) 15 (old)	Total isoflavones	56	Rats (Sprague–Dawley)

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
10960 Bahr et al., 2005	Soy protein isolated from soybean (Clinical 670 blend, lot # 019-99, DuPont Protein Technologies, St. Louis, MO, USA) containing 2.14 mg/g of aglycone from isoflavones (genistein, 1.10 mg/g; daidzein, 0.84 mg/g; and glycitein, 0.20 mg/g)	Reported in the publication	1.3:1:0.2	21.6	Aglycones	84	Rats (Sprague–Dawley)
				10.5			
	Alcoholic extract from SPI (Soy product, lot# 2762–9–1, DuPont Protein Technologies) containing 11.37 mg/g of aglycone from isoflavones (genistein, 7.09 mg/g; daidzein, 3.81 mg/g; and glycitein, 0.47 mg/g).		1.9:1:0.1	19.3			
				9.4			
2803 Tansey et al., 1998	Soy bean extract (SBE) isolate SUPRO 670® (Protein Technologies International, St. Louis, MO, USA) added at 16 g/100 g diet. Authors report dose equal to 117.8 mg/1 800 kcal	The conversion has been calculated assuming the daily caloric intake of a rat is 60 cal and that average weight is 250 g	–	15.7	Total isoflavones	60	Rats (Sprague–Dawley)
	SBE isolate (alcohol extracted) SUPRO 670-IF® (Protein Technologies International, St. Louis, MO, USA) added at 15.40 g/100 g diet. Authors report dose equal to 11.6 mg/1 800 cal		–	1.5			
Daidzein-rich isoflavones							
1618 Mosquette et al., 2007	A fermented soybean extract rich in the major isoflavone aglycones, genistein and daidzein (Zhonshan Road, Dalian, China) containing 42.6 % isoflavones (approximately 36 % were genistein/genistin, 62 % were daidzein/daidzin and 2 % glycitein/glycitin)	Reported in the publication	0.6:1:0.03	255.6	Total isoflavones	21	Rats (Wistar)
				127.8			
				42.6			
				21.3			
				4.3			
15224 Hidaka et al., 2003	Fujiflavones P40 (Fujicco Co Ltd, Kobe, Japan) containing 46.63 % (w/w) total isoflavones	Reported in the publication	0.2:1:0.7	64–83	Aglycones	49	Rats (Sprague–Dawley)

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
12969 Carbonel et al., 2011b	Soy extract (Zhonshan Road, Dalian, China) containing 42.6 % isoflavones (approximately 36 % were genistein/genistin, 62 % were daidzein/daidzin and 2 % glycitein/glycitin)	Reported in the publication	0.6:1:0.03	250	Genistein equivalent	30	Rats (Wistar EPM-1)
				125			
				42			
14909 Carbonel et al., 2011a	A fermented soybean extract rich in the major isoflavone aglycones, genistein and daidzein (Zhonshan Road, Dalian, China) containing 42.6 % isoflavones (approximately 36 % were genistein/genistin, 62 % were daidzein/daidzin and 2 % glycitein/glycitin) administered at 46 mg/kg bw/day and 120 mg/kg bw/day	Calculated on the basis of the isoflavone content, as reported in the publication	0.6:1:0.03	53.25	Total isoflavones	21	Rats
				19.6			
454 Francisco et al., 2013	A fermented soybean extract rich in the major isoflavone aglycones, genistein and daidzein (Zhonshan Road, Dalian, China) containing 42.6 % isoflavones (approximately 36 % were genistein/genistin, 62 % were daidzein/daidzin and 2 % glycitein/glycitin) administered at 46 mg/kg bw/day and 120 mg/kg bw/day	Reported in the publication. The Panel, however, noted that, according to the percentage of isoflavones reported (42.6%), the calculated dose of isoflavones would be 19.6 mg and 51.1 mg	0.6:1:0.03	42.6	Total isoflavones		
				21.3			
1251, Baeza et al., 2009	Dry ethanolic extract of *Glycine max* hypocotyls (Phytosoya®, Arkochim, Spain) containing 9–11 % isoflavones (4–6 % daidzein, 2–4 % glycitein, 1–3 % genistein) at a dose of 300 mg/kg bw/day.	Isoflavones dose calculated by the Panel assuming the extract contained 10 % of total isoflavones	0.5:1:0.6	30	Total isoflavones	70	Rats (Wistar)
14918 Castillo et al., 2006	Dry ethanolic extract of *Glycine max* hypocotyls (Phytosoya®, Arkochim, Spain) containing 9–11 % isoflavones (4–6 % daidzein, 2–4 % glycitein, 1–3 % genistein) at a dose of 300 mg/kg bw/day	Isoflavones dose calculated by the Panel assuming the extract contained 10 % of total isoflavones	0.5:1:0.6	30	Total isoflavones	70	Rats (Wistar)

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
5420 Lee et al., 2004	Soy hypocotyl extract (SHE) administered at 6.25 g/kg diet and providing 521.94 mg/kg diet of total isoflavones. The authors report that 6.25 g of isoflavone extract contained: genistein 1.79 mg; daidzein 3.06 mg; glycitein, 8.36 mg; genistin, 57.63 mg; daidzin, 258.99 and glycitin 171.31 mg	Dose calculated using the EFSA default value of 0.05 for chronic studies in rats (EFSA Scientific Committee, 2012)	0.2:1:0.7	26	Total isoflavones	112	Rats (Sprague–Dawley)
14919 Catania et al., 2002	Soy fraction with mainly isoflavones SOYPH (Pharmalife Research), containing 42.3 % isoflavones (genistein and genistin, 17%; daidzein and daidzin, 22%, glycitein and glycitin, 3.1%) and administered at a dose of 5 mg/kg/day	Dose calculated by the Panel on the basis of the isoflavone percentage reported	0.8:1:0.1	2.1	Total isoflavones	28	Rats (Sprague–Dawley)
Red clover extract							
4723 Overk et al., 2008	Standardised extract of *T. pratense* (Naturex, South Hackensack, NJ, USA) to a minimum of 30 % isoflavone content by weight of four isoflavones—genistein, 0.41 %; daidzein, 0.23 %; biochanin A, 14.47 %; and formononetin, 14.26 %—as hydrolysed aglycones. The dose of 40 mg/kg bw/day were based on a clinical dose of 120 mg/day for a woman contained in 400 mg/day of *T. pratense* extract	Dose calculated by the Panel on the basis of the isoflavone percentage reported	n.a.	120 12 1.2	Aglycones	21	Rats (Sprague–Dawley)
11684 Burdette et al., 2002	Red clover extract, standardised to a minimum 15 % isoflavone content by weight of four isoflavones: genistein, 0.850 %; daidzein, 0.349 %; biochanin A, 6.57 %; and formononetin, 8.56 %, as hydrolysed aglycones. The extract was administered at doses of 250, 500 and 750 mg/kg/day. According to the	Dose of isoflavones calculated on the basis of the percentage (15 %) reported by the authors.	n.a.	112.5 75	Aglycones	21	Rats (Sprague–Dawley)

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
	authors the doses chosen were based on a clinical dose of 40 mg isoflavones/day.			37.5			
19187 Kang et al., 2015	Red clover extract (Health-Love Co, Ltd, Anyang, Korea) standardised to contain 8 % of total isoflavones (genistein, 0.62%; biochanin A, 5.43%; formononetin, 3.66%; and daidzein, 0.47%) and administered at 40 mg/kg bw/day	Dose of isoflavones calculated on the basis of the percentage (8%) reported by the authors.	n.a.	3.2	Total isoflavones	84	Rats (Sprague–Dawley)
15353 Kawakita et al., 2009	Red clover extract (Menoflavon Forte®, Named, Lesmo, Italy) standardised to contain 40 % isoflavones by weight (genistein, daidzein, biochanin A and formononetin present as hydrolysed aglycones) and administered at 6 mg/kg bw/day	Dose of isoflavones calculated on the basis of the percentage (8%) reported by the authors.	n.a.	2.4	Aglycones	90	Rats (Sprague–Dawley)
14764 Alves et al., 2008	Standardised extract of *Trifolium pratense* (Galena Química e Farmacêutica Ltda, Campinas, São Paulo, Brazil) containing 9.7 % of isoflavones. According to the authors the dose chosen was equivalent human dose of 80 mg/day	Reported in the publication	n.a.	1.15	Total isoflavones	28	Rats (Wistar)
14737 Adaikan et al., 2009	A 0.6 % red clover extract	Estimated by the authors	n.a.	0.1	Daidzein equivalent	84	Rabbits (New Zealand White)
Kudzu root							
19076 Dong et al., 2014	Water extract of *Puerariae radix* (Huangshan Tianjian Sci-Tech Ltd, Anhui Province, China) containing 18.7 % total isoflavones (the two main isoflavones puerarin and daidzin accounted for 9.7 % and 2.3%, respectively) administered at 300 mg/kg/day	Dose calculated by the Panel on the basis of the percentage reported in the publication, assuming the dosing of the extract is expressed as mg/kg bw/day		56	Total isoflavones	84	Rats (Sprague–Dawley)

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
11449 Wang et al., 2003	Crude *Puerariae radix* powder (100 mesh) from Nihon Funmatsu, Osaka, Japan containing daidzein at 8.03 mg/g and genistein 1.01 mg/g. The powder was administered at 5%, 10 % and 20 % in the diet providing daily isoflavones intake of 1.8, 3.6 and 7.2 mg/day	Dose conversion calculated by the Panel on the basis of the information reported on the approximate mean weight of the animals (30 g)		240	Total isoflavones	28	Mice (ddY)
				120			
				60			
4121 Cho et al., 2012	Isoflavones from *P. Lobata* extracted from the dried powder of *P. lobata* (Willd) Ohwwi with 60 % aqueous ethanol. Compositional analysis of the extract showed that total isoflavones were 13.6 % (puerarin, 7.5 %; daidzin, 4.2 %; and genistin, 1.9 %)	Dose calculated on the basis of the percentage of isoflavones in the extract reported in the publication		68	Total isoflavones	28	Mice (ICR)
				2			
Genistein							
16299 Wuttke et al., 2006	In the publication it is reported that the dose administered to the animals was 53 mg/day. The first author was contacted and clarified that the weight of the animals used in the study was 230–250 g (Chair of Working Group Isoflavones, personal communication, May 2015)	Dose conversion calculated by the Panel on the basis of the information received	1:-:–	221	Aglycone	90	Rats (Sprague–Dawley)
15958 Santell et al., 1997. Expt. 1	Genistein synthesised by the demethylation of biochanin A or from organic precursor. Chemical identity assessed by nuclear magnetic resonance and purity assessed at > 98 %. Animals administered at doses of 150, 375 and 750 µg/g diet	Dose calculated using a default value of 0.12 for sub-acute studies in rats (EFSA Scientific Committee, 2012)	1:-:–	90	Aglycone	5	Rats (Sprague–Dawley)
				45			
15958 Santell et al., 1997. Expt. 2						21	Rats (Sprague–Dawley)
				18			
4863 Rimoldi et al., 2007	Genistein, purity of 98.5 %; commercial product of Chemos (Regensburg, Germany)	As reported in the publication	1:–:–	54	Aglycone	90	Rats (Sprague–Dawley)
				5.4			
19012 Carbonel et al., 2015	Genistein obtained from Glycine max (soybean, Sigma Aldrich, St. Louis, MO, USA)	As reported in the publication	1:-:-	50	Aglycone	30	Rats

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
11367 Li and Yu, 2003	Genistein (Sigma Chemical Co, Saint Louis, MO, USA)	As reported in the publication	1:-:-	45	Aglycone	84	Rats (Sprague–Dawley)
15219 Hertrampf et al., 2009	Genistein (Sigma Aldrich, Deisenhof, Germany)	As reported in the publication	1:-:-	42	Aglycone	84	Rats (Wistar)
2232 Ye et al., 2003 Expt. 2	Genistein (Sigma Chemical Co., St. Louis, MO, USA)	As reported in the publication	1:-:-	25	AGLYCONE	21	Rats (Wistar)
1354 Wang et al., 2008	NO-releasing pro-drug of genistein. Doses are equimolar of genistein.	As reported in the publication	1:-:-	18	aglycone	84	Rats (Sprague–Dawley)
				9			
				4.5			
	Genistein (Sigma Chemical Co, Saint Louis, MO, USA)			9			
13554 Mathey et al., 2007	Genistein (Pharmaceutical Research Institute, Warsaw, Poland)	As reported in the publication	1:-:–	10	Aglycone	90	Rats (Wistar)
16713 Phrakonkham et al., 2007	Genistein (Pharmaceutical Research Institute, Warsaw, Poland)	As reported in the publication	1:-:–	10	Aglycone	90	Rats (Wistar)
10284 Vera et al., 2006	Genistein	As reported in the publication	1:-:–	10	Aglycone	35	Rats (SHR)
14841 Bitto et al., 2009	Genistein aglycone (Sigma). Dose equivalent to human dose of 54 mg/day	As reported in the publication	1:-:–	4.8	Aglycone	28	Rats (Wistar Kyoto)
4038 Nguyen et al., 2013	Genistein-enriched diet (Ssniff, Soest) at a concentration of 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 g/kg food	Dose calculated using a default value of 0.2 for sub-chronic/sub-acute studies in mice (EFSA Scientific Committee, 2012)	1:-:–	2 000	Aglycone	84	Mice (C57BL/6J)
				600			
				200			
				60			
				20			
				6			
4676 Chen et al., 2009	Genistein (Sigma) enriched diet at a concentration of 0.2 g/kg diet	Dose calculated using a default value of 0.2 for sub-chronic/sub-acute studies in mice (EFSA Scientific Committee, 2012)	1:-:–	400	Aglycone	84	Mice (BALB/c)

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
4561 Zhang et al., 2009	Genistein at a dose of 0.5 g/kg diet			100	Aglycone	35	Mice (C57BL/6J)
Genistin							
14256 Uesugi et al., 2001	Genistin isolated from soybeans (method described in the publication)	As reported in the publication	n.a.	100 50	Glycoside	28	Rats (Sprague–Dawley)
Daidzein							
14737 Adaikan et al., 2009	Daidzein (Sigma Aldrich Ltd, St. Louis, MO, USA)	As reported in the publication	–:1:–	0.1	Aglycone	84	Rabbits (New Zealand White)
16809 Rachoń et al., 2007b	Daidzein 98 % purity, Changzhou Dahua Importing and Exporting Group, China, at concentrations of 250 and 1 000 mg/chow	Dose calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012).	–:1:–	90 22.5	Aglycone	90	Rats (Sprague–Dawley)
13554 Mathey et al., 2007	Daidzein supplied by the University of Athens, Division of Pharmacognosy, Greece (Athens, Greece)	As reported in the publication	–:1:–	10	Aglycone	90	Rats (Wistar)
16713 Phrakonkham et al., 2007	Daidzein supplied by the University of Athens, Division of Pharmacognosy, Greece (Athens, Greece)	As reported in the publication	-:1:-	10	Aglycone	90	Rats (Wistar)
Daidzin							
14256 Uesugi et al., 2001	Genistin isolated from soybeans (method described in the publication)	As reported in the publication	n.a.	50 25	Glycoside	28	Rats (Sprague–Dawley)
Glycitin							
14256 Uesugi et al., 2001	Genistin isolated from soy hypocotyls (method described in the publication)	As reported in the publication	n.a.	50 25	Glycoside	28	Rats, Sprague–Dawley
Equol							
1714 Wood et al., 2006	96.0 % pure racemic mixture of *S*- and *R*-equol enantiomers. Actual dose administered 1020 mg/1 800 kcal			68	Racemic equol	30	Monkeys *(Macaca fascicularis*)

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
16269 Wood et al., 2008	96.0 % pure racemic mixture of *S*- and *R*-equol enantiomers. Actual dose administered equivalent to a human equivalent dose of 105 mg/day			7.2	Racemic equol	240	Monkeys (*Macaca fascicularis*)
643 Rachoń et al., 2008	Racemic mixture, 98 % purity, Changzhou Dahua Importing and Exporting Group, China at concentrations of 50 and 400 mg/chow	Dose calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012).	n.a.	36	Racemic equol	90	Rats (Sprague–Dawley)
15876 Rachoń et al., 2007a				4.5			
1203 Legette et al., 2009	Equol powder (50 % *R*-equol, 50 % *S*-equol) at concentrations of 50, 100 and 200 mg/diet	Dose calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012)	n.a.	18	Racemic equol	56	Rats (Sprague–Dawley)
				9			
				4.5			
16333 Yoneda et al., 2011	The fermented soy germ product SE5-OH (Otsuka Pharmaceuticals Co Ltd, Tokushima, Japan) was administered at doses of 2 000 mg/kg bw/day	As reported in the publication	n.a.	11.7	*S*-equol	38	Rats (Sprague–Dawley)
	Purified *S*-equol extracted from SE5-OH (Otsuka Pharmaceuticals Co. Ltd, Tokushima, Japan)						
13554 Mathey et al., 2007	Equol provided by the ENITA Unité Micronutriments-Reproduction-Santé (Bordeaux, France)	As reported in the publication	1:-:-	10	Racemic equol	90	Rats (Wistar)
16713 Phrakonkham et al., 2007	Racemic mixture of R and *S*-equol was provided by the ENITA Unité Micronutriments-Reproduction-Santé (Bordeaux, France)	As reported in the publication	n.a.	10	Racemic equol	90	Rats (Wistar)
Puerarin							
16809 Rachoń et al., 2007b	Puerarin 98 % purity (Changzhou Dahua Importing and Exporting Group, China) at concentrations of 600 and 3000 mg/chow	Dose calculated using a default value of 0.09 for sub-chronic studies in rats (EFSA Scientific Committee, 2012)	-:1:-	270	Aglycone	90	Rats (Sprague–Dawley)
				54			

Ref. ID Authors, year	Description	Dose conversion to mg/kg bw/day	Ratio GEN:DAI:GLY	Dose (mg/kg bw/day)	As	Duration (days)	Animal species
Biochanin A							
12777 Su et al., 2013	Biochanin A (Sigma Aldrich Ltd, St. Louis, MO, USA)	As reported in the publication	n.a.	25	Aglycone	98	Rats (Sprague–Dawley)
Formononetin							
15689 Mu et al., 2009	Formononetin prepared from dried red clover (method described in the publication)	As reported in the publication	n.a.	500 50		180	Mice (Kunming)

Exposure estimates from food supplements

On the basis of the information gathered from the data providers, the recommended daily doses of isoflavones from food supplements range from 20 to 150 mg/day for soy supplements (with the exception of one product providing 300 mg/day) and from 40 to 80 mg/day for red clover supplements, for use by peri- and post-menopausal women. For food supplements containing powder or extracts of kudzu root, the recommended daily doses of isoflavones ranged from 3 to 80 mg/day (see section 1.7.1).

Only capsules and tablets were mentioned as the type of formulations used by the data providers, the recommended number of servings per day ranging from 1 to 2.

Limits on the duration of intake of these food supplements were not mentioned by any of the data providers; on the contrary, some food supplements bear recommendations for long-term usage.

The information gathered from the relevant food sector business operators was compared with some data available from the published literature investigating the composition of food supplements available on the market.

The actual isoflavones composition of 15 food supplements marketed for the relief of menopausal symptoms, as determined by high-performance liquid chromatography (HPLC), was compared with their labelled information (Almeida et al., 2015). According to the information on the label of the marketed supplements, the recommended daily doses of isoflavones from food supplements ranged 30–80 mg/day for soy extract, and is 40 mg/day for red clover extract. Only 4 out of the 15 food supplements analysed in this study contained isoflavones from soy or red clover alone, the others being multi-ingredient preparations in which soy extracts are mixed with a number of other botanicals. The Panel noted, however, that the range of recommended daily doses is broader (14.6–110.9 mg/day, expressed as aglycones) when the analytical results are taken into account (the actual content of isoflavones ranging 41.9–138.6 % with respect to the content claimed on the label).

The isoflavone content of six food supplements containing soy extract was determined by HPLC after hydrolysis, both as aglycones and as malonylglycosides (Csupor et al., 2015). In the first case, the isoflavone content determined as aglycones was always lower than the amount declared on the label, ranging 55.9–87.0 % of the content reported on the package. After calculation of the equivalent content expressed as malonyl derivatives, the quantified amount turned out to be higher than the content reported on the label, with values ranging 108.5–168.5 %. In this study the ratio of the individual isoflavones genistein, daidzein and glycitein was also examined. Daidzein and genistein were the major aglycones in three products each, whereas glycitein was detected in small quantities in four of the six samples analysed. No indication is provided on the recommended daily doses for the food supplements analysed in this study.

The discrepancies between the amount of isoflavones claimed on the label and the actual content, as determined by analytical testing, was consistent with the findings from a previous study in which the concentrations of isoflavones in a number of food supplements available on the market in the UK, Italy and Canada were measured by means of HPLC–mass spectrometry (MS) (Clarke et al., 2008). According to the information in the label of the marketed supplements, the recommended daily doses of isoflavones from food supplements range from 20 to 60 mg/day for soy extract, with the exception of one product reporting a recommended daily dose of 2.2 mg/day. In this case the actual quantity of soy isoflavones was always lower than the amount claimed on the label of the products (from 5 %to 69 % of the content claimed on the label) and therefore the corresponding daily intake of isoflavones, calculated according to the recommended number of serving, would be in the range 1–40 mg/day of soy isoflavones (expressed as aglycones).

Using a newly established reversed-phase ultra-performance liquid chromatography method, 11 soy-based supplements retailed in Austria (capsules and tablets, with the exception of one liquid formulation) were analysed for their isoflavone content (Fiechter et al., 2010). The total amount of aglycones per serving (capsule/tablets) ranged 16.24–156.25 mg, compared with labelled amounts of 20–100 mg/serving. In terms of deviation from the labelled content, the measured amounts ranged 40.6–72.1 % of the labelled content, with the exception of one sample corresponding to 95.3 % of the declared amount. The only liquid sample analysed had the lowest concentration of isoflavones (0.55 mg/10 ml serving size), corresponding to 61.5 % of the amount on the label. No information is

provided on the recommended number of servings of the samples analysed. With respect to the distribution of the individual isoflavones, daidzein was the major aglycone in 6 of the 11 products analysed, genistein was predominant in four, whereas genistein and daidzein were present in equal proportion in the remaining product.

Quantification of isoflavone aglycones in 14 food supplements marketed in Italy and targeted at peri- and post-menopausal women was performed by means of HPLC analysis after hydrolysis (Boniglia et al., 2009). Most of the samples were described as containing extracts of soy seed or soy isoflavones, with the exception of one product containing isoflavones from *Pueraria lobata* and two products containing no better specified isoflavones. Also, in this case, the products showed variability with respect to the amount declared on the label, which the authors interpret as possibly due to the difference in weight between the aglycones and the glycosylated form. The content of isoflavones, expressed as aglycones, was in most of the cases lower than the values reported on the labels. The recommended daily doses, as claimed on the label, ranged 33.75–80 mg/day; however, the actual content of the food supplements ranged 5.40–107.40 mg/day.

The isoflavone content of 49 food supplements containing soy isoflavones and retailed in France was determined using an enzyme-linked immunoassay (Vergne et al., 2008). The measured content of isoflavones was found to be extremely variable from brand to brand, ranging 0.03–121 % of the labelled content, with few exceptions. According to recommendations provided on the packaging of these products the actual intake of isoflavones (expressed as aglycones) would range 0.07–92.8 mg/day.

In the case of food supplements containing red clover extracts, the amount of isoflavones determined was more or less comparable to the quantity claimed on the label, with percentages ranging 84–133 % (Clarke et al., 2008; Almeida et al., 2015). On the basis of the recommended daily doses reported for red clover extract of 40 mg/day, it can be estimated that the actual intake of red clover isoflavones would be in the range 37–53 mg/day.

Three samples of food supplements containing isoflavones from kudzu root were also tested in the study by Clarke et al. (2008). The recommended daily dose of isoflavones from kudzu root reported was in the range 35–55 mg/day; however, the actual content of isoflavones was lower than claimed in two of the samples tested (40 % and 46 %), whereas it was higher in the third (115 % of the label claim). The corresponding daily intake of isoflavones from kudzu root based on the measured content and the recommended number of servings reported in the publication would range 16–46 mg/day.

The composition of 11 food supplements derived from either soy or red clover and available on the German market was also determined using HPLC/MS/MS and the results of the analysis were compared with the quantity of isoflavones reported by the manufacturer (Andres et al., 2015). In this study, the overall isoflavone content determined in 9 out of the 11 sampled supplements was comparable to the information reported by the manufacturer (for soy-based supplements the isoflavones content was 92.2–107.2 % and for red clover supplements the amount ranged 92.2–100.4%). However when taking into account the isoflavones aglycone equivalent values of the soy-based supplements analysed, these were approximately 40–70 % of the labelled isoflavones content. No information is reported on the recommended daily doses for the individual products analysed in this study; however, it is stated that manufacturers recommended intake of 1–2 capsules per day, amounting to 40–100 mg isoflavones. On the basis of the information provided it can be assumed that the actual intake of soy isoflavones would range 31.9–107.2 mg/day whereas, in the case of red clover isoflavones, the actual intake may vary between 34.9 and 158.6 mg/day.

On the basis of the information reported in the studies described above, an estimate of the actual isoflavone intake was calculated and is reported in Table 29.

Table 29: Estimated daily intake of isoflavones from food supplements based on measured content and recommended number of servings.

Reference	Estimated intake (mg/day) based on measured content of isoflavones in food supplements and recommended number of servings	
	Min	Max
Soy isoflavones		
Almeida et al., 2015	14.6	110.9
Andres et al., 2015	31.9	107.2
Boniglia et al., 2009	5.4	107.4
Clarke et al., 2008	1	40
Vergne et al., 2009	0.07	92.8
Red Clover		
Almeida et al., 2015	44.6[(a)]	
Andres et al., 2015	34.9	158.6
Clarke et al., 2008	37	53
Kudzu Root		
Clarke et al., 2008	16	46

(a): Only one value was reported in the publication.

3.6.3. Discussion on exposure assessment

On the basis of the published studies included in this assessment and measuring the actual content of isoflavones in a number of food supplements available in the market, it can be estimated that intake of isoflavones from food supplements is extremely variable and ranges from approximately 0.1 to 100 mg/day for soy isoflavones, from 30 to 160 mg/day for isoflavones from red clover and from 20 to 50 mg/day for isoflavones from kudzu root (all the values above are expressed as aglycones).

The values above are in line with information provided by the relevant food sector business operators, with respect to the recommended daily doses of isoflavones in products mainly targeted at menopausal women. However, these studies highlight important uncertainties with respect to the actual content and the relative intake of isoflavones from food supplements, which in fact could be either underestimated or overestimated.

The overall intake of isoflavones from the diet in women (0.27–1.43 mg/day) is lower than the lowest recommended daily intake of soy isoflavones from food supplements (20–35 mg/day) although in women with special dietary habits (e.g. soy consumers and vegetarians) the intake of isoflavones from the diet could be within the range of exposure from food supplements. However, it is noted that the presence of soy in several processed foods may lead to an additional intake of isoflavones which is not taken into account in the current assessment because of lack of information. Therefore, in the current assessment, exposure to isoflavones from the diet may be underestimated.

The Panel noted that consumption of certain soy-based food products (e.g. soy drink, soy yoghurt, tofu) might contribute to the intake of isoflavones with the same order of magnitude estimated for the food supplements. The Panel noted that the number of consumers in the EFSA Comprehensive Database reporting consumption of soy based products (see Table 26) is quite low in the target population of women aged > 40 years. However, for example, the intake assessment carried out showed that a consumer of soy drink or yoghurt could present an intake of isoflavones which would be in the high range of the doses of isoflavones used in food supplements.

3.7. Identification of doses and duration of exposure without adverse effects on the three target organs

Having reviewed the available evidence from human interventional studies, the Panel considered that the highest doses tested for each target organ without adverse effects and the maximum duration of the treatment could serve as a starting point to develop guidance for the use of food supplements containing isoflavones.

Only for preparations containing soy isoflavones/soy extracts, soy protein, daidzein-rich isoflavones, genistein alone and red clover extracts were there sufficient data to cover all the three target organs

of interest. The lowest dose that did not cause adverse effects in all the three target organs was identified, as was the minimum duration of the studies. The Panel considered that this was the most conservative approach that could be applied to the available dataset. These values are given in Table 30 The Panel considered that these values could be used as guidance for dosing and duration of intake.

Two doses of preparations containing purified aglycones were identified (235 mg/day and 900 mg/day) as the highest doses tested without an effect on mammary gland and thyroid, respectively (Pop et al., 2008; Khan et al., 2012). The Panel considered that these two studies could not be used to provide guidance for the intake of isoflavones from food supplements since they do not reflect the typical composition of commercial products available on the market (see Table 5) and the isoflavones doses provided are outside the range of doses typically used.

Table 30: Overview of doses of isoflavones and duration of intake with no evidence of adverse effects on the three target organs in humans

Type of preparation	Daily dose without effect (mg/day) Duration of intake (months)			Dose without effect in all three target organs (mg/day)	Duration of intake without effect in all three target organs (months)
	Target organ: mammary gland	Target organ: uterus	Target organ: thyroid		
Soy isoflavones/ soy extract	100 (total) 10	150 (total) 30	200 (aglycones) 24	100 (total isoflavones)	10
Soy protein	99 (aglycones) 12	120 (aglycones) 6	132 (aglycones) 3	99 (aglycone)	3
Daidzein rich isoflavones	120 (total) 24	72 (total) 6	120 (aglycones) 24	72 (total)	6
Genistein	54 36	54 36	54 36	54[(a)]	36
Red clover	43.5 (total) 12	80 (aglycones) 3	120 (aglycones) 12	43.5 (total isoflavones)	3

(a): From the same clinical trial.

4. Conclusions

4.1. Mode of action

The International Programme on Chemical Safety (IPCS) published frameworks for analysing the relevance of cancer and non-cancer modes of action for humans (Boobis et al., 2006, 2008). The Panel considered the application of these frameworks.

The Panel noted that potential modes of action can be hypothesised based on the available evidence, e.g. oestrogenic activity and inhibition of TPO (see introductory section 1.5.). However, given the absence of an association in the human studies between exposure to isoflavones from food supplements and adverse effects in the three target organs addressed in this opinion, it was not possible to define a mode of action for causation which can be evaluated by these frameworks.

4.2. Uncertainties in the assessment

In this opinion, data from human and animal studies have been assessed; however, the Panel noted the following limitations which could introduce uncertainties in the evaluation.

Owing to the nature of the uncertainties described below, it was not always possible to state in which direction they might have influenced the conclusions.

4.2.1. Uncertainties associated with composition of interventions/test material

The studies were grouped together according to the composition of the test material used. However, the Panel recognised that in some studies only limited information on the composition of the test material was available and that there was a lack of standardised reporting of the isoflavone content of the different interventions (e.g. whether or not the content was expressed as aglycones). For this reason the Panel considered that a formal meta-analysis of the studies included in this assessment was not feasible.

The presence of unidentified or uncharacterised isoflavones in the soy or red clover extract would underestimate the total isoflavones load of the extract. This would over estimate the dose–response effect if activity were due to the total isoflavones content. However, if activity is related to a specific isoflavone, two scenarios exist. Firstly, if the unidentified or uncharacterised isoflavone is responsible for activity, the risk would not be accurately estimated. Secondly, if another isoflavone is solely responsible for the activity, the presence of unidentified or uncharacterised isoflavone has no influence on the risk.

Other biological active components could be present in the extract (e.g. coumestrol in red clover extracts), and isoflavones exposure in the study may be a surrogate for these exposures. The risk would be related to the other biological component, and deriving a dose–response relationship from the isoflavone component would not be representative of the actual risk, if a risk existed.

The possibility of an interaction between isoflavones and other biological components resulting in antagonism, additivity or synergy of effects could not be estimated. This could lead to either under- or overestimation of the risk.

4.2.2. Uncertainties associated with intervention/exposure

In order to overcome the difficulties, an effort was made to convert the doses in animal studies, as reported in the publications, to doses expressed as mg/kg bw/day. In doing so, the Panel on several occasions applied default values or based its judgement on its own interpretation of the data. Although the assumptions made by the Panel are clearly documented in the opinion, these may have introduced an additional source of uncertainty around the observations from the animal studies included in this review and therefore caution should be exercised when interpreting findings from the animal studies in comparison with the doses used in humans. In addition, as pointed out above, the isoflavone doses reported in this opinion are in the same form as reported in the publications, meaning that in some cases they are given as aglycone doses, whereas in other cases they are expressed as the amount of glycosides; in yet other cases this information is not available.

With respect to the observational studies, the known limitations of self-reporting and the lack of information on the composition of the supplements and the doses and the exact duration of the exposure introduce important uncertainties. These uncertainties might affect the exposure estimate between the subjects and therefore might reduce the statistical power of the studies to detect small differences.

Variability in isoflavone content in soy and soy-based products is the major source of uncertainty in the estimates related to the background exposure to isoflavones among consumers of these foods. In certain countries, the overall intake of isoflavones from the diet might be slightly underestimated by the use of soy enzymes in the processing of flour.

When comparing doses in animals with those in humans, it should also be noted that in plasma the fraction of the active unconjugated isoflavones is several-fold higher in animals than in man, e.g. for genistein there is 20-fold and 23-fold difference between rats and mice, respectively, and man.

4.2.3. Uncertainties around the endpoints

Four observational studies investigating the association between use of food supplements containing isoflavones and risk of breast cancer were found. However, no such studies were available for uterine cancer and thyroid disease, thus limiting the current assessment to the evaluation of human interventional studies investigating surrogate endpoints (e.g. endometrial thickness, changes to thyroid hormone levels).

Surrogate endpoints defined in the protocol for breast and uterus are qualitatively related to the disease of interest; however, no quantitative relationship has been derived so far. In addition, these surrogate endpoints may not always be an event on the mechanistic pathway leading to the adverse effects.

The Panel noted that the endpoints considered for this risk assessment were secondary rather than primary endpoints in the majority of the human interventional and animal studies included in this opinion. Therefore, the power calculations in the human interventional studies were not necessarily based on detecting significant changes in the endpoints relevant for this assessment. The Panel could not ascertain whether all the studies were sufficiently powered to detect small changes in the relevant endpoints.

4.2.4. Uncertainties around the target population

Human data, from observational and interventional studies, in the relevant population (peri- and post-menopausal women) were considered the most relevant. One case–control study included pre-menopausal women, but most of the interventional studies predominantly included post-menopausal women. The Panel noted that subjects enrolled in the studies would be representative of the target population unless specific exclusion criteria applied by the investigators excluded certain sub-groups (e.g. women with previous history of breast cancer, women on medication for thyroid diseases or other chronic conditions). Underrepresentation of certain groups in the total study population could be a source of uncertainty for the generalisation of the findings.

There is a progression from pre- through peri- to post-menopause. During this progression there are changes in physiology and receptor density. However, there is an overlap, particularly between peri- and post-menopause. The possibility of a difference in sensitivity between peri- and post-menopausal women exists as a result of differences in oestrogen receptor numbers. The Panel noted that oestrogen receptor numbers would be even higher in pre-menopausal women.

The interval between ovariectomy and the start of intervention with isoflavones was different in the animal species. In rats, the interval was typically quite short (1 day to few weeks), whereas in the studies conducted in monkeys ovariectomy had been performed 4.5 years before the start of the intervention. In comparing results among animal studies and those in the human target population, the Panel acknowledged that there could be differences in the sensitivity between species. However, this should not represent a source of uncertainty given that only human studies were eventually considered for the conclusions.

4.3. Conclusions

In line with the Terms of Reference, this risk assessment was focused on three target organs, mammary gland, uterus and thyroid, in a sub-group of the general European population, peri- and post-menopausal women. The assessment was limited to isoflavones ingested as food supplements at doses used in the human studies available in the published scientific literature and following a request for information from relevant interested parties.

The Panel considered that results obtained from the human studies and the studies in ovariectomised animals were most relevant for the target population. Furthermore, the Panel noted that, in addition to all the limitations described for the relevant studies, studies in other human populations (e.g. males) or animal models (e.g. juvenile animals, transgenic models) would not provide additional relevant information on the specific risks being assessed in peri- and post-menopausal women. Therefore, the result from this assessment cannot be extrapolated to other groups and other situations in the general population.

Based on the data reviewed and presented in the current opinion and taking into account the uncertainties described above, the Panel reached the following conclusions:

1) The Panel concluded that in addressing the Terms of Reference an assessment could be provided only for human studies in the relevant population of peri- and post-menopausal women or from animal studies in OVX animals investigating the relevant pre-defined endpoints in mammary gland, uterus and thyroid (details of the isoflavone composition of the preparations tested in the human and animal studies are given in Table 27 and Table 28).

2) In assessing the effects of isoflavones on the three target organs, the Panel decided that differences in functions, receptor density, proportions of ERα and ERβ and effects of receptor activation meant that it was not possible to directly extrapolate observations from any one organ to the others. The Panel noted differences in biological effects and activity between isoflavones from different sources and in different organs and, therefore, concluded that currently it is not generally feasible to apply a read-across approach either between different preparations or between similar preparations in different organs. Hence, a full evaluation is possible only if study results are available for all three target organs.

3) There is overlap between peri- and post-menopause. The World Health Organization defines 'perimenopause' as the period immediately prior to the menopause (when the endocrinological, biological and clinical features of approaching menopause commence) and the first year after menopause and 'postmenopause' as the period dating from the final menstrual period. Although women progress through peri-menopause to post-menopause, it may not be possible to definitively categorise them as peri- or post-menopausal. Only a small proportion of the participants included in the interventional studies would be classified as peri-menopausal women according to the definition above. Despite the uncertainties and limitations described, and given the overlap in the definitions, the Panel considered that the data on mammary gland and thyroid allow conclusions that are applicable to post- and peri-menopausal women.

   With respect to the data on uterus, the Panel considered that the database is not sufficient to draw conclusions on peri- menopausal women.

   Because not all three target organs were covered by the intervention studies in the peri-menopausal population, the overall conclusions of this opinion apply only to post-menopausal women.

4) For the target organ mammary gland, three case–control studies and one prospective cohort study did not support the hypothesis of an increased risk of breast cancer associated with the intake of isoflavones from food supplements. The Panel acknowledged that the central tendency was around 1 consistently across all the studies included in the review, and the upper limit of the confidence interval for the estimated odds ratio was always below 1.67.

   Based on interventional trials encompassing 816 women, the Panel concluded that neither enhanced breast density (741 women) nor histopathological changes (75 women) were observed for soy isoflavones/soy extracts, soy protein, daidzein-rich isoflavones, genistein and red clover extract. The Panel concluded that on the basis of the evidence reviewed there is no indication for adverse effects on the mammary gland in post-menopausal women from isoflavones when taken in doses and for durations as described above.

   The information on women with breast cancer obtained from this systematic review is limited, therefore, the opinion cannot conclude on the risk of oestrogenic isoflavones-based food-supplements in post-menopausal women with a current diagnosis or history of oestrogen-dependent cancer.

5) For the target organ uterus, neither changes in endometrial thickness (studies involving, in total, 1 484 participants) nor remarkable histo(patho)logical findings (studies encompassing 677 participants) were observed in any of the human interventional studies, with the highest isoflavone dose being 150 mg/day administered for a period of 2.5 years. An effect on uterine weight was found in rats with doses of various isoflavones of between 10 mg/kg bw/day and 100 mg/kg bw/day. The Panel considered that this was not an adverse effect.

   On the basis of the evidence from human studies and the considerations on the findings from the animal studies, the Panel concluded that soy isoflavones/soy extract, soy protein, daidzein-rich isoflavones, glycitein-rich isoflavones, genistein and red clover extract have no adverse effects on the uterus in post-menopausal women when taken in doses and for durations as described above.

   No information on women with uterine cancer was obtained from this systematic review; therefore, the Panel cannot conclude on the risk of oestrogenic isoflavones-based food-

supplements in post-menopausal women with a current diagnosis or a history of oestrogen-dependent cancer.

6) The assessment of effects on the thyroid function was exclusively based on the results from human interventional studies. Based on these studies (involving 925 participants taking isoflavones and 576 serving as controls), the Panel concluded that there are no statistically significant changes to indicate that food supplements containing soy isoflavones/soy extract, soy protein, daidzein-rich isoflavones, genistein or red clover extract exert a hypothyroid effect in post-menopausal women with normal thyroid function.

7) Human studies investigating effects on the three target organs of intake of food supplements containing extracts from kudzu root and the isoflavones: genistin, daidzin, daidzein, glycitin, glycitein, puerarin, biochanin A and formononetin were not retrieved. No animal studies other than those evaluating their effect on uterine weight were available. Taken together, these findings preclude an assessment of these substances and mixtures.

8) For genotoxicity, positive findings expressed *in vitro* in mammalian cells by the two catecholic oxidative metabolites of daidzein 3′,4′,7-trihydroxyisoflavone and 4′,6,7-trihydroxyisoflavone and by genistein through the stabilisation of the 'cleavable complex' and generation of DNA double-strand breaks (DSBs) at topoisomerase II–DNA binding sites, for which a thresholded mechanism of action has been demonstrated, have not been reproduced in valid *in vivo* micronucleus tests in rats and mice and in comet assay and micronucleus test in human studies. On these bases, the use of isoflavones in food supplements is not of genotoxic concern.

9) A comparison of the estimated intake of isoflavones from food supplements with estimates of intake based on food consumption data showed that the levels of daily intake of soy isoflavones from food supplements may be achieved by consumers of specific soy foods, such as tofu, soy yoghurt, soy milk and drinks.

Overall, the Panel concluded that it was not possible to derive a single health-based guidance value (HBGV) or a safe intake level for food supplements containing isoflavones. The doses and duration of treatment in the interventional studies for the individual preparations may serve as guidance for a dose and duration of use at which no effect has been observed in all three target organs (see Table 30) in the evidence considered for this opinion. The Panel noted that recommended daily doses of the marketed food supplements, with the exception of one product containing soy isoflavones and one product containing red clover seem to fall within these ranges (see Table 5 and Table 29), albeit the food supplements do not bear any clear indication with respect to the recommended duration of use. For products containing kudzu root, no data were available which could guide their use in post-menopausal women.

The proposed values are applicable only to post-menopausal women without a current diagnosis or history of oestrogen-dependent breast or uterine cancer.

The Panel noted that the conclusions drawn in this opinion are based on the assumption that intake of isoflavones from the use of food supplements represents the major contribution to the intake of isoflavones, as has been the situation in the human interventional studies.

5. Recommendations

The Panel considered that more data on the doses and duration of consumption should be generated which would improve the available database on the safety of prolonged use of food supplements containing isoflavones.

This assessment identifies the need for a harmonised way of reporting the isoflavone content of food supplements.

Future studies should use a standardised description of the isoflavones and should explicitly state whether the content of isoflavones is expressed as aglycones or not and should report the ratio of the individual isoflavones.

Documentation provided to EFSA

1. AESGP (Association of the European Self-Medication Industry) May 2014 and June 2015. Response to EFSA request for information on food supplements containing isoflavones.

2. EHPM (European Federation of Associations of Health Product Manufacturers), April 2014 and June 2015. Response to EFSA request for information on food supplements containing isoflavones.

3. FSE (Food Supplements Europe), July 2014 and June 2015. Response to EFSA request for information on food supplements containing isoflavones.

4. Intertek, May 2014. Response to EFSA request for information on food supplements containing isoflavones.

5. Linnea SA, April 2014. Response to EFSA request for information on food supplements containing isoflavones.

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Abbreviations

AP	apurinic/apyrimidinic
AUC	area under the concentration–time profile
BCRP	breast cancer resistance protein
CEE	conjugated equine oestrogen
DB	double-blind
DSB	double-strand break
E2	17β-oestradiol
EQ	equol
ERα	oestradiol receptor α
ERβ	oestradiol receptor β
ERγ	oestradiol receptor γ
ERRα	oestrogen-related receptor α
ERRβ	oestrogen-related receptor β
FMP	final menstrual period
FSP	fermented soy pulp
GEN	genistein
HED	human-equivalent dose
3-HO-DAI	3-hydroxy-daidzein
6-HO-DAI	6-hydroxy-daidzein
HPLC	high-performance liquid chromatography
HRT	hormone replacement therapy
HT	hormone therapy
IA	isoflavone aglycone
IF/ISO	isoflavones
i.v.	intravenous
MLL	myeloid/lymphoid leukaemia
MS	mass spectometry
OVX	ovariectomised
PCE	polychromatic erythrocyte
PCNA	proliferating cell nuclear antigen
PUE	puerarin
RCE	red clover extract
RCT	randomised clinical trial
(f)T_3	(unbound) triiodothyronine
(f)T_4	(unbound) thyroxine
s.c.	subcutaneously
SPI	soy protein isolate
SSE	standardised soy extract
TBG	thyroxine-binding globulin
TPO	thyroid peroxidase
TSH	thyroid-stimulating hormone
UPLC	ultra-performance liquid chromatography

Appendix A – Summary of human intervention studies included in the systematic review

Alekel et al., 2015 – Ref ID: 14945

Study characteristics and population	**Study design**	double-blind, randomized, placebo-controlled study
	N	255 allocated to treatment (placebo: 83; isoflavones 80 mg/day: 87; isoflavones 120 mg/day: 85); 216 completed treatment (placebo: 74; isoflavones 80 mg/day: 72; isoflavones 120 mg/day: 70); 224 ITT analysis (placebo: 74; isoflavones 80 mg/day: 77; isoflavones 120 mg/day: 73)
	Location	Iowa and California, USA
	Sampling time frame	2003 through 2005
	Menopausal status	Cessation of menses from 1 through 8 y
	Age at baseline [median (min-max)]	Total: 54.3 (45.8-65.0) placebo: 54.2 (45.8-61.4) isoflavones: 80 mg/day: 54.3 (48.2-65.0); isoflavones: 120 mg/day: 54.7 (46.5-62.0)
	Age at menopause	Total: 51.0 (41.0-60.0) placebo: 51.0 (42.0-57.0) isoflavones 80 mg/day: 50 (44.0-60.0); isoflavones 120 mg/day: 51.0 (41.0-59.0)
	Time since menopause: years [median (min-max)]	Total: 2.8 (0.8-10.0) placebo: 2.7 (0.8-7.9) isoflavones 80 mg/day: 3.0 (0.9-10.0); isoflavones 120 mg/day: 2.8 (1.0-8.0)
	Inclusion criteria	Healthy postmenopausal women younger than 65 years who had undergone natural menopause were not experiencing excessive vasomotor symptoms, were nonsmokers, and had a BMI from 18.5 through 29.9 kg/m2
	Exclusion criteria	vegans • high alcohol intake (97 servings/wk) • women diagnosed with a chronic disease (laboratory evidence of diabetes mellitus; abnormal renal, liver, and/or thyroid function; or abnormal lipid profile) • first-degree relative with breast cancer • on long-term medications (current: cholesterol-lowering or antihypertensive drugs; past 3 mo: antibiotics; past 6 mo: calcitonin, estrogen/progestogen creams; past 12 mo: oral hormones/estrogen or selective estrogen receptor modulators; ever: bisphosphonates) • women with endometrial thickness greater than 5.0 mm at baseline, except for those with 5.0 to 6.0 mm endometrial thickness who underwent endometrial biopsy that yielded normal results.
	Funding source	Grant (RO1 AR046922) from the National Institute of Arthritis and Musculoskeletal and Skin Diseases and was also supported by the Nutrition and Wellness Research Center at Iowa State University; US Department of Agriculture-Agricultural Research Service Western Human Nutrition Research Center (5306-51530-006-00D); Clinical and Translational Science Center, Clinical Research Center, University of California (1M01RR19975-01); and National Center for Medical Research (UL1 RR024146).
	Authors conflicts of interest	One of the authors declared being a member of the Soy Nutrition Institute Scientific Advisory Board. The other authors declare no conflicts of interest.
	Clinical trial registration and/or acronym	Clinical trial registration: NCT00043745 SIRBL study
Intervention/ exposure	**Intervention**	Placebo
		Soy isoflavones 80 mg/day
		Soy isoflavones 120 mg/day
	Duration	3 years

Statistical analysis	**Statistical analysis**	Power analysis was based on the primary bone outcomes of interest for the overall SIRBL Study (BMD) Results considered statistically significant (two-sided) at $P < 0.05$. Change in endometrial thickness (mm) data is presented at each time point (baseline, 12 mo, and 36 mo) separately according to site because of site differences in ultrasound equipment. The primary analysis was intent-to-treat analysis,18 which included all data from all women who had a follow-up BMD at 36 months (N = 224) regardless of treatment compliance. Because the assumption of normality was not supported by the data, we used nonparametric analysis of variance (ANOVA) to determine the effects of treatment on circulating hormone concentrations and endometrial thickness. To determine whether treatment differences were consistent across all time points, we used parallel-profile tests (based on Wilks' L criterion) for changes in endometrial thickness at each site. To determine the effects of treatment on adverse events, we used a Poisson regression model with an extra dispersion parameter that links the natural logarithms of the rates of occurrence of adverse events to treatment and site effects. The extra dispersion parameter is needed to account for overdispersion in the response variable (ie, its variance is larger than its mean).	
Results	**Uterus**		
	Endometrial thickness (mm)	Endometrial thickness decreased for each site during treatment. Graphical display and statistical analysis remained separate for each geographic site because endometrial thickness values between sites were statistically different at each time point owing to differences in ultrasound equipment. Non-parametric analysis of variance for treatment differences (absolute) among groups showed no differences at any time point (for more details see Figure n. 2 in the original publication).	
	Thyroid		
	TSH, µIU/mL [median (min-max)]	Baseline	2.46 (0.34-10.76)
		6 months	2.30 (0.03-9.03)
		12 months	2.54 (0.01-23.26)
		24 months	2.35 (0.21-13.00)
		36 months	2.51 (0.54-13.76)
	Free thyroxin, ng/dL [median (min-max)] only in those samples with TSH < 0.35 or > 5.5 µIU/mL	Baseline (n=22)	1.14 (0.65-1.39)
		6 months (n=24)	1.13 (0.72-1.39)
		12 months (n=33)	1.06 (0.73-2.08)
		24 months (n=10)	1.05 (0.52-1.69)
		36 months (n=13)	1.05 (0.75-1.29)
		Nonparametric ANOVA indicated no differences in circulating hormone concentrations at various time points among treatment groups.	

Risk of Bias appraisal			**Tier: 2 (uterus)/ Tier: 1 (thyroid)**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Subjects were randomised, but no method given. They were stratified according to bone health (as this was primary endpoint)
	Was allocation to study groups adequately concealed?	+	Study described as double-blind, but no more information given.
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Groups were largely comparable. Background diet and other factors assessed, but not equol producer status. Study appears to be designed for bone health, and this might have an adverse effect.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Only direct comparison, but comparable groups

Bias domain	Question	Score	Judgement
Performance	Did deviations from the study protocol impact the results?	+	none reported
	Were the research personnel and human subjects blinded to the study group during the study?	-	Not clear whether active and placebo were distinguishable or not.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Low attrition, fully explained
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Endometrial thickness: No information given, but described as double blind
		+	Thyroid: No information given, but described as double blind.
	Key question B: Can we be confident in the outcome assessment?	-	Endometrial thickness: Standard method, but no details on QC and validation. Differences between study sites could affect outcome.
		+	Thyroid: Standard method, but no details on QC and validation. Differences between study sites could affect outcome.
Selective reporting	Were all measured outcomes reported?	+	Protocol not available, but probably yes

Atkinson et al., 2004

Study characteristics and population	**Study design**	Randomized controlled trial
	N	Total randomized: 205 women (RCE, n=102; placebo, n=103) Completed trial: 177 (RCE n=86; placebo, n=91) Analysed: 162 (RCE, n=77; placebo, n=85)
	Location	Cambridge, UK
	Sampling time frame	November 1997 and May 1999
	Menopausal status	Pre-menopausal: FSH < 30 IU/l and oestradiol > 100 pmol/l (16% in the RCE group; 17% in the placebo) Post-menopausal: FSH > 30 IU/l and oestradiol < 100 pmol/l (67% in the RCE group; 68% in the placebo) Peri-menopausal: FSH> 30 IU/l and oestradiol > 100 pmol/l, or if FSH < 30 IU/l and oestradiol < 100 pmol/l. However, if a woman had noted on her questionnaire at the initial home visit that she was currently menstruating, but her baseline hormone profile was that of a postmenopausal woman (i.e. FSH > 30 IU/l and oestradiol < 100 pmol/l), then she was classed as perimenopausal (14% in the RCE, 16% in the placebo)
	Age at baseline	RCE: 55.1 ± 4.7 Placebo: 55.2 ± 4.9
	Age at menopause	Not reported
	Time since menopause:	Not reported
	Inclusion criteria	Women with Wolfe P2 or DY breast patterns
	Exclusion criteria	Women with a history of breast cancer and/or major breast surgery • Women taking HRT
	Funding source	Study supported by grants from the Food Standards Agency FS2034 and the Medical Research Council.
	Authors conflicts of interest	One of the authors was in receipt of research support from Novogen Ltd (Australia), who also supplied the Promensil and placebo tablets and performed the urinary isoflavone analyses. All other authors have no competing interests to declare.
	Clinical trial registration and/or acronym	ISRCTN42940165

Intervention/ exposure	**Intervention(s)**	RCE: isoflavone tablet (Promensil®) Placebo	
	Duration	12 months	
Statistical analysis	**Statistical analysis**	The authors estimated that 20 % of women in the isoflavone group and 5 % of the women in the placebo group would change to a more lucent Wolfe pattern. Thus, to yield 80 % power to detect a difference between treatment groups at the 5% significance level (two-tailed), a sample size of 76 women per treatment arm would be needed. Changes in estimated percentage breast density, were expressed as absolute change (i.e. 12-month data – baseline data). Differences between treatment groups for changes in breast density were tested using an unpaired t-test.	
Results	**Mammary gland**		
	Change in % breast density (mean ± SD) (*P* = 0.73)	Placebo:	–3.9 ± 11.7%:
		RCE	–3.2 ± 11.7%

Risk of Bias Appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement/justification**
Selection	Was the administered dose adequately randomised?	+	Randomization was performed using random number generation in Microsoft Excel
	Was allocation to study groups adequately concealed?	+	Randomization was performed by the Outpatient Pharmacy of the investigation site.
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Interactions between treatment group and menopausal status for changes in estimated percentage breast density were non-significant (P > 0.05), and so changes were assessed for the combined group.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	HRT was a criteria for exclusion at baseline and of withdrawl from the study. Confounding factors were not included, but groups were similar (no stat. sig. diff).
Performance	Did deviations from the study protocol impact the results?	+	No deviation described.
	Were the research personnel and human subjects blinded to the study group during the study?	++	Researchers and study participants remained blinded to tablet allocation throughout the study.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	Loss similar between groups, less than <20%
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	Independently of each other, and blinded to intervention or placebo status, two radiologistsassigned Wolfe patterns and visually estimated the percentage density on each set (left and right mediolateral oblique views) of mammograms.
	Key question B: Can we be confident in the outcome assessment?	+	Detailed description, including validation/QC
Selective reporting	Were all measured outcomes reported?	+	All reported

Bruce et al., 2003 – Ref ID: 14889

Study characteristics and population	**Study design**	Nested double blind, placebo-controlled study.
	N	42 included in the study (25 soy protein; 17 placebo); 38 included in the analysis (22 soy isoflavones ; 16 placebo)
	Location	USA
	Sampling time frame	Not specified
	Menopausal status	Postmenopausal
	Age at baseline [mean and SE values]	Isoflavone supplement: 68.9 (1.03) Placebo: 69.9 (0.98)
	Age at menopause	Not reported
	Time since menopause:	Not reported
	Inclusion criteria	At least 60 years of age at study entry
	Exclusion criteria	Concomitant treatment with HRT • history of breast cancer • development of major illness, such as cancer, parathyroid disease, diabetes, or kidney or heart disease or experience of a major clinical event, such as myocardial infarction • mental condition that would make them unsuitable to participate in the study • BMI ≥ 35.
	Funding source	Grant from Archer Daniels Midland Company
	Authors conflicts of interest	Not reported
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Isoflavone supplement 50 mg (30 mg aglycone), three times a day Placebo: maltodextrin with 10% caramel colour
	Duration	180 days
Statistical analysis	**Statistical analysis**	All data are presented as mean ± SE. Standard statistical techniques were used to assess two-sample means. A non-paired *t* test was used to compare differences in thyroid function between the supplement and placebo groups. Generalized linear modelling was used to compare fasting serum levels of thyroid function at baseline and at 3 and 6 months. Level of significance was set at P < .05.

Results — **Thyroid**

		Baseline	90 days	180 days
TSH (µU/mL) [mean (SE)]	Placebo	3.35 (0.51)	3.91 (0.78)	3.63 (0.57)
	ISO	3.00 (0.44)	3.44 (0.50)	3.49 (0.52)
T_4 (nM) [mean (SE)]		Baseline	90 days	180 days
	Placebo	145.39 (6.69)	148.25 (6.01)	153.77 (6.64)
	ISO	149.00 (5.04)	149.45 (5.06)	154.52 (2.09)
T_3 (nM) [mean (SE)]		Baseline	90 days	180 days
	Placebo	1.55 (0.18)	1.65 (0.12)	1.75 (0.10)
	ISO	1.53 (0.13)	1.56 (0.13)	1.78 (0.12)

Risk of Bias appraisal — **Tier: 2**

Bias domain	Question	Score	Judgement
Selection	Was the administered dose adequately randomised?	-	No information given on randomisation
	Was allocation to study groups adequately concealed?	-	Study was described as blinded - no further information given
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Subjects were advised to maintain their diet, but many factors were not included (e.g. equol producer status). Some subjects were on thyroid medication at baseline
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Only direct comparisons were conducted

Bias domain	Question	Score	Judgement
Performance	Did deviations from the study protocol impact the results?	+	non reported
	Were the research personnel and human subjects blinded to the study group during the study?	-	No information given, but placebo/active might have been similar, not identical as placebo was coloured artificially (and it is not clear whether this was successful)
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	No information given
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	No information given
	Key question B: Can we be confident in the outcome assessment?	+	No information on QC/validation – however standard method
Selective reporting	Were all measured outcomes reported?	+	No protocol available

Carmignani et al., 2010 – Ref ID: 1103

Study characteristics and population	**Study design**	Double-blind, placebo-controlled, randomised clinical trial
	N	60
	Location	Brazil, two investigation sites
	Sampling time frame	January and October 2007.
	Menopausal status	postmenopausal women between 40 and 60 years of age who had their last menstrual period more than 12 months previously, had FSH levels >30 mUI/ml and estradiol levels <20 pg/ml, who were having more than 8 hot flashes in 24 h.
	Age at baseline, years [Mean ± SD]	52.4 ± 3.9
	Age at menopause, years [Mean ± SD]	48±3.7
	Time since menopause, years [Mean ± SD]	4.1±3.3
	Inclusion criteria	postmenopausal women between 40 and 60 years
	Exclusion criteria	any form of hormonal treatment during the previous 6 months • current use of any lipid-lowering and/or antidiabetic drugs • current use of soybean derived products or herbal supplements • previous hysterectomy • chronic gastrointestinal disorder • any contraindication to hormone therapy • patients participating in a conflicting clinical trial • known allergy or hypersensitivity to soy or cow milk • not willing to cease consumption of soy products
	Funding source	São Paulo Foundation for the Support of Research (FAPESP), grant # 03/04464-0.
	Authors conflicts of interest	None reported.
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Hormone therapy (n = 20): one tablet containing 1mg of estradiol and 0.5 mg of norethisterone acetate (Activelle®, Medley Pharmaceuticals, Campinas, São Paulo, Brazil), in addition to 2 portions/day of placebo powder.
		Soy group (n = 20): one placebo tablet plus 2 portions/day of dietary soy supplementation powder containing 45mg of isoflavone per portion, making a total of 90mg of isoflavone/day (Previna®, Sanavita Functional Foods, Piracicaba, São Paulo, Brazil).
		Placebo group (n = 20): one placebo tablet and 2 portions/day of placebo powder.
	Duration	16-week

Statistical analysis	**Statistical analysis**	Data analysed according to the intention-to-treat principle, and included all the original participants in the group to which they were randomly assigned. The mean percentage variation between groups was compared using the Kruskal–Wallis test. An alpha error of 0.05 was chosen; only p-values <0.05 were considered significant.

Results	**Uterus**			
	endometrial thickness mean (mm) (±SD)		Baseline	Post-treatment
		Placebo:	3.9 (±2.2)	3.5 (±2.6)
		Soy:	4.2 (±2.3)	4.1 (±2.0)
		HT:	3.2 (±1.3)	3.8 (±1.6)
			(P = 0.11)	(P = 0.16)

Risk of Bias appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Authors state that allocation to treatment group was performed by computer-generated randomisation list
	Was allocation to study groups adequately concealed?	++	Authors state that for duration of the study the subject and personnel remained blinded with respect to treatment.
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Endometrial thickness was only assessed as adverse event. No differences in main baseline characteristics. No stratified randomisation, no multivariate analysis. Background diet was not assessed.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Background diet, parity etc were not taken into consideration for analysis. Soy group had considerably more subjects with <2 children and fewer smokers.
Performance	Did deviations from the study protocol impact the results?	+	No deviations reported.
	Were the research personnel and human subjects blinded to the study group during the study?	++	The authors reported that for the duration of the study, the subjects and study personnel remained blinded with respect to the treatment modality..
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No drop-out or exclusions reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	The authors reported that the operator who performed all the exams did not know patient clinical data
	Key question B: Can we be confident in the outcome assessment?	+	The authors report that the endometrial stripe was measured at its maximum anteroposterior thickness along the longitudinal axis (1/3) of the uterine body from the echogenic interface of the endometrial–myometrial junction on both sides. No information on reproducibility of the test.
Selective reporting	Were all measured outcomes reported?	++	All data reported.

Cheng et al., 2007 – Ref ID: 3158

Study characteristics and population	**Study design**	Double-blind
	N	60 allocated to treatment (N soy protein; N placebo); 51 completed (N soy protein; N placebo)
	Location	Sweden
	Sampling time frame	
	Menopausal status	At least 1 year since last period, FSH > 30 IU/mL
	Age at baseline [mean ± SD)	Placebo: 56.9 ± 4.2 Isoflavones: 58.4 ± 5.0
	Age at menopause	-
	Time [Years] since menopause:	Placebo: 7.0 ± 3.8 Isoflavones: 8.4 ±5.3
	Inclusion criteria	Healthy postmenopausal women aged 49-69 at least 6 months without taking HRT
	Exclusion criteria	-
	Funding source	Grants from the Stockholm County Council (ALF), the Swedish Cancer Society, the European Union Specific Targeted Research Project (grant EWA) and the Susan G. Komen Breast Cancer Foundation
	Authors conflicts of interest	One of the authors declared being co-founder, deputy board member, shareholder, grant recipient and consultant of KaroBio AB.
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Isoflavones 60 mg/day in a fruit drink Placebo: matched fruit drink
	Duration	12 weeks
Statistical analysis	**Statistical analysis**	Differences between groups were analysed with Student's *t* test and a paired-sample t test. A *P* value less than 0.05 was considered significant.
Results	**Mammary gland**	
	Proliferation marker Ki-67	Observed in 0% to 0.5% of samples. No significant change was induced by isoflavone treatment.
	Uterus	
	Proliferation marker Ki-67 (endometrium)	Observed in 0% to 3% of samples. No significant change was induced by isoflavone treatment.
	Uterus	
	Endometrial thickness mean (mm) (±SD)	

	Baseline	Post-treatment
Placebo	2.0 ± 1.0	3.2 ± 1.8
Isoflavones	2.3 ± 1.1	2.6 ± 1.2
	P > 0.05	

Risk of Bias appraisal			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	There is insufficient information - participants were allocated by computer, but no information on how this was performed.
	Was allocation to study groups adequately concealed?	+	Method states blind study, but description of blinding is insufficient.
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Data analysis was performed by comparing groups without adjustment; no stratified randomisation. Authors do not justify this approach.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	There is no information about background diet, no multivariate analysis or stratification. But it appears unlikely that these introduce bias.

Bias domain	Question	Score	Judgement
Performance	Did deviations from the study protocol impact the results?	+	Authors do not report deviation.
	Were the research personnel and human subjects blinded to the study group during the study?	+	There is insufficient information about the blinding method used.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Loss of subject was well document and does not seem to affect outcome.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	--	There is insufficient information about the blinding method used.
	Key question B: Can we be confident in the outcome assessment?	--	There are no information on the reliability and reproducibility of the method.
Selective reporting	Were all measured outcomes reported?	-	Information only given in 31 out of 60

Chilibeck et al., 2013 – Ref ID: 14960

Study characteristics and population	**Study design**	Double blind, parallel-group, randomized controlled trial
	N	Total randomised: 351 (Ex = 86, Iso = 90, ExIso = 87, control = 88) Total analysed after 2 years: 298 (Ex =77, Iso = 76, ExIso = 72, control = 73).
	Location	Saskatoon, SK, Canada. Single centre.
	Sampling time frame	Participants were recruited from November 2004 to January 2006 and all had completed the intervention by June 2008
	Menopausal status	Questionnaire about LMP. If women reported that they were less than 2 years postmenopause, menopausal status was verified by determining levels of FSH and LH.
	Age at baseline	Group 1 (Ex): 55.3 (6.3) Group 2 (Iso): 56.7 (6.5) Group 3 (ExIso): 55.8 (5.0) Group 4 (Control): 56.4 (7.1)
	Age at menopause	Not reported
	Time since menopause:	Partcipants were stratified as either 1 to 9 years postmenopause or over 9 years postmenopause before randomisation.
	Inclusion criteria	Postmenopausal women
	Exclusion criteria	previous fragility fractures (defined as fractures resulting from minimal trauma) ● treatment with bisphosphonates, hormone replacement therapy, selective estrogen receptor modulators (Raloxifene), parathyroid hormone, or calcitonin within the past 12 months ● currently taking corticosteroids or thiazide diuretics ● Crohn's disease ● Cushing's disease ● kidney disease ● allergy to soy ● severe osteoarthritis ● currently involved in vigorous exercise training (defined as jogging or resistance training for more than 20 minutes per session, more than twice per week) ● planning to travel outside of Saskatoon for an extended period during the study ● osteoporosis (lumbar spine or proximal femur BMD 2.5 SD below the young adult mean (ie, T-score of –2.5 or lower) ● current or previous breast cancer ● current or previous endometrial cancer.
	Funding source	Grant from the Canadian Institutes of Health Research (application 124322)
	Authors conflicts of interest	None stated
	Clinical trial registration and/or acronym	Trial registered with clinicaltrials.gov with number NCT00204425.

Intervention/ exposure	**Intervention**	Group 1 was exercise training (combined weight training and walking) plus isoflavone placebo (Ex);
		Group 2 was exercise training placebo (flexibility program) plus isoflavone therapy (Iso; 165 mg total isoflavone/d or 105 mg aglycone equivalent/d);
		Group 3 was exercise training plus isoflavone therapy (ExIso);
		Group 4: exercise training placebo plus isoflavone placebo (control).
	Duration	2 years
Statistical analysis	**Statistical analysis**	Data were analyzed on an intent-to-treat basis; ie, an attempt was made to follow up participants that did not adhere to the exercise or supplementation. Adverse events across groups were compared by chi-square analysis. Baseline data are presented as means (SD). All other data are presented as absolute change scores and their 95% confidence intervals. Significance was set at alpha = 0.05.
Results	**Mammary gland**	
	Adverse events detected with mammography	Ex group: one mastectomy of both breasts after a lump was found after the intervention (serious, severe, not related), one abnormal mammogram requiring biopsy that resulted in benign results (mild, possibly related). Iso group: 1 participant had a cyst on her breast (mild, unrelated). ExIso group: 1 participant had calcification of breast tissue (mild, unrelated), Control group: 1 subject had breast calcification and1 had a ruptured breast implant (both mild, unlikely).

Uterus			
Endometrial thickness mean (mm) (±SD) p > 0.05		Baseline	Post-treatment
	Placebo:	2.5 (±1.6)	2.2 (±1.3)
	Isoflavones:	3.0 (±2.5)	2.3 (±1.4)

Risk of Bias appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	++	Computer randomisation by external group (pharmacy)
	Was allocation to study groups adequately concealed?	++	Concealment clearly described
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Many confounding factors were addressed (eg diet), but not equol producer status. Groups were similar however.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Direct comparison, but groups were similar
Performance	Did deviations from the study protocol impact the results?	+	None reported
	Were the research personnel and human subjects blinded to the study group during the study?	++	All blinded - explicitly stated
Attrition/exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	Loss of subjects were clearly addressed
Information/detection	Were the outcome assessors blinded to study group or exposure level?	++	Assessors were blinded
	Key question B: Can we be confident in the outcome assessment?	+	Method is standard method, but no information on validation and QC
Selective reporting	Were all measured outcomes reported?	+	All reported

Colacurci et al., 2013 – Ref ID: 3071

Study characteristics and population	**Study Design**	Randomised, placebo controlled trial.
	N	130 allocated to treatment (65/group) 124 included in the analysis (62/group)
	Location	Italy
	Sampling time frame	Not reported
	Menopausal status	Post-menopausal
	Age at baseline	Isoflavone group: 55.3 ± 7.6 years Placebo group: 55.7 ± 7.7 years
	Age at menopause	Not reported
	Time since menopause	Isoflavones: 3.2 ± 2.8 years (amenorrhoea) Placebo: 2.8 ± 2.6 years
	Inclusion criteria	Age 45–65 years, amenorrhoea > 12 months, FSH > 30 mIU/mL, E2 < 20 pg/mL.
	Exclusion criteria	Use of antibiotic in the last 6 months ● HRT or treatments for climacteric symptoms ● dietetic regimens such as strict vegetarian, high fibre or high soy diet, regular consumption of vitamin and mineral supplementation greater than the Recommended Dietary Allowances ● chronic disorders ● benign breast disease ● endometrial thickness > 5 mm ● BMI > 30.
	Funding source	Not reported
	Authors conflicts of interest	The authors have no financial affiliation (e.g. employment, direct payments, stock holdings, retainers, consultantship, patent-licensing arrangements, or honoraria) or involvement with any commercial organization with direct financial interest in the subject or material discussed in this manuscript. The authors have no financial interest in any aspect of the work and did not receive any financial support. One of the authors was a consultant for Rottapharm Madaus, the supplier of the test materials.
	Clinical trial registration and/or acronym	None reported.
Intervention/ exposure	**Intervention**	Soy isoflavones: 60 mg/day Placebo (calcium 240 mg, vitamin D3 5 µg)
	Duration	12 months
Statistical analysis	**Statistical analysis**	The statistical analysis was carried out with χ2, Fisher exact's test and ANOVA when appropriate.
Results	**Mammary gland**	

Mammographic density Density score		Baseline:	Post-treatment
	Placebo:	1.75 ± 0.85	1.58 ± 0.84
	Isoflavones:	1.89 ± 0.96	1.80 ± 0.95
Adverse events:	Mastodynia and mammary tension reported by the patients were classified as mild, moderate and severe. No difference observed between the groups		
Uterus			
Endometrial thickness		Baseline	Post-treatment
	Placebo:	3.47 ± 1.07	3.12 ± 0.73
	Isoflavones:	3.35 ± 0.95	3.08 ± 0.62

Risk of Bias appraisal:		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Randomization list balanced in blocks of 10 provided for each centre,
	Was allocation to study groups adequately concealed?	++	

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Many factors addressed, including high soy diet, but not general background diet. Baseline characteristics were described as similar but only limited
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Baseline characteristics were described as similar, but data not included in analysis
Performance	Did deviations from the study protocol impact the results?	+	No deviations reported
	Were the research personnel and human subjects blinded to the study group during the study?	-	Appears to be open study
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Only 3 patients per each group discontinued treatment and were excluded from analysis.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	
	Key question B: Can we be confident in the outcome assessment?	+	Insufficient information on QC and validation
Selective reporting	Were all measured outcomes reported?	+	All information given

Del Manto et al., 2013 – Ref ID: 16401

Study characteristics and population	**Study Design**	Randomised, double-blind, placebo-controlled trial.
	N	80 allocated to treatment (40/group) 66 completed (32 isoflavones; 34 placebo)
	Location	Sao Paulo, Brasil
	Sampling time frame	Not reported
	Menopausal status	Post-menopausal
	Age at baseline: [mean (SD)]	Isoflavones: 55.1 (6.0) Placebo: 56.2 (7.7)
	Age at menopause [mean (SD)]	Isoflavones: 48.4 (3.7) Placebo: 47.7 (3.5)
	Time since menopause [years (SD)]	Isoflavones: 6.6 (4.8) Placebo: 7.1 (4.2)
	Inclusion criteria	Age > 45 years, spontaneous amenorrhea for at least 12 months, 5 or more vasomotor symptoms/day
	Exclusion criteria	Strict vegetarian diet • high fiber or high soy diet • history of at least one of the following conditions: breast cancer, reduction mammoplasty or mammary protesis, endometrial carcinoma, cardiovascular disease, thromboembolic disorder, chronic alcoholism, or chronic gastrointestinal disease • concomitant or past (6 months) use of HRT or any phytoestrogen
	Funding source	Investigation supported by Ativus Farmaceutica, Brasil and by grants from the Lucentis Foundation for Supporting Culture, Teaching, Research and Extension.
	Authors conflicts of interest	None reported
	Clinical trial registration and/or acronym	None reported
Intervention/ exposure	**Intervention**	Isoflavones: 100 mg/day
		Placebo: lactose
	Duration	10 months
Statistical analysis	**Statistical analysis**	Only those women who had mammographic and ultrasound at the end were included in the analysis. For data analysis, means (SD) were calculated for quantitative variables and medians were calculated for qualitative variables. Independent *t* test was used to

		compare groups on quantitative characteristics (clinical, anthropometric, and biochemical). Mann-Whitney *U* test was used to compare categorical variables between groups and $\chi 2$ trend test to compare moments. Statistical tests were two-tailed and the significance level adopted was 5%.

Results

Mammary gland

Mammographic density median score (difference between groups)		Baseline p=0.238	Post-treatment p=0.393
	Placebo:	2.0	2.0
	Isoflavones:	1.5	1.0

Classification of mammographic density	Classification	Baseline		Post-treatment	
		ISO	Control	ISO	control
	1	16	13	17	13
	2	10	10	8	13
	3	6	10	7	8
	4	0	1	0	0

Risk of Bias appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Participants were randomised in a pre-established numerical sentence. A computerised randomisation process was performed using specific statistical software.
	Was allocation to study groups adequately concealed?	+	Examiners and women had no previous knowledge of group assignment. The only person not blinded to the treatment was the study statistician
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No data on equol producer status or background diet, but similar groups
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Groups were comparable
Performance	Did deviations from the study protocol impact the results?	+	none reported
	Were the research personnel and human subjects blinded to the study group during the study?	+	Participants were blinded, researchers appear to have been blinded
Attrition/exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Loss to follow-up less than 20%
Information/detection	Were the outcome assessors blinded to study group or exposure level?	+	Mammographic density was classified by a single evaluator who was blinded to the examination date and study group.
	Key question B: Can we be confident in the outcome assessment?	+	Standard method, but insufficient information on quality control
Selective reporting	Were all measured outcomes reported?	+	protocol not available

Duncan et al., 1999 – Ref ID: 3179

Study characteristics and population	**Study design**	Randomised cross-over
	N	23 enrolled; 18 completed
	Location	Minnesota, USA
	Sampling time frame	Not reported
	Menopausal status	Postmenopausal

	Age at baseline [mean ± SD]	56.9 ± 5.8
	Age at menopause	Not reported
	Time since menopause (years) [mean ± SD]	7.6 ± 4.7
	Inclusion criteria	Postmenopausal women
	Exclusion criteria	Strict vegetarian, high fiber, high soy, or low fat diets • regular consumption of vitamin and mineral supplementation greater than the Recommended Dietary Allowances • athleticism • cigarette smoking • antibiotic or hormone use within six months • menstrual bleeding within 12 months • hysterectomy or oophorectomy • FSH concentration < 25 IU/L • history of chronic disorders including endocrine or gynecological diseases • benign breast disease • regular use of medication known to interfere with study endpoints (including aspirin) • < 90% or > 120% ideal body weight • weight change of more than 10 lb within the previous year • inability to abstain from alcoholic beverages during the study
	Funding source	Work supported by NIH Grant CA-66016 and General Clinical Research Center Grant MO1-RR00400 from the National Center for Research Resources. The soy powders used in the study were donated by Protein Technologies International, St. Louis, Missouri.
	Authors conflicts of interest	Not reported
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Control: soy protein isolate isoflavone-free (7.1 ± 1.1 mg/day expressed as aglycones)
		Low-ISO: soy protein isolate providing 65 ± 11 mg/day isoflavones expressed as aglycones)
		High-ISO: soy protein isolate providing 132 ± 22 mg/day isoflavones expressed as aglycones)
	Duration	Three 93-day diet periods, separated by 26-day washouts.
Statistical analysis	**Statistical analysis**	In order to allow adaptation to each diet, days 36–38 plasma pools were excluded from the statistical analyses of the plasma hormones. Repeated measures ANOVA were performed on plasma hormones, controlling for subject, diet, and time of collection (days 64–66 vs. days 92–94). There were no significant interactions between diet and time of collection. Repeated measures ANOVA were performed on anthropometric, food record, vaginal cytology, and endometrial biopsy endpoints, controlling for subject and diet. Comparisons between each diet and baseline were evaluated using paired t-tests. Results are expressed as mean± sd or mean ± se. In the event of missing data, least squares means (lsmean) are presented to account for the imbalance. Significance was considered at P < 0.05.
Results	**Uterus**	
	Endometrial biopsy	8/18 inactive at baseline and at high-iSO
		4/18 proliferative at baseline and at high-iSO 2/18 proliferative at baseline, inactive at high-ISO

Thyroid				
[lsmean ± SE]	Baseline	Control	Low-ISO	High-ISO
Free T3 (pmol/L)	3.51 ± 0.09	3.39 ± 0.06	3.53 ± 0.06	3.39 ± 0.06
Total T3 (nmol/L)	0.030 ± 0.0003	0.025 ± 0.0003	0.026 ± 0.0003	0.026 ± 0.0003
Free T4 (pmol/L)	15.20 ± 0.39	14.95 ± 0.26	14.97 ± 0.26	15.60 ± 0.26
Total T4 (nmol/L)	93.1 ± 1.16	90.9 ± 1.03	90.7 ± 1.03	93.6 ± 1.03
TSH (mU/L)	3.48 ± 0.15	3.25 ± 0.13	3.33 ± 0.14	3.49 ± 0.13
TBG (nmol/L)	605.9 ± 13.6[(a)]	559.3 ± 12.2[(b)]	600.4 ± 12.8[(a)]	554.1 ± 12.2[(b)]

Different surperscripts are significantly diifferent (P < 0.05).

Risk of Bias appraisal			Tier: 2 (uterus) / Tier: 1 (thyroid)
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Method of randomisation is not clear. Cross-over study, so might not affect outcome too much.
	Was allocation to study groups adequately concealed?	+	Described as blinded
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Background diet was assessed, cross-over design means subjects were own controls.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	cross-over design, confounders were also included in analysis
Performance	Did deviations from the study protocol impact the results?	+	Not reported.
	Were the research personnel and human subjects blinded to the study group during the study?	-	Supplied in packets. Study claims to be blinded, but not clear whether powders were really identical.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	No information on attrition
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Probably yes, but no information given
	Key question B: Can we be confident in the outcome assessment?	-	Uterus: Validation data given, but not clear whether quality controls were used.
		+	Thyroid: Validated method, all information given
Selective reporting	Were all measured outcomes reported?	-	All data reported, but not in tabular form.
		+	All data reported.

Evans et al., 2011 – Ref ID: 15095

Study characteristics and population	**Study design**	Randomised, double-blind, placebo-controlled
	N	N=84 allocated to treatment (genistein, n=41; placebo, n=42); N=82 included in the ITT analysis (genistein, n=40; placebo, n=42); N=68 included in the PP analysis (genistein, n=32; placebo, n=36);
	Location	Five sites, Ontario, Canada.
	Sampling time frame	Not reported.
	Menopausal status	Post-menopausal (surgical in 13/41 genistein and in 13/40 in placebo).
	Age at baseline [mean ± SD]	Placebo: 53.50 ± 4.44 Genistein: 53.39 ±5.05
	Age at menopause	Not reported
	Time since menopause:	Placebo: 1-5 years, 20/41; 6-10 years, 10/41; > 10 years, 11/41 Genistein: 1-5 years, 16/40; 6-10 years, 13/40; > 10 years, 11/40
	Inclusion criteria	Minimum of 40 hot flushes per week • age 40-65 • in a physiological state of natural or surgical menopause. In the case of natural menopause, women were required to be amenorrheic for ≥3 months and have serum FSH levels > than 35 IU/ml whereas surgically menopausal women had to be >42 days post-surgery.
	Exclusion criteria	clinical or laboratory abnormalities identified • had used conventional hormone therapy or selective oestrogen receptor modulators within 4 weeks of study start • known allergy or hypersensitivity to soy, peanuts, purified isoflavones, genistein, lactose and/or cow's milk • had consumed soy products within 4 weeks prior to the screening visit • reported unpredictable vaginal

		bleeding (i.e., leiomyoma or endometrial polyps), uterine fibroids or endometriosis that required treatment • untreated polycystic ovary syndrome (PCOS) • history of abnormal pap smear • use of gonadotropin agonists within 24 weeks • glucocorticoids or chronic high dose (>7.5 mg/day) prednisone or equivalent for the past 12 weeks.
	Funding source	DSM Nutritional Products, Inc., the manufacturer of the genistein tested, fully funded the study but played no role in its execution and analysis of findings.
	Authors conflicts of interest	Two of the authors are employees of DSM Nutritional Products, Inc. the company which funded the study. The other authors have no competing interest.
	Clinical trial registration and/or acronym	Not reported.
Intervention/exposure	**Intervention**	Genistein: 30 mg/day Placebo:
	Duration	12 weeks
Statistical analysis	**Statistical analysis**	Statistical analysis was performed using analysis of covariance (ANCOVA). *P* values less than 0.05 are significant.
Results	**Uterus**	

		Baseline	12-week
Endometrial thickness, mm [mean (±SD)]	Placebo	3.66 (1.21) n=28	4.28 (1.98) n=14
	Genistein	4.18 (1.92) n=25	4.89 (2.76) n=18
	P value 0.548		

Risk of Bias appraisal:			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Randomisation appears to be good, using block randomisation and number generator
	Was allocation to study groups adequately concealed?	++	Study was blinded - no more details given
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	no information on background and anthropometric assessment, but groups appear to be similar
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	direct comparison, but groups appear similar
Performance	Did deviations from the study protocol impact the results?	+	none reported
	Were the research personnel and human subjects blinded to the study group during the study?	+	Packaging and tablets were identical
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Attrition was explained and within acceptable range
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Described as double blind, but no information given
	Key question B: Can we be confident in the outcome assessment?	-	No information on method, QC and validation
Selective reporting	Were all measured outcomes reported?	+	protocol not available, but apparently yes

Geller et al., 2009 – Ref ID: 19610

Study characteristics and population	**Study design**	randomized, double-blinded, placebo-controlled trial
	N	Total 89 randomised (Placebo: 22; CEE/MPA:23; RCE: 22; BC: 22); 88 included in the analysis (Placebo: 22; CEE/MPA:23; RCE: 22; BC: 21);
	Location	Illinois, USA
	Sampling time frame	February 2003 to December 2007.
	Menopausal status	Peri- and postmenopausal
	Age at baseline	Placebo: 52.0 (4.2)
		CEE/MPA: 53.3 (4.0)
		RCE: 52.4 (4.6)
		BC: 54.4 (3.9)
	Age at menopause	Not reported
	Time since menopause (years) [mean, SD]	Placebo: 2.8 (2.9)
		CEE/MPA: 3.6 (2.9)
		RCE: 4.1 (2.8)
		BC: 3.4 (2.6)
	Inclusion criteria	Perimenopausal or postmenopausal woman with intact uterus • experiencing at least 35 vasomotor symptoms per week • amenorrhea > 6 months < 10 years duration • FSH > 40 mIU/mL • HT not contraindicated • able to give informed consent
	Exclusion criteria	previous hysterectomy • fewer than 35 vasomotor symptoms (HF+NS) per week • LMP > 10-y duration • positive pregnancy test or breastfeeding • obesity, BMI > 38 kg/m^2 • previous history of endometrial hyperplasia/neoplasia • previous history of cancers of the breast or reproductive tract • history of presence of myocardial infarction or stroke • history of severe recurrent depression, or severe psychiatric disturbance • history or presence of cerebrovascular accident, severe varicose veins, sickle cell anemia • history of alcohol or drug abuse • abnormal vaginal bleeding of undetermined cause • untreated or uncontrolled hypertension defined as systolic blood pressure > 165 mm Hg or diastolic blood pressure > 95 mm Hg • concurrent administration of medication containing estrogen, progestin, SERM, St. John's wort, bisphosphonates, or dietary phytoestrogens • history of migraine associated with hormone use • history or presence of deep vein thrombosis, thrombophlebitis or thromboembolic disorder • current participation in any other clinical trial within 30 days of enrolment • >5 alcoholic drinks per week • smoker • diabetes • abnormal transvaginal ultrasound defined as >7-mm thickness • abnormal endometrial biopsy or mammogram • vegans (vegetarians who tend to consume greater than average doses of phytoestrogens)
	Funding source	Work supported by NIH grant P50 AT000155 funded jointly by the Office of Dietary Supplements (ODS), the National Center for Complementary and Alternative Medicine (NCCAM), the National Institute for General Medical Sciences (NIGMS), and the Office for Research on Women's Health (ORWH).
	Authors conflicts of interest	One of the authors was a paid consultant for Pharmavite.
	Clinical trial registration and/or acronym	NCT00066144
Intervention/ exposure	**Intervention**	Placebo
		CEE/MPA: 0.625 mg conjugated equine estrogens plus 2.5 mg medroxyprogesterone acetate
		Red clover (RCE): ethanolic extract of the aerial parts of red clover (398 mg/d standardized to 120 mg isoflavones)
		Black cohosh (BC): ethanolic extract of black cohosh below-ground parts (128 mg/d standardized to 7.27 mg triterpene glycosides)
	Duration	12 month

Statistical analysis	**Statistical analysis**	The sample size was calculated for the primary outcome (reduction in vasomotor symptoms). A one-way analysis of variance was used for the analyses of primary and secondary data. The Fisher's least significant difference procedure was used for pairwise comparison of the treatment groups. The primary and secondary data were analyzed post hoc, adjusting for baseline covariates, but this exploratory analysis yielded no clinically meaningful or statistically significant findings. Missing data were imputed using the last-observation-carried-forward method. All data were summarized as mean (SD), and P values of less than 0.05 were considered statistically significant.
Results	**Uterus**	
	Endometrial thickness, Difference in mean reduction	Placebo vs RCE
		-0.005 (0.569), *P* = 0.99
	Thyroid	
	TSH, Difference in mean reduction	Placebo vs RCE
		0.64 (0.48), *P* = 0.19

Risk of Bias appraisal			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	++	Well described and appropriate.
	Was allocation to study groups adequately concealed?	++	Information suggests that study was well blinded and randomised.
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Safety was only secondary endpoint and therefore not taken into consideration. No information on equol producer status or background diet. However, groups (red clover vs placebo) were comparable.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Some differences in menstrual reproductive years at baseline
Performance	Did deviations from the study protocol impact the results?	+	Non reported
	Were the research personnel and human subjects blinded to the study group during the study?		Uterus
		++	Thyroid
Attrition/exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	Less than 10% drop out for red clover/placebo
Information/detection	Were the outcome assessors blinded to study group or exposure level?	-	Uterus: no details in the text
		NA	Only adverse effect reporting -
	Key question B: Can we be confident in the outcome assessment?	-	no details given on how the outcome was measured
		NA	Only adverse effect reporting
Selective reporting	Were all measured outcomes reported?	++	
		NA	Only adverse effect reporting

Hale et al., 2001 – Ref ID: 16405

Study characteristics and population	**Study design**	Double-blind, randomised, placebo-controlled trial
	N	30 allocated to treatment (RCE, n=15, placebo, n=15); 24 ITT included in the analysis (RCE, n=11, placebo, n=13);
	Location	California, USA
	Sampling time frame	Not reported
	Menopausal status	Late reproductive aged women
	Age at baseline [mean (SD)]	Placebo: 46.5 (1.8) RCE: 47.9 (3)
	Age at menopause	-
	Time since menopause:	-

	Inclusion criteria	Age 45-53 • at least 2 menstrual period over the last 6 months • no oral contraceptive pill within the last 6 months • no IUD use within the last 2 months • no more than 2 hot flushes per week over the last 6 months
	Exclusion criteria	Chronic disease or malignancy (excluded localised skin malignancy) • more than 2 standard alcoholic beverages per day • smoking within the last 12 months • antibiotics within the past 6 months • H2 antagonists, proton pump inhibitors, benzodiazepines, bone sparing agents, lipid lowering agents, or isoflavones supplements within the last 6 weeks • a low isoflavone-containing diet within the last 6 weeks •maintenance of body weight within 5 pounds throughout the study period.
	Funding source	Funded by a grant from Novogen (producer of the red clover extract tested) and from General Clinical Research Center Grant M01-RR00425, National Center for Research Resources.
	Authors conflicts of interest	Not reported
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	RCE: purified red clover, 50 mg/day Placebo
	Duration	3 months
Statistical analysis	**Statistical analysis**	Continuous parameters were tested for differences using two sample t tests or their non-parametric equivalent and categorical parameters using χ^2 or Fisher's exact tests. Relationships between pairs of variables were examined using either Pearson's correlation or a non-parametric Spearman's ranked correlation. Covariance models tested the differences between treatment groups at month 3, while adjusting for values at baseline. A non-parametric ranked analysis of covariance was used when the data were abnormally distributed at either time point.

Results

Uterus

		Baseline	3 months	Change
Ki-67 (%) mean (SD) *P* =0.22	Placebo (n=12)	44.42 (12.16)	40.84 (12.21)	-3.58 (12.63)
	RCE (n=10)	42.56 (10.44)	46.16 (10.91)	+3.61 (15.15)
Endometrial thickness (mm) [mean (SD)] *P* = 0.35	Placebo (n=13)	6.46 (1.72)	6.05 (2.20)	-0.42 (2.77)
	RCE (n=11)	7.09 (2.85)	7.25 (2.88)	+0.15 (2.88)

Risk of Bias appraisal: **Tier:1 (ki-67)**
Tier 2: endometrial thickness

Bias domain	Question	Score	Judgement
Selection	Was the administered dose adequately randomised?	+	No method described, but subjects were randomised
	Was allocation to study groups adequately concealed?	+	Described as blinded, but no more information given
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No equol producer status or background diet, but similar in composition.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Direct comparison, but similar in composition.
Performance	Did deviations from the study protocol impact the results?	+	none reported
	Were the research personnel and human subjects blinded to the study group during the study?	+	Described as blinded
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	-	10 (33%) loss

Bias domain	Question	Score	Judgement
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Not clear whether pathologist was blinded - but method appears to be robust
	Key question B: Can we be confident in the outcome assessment?	+	Ki-67
		-	Endometrial thickness
Selective reporting	Were all measured outcomes reported?	+	Protocol not available

Han et al., 2002 – Ref ID: 2414

Study characteristics and population	**Study design**	Double-blind, placebo controlled randomised trial
	N	82 allocated to treatment (soy isoflavones, N=41; placebo, N=41); 80 completers included in the analysis (soy isoflavones, N=40; placebo, N=40)
	Location	São Paulo, Brazil
	Sampling time frame	August 1999 February 2000
	Menopausal status	Post-menopausal
	Age at baseline [mean ± SEM]	Placebo: 49 ± 1.3; Isoflavones: 48 ± 1.1
	Age at menopause	Not reported
	Time since menopause: Years [mean ± SEM]	Placebo: 2 ± 0.3; Isoflavones: 1.8± 0.2
	Inclusion criteria	Women aged 45-55, in menopause for at least 12 months with intact uterus, FSH in blood serum > 25 U/L; oestradiol level < 20 pg/mL; presence of hut flashes.
	Exclusion criteria	Any type of hormonal treatment during the previous 12 months • currently using lipid-lowering drugs, antidiabetic medications • history of uncontrolled hypertension, stroke or transient ischemic attack • cancer diagnosed less than 5 years ago • previous myocardial infarction
	Funding source	Not reported
	Authors conflicts of interest	Not reported
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Isoflavones: 100 mg/day Placebo:
	Duration	4 months
Statistical analysis	**Statistical analysis**	The natural pairing of observations (baseline and after treatment) in the same group was compared with a Student paired, two-tailed *t* test. A Student unpaired, two-tailed *t* test was used for comparison of between groups data. The level of significance was set at P < 0.05. All values expressed as mean ± SEM
Results	**Uterus**	
	Endometrial thickness mean (mm) (±SD)	Baseline
		Placebo: 2.6 ± 0.1
		Isoflavones: 3.3 ± 0.1
		Post-treatment
		Placebo: 2.6 ± 0.1
		Isoflavones: 3.1 ± 0.1

Risk of Bias appraisal			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Computerised randomsation - no more details given
	Was allocation to study groups adequately concealed?	+	Study is described to be blinded, but no further information given. Treatment appears to have been concealed.

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	--	Background diet was not included, but no subjects using soy-containing foods were included. Otherwise no adjustment for confounders. Baseline characteristics between groups for endometrial thickness appear to be different, and it is not clear whether these have been tested for statistical difference.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	No adjustment
Performance	Did deviations from the study protocol impact the results?	+	No deviation reported
	Were the research personnel and human subjects blinded to the study group during the study?	+	Described as blinded.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No attrition reported - seems no attrition.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	The operator who perfomed all examinations did not know the patient's clinical data. Occurrence of siude effects recorded by an independent gynaecologist. Follow-up conducted by a gynaecologist who did not participate in the screening part of this study or the distribution of the drugs.
	Key question B: Can we be confident in the outcome assessment?	+	Transvaginal sonography was performed to evaluate the endometrial cavity. Measuring the anteroposterior direction from the echogenic interface of the endometrium-myometrium junction on both sides in the most endometrial thickness area in the 1/3 of the uterine body. Insufficient details about quality control
Selective reporting	Were all measured outcomes reported?	+	All data reported - no protocol available

Imhof et al., 2006 – Ref ID: 15280

Study characteristics and population	**Study design**	Randomised, double-blind, placebo-controlled , cross-over trial
	N	109 allocated to treatment (Group A: RCE-placebo, n=50; Group B: placebo-RCE, n=59); 92 included in the analysis (Group A, n=41; Group B, n=50);
	Location	Two sites, Vienna, Austria
	Sampling time frame	Not reported
	Menopausal status	Post-menopausal women (hysterectomy: 18% in the Group A group; 13.6% in the Group B)
	Age at baseline	All: 53.5 ± 7.1 Group A: 54.5 ± 6.2 Group B: 53.7 ± 7.8
	Age at menopause	Not reported
	Time since menopause:	Not reported
	Inclusion criteria	postmenopausal status (amenorrhea > 12 months) • 40 years or older •negative pregnancy test • willingness for adherence to the control dates, and to take the prescribed preparations • moderate to severe menopausal symptoms (Kupperman index ≥ 15)..
	Exclusion criteria	Women under constant HRT • known isoflavone hypersensitivity

	Funding source	Not reported
	Authors conflicts of interest	Not reported
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	RCE: MF11RCE standardised red clover extract providing 80 mg/day Placebo
	Duration	3 months followed by 7-day wash-out period.
Statistical analysis	**Statistical analysis**	Due to different absolute values at the baseline points of the two phases, comparison was performed with regard to the observed changes. Differences between verum and placebo phases were assessed by Wilcoxon rank test. Changes within each of the treatment phases were assessed using paired *T*-test. A p-value <0.05 was considered statistically significant.
Results	**Uterus**	
	endometrial thickness (mm) [mean ± ±SD] and % change	Before RCE: 3.8 ± 1.9
		After RCE: 3.2 ± 1.5
		Difference: -14.69 %, p = 0.001

Risk of Bias appraisal:			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	-	No information on randomisation method
	Was allocation to study groups adequately concealed?	+	Described as blinded, but no more information given
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No information on background diet or equol producer status, however, groups were comparable and design was cross-over. Groups were unbalanced (50 vs 59)
	Did researchers adjust or control for other exposures that are anticipated to bias results?		Direct comparison, but cross-over design
Performance	Did deviations from the study protocol impact the results?	+	none reported
	Were the research personnel and human subjects blinded to the study group during the study?	+	Blinded, but no more information given
Attrition/exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	low attrition, drop out because of start of HRT
Information/detection	Were the outcome assessors blinded to study group or exposure level?	+	Blinded, but no more details
	Key question B: Can we be confident in the outcome assessment?	-	insufficient information on QC/validation and method
Selective reporting	Were all measured outcomes reported?	+	no protocol available

Irace et al., 2013 – Ref ID: 256

Study characteristics and population	**Study design**	Nested in a larger randomized, doubled blinded, placebo controlled trial.
	N	20 allocated to treatment (genistein, 10; placebo, 10); 20 included in the analysis (genistein, 10; placebo, 10)
	Location	Italy
	Sampling time frame	Not reported
	Menopausal status	Post-menopausal
	Age at baseline [mean ± SD/ES]	Placebo: 57.5 ± 8.6 Genistein: 60.1 ± 5.9
	Age at menopause	Not reported
	Time since menopause:	At least 12 months

	Inclusion criteria	Postmenopause for at least 12 months at the time of the inclusion in the study • FSH > 50 IU/L and a serum beta-oestradiol level ≤ 100 pM or less (< 27 pg/mL) • age range 49–67 years • presence of metabolic syndrome • stable pharmacological treatment (previous 6 months).
	Exclusion criteria	surgically induced menopause • clinical or laboratory evidence of confounding systemic diseases (e.g. chronic renal or hepatic failure, chronic inflammatory diseases), as well as CVD defined as documented myocardial infarction, ischaemic heart disease, coronary heart bypass, coronary angioplasty, cerebral thromboembolism and peripheral amputations, or by Minnesota codes 1°1-3, 4°1-4, 5°1-3 at a standard ECG performed in the 12 months preceding the study • coagulopathy • use of oral or transdermal oestrogen, progestin, androgens, selective oestrogen receptor modulators or other steroids • treatment in the preceding 6 months with polyunsaturated ω-3 fatty acids supplements, use of nonsteroidal anti-inflammatory drugs (NSAIDs) or steroids, that would interfere with evaluation of the study medication • smoking habit of more than two cigarettes daily • alcohol or drug abuse history • participation in another clinical study at the beginning of the study or during the last month • proven hypersensitivity to genistein or related drugs.
	Funding source	Grant from the Italian Ministry of Education, University, and Research (protocol 20073XZSR3) and by the University of Messina, Italy.
	Authors conflicts of interest	None reported.
	Clinical trial registration and/or acronym	NCT00541710
Intervention/ exposure	**Intervention**	Placebo
		Genistein: 54 mg/day
	Duration	6 months
Statistical analysis	**Statistical analysis**	The t-test for paired data and the Wilcoxon signed-rank test were used to compare variables at baseline and follow-up visit. The t-test for unpaired data and the Mann–Whitney U-test were applied to compare variables between genistein and placebo group at baseline and 6 month. The χ^2 test was used to compare prevalence among groups. A *P* value of 005 or less was considered statistically significant.
Results	**Uterus**	
	Endometrial thickness mean (mm) (±SD)	No significant change in endometrial thickness compared with placebo (data not shown)

Risk of Bias appraisal			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Randomisation was performed by a computer generated random sequence, however, no more details are given.
	Was allocation to study groups adequately concealed?	+	Study is described as double blind, and it is stated that subjects and researches were blinded. However, no information about blinding/unblinding and data handling are given.
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	-	Endometrial thickness was only included as adverse effect and therefore no detailed analysis was conducted. No information is given on background diet.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Endometrial thickness was only included as adverse effect and therefore no detailed analysis was conducted. No information is given on background diet.

Bias domain	Question	Score	Judgement
Performance	Did deviations from the study protocol impact the results?	+	No information about any deviation given.
	Were the research personnel and human subjects blinded to the study group during the study?	+	The study is described as double-blind, but not more information given.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	No information is given.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Study is described as double-blind, but no more information is given.
	Key question B: Can we be confident in the outcome assessment?	-	Endometrial thickness was measured by ultrasound - no information is given on the validation and accuracy of the method.
Selective reporting	Were all measured outcomes reported?	-	No quantitative data given ("papers states 'no significant change').

Kaari et al., 2006 – Ref ID: 1640

Study characteristics and population	**Study design**	single-center, randomized, double-blind, oestrogen-controlled trial			
	N	79 allocated to treatment (40 isoflavones; 39 oestrogen); 68 completed (33 isoflavones; 35 oestrogen)			
	Location	São Paulo, Brazil			
	Sampling time frame	July 2001 to November 2002			
	Menopausal status	postmenopausal			
	Age at baseline [mean ± SE]	Isoflavones: 53.9 ± 0.9 Oestrogen: 53.7 ± 0.9			
	Age at menopause [mean ± SE]	Isoflavones: 48.6 ± 0.8 Oestrogen: 46.8 ± 0.9			
	Time since menopause: [mean ± SE]	Isoflavones: 5.3 ± 0.5 Oestrogen: 6.7 ± 0.5			
	Inclusion criteria	Postmenopausal women aged 45 years or older with good overall health, no menses for at least 12 months, FSH levels of 30 mU/mL or more, intact uterus, echo endometrial thickness less than 5 mm and atrophic endometrium by biopsy			
	Exclusion criteria	strict vegetarian • high fiber • high soy diet • regular consumption of vitamin and mineral supplementation greater than the RDAs • antibiotic or hormone use within 6 months • history of chronic disorders including endocrine or gynaecological diseases or neoplasia, as well as benign breast disease • regular use of medication known to interfere with study endpoints • body mass index over 30 • patients with cervico-vaginal cytology classified as Class III of Papanicolaou or more • subjects with hypertension using two or more antihypertensive drugs.			
	Funding source	Not reported			
	Authors conflicts of interest	Not reported			
	Clinical trial registration and/or acronym	Not reported			
Intervention/ exposure	**Intervention**	Isoflavones: 120 mg/day			
		E2: 0.625 mg/day conjugated equine oestrogens			
	Duration	6 months			
Statistical analysis	**Statistical analysis**				
Results	**Uterus**				
	Endometrial thickness mean (mm) ($p < 0.01$ between groups)		Baseline	3 months	6 months
		Isoflavones	3.2	-	3.0
		E2	2.8	6.2	5.9

	Histo(patho)logical changes		Baseline	6 months
		Isoflavones N=33	Atrophy: 26 Proliferative: 0 Hyperplasia: 0 Insufficient tissue: 7	Atrophy: 29 Proliferative: 1 Hyperplasia: 0 Insufficient tissue: 3
		E2 N=35	Atrophy: 21 Proliferative: 0 Hyperplasia: 0 Insufficient tissue: 14	Atrophy: 19 Proliferative: 10 Hyperplasia: 1 Insufficient tissue: 5
		Fisher's exact test: estrogens group, comparison between baseline and 6 months: $p < 0.01$. Isoflavones group, comparison between baseline and 6 months: $p = 0.30$.		
	Pap smear (percentage of superficial, intermediate and parabasal cells)	No differences were observed in the isoflavone group in the percentage of superficial, intermediate and parabasal cells during the trial. In the oestrogen group, there was a statististically significant increase in the percentage of superficial cells after 3 months and at the end of the study when compared with the baseline in the same group and with isoflavones at 3 and 6 months (see Figure 5 in the original publication).		

Risk of Bias appraisal			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	-	No information on randomisation given
	Was allocation to study groups adequately concealed?	-	No information given.
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Many confounding factors were assessed, but not clear how background diet was assessed. Groups are largely comparable.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Not included, but groups were comparable
Performance	Did deviations from the study protocol impact the results?	+	No deviation reported.
	Were the research personnel and human subjects blinded to the study group during the study?	-	Intervention and placebo were only similar, not identical. No information on blinding/unblinding were given.
Attrition/exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Low attrition rate, fully documented.
Information/detection	Were the outcome assessors blinded to study group or exposure level?	-	No information given, but study is described as double-blind.
	Key question B: Can we be confident in the outcome assessment?	-	No detailed information on QC/validation given
Selective reporting	Were all measured outcomes reported?	+	Appears to be - study protocol not available.

Khan et al., 2012 – Ref ID: 16409

Study characteristics and population	**Study design**	Randomised, double-blind, placebo-controlled trial.
	N	126 allocated to treatment; 98 included in the analysis (48 soy group; 50 placebo)
	Location	Chicago, USA
	Sampling time frame	Not reported
	Menopausal status	Pre- and post-menopausal women Women were defined post-menopausal if plasma FSH > 30 mIu/mL, oestradiol < 30 pg/mL and progesterone < 1 pg/mL with no menstrual period within 6 months.
	Age at baseline: [interquartile range]	Soy group: 48 (43-53) Placebo: 50 (46-55)

	Age at menopause	Not reported
	Time since menopause:	Not reported
	Inclusion criteria	Healthy, non-pregnant and non-lactating women at increased risk of breast cancer or women with a history of unilateral minimal risk of breast cancer (Tis, or T1a-b, N0 breast cancer, when only the unaffected breast was studied). Age between 25 to 55 years ● 5-year Gail or Claus model risk estimate ≥ 1.66% for women older than 40 years, ≥ 1.0% for those aged between 30 and 39, and ≥ 0.1% for women aged between 20 and 29 ● Adequate bone marrow, liver, kidney, and thyroid function.
	Exclusion criteria	Participants were asked to avoid soy-containing foods and supplements, hormonal contraceptives, and hormone therapy ● Participants excluded from the analysis if had less than 4 000 epithelial cells in rFNA samples pre- and post-intervention.
	Funding source	Supported by NIH Grant N01-CN-35157
	Authors conflicts of interest	No potential conflicts of interest were disclosed by the authors.
	Clinical trial registration and/or acronym	NCT00290758
Intervention/ exposure	**Intervention**	PTIG-2535
		matched placebo pills
	Duration	6 months
Statistical analysis	**Statistical analysis**	The authors planned to accrue 150 women and randomize 120, expecting that 80% of subjects would yield sufficient epithelial cells for analysis (≥ 4 000 cells). With a 28% dropout rate (including women who had insufficient cells for analysis the 6-month time point), we planned a total of 90 women (45 per group) for final analysis. We estimated a median post-intervention decrease in the primary endpoint (Ki-67 labeling index of epithelial cells) of 1.5% in the soy group, compared with a median change of zero in the control group. Assuming an SD of 1.5% to 2%, this would provide more than 90% power with 45 subjects per group. Interim analyses were planned to identify evidence for a systemic estrogenic effect of the soy isoflavone supplement, defined as an increase in the 1-month plasma in SHBG of 1.5 times the baseline level. The baseline demographic characteristics between treatment and control groups were compared using the Wilcoxon rank-sum test for continuous variables and Fisher exact test for categorical variables. Analyses of cellular parameters were adjusted for cell number. The effects of treatment were assessed within groups (month 6 – baseline) using the signed-rank test and between groups (treated difference – control difference) using the Wilcoxon rank-sum test. Women with plasma equol concentrations >5 ng/mL were designated as equol producers. The month 6 minus baseline differences between groups were tested using the unpaired t test, whereas differences within groups were tested using the unpaired t test, whereas differences within groups were tested using the paired t test.We adjusted P values from these tests via the Benjamini-Hochberg approach.
Results	**Mammary gland**	

Ki67-labeling [median (interquartile range)]		Baseline	Post-treatment
All subjects (N= 98)	Placebo	0.97 (0.70-1.90)	0.92(0.59-1.96)
	Isoflavones	1.17 (0.66-1.93)	1.09 (0.75-2.33)
	Between groups:	-0.03 (-0.42 to 0.08) P=0.24	
Post-menopausal (n=45)	Placebo	0.70 (0.57-1.07)	0.63 (0.42-0.98)
	Isoflavones	0.63 (0.52-1.08)	0.77 (0.35-0.94)
	Between groups:	-0.12 (-0.37 to 0.23) P=0.74	

	Pre-menopausal (n=53)	Placebo	1.90 (0.88-2.33)	1.94 (0.92-2.55)
		Isoflavones	1.71 (1.12-2.35)	2.18 (1.18-3.04)
		Between groups:	0.19 (-0.46 to 1.07) P=0.31	
	Atypical cytology [% of women]		Baseline	Post-treatment
	All subjects (N= 98)	Placebo	40.8 %	53.1%
		Isoflavones	42.9 %	53.1 %
		Between groups:	-2.1 % *P*=0.83	
	Post-menopausal (n=45)	Placebo	33.3 %	33.3 %
		Isoflavones	33.3 %	23.8 %
		Between groups:	-9.5% *P*=0.72	
	Premenopausal (n=53) Atypical cytology (% of women)	Placebo	75.0 %	72.0 %
		Isoflavones	50.0 %	48.0 %
		Between groups:	1.0% *P*=0.99	

Risk of Bias appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Insufficient information on randomisation process, but groups appear to be similar
	Was allocation to study groups adequately concealed?	-	No information on allocation or control
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Information on background diet etc collected, no equol producer status assessed
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Direct comparison, but groups were similar
Performance	Did deviations from the study protocol impact the results?	+	none reported
	Were the research personnel and human subjects blinded to the study group during the study?	-	No information given
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Low attrition, explained
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	No information given
	Key question B: Can we be confident in the outcome assessment?	+	Full QC and validation data given
Selective reporting	Were all measured outcomes reported?	+	protocol not available

Khaodiar et al., 2008 – Ref ID: 3195

Study characteristics and population	**Study design**	randomized, double-blind, placebo-controlled trial
	N	191 allocated to treatment; 142 included in the analysis (placebo, n=45;DRI-40, n=48; DRI-60, n=49)
	Location	Single centre, Boston, MA, USA.
	Sampling time frame	Not reported
	Menopausal status	Menopausal (119 postmenopausal, 28 perimenopausal)
	Age at baseline [mean ± SD]	Placebo: 53.8 ± 5.1
		DRI-40: 52.2 ± 4.8
		DRI-60: 53.2 ± 5.6
	Age at menopause	Not reported
	Time since menopause (years): [mean ± SD]	Placebo: 5.6 ± 5.9
		DRI-40: 4.0 ± 4.9
		DRI-60: 5.8 ± 6.1

	Inclusion criteria	Postmenopausal with no menses for at least the previous 6 months, between the ages of 38 and 60, and experiencing hot flashes four or more times per day but 15 times or less per day
	Exclusion criteria	active smokers (more than one pack of cigarettes per day) • use of dietary supplements containing soy isoflavones, vitamin E, flaxseed, or red clover within 6 weeks prior to start of the study • concomitant use of HT or any medications for treatment of hot flashes (clonidine, selective serotonin reuptake inhibitors) within 6 weeks before enrolment • BMI > 40 kg/m^2 • history of breast, endometrial, or cervical cancer • positive pregnancy test • history of undiagnosed abnormal vaginal bleeding, thromboembolic disease, cardiovascular disease, liver or kidney disease, diabetes mellitus, or other major illnesses.
	Funding source	Investigator-initiated study supported by a research grant by Nichimo Co, Ltd, Tokyo, Japan, who made daidzein-rich isoflavone aglycone extract from soy germ fermentation with Koji fungus Effisoy.
	Authors conflicts of interest	Not reported.
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Placebo
		DRI-40: daidzein rich extract providing 40 mg/day of isoflavone aglycones
		IF-60: daidzein rich extract providing 60 mg/day of isoflavone aglycones
	Duration	12 weeks
Statistical analysis	**Statistical analysis**	The primary measure of efficacy was the percentage of change in hot flash frequency. Statistical analysis not described for other endpoints. A *P* value less than 0.05 was considered significant.
Results	**Thyroid**	

[mean ±SD]		Baseline	12-wk
freeT3 (pg/mL)	Placebo	2.8 ± 0.9	2.6 ± 1.0
	DRI-40	2.7 ± 0.8	2.7 ± 0.9
	DRI-60	2.9 ± 1.1	2.8 ± 1.0
total T3 (ng/mL)	Placebo	85.6 ± 31.3	83.4 ± 30.5
	DRI-40	82.7 ± 30.2	81.9 ± 29.5
	DRI-60	84.5 ± 25.4	83.1 ± 25.0
freeT4 (pg/mL)	Placebo	1.1 ± 0.2	1.0 ± 0.2
	DRI-40	1.0 ± 0.2	1.0 ± 0.3
	DRI-60	1.1 ± 0.3	1.1 ± 0.3
total T4 (µg/mL)	Placebo	6.3 ± 1.7	6.0 ± 1.6
	DRI-40	6.4 ± 2.2	6.3 ± 1.6
	DRI-60	6.6 ± 1.8	6.4 ± 2.1
TSH (µIU/mL)	Placebo	3.2 ± 3.6	3.5 ± 3.8
	DRI-40	4.3 ± 11.0	4.9 ± 11.6
	DRI-60	3.6 ± 3.2	3.4 ± 2.6
Thyroglobulin (ng/mL)	Placebo	17.6 ± 19.1	24.8 ± 32.1
	DRI-40	25.6 ± 24.2	26.3 ± 23.9
	DRI-60	38.8 ± 93.3	41.0 ± 88.6

Risk of Bias appraisal			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	It is not clear how participants were randomised
	Was allocation to study groups adequately concealed?	+	There is no information about blinding, but study is described as blinded.

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Many confounders were addressed by restrictive inclusion criteria; potential confounders were not included in analysis.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Background diet was not included
Performance	Did deviations from the study protocol impact the results?	+	Authors do not report any deviation.
	Were the research personnel and human subjects blinded to the study group during the study?	-	There is no information about blinding of researchers
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Loss of subjects is well documented.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	No information given
	Key question B: Can we be confident in the outcome assessment?	-	No information about quality of method - no validation parameter
Selective reporting	Were all measured outcomes reported?	+	All information reported

Lappe et al., 2011 – Ref ID: 15431

Study characteristics and population	**Study design**	Randomised, double-blind, placebo-controlled
	N	70 allocated to treatment (genistein, 35; placebo, 35); 58 included in the analysis (genistein, 28; placebo, 30)
	Location	Nevada, USA.
	Sampling time frame	January 2007-April 2008
	Menopausal status	Early post-menopausal
	Age at baseline [mean ± SD]	All: 54.7 ± 2.4 Genistein: 54.8 ± 2.5 Placebo: 54.7 ± 2.3
	Age at menopause	Not reported
	Time since menopause:	All: 2.1 ± 0.8 (n = 62) Genistein: 2.2 ± 0.8 (n = 30) Placebo: 2.1 ± 0.8 (n = 32)
	Inclusion criteria	Age 45-55 years • 1–3 years since the last spontaneous menstrual bleeding and FSH > 75 IU/mL and 17β-oestradiol (E2) < 20 ng/L • natural menopause or total hysterectomy • smoking\10 cigarettes/days
	Exclusion criteria	T-score <–2.5 at total hip and spine (either or both) • BMI > 30 or <21 • use of HT within the previous 6 months • use of any drug that might interfere with bone metabolism within the previous 12 months • extreme dietary habits • use of dietary supplements while on study except multi-vitamins • total genistein blood concentrations >100 ng/mL measured at pre-study examination • unexplained weight loss or weight gain of >5 kg in the 3 months prior to the study • history of liver or pancreatic diseases, cardiovascular disease, history of breast cancer, endometrial cancer or other malignancy except basal and squamous cell skin cancer, history of thromboembolism, any fractures within the past year except for fingers, toes and facial bones • susceptibility to fractures •endometrial thickness >6 mm, endometrial polyps • insulin-dependent diabetes mellitus • any condition that might interfere with the absorption of the investigational product • co-medications.
	Funding source	Study sponsored by DSM, the producer of the genistein tested.
	Authors conflicts of interest	Some of the co-authors were employed by the company producing the genistein tested

	Clinical trial registration and/or acronym	NCT 00698984
Intervention/ exposure	**Intervention**	Genistein (30 mg/day), vitamin D3, vitamin K1 and PUFAs
		Placebo
	Duration	6 months
Statistical analysis	**Statistical analysis**	Differences were considered significant if p <0.05. Safety data were evaluated by descriptive statistics, and statistically significant differences were determined by *t* tests (within the groups by paired t test)
Results	**Uterus**	

Endometrial thickness (mm)		Baseline	6-months
	Placebo	2.2	2.3
	Genistein	2.3	1.8[(a)]
	(b): Significanly decreased compared to baseline (p = 0.007), however no difference between the groups.		

Risk of Bias appraisal: **Tier: 1**

Bias domain	Question	Score	Judgement
Selection	Was the administered dose adequately randomised?	++	Randomisation by four-block randomisation, conducted by outside company
	Was allocation to study groups adequately concealed?	++	Research team not involved in randomisation; unblinding after end of study
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No background diet or equol producer status, but groups were comparable.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	only direct comparisons, but groups were comparable
Performance	Did deviations from the study protocol impact the results?	+	none reported
	Were the research personnel and human subjects blinded to the study group during the study?	++	Completely blinded
Attrition/exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Low attrition
Information/detection	Were the outcome assessors blinded to study group or exposure level?	+	Blinding by outside group
	Key question B: Can we be confident in the outcome assessment?	+	No QC/Validation data, but standard method
Selective reporting	Were all measured outcomes reported?	-	Reported only as text.

Levis et al., 2011 - Ref ID: 16411

Study characteristics and population	**Study design**	randomized, placebo-controlled, double-blind clinical trial
	N	248 allocated to treatment (soy isoflavones, 122; placebo, 126); 182 included in the analysis (soy isoflavones, 99; placebo, 83);
	Location	Single-centre, South Florida, USA
	Sampling time frame	July 1, 2004, through March 31, 2009
	Menopausal status	Peri and post-menopausal women
	Age at baseline [mean SD]	Soy isoflavones: 53 (3.3) Placebo: 52 (3.3)
	Age at menopause	Not reported
	Time since menopause:	Not reported
	Inclusion criteria	Women aged 45 to 60 years who had been menopausal for 1 to 5 years or for 6 to 12 months and had FSH ≥ 40 mIU/mL

	Exclusion criteria	Osteoporotic fractures • BMD T score in the lumbar spine or total hip of less than −2.0 • BMI ≥ 32 • abnormal mammogram findings • cancer in the past 10 years (except for skin cancer) • Concomitant medications: bone active drugs, corticosteroids, or herbal products. • menopausal hormone therapy since menopause discontinued for less than 6 months.
	Funding source	National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, grant RO1AR048932-01A1
	Authors conflicts of interest	None reported
	Clinical trial registration and/or acronym	NCT00076050 SPARE (Soy Phytoestrogens As Replacement Estrogen)
Intervention/ exposure	**Intervention**	Soy isoflavones extracted from soy protein: 200 mg/day
		Placebo: identical tablets
	Duration	2 years
Statistical analysis	**Statistical analysis**	The sample size and power calculation was based on testing the primary hypothesis that soy isoflavone tablets prevent bone loss, as assessed by BMD, among women in the early years of menopause. A random-effects model based on an unstructured covariance matrix was used to analyze the effect of the treatment on other outcomes, including thyroid outcomes. Potential confounding factors and effect modifiers discussed in the publication were adjusted for in the models
Results	**Thyroid**	
	Thyrothropin (mIU/L) [mean (SE)]	Baseline to Year 1
		Placebo (n=81) −0.15 (0.78)
		Soy isoflavones (n=96) 0.32 (0.08)
		Baseline to Year 2
		Placebo (n=75) −0.61 (0.81)
		Soy isoflavones (n=93) 0.04 (0.08)

Risk of Bias appraisal			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	-	No information on randomisation
	Was allocation to study groups adequately concealed?	+	Study described as double blind, but no more information given
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Controls are younger, no other significant difference; background diet, equol producer status and others not included.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Confounding factors not included, but comparable groups
Performance	Did deviations from the study protocol impact the results?	+	none reported
	Were the research personnel and human subjects blinded to the study group during the study?	-	Study described as double blind, but no more information given.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Attrition within acceptable range
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Stated as double blind, but no detailed information
	Key question B: Can we be confident in the outcome assessment?	-	Standard method, but no information on quality control etc
Selective reporting	Were all measured outcomes reported?	+	no protocol available

Marini et al., 2007 – Ref ID: 4922; Marini et al., 2008 – Ref ID: 3138; Bitto et al., 2010 – Ref ID: 16393

Study characteristics and population	**N**	389 allocated to treatment (placebo, n=191; genistein, n=198); 304 subjects completed the 2-yr program (placebo, n =154; genistein, n = 150 (reported in Marini et al., 2007) 138 subjects chose to continue the study through 3 yr (placebo group, n = 67; genistein group, n = 71) (reported in Marini et al., 2008 and Bitto et al., 2010)
	Location	Italy (Messina)
	Sampling time frame	Not reported
	Menopausal status	Post-menopausal
	Age at baseline	Placebo: 53.5 ± 2.0 Genistein: 53.8 ± 2.9
	Age at menopause	Not reported
	Time since menopause, months:	Marini et al., 2007. At baseline: Placebo: 59.1 (38.4) Genistein: 66.8 (45.8)
		Marini et al., 2008. At baseline, women who were enrolled in the 3-year study Placebo: 42.8 ± 26. 3 Genistein: 42.9 ± 35.9
	Inclusion criteria	Women aged 49-67 years of age • post-menopausal for at least 12 months • good general health • not had a menstrual period in the preceding year • not undergone surgically induced menopause • FSH > 50 IU/L and serum 17β-oestradiol ≤ 100 pmol/L (27 pg/mL)
	Exclusion criteria	clinical or laboratory evidence of confounding systemic diseases, such as cardiovascular, hepatic, or renal disorders; coagulopathy • use of oral or transdermal estrogen, progestin, androgens, selective estrogen receptor modulators, or other steroids; use of biphosphonates, cholesterol-lowering therapy, or cardiovascular medications (including antihypertensive drugs) in the preceding 6 months • smoking habit of more than 2 cigarettes daily • treatment in the preceding year with any drug that could have affected the skeleton • family history of oestrogen-dependent cancer • BMD at the femoral neck > 0.795 g/cm^2 (which corresponds to a T-score of -1.0 SD).
	Funding source	This work was supported by the Italian Ministry of Education, University and Research; by the University of Messina, Italy; and by Primus Pharmaceuticals, Inc. Scottsdale, Arizona.
	Authors conflicts of interest	Two of the authors in the Marini et al., 2008 stduy work for Primus Pharmaceuticals. All other authors have nothing to declare. Furthermore, all authors are independent from funders.
	Clinical trial registration and/or acronym	ClinicalTrials.gov identifier: NCT00355953 (Marini et al., 2007) ClinicalTrials.gov identifier: NCT00626769 (Marini et al., 2008; Bitto et al., 2010).
Intervention/ exposure	**Intervention**	Genistein, 54 mg/day
		Placebo
	Duration	24 months (Marini et al., 2007) + 1-year extension (Marini et al., 2008; Bitto et al., 2010).
Statistical analysis	**Statistical analysis**	Two-way ANOVA with post hoc comparisons were carried out to assess the main effect of treatment, time, and treatment X time interaction; P < 0.05 was considered to be statistically significant. The study had 80% power to detect a 1% change in IMI at a significance level of 5%.

Results Marini et al., 2007

Uterus

Endometrial thickness, mm [Mean (SD] N measured		Baseline	1-year	2-year
	Placebo	3.2 (1.8) N=183	3.0 (1.5) N=164	3.0 (1.1) N=154
	Genistein	3.1 (1.5) N=186	3.0 (1.4) N=166	3.2 (1.4) N=150

Results Marini et al., 2008	**Mammary gland**						
	Mammographic density: [mean (95% CI)]	No significant difference in IMI breast density was observed between groups at different timepoints (baseline, 2-year, 3-year). Mammographic density was significantly decreased in both groups after three years of treatment ($P < 0.001$ vs. placebo and genistein baseline). For more details see Figure 2A in the original publication.					
	Classification of mammographic density	Wolfe class	Baseline		2-year		3-year
			GEN	control	GEN	control	GEN	control
		N1	53	57	64	63	69	66
		P1	16	10	7	4	2	1
		P2	2	0	0	0	0	0
		DY	0	0	0	0	0	0
	Uterus							
	Endometrial thickness [Mean (95% CI)]	No significant difference in endometrial thickness was observed between the two groups at different time-points (baseline, 2-year, 3-year). Endometrial thickness was significantly decreased in both groups after three years of treatment ($P < 0.001$ vs. placebo and genistein baseline). For more details see Figure 3B in the original publication.					

Results Bitto et al., 2010	**Thyroid**			
	TSH, µg/mL [mean ± SEM]		Baseline	3-year
		Placebo	1.69 ± 0.10	1.7 ± 0.08
		Genistein	2.04 ± 0.10	2.02 ± 0.10
	freeT3, pg/mL [mean ± SEM]		Baseline	3-year
		Placebo	2.80 ± 0.06	2.77 ± 0.06
		Genistein	2.73 ± 0.06	2.65 ± 0.07
	freeT4, pg/mL [mean ± SEM]		Baseline	3-year
		Placebo	13.50 ± 0.16	13.90 ± 0.14
		Genistein	13.20 ± 0.20	13.60 ± 0.11
	TPO (IU/mL) [mean ± SEM]		Baseline	3-year
		Placebo	20.33 ± 0.41	19.76 ± 1.53
		Genistein	18.34 ± 0.53	19.35 ± 1.09
	TG (IU/mL) [mean ± SEM]		Baseline	3-year
		Placebo	32.90 ± 2.97	30.50 ± 2.46
		Genistein	29.30 ± 2.12	29.0 ± 1.68
	TMA (IU/mL) [mean ± SEM		Baseline	3-year
		Placebo	23.60 ± 1.11	22.60 ± 1.02
		Genistein	18.30 ± 1.30	19.50 ± 1.22

Marini et al., 2007			**Ref ID: 4922**
Risk of Bias appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	++	Block randomisation, stratified by centre
	Was allocation to study groups adequately concealed?	++	Placebo and genistein were identical in appearance and taste.
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Subjects were advised to reduce phytoestrogen intake, although this was not monitored. Groups were comparable.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Confounders were not included, but groups were comparable
Performance	Did deviations from the study protocol impact the results?	+	No deviation reported
	Were the research personnel and human subjects blinded to the study group during the study?	-	No information given
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Detailed information given - unlikely to introduce bias

Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	No information given
	Key question B: Can we be confident in the outcome assessment?	+	No detailed information given - no QC/validation data
Selective reporting	Were all measured outcomes reported?	+	All data reported
Marini et al., 2008			**Ref ID: 3138**
Risk of Bias appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	++	Block randomisation stratified by centre (see Marini et al., 2007)
	Was allocation to study groups adequately concealed?	++	Se Marini et al., 2007
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Groups were comparable in terms of baseline characteristics.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Adjustment
Performance	Did deviations from the study protocol impact the results?	+	No deviation reported
	Were the research personnel and human subjects blinded to the study group during the study?	-	Intervention and placebo were only similar and not identical, suggesting that subjects and researchers could identify them.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Loss of subjects was addressed,
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	All mammograms were assessed and discussed by two independent radiologists who were blinded to treatments.
	Key question B: Can we be confident in the outcome assessment?	+	Detailed description of method given
Selective reporting	Were all measured outcomes reported?	+	All data reported
Bitto et al., 2010			**Ref ID: 16393**
Risk of Bias appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	++	Computerised randomisation stratified by centre
	Was allocation to study groups adequately concealed?	++	no information given, but described as double blind
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Diet was under supervision of nutritionist, not clear how other factors were assessed, but groups largely comparable
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Confounders were not included but groups were comparable groups
Performance	Did deviations from the study protocol impact the results?	+	No deviation reported
	Were the research personnel and human subjects blinded to the study group during the study?	-	no information given, but described as double blind

Bias domain	Question	Score	Judgement
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	--	Third year of study is only voluntary follow-up and only ~1/3 participate.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	No information give, not clear whether analysis was performed by outside lab or by researchers
	Key question B: Can we be confident in the outcome assessment?	+	No information on method validation and quality control
Selective reporting	Were all measured outcomes reported?	+	No protocol available

Maskarinec et al. 1999 – Ref ID: 1199; Steinberg et al., 2011 – Ref ID: 4366

Study characteristics and population	**Study design**	multi-site, randomised, double-blinded, placebo-controlled, intent-to-treat clinical trial with bone density as the primary endpoint
	N	406 allocated to treatment (n=135 isoflavones 80 mg/day; n=136 isoflavones 120 mg/day; n=135 placebo); 373 in study after 1 year (n=122 isoflavones 80 mg/day; n=123 isoflavones 120 mg/day; n= 128, placebo); 362 in study after after 2 year (n= 119 isoflavones 80 mg/day; n=117 isoflavones 120 mg/day; n=126, placebo).
	Location	USA (three sites in Georgia, Texas and California)
	Sampling time frame	2003–2004
	Menopausal status	Postmenopausal.
	Age at baseline	Isoflavones 80 mg/day: 55.2 ± 4.0 [mean ± SD] Isoflavones 120 mg/day: 54.7 ± 3.8 Placebo: 54.8 ± 3.6
	Age at menopause [mean ± SD]	Isoflavones 80 mg/day: 48.5 ± 5.7 Isoflavones 120 mg/day: 47.9 ± 6.2 Placebo: 48.3 ± 5.2
	Years since menopause [mean ± SD]	Isoflavones 80 mg/day: 6.7 ± 5.4 Isoflavones 120 mg/day: 6.9 ± 6.7 Placebo: 6.5 ± 5.2
	Inclusion criteria	Menopausal women between the ages of 40 and 60 y with a serum FSH concentration of > 30 IU/mL and 12 months of amenorrhea.
	Exclusion criteria	Allergic reactions to soy products and vegetarians ● soy food consumption of >1 serving/week ● smoking or having quit smoking,5 y prior to enrolment ● highly physically active or completely sedentary ● T-scores for lumbar spine bone mineral density outside the normal range ● exercise or drug treatment for bone disease ● BMI ≥ 30 kg/m2 ● current use of bisphosphonates, calcitonin, fluoride, corticosteroids, Tamoxifen, Raloxifene, Farestron, Letrozole, Premarin, and any other hormone replacement therapy ● current use of supplements, including black cohosh, blue cohosh, dong quai, Caltrate, 6001Soy, Estroven, ginseng,HealthyWomen, NaturalEstrogen,Opti-Soy, PhytoFem, Probalance, Promensil, Remifemin, Rimostil, or Trinovin ● medical illnesses, including osteoporosis, spine and/or hip fractures, cancer, as well as liver, kidney, gallbladder, and heart disease.
	Funding source	Supported by the Initiative for Future Agriculture and Food Systems grant no. 2001-52102-11255 from the USDA Cooperative State Research, Education, and Extension Service and R03 CA121879 from the National Cancer Institute.
	Authors conflicts of interest	None disclosed.
	Clinical trial registration and/or acronym	NCT00665860 OPUS (Osteoporosis Prevention Using Soy) study
Intervention/ exposure	**Intervention**	80 mg/day isoflavones (total) 120 mg/day isoflavones (total) Placebo

	Duration	24 months
Statistical analysis	**Statistical analysis**	Maskarinec et al., 1999: All analyses investigating the intervention effect followed the intent-to-treat principle, i.e. participants were analysed as part of their assigned treatment group. We computed means ± SD by group at baseline and used ANOVA and chi-square tests to assess the differences for significance. A mixed general linear model using maximum likelihood estimation was applied to evaluate whether isoflavone supplementation modified mammographic density. The repeated measurements were modelled as random effects. The model included a fixed effect portion to test for a change in breast density over time and an interaction between group assignment and time. After the significant difference in breast density across study site was noted, we repeated the mixed models with study site and baseline density as covariates. We also assigned a variable to indicate the dosage level (0, 80, and 120) to test for a dose-response relation. Participants with missing mammograms were part of the overall analysis, but we repeated the models with the 303 women who had a complete set of 3 mammograms and again after exclusion of all 333 density readings from digital mammograms. To explore whether any subgroups responded differently to isoflavone exposure, we introduced an interaction term into the model and stratified by BMI (<25 and ≥25 kg/m^2) and age group (<55 and ≥55 y). Steinberg et al., 2011: Treatment groups were compared with respect to baseline demographic and clinical characteristics for potential confounding. Those characteristics shown to be different between groups to a clinically important degree were included as covariates in the analyses. Each outcome was assessed by using generalized estimating equations with respect to the effects of treatment group, time, and interaction between treatment and time with clinical site, confounders, and the corresponding baseline value of the outcome variable accounted for. Log transformations of skewed data were carried out as needed, and the statistical analyses were repeated. A significant treatment-by-time interaction was followed by group comparison at years 1 and 2. If the interaction was not significant, that term was dropped from the model and the analysis was repeated to assess the main effect of treatment. Bonferroni adjustments were made for testing multiple comparisons. For effects on the thyroid, *P* values for baseline and years 1 and 2 were derived from a multivariate model ANOVA with adjustment for study site, soy intake, and pre-treatment values. The interaction terms were not significant. For all significance tests, an α of < 0.05 was considered significant.

Results Maskarinec et al., 1999

Mammary gland

Mammographic density Percent density (%) (±SD)		Baseline:	1-year *P*-value:)	2-year *P*-value: (
	Placebo	32.0 ±17.5	30.0 ± 17.2	29.3 ±18.0
	IF-80:	28.9 ± 17.4	27.2 ± 17.6	25.8 ± 17.4
	IF-120	32.3 ± 19.0	31.4 ± 18.4	30.6 ± 19.2
	P-value (group; time; interaction)	0.91; 0.006; 0.59	0.69; < 0.001; 0.79	0.17; < 0.001; 0.85

Results Steinberg et al., 2011

Uterus

Endometrial thickness (mm) [mean ± SD] (measured in a sub-cohort of women from California site, n=116)		Baseline	2-year
	Placebo	2.0 ± 1.22	1.5 ± 1.25
	IF-80	1.9 ± 1.67	1.3 ± 0.74
	IF-120	1.8 ± 0.98	1.0± 0.58

	Uterine fibroids (n) (measured in a sub-cohort of women from California site, n=116	Only 13 women had detectable uterine fibroids. No growth of any uterine fibroids and no statitistical differences between the treatment groups over the 2-year interval were observed.			
	Thyroid				
	TSH (normal range: 0.35-5.5 µIU/mL) [mean ± SD]		Baseline (P = 0.612)	1-year (P = 0.850)	2-year (P = 0.264)
		Placebo	2.8 ± 7.4 (n=134)	2.6 ± 2.4 (n=128)	2.4 ± 1.4 (n=126)
		IF-80	2.2 ± 1.5 (n= 135)	2.5 ± 1.6 (n=122)	2.4 ±1.3 (n=119)
		IF-120	2.4 ± 2.9 (n=134)	2.6 ± 3.2 (n=123)	2.2 ± 1.2 (n=117)
	freeT4 (normal range: 0.80-1.80 ng/dL) [mean ± SD]		Baseline (P = 0.810)	1-year (P = 0.739)	2-year (P = 0.052)
		Placebo	1.1 ± 0.2 (n=134)	1.1 ± 0.2 (n=128)	1.2 ± 0.2 (n=126)
		IF-80	1.2 ± 0.2 (n= 135)	1.2 ± 0.2 (n=122)	1.1 ± 0.1 (n=119)
		IF-120	1.2 ± 0.2 (n=134)	1.2 ± 0.2 (n=123)	1.1 ± 0.2 (n=117)

Maskarinec et al., 1999

Risk of Bias appraisal			**Tier:1**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	-	The detailed protocol is "published elsewhere" but could not be found. The study is described as randomised and double blind.
	Was allocation to study groups adequately concealed?	-	It is not clear how study groups were concealed as the information was not included (it is included as "not published" data)
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Potential confounders were assessed and included in the analysis. Background diet was assessed.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	Subjects were advised not to consume supplements and less than one serving of soy per week.
Performance	Did deviations from the study protocol impact the results?	+	No statement but authors do not report any deviation.
	Were the research personnel and human subjects blinded to the study group during the study?	-	No information given - protocol is unpublished.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	Study designed as intention to treat – sensitivity analyses were conducted.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	Randomised and blinded, sufficient information given
	Key question B: Can we be confident in the outcome assessment?	+	Method well described, some validation data given.
Selective reporting	Were all measured outcomes reported?	++	All data provided

Steinberg et al., 4366			
Risk of Bias appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	There is insufficient information. Participants were allocated using time blocks, but no information on how they were assigned.
	Was allocation to study groups adequately concealed?	+	Study states that all were blinded
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No equol producer status, but soy intake was assessed by questionnaire and included in analysis
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Multivariable techniques were used to adjust for confounders. Groups were comparable
Performance	Did deviations from the study protocol impact the results?	+	None reported
	Were the research personnel and human subjects blinded to the study group during the study?	+	Study described as blinded, tablets were identical - no information about data handling
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Attrition fully explained
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	no information given - but lab method
	Key question B: Can we be confident in the outcome assessment?	+	Insufficient information on QC and validation
Selective reporting	Were all measured outcomes reported?	+	no information given

Morabito et al., 2002 - Ref ID: 2282

Study characteristics and population	**Study design**	Randomized double-blind placebo-controlled study
	N	90 allocated to treatment (30/group)
	Location	Italy, Messina (single centre)
	Sampling time frame	Not reported
	Menopausal status	Healthy women, aged 47–57 years, not had a menstrual period in the preceding year, and FSH level > 50 IU/L and a serum 17β-estradiol (E2) level of ≤ 100 pmol/liter.
	Age at baseline [mean ± SD]	genistein: 52 ± 3 HRT: 52 ± 5 placebo: 51 ± 4
	Age at menopause	Not reported
	Time since menopause: [mean ± SD]	genistein: 7 ± 6 HRT: 7 ± 3 placebo: 6 ± 5
	Inclusion criteria	
	Exclusion criteria	Surgically induced menopause • clinical or laboratory abnormalities that suggested cardiovascular, hepatic, or renal disorders • coagulopathy • use of oral or transdermal estrogen, progestin, androgen, or other steroids in the preceding year • smoking habit of more than two cigarettes per day • previous treatment with any drug that could affect the skeleton • a family history of estrogen-dependent cancer • BMD at the femoral neck > 0.795 g/cm2.
	Funding source	Not reported
	Authors conflicts of interest	None stated.
	Clinical trial registration and/or acronym	Not reported

Intervention/ exposure	**Interventions**	placebo (n = 30) genistein (n =30; 54 mg/day) HRT (n = 30; 1 mg/day of E2 combined with norethisterone acetate; Activelle, Norvo Nordisk, Logenhagen, Denmark),	
	Duration	12 months	
Statistical analysis	**Statistical analysis**	The evaluation of the incidence of side effects in the several groups of postmenopausal women was carried out with the Fisher's exact probability test. All statistical tests were two sided. All data are reported as means and SD. A value of $p < 0.05$ was considered statistically significant.	
Results	**Mammary gland**		
	Mammographic density	No patient in any of the groups showed significant change in the mammography exams at 1 year of follow-up. No further data provided.	
	Adverse events: breast tenderness	Placebo	1/30 (3.3%)
		Genistein	3/30 (10%)
		HRT	6/30 (20%)[(a)]
	Uterus		
	Endometrial thickness > 5 mm (n of patients/group) p > 0.05	Placebo	3/30 (10%)
		Genistein	3/30 (10%)
		HRT	2/30 (6.6%)
	Adverse events: vaginal bleeding	Placebo	1/30 (3.3%)
		Genistein	1/30 (3.3%)
		HRT	6/30 (20%)[(a)]
		(a): $p < 0.05$ versus placebo	

Risk of Bias appraisal			**Tier:2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	-	No information on randomisation method
	Was allocation to study groups adequately concealed?	-	described as double blind, no more details given
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Background diet was restricted to low soy intake. It is not clear how other confounders were addressed, but groups were comparable.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Confounders were not included in analysis, but groups were comparable.
Performance	Did deviations from the study protocol impact the results?	+	No deviation given
	Were the research personnel and human subjects blinded to the study group during the study?	-	No information given, but study described as double blind.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	No information about drop outs
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	No information given
	Key question B: Can we be confident in the outcome assessment?	-	
Selective reporting	Were all measured outcomes reported?	-	Only limited reporting for mammographic density.

Murray et al., 2003 – Ref ID: 11323

Study characteristics and population	**Study design**	Double-blind, randomized, placebo controlled trial
	N	39 enrolled; 30 included in the analysis (Low-E2: 7; High-E2: 7; low-E2 + SPI: 8; high-E2 + SPI: 8)
	Location	USA
	Sampling time frame	Not reported

	Menopausal status	Post-menopausal		
	Age at baseline [mean ± SD]	Low-E2: 53.0 ± 3.4		
		High-E2: 53.4 ± 4.1		
		Low-E2 + SPI: 56.3 ± 7.4		
		High-E2 + SPI: 56.6 ± 9.1		
	Age at menopause	Not reported.		
	Time since menopause:	Not reported.		
	Inclusion criteria	Age > 45 years • FSH level > 40 mIU/mL • intact uterus		
	Exclusion criteria	Use of HRT within 3 months of initiating the study • obesity (BMI < 30 kg/m^2) • endometrial hyperplasia • cancer • breast cancer • cerebrovascular accident • myocardial infarction • cardiovascular disease • gallbladder disease • history of thrombosis • active liver disease • undiagnosed genital bleeding • excessive alcohol use • drug abuse • cigarette smoking • allergy to xylocaine		
	Funding source	Partly funded by a grant from Protein Technologies International Inc.		
	Authors conflicts of interest	Not reported		
	Clinical trial registration and/or acronym	Not reported		
Intervention/ exposure	**Intervention**	Low-E2: 0.5 mg/day oestradiol + placebo		
		High-E2: 1 mg/day oestradiol + placebo		
		Low-E2 + SPI: 0.5 mg/day oestradiol + 38 g SPI (120 mg/day isoflavones aglycones)		
		High-E2 + SPI: 1 mg/day oestradiol + 38 g SPI (120 mg/day isoflavones aglycones)		
	Duration			
Statistical analysis	**Statistical analysis**	A sample size of 18 participants in each group was deemed necessary to detect a 10% decrease in ki67 Hscores with α = 0.05 and power of 0.80. The non-parametric Kruskal-Wallis test was used to detect differences among the four groups at a given time point. The Wilcoxon two-sample paired signed rank test was used to detect differences in matched pairs, comparing baseline to the 6-month effect. Level of significance was set at *P* < 0.05. Data analysed with intention to treat.		
Results	**Uterus**			
	Endometrial thickness (mm) [mean ±SD]		Baseline:	6-month:
		Low-E2	3.6 ± 1.6	12.0 ± 7.1[(a)]
		High-E2	3.6 ± 1.6	11.6 ± 3.3[(a)]
		Low-E2 + SPI	3.0 ± 1.0	6.8 ± 4.1[(a)]
		High-E2 + SPI	3.0 ± 1.0	7.1 ± 4.0[(a)]
	Hyperplasia (n)		Baseline:	6-month:
		Low-E2	0	1
		High-E2	0	4
		Low-E2 + SPI	0	4
		High-E2 + SPI	0	4
	Endometrial Ki67 stroma (H Score) [mean ±SD]		Baseline:	6-month:
		Low-E2	0.08 ± 0.08	0.33 ± 0.45
		High-E2	0.13 ± 0.19	0.63 ± 0.23
		Low-E2 + SPI	0.50 ± 0.80	0.74 ± 0.58
		High-E2 + SPI	0.22 ± 0.29	0.51 ± 0.66
	Endometrial Ki67 glands (H Score) [mean ±SD]		Baseline:	6-month:
		Low-E2	0.18 ± 0.18	0.64 ± 0.73
		High-E2	0.56 ± 0.98	1.43 ± 0.90
		Low-E2 + SPI	0.50 ± 0.78	1.25 ± 0.97[(a)]
		High-E2 + SPI	0.22 ± 0.29	0.51 ± 0.66
		(c): Significant increase (P < 0.05) within group over time		
Risk of Bias appraisal			**Tier: 3**	

Bias domain	Question	Score	Judgement
Selection	Was the administered dose adequately randomised?	+	Computerised randomisation
	Was allocation to study groups adequately concealed?	-	Investigators were blinded, but no more information
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	-	No background diet or equol producer status, groups appear to have differences in BMI and FSH, but stated as not significant.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Direct comparison. Groups appear to have differences in BMI and FSH, but stated as not significant.
Performance	Did deviations from the study protocol impact the results?	+	none reported
	Were the research personnel and human subjects blinded to the study group during the study?	-	Described as blinded, but differences could be seen in SPI and E2 tablets
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	-	Attrition of 25% (9/40). One subject with 2-week hiatus (following combustion of SPI) included
Information/detection	Were the outcome assessors blinded to study group or exposure level?	+	Described as masked, no further information available
	Key question B: Can we be confident in the outcome assessment?	-	No information on quality control, validation.
Selective reporting	Were all measured outcomes reported?	+	Protocol not available

Nahas et al., 2007 – Ref ID: 10231

Study characteristics and population	**Study design**	double-blind, randomized, placebo-controlled study
	N	80 allocated to treatment (40 soy isoflavones; 40 placebo); 76 completed and included in the analysis (38 soy isoflavones; 38 placebo)
	Location	Sao Paulo, Brazil
	Sampling time frame	Not reported
	Menopausal status	Post-menopausal women,
	Age at baseline [mean]	55.7 (soy isoflavones: 55.1 ± 6.0; placebo: 56.2 ± 7.7)
	Age at menopause	soy isoflavones: 48.4 ± 3.7; placebo: 47.7 ± 3.5)
	Time since menopause [years]:	soy isoflavones: 6.6 ± 4.8; placebo: 7.1 ± 4.2
	Inclusion criteria	postmenopausal women aged 45 years or older with good overall health, spontaneous amenorrhea for at least 12 months, follicle-stimulating hormone level greater than 40 mIU/ml, and average of five or more vasomotor symptoms per day.
	Exclusion criteria	strict vegetarian • high-fiber or high-soy diet • and history of breast cancer • endometrial carcinoma • cardiovascular disease • thromboembolic disorders • undiagnosed vaginal bleeding • chronic alcoholism • chronic gastrointestinal diseases • HT or phytoestrogens within the preceding 6 months • thyroid dysfunctions
	Funding source	Ativus Farmaceutica, Brazil, and grants by Fundação Lucentis de Apoio a Cultura, Ensino, Pesquisa e Extensão.
	Authors conflicts of interest	Not reported
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Soy isoflavone extract : 100 mg Placebo identical in appearance

	Duration	9 months treatment, 10 months follow up
Statistical analysis	**Statistical analysis**	Normally distributed variables were reported as mean ± standard deviations. Group interaction was assessed by an independent t-test. Differences between baseline and post-treatment values were analyzed by the paired t test. Timing effect was assessed by one-way repeated measures analysis of variance (ANOVA). When differences were detected, a Tukey's post hoc test was performed to determine pair wise differences. For the variables that showed abnormal distribution, the nonparametric Wilcoxon test was used and the results were expressed as median with 25th and 75th percentiles. Exact p values were obtained from the tests employed. Statistical tests were two-tailed and significance was set at 5%
Results	**Uterus**	
	Endometrial thickness median (mm) [25th and 75th percentile]	Baseline
		Placebo: 3.7 (2.6-4.1)
		Isoflavones: 3.0 (2.2-4.1)
		Post-treatment
		Placebo: 2.8
		Isoflavones: 2.4

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	No information on randomisation method, but randomisation was conducted by uninvolved statistician
	Was allocation to study groups adequately concealed?	+	Centralized computerized subject randomization process was conducted using specific software by statistician unaware of the study protocol.
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No information on background diet and equol producer status, but groups were comparable
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Direct comparison, but groups were comparable
Performance	Did deviations from the study protocol impact the results?	+	none reported
	Were the research personnel and human subjects blinded to the study group during the study?	++	Examiners and subjects had no previous knowledge of group assignment. The only unblinded person was statistician responsible.
Attrition/exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	low attrition (2/group)
Information/detection	Were the outcome assessors blinded to study group or exposure level?	+	blinded by statistician
	Key question B: Can we be confident in the outcome assessment?	+	standard method, but no validation
Selective reporting	Were all measured outcomes reported?	+	protocol not available

Nikander et al., 2005 – Ref ID: 1639

Study characteristics and population	**Study design**	Double-blind, randomized, placebo-controlled crossover trial.
	N	62 randomised (isoflavones: 32; placebo: 30); 56 completed (isoflavones: 28; placebo: 28);
	Location	Helsinki, Finland.
	Sampling time frame	September 1, 1999 and October 10, 2000.
	Menopausal status	Post-menopausal

	Age at baseline [mean ± SD (range]	54 ± 6 (35–69)		
	Age at menopause	Not reported		
	Time since menopause [mean ± SD (range]	5.3 ± 5.5 (0.6-27.0)		
	Inclusion criteria	Post-menopausal women (serum FSH levels > 30 IU/L) who had undergone surgery for breast cancer > 6 months before enrolment.		
	Exclusion criteria	Treatment with HT, statins, natural products with presumed oestrogenic activity or drugs possibly affecting climacteric symptomps, or metabolism and absorption of phytoestrogens (e.g. antibiotics during the previous 3 months)		
	Funding source	Grants from the research funds of Jorvi Hospital and Helsinki University Central Hospital, the Research Foundation of Orion Corporation, and the Juho Vainio Foundation		
	Authors conflicts of interest	Not reported.		
	Clinical trial registration and/or acronym	Not reported.		
Intervention/ exposure	**Intervention**	Isoflavones: 114 mg/day (glycitein-rich)		
		Placebo: similar looking tablets		
	Duration	3-month treatment period with		
Statistical analysis	**Statistical analysis**	All the data were first analysed separately for the first and second treatment phase, and because the order of treatment was not a confounding factor, the data were pooled to form a single phytoestrogen and a single placebo group. No carry-over effect was detected and, hence, nonparametric (sign) tests were used to determine any changes (difference between baseline and post-treatment values) in MIs and in endometrial thickness. These tests were also used to compare the effects of isoflavones and placebo treatment. The McNemar test was used to determine changes in Ki-67 and the marginal homogenicity test for changes in estrogenic effects on endometrial histology, and endometrial expression of ER and PR between baseline and post-treatment values. A P value of < 0.05 was considered significant.		
Results	**Uterus**			
	Endometrial thickness (mm) [mean ±SD]		Baseline:	3-month:
		Placebo	2.1 ± 1.1	2.2 ± 1.4
		Isoflavones	2.4 ± 1.5	1.9 ± 0.8
	Ki67 stroma [n, %]		Baseline	3-month
		Placebo		
		Detectable:	0/24	0/24
		Undetectable:	21/24 (87.5%)	23/24 (95.8%)
		Insufficient sample:	3/24 (12.5%)	1/24 (4.2%)
		Isoflavones		
		Detectable:	1/24 (4.2%)	0/24
		Undetectable:	22/24 (91.7%)	23/24 (95.8%)
		Insufficient sample:	1/24 (4.2%)	1/24 (4.2%)
	Ki67 epithelial [n, %]		Baseline	3-month
		Placebo		
		Detectable:	0/24	0/24
		Undetectable:	20/24 (83.3%)	23/24 (95.8%)
		Insufficient sample:	4/24 (16.7%)	1/24 (4.2%)
		Isoflavones		
		Detectable:	1/24 (4.2%)	3[(a)]/24 (12.5%)
		Undetectable:	23/24 (95.8%)	20/24 (83.3%)
		Insufficient sample:	0/24	1/24 (4.2%)
		(d): One of the three women with detectable levels of Ki-67 reported bleeding. No changes were statistically significant.		

	Papanicolau smears (% of parabasal, intermediate and superficial cells)	No changes in the percentage of cells in the parabasal, intermediate and superficial layers during the isoflavones regimen. A trend for an increase in the percentage of parabasal cells was observed during the placebo regimen (see Figure 1 in the original publication).		
	Endometrial histology		Baseline	3-month
		Total atrophy		
		Placebo	13/27	11/27
		Isoflavones	12/27	8/27
		Estrogenic status		
		Placebo	Minimal: 7/27 Modest: 7/27 Proliferation:0/27	Minimal: 8/27 Modest: 7/27 Proliferation: 1/27
		Isoflavones	Minimal: 6/27 Modest: 5/27 Proliferation: 4/27	Minimal: 12/27 Modest: 6/27 Proliferation: 1/27

Risk of Bias appraisal			**Tier: 1 (endometrial thickness)/ Tier: 2 (histopathological changes)**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Subjects were computer-randomised, but no other information given.
	Was allocation to study groups adequately concealed?	-	Study was described as double blind
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Study followed a cross-over design - tests for period effect were negative.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	Crossover study, so subjects acted as own control
Performance	Did deviations from the study protocol impact the results?	+	Authors did not report deviation
	Were the research personnel and human subjects blinded to the study group during the study?	-	Placebo was not identical but only similar - it is very likely that subjects and researchers could break blinding.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Small attrition
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	No information given on blinding
	Key question B: Can we be confident in the outcome assessment?	+	Endometrial thickness: Only brief information given, no data on reliability
		-	Histopathological changes: Only brief information given, no data on reliability etc
Selective reporting	Were all measured outcomes reported?	+	All data given - no protocol available.

Penotti et al., 2003 – Ref ID: 3110

Study characteristics and population	**Study design**	Double-blind, randomized, placebo-controlled trial
	N	62 allocated to treatment (soy isoflavones: 34; placebo, 28);
	Location	Italy
	Sampling time frame	Not reported
	Menopausal status	Postmenopausal
	Age at baseline [mean ± SD (range)]	Placebo: 52.5 ± 2.3 (49–57) Isoflavones: 52.5 ± 2.5 (49–58)
	Age at menopause	-

	Time since menopause: [mean ± SD (range)]	Placebo: 2.35 ± 1.5 (0.5–5.8) Isoflavones: 2.4 ± 1.2 (0.5–4.3)
	Inclusion criteria	Age between 45-60 years, postmenopausal for at least 6 months, FSH and 17β-E2 levels within postmenopausal range (not given): LDL cholesterol levels below 160 mg/dL. Subjects had to be experiencing ≥ seven hot flushes/day; computerized bone mineralometry T score of more than -2.5 at the level of the lumbar spine.
	Exclusion criteria	Suffering from any major disease, such as hypertension, heart disease, diabetes, or renal or peripheral vascular diseases
	Funding source	Not reported
	Authors conflicts of interest	Not reported
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Isoflavones: 72 mg/day Placebo: tablets identical in appearance and packaging
	Duration	6 months
Statistical analysis	**Statistical analysis**	The study had an 80% power to detect a 35% difference between the two groups. The between-group differences were measured by means of one-way analysis of variance (ANOVA) or Student's *t* test, as appropriate. Fisher's least significant difference test was used post hoc to determine significant differences. A *P* value of <0.05 was considered statistically significant.

Results

Uterus

Endometrial thickness mean (mm) (±SD), (min-max)		Baseline	3-month	6-month
	Placebo	3.2 ± 1.8 (2–7.5)	3.6 ± 2.4 (1.8–9.8)	3.4 ± 1.7 (1.4–6.8)
		N=30	N=24	N=21
	Isoflavones	2.6 ± 1.8 (1–9.5)	2.6 ± 1.2 (1–5.6)	2.2 ± 0.9 (1.3–4.3)
		N=27	N=20	N=17

Risk of Bias appraisal — **Tier: 1**

Bias domain	Question	Score	Judgement
Selection	Was the administered dose adequately randomised?	+	Randomization list balanced in blocks of 10t
	Was allocation to study groups adequately concealed?	+	Described as blinded, intervention and placebo were identical, but no more details
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No information on background diet, no information on how anthropometric data was assessed, but apparently no sig. difference
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Confounding factors were not included in analysis, but groups were similar
Performance	Did deviations from the study protocol impact the results?	+	No deviation described
	Were the research personnel and human subjects blinded to the study group during the study?	-	No information given about method, but study described as blinded
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	-	High percentage of patients excluded from analysis
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	No information given
	Key question B: Can we be confident in the outcome assessment?	+	Appears to be standard method, but no information on QC/validation
Selective reporting	Were all measured outcomes reported?	++	All data reported

Persky et al., 2002

Study characteristics and population	**Study design**	D Double-blind, randomized, placebo-controlled trial
	N	81 enrolled (N soy protein; N placebo); 73 included in the analysis (24 ISP56; 22 ISP90: 25 control)
	Location	Illinois, USA
	Sampling time frame	cohort 1: summer and fall of 1994 (n = 24), cohort 2: spring and summer of 1995 (n = 25),
	Menopausal status	Postmenopausal
	Age at baseline (years)	ISP56: 59.3 ISP90: 61.9 Control: 61.0
	Age at menopause	Not reported
	Time since menopause:	Not reported
	Inclusion criteria	≥1 year since the LMP • total plasma cholesterol concentration of 6.2–7.8 mmol/L.
	Exclusion criteria	Concomitant HRT • concomitant use of other medication known to lower cholesterol • history of diabetes mellitus or thyroid disease • chronic illness that might affect lipid measurements or limit their ability to participate in the study • allergy to soybean protein.
	Funding source	Supported by the Illinois Soybean Program and the National Cancer Institute (RO3CA64459-01A1), NIH Instrument Grant (S10RR06487) and the University of Alabama at Birmingham (UAB). Soy protein products provided by Protein Technologies International, St Louis
	Authors conflicts of interest	Not reported
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	ISP56: Moderate isoflavone (40 g test protein providing 56 mg isoflavone aglycones/day)
		ISP90: High isoflavone (40 g test protein providing 90 mg isoflavone aglycones/day)
		Control: 40 g casein from non-fat dry milk
	Duration	6 months
Statistical analysis	**Statistical analysis**	Geometric means are presented for the values of all TSH which were log-transformed before analyses to meet the normal distribution assumption of the statistical test. Arithmetic means for the other parameters investigated. The effects of diet on hormones were evaluated for the ISP56 diet (ISP56 compared with control) and for the ISP90 diet (ISP90 compared with control) by random-effects regression models after adjustment for clustering of hormone measures within participants at 3 and 6 mo, for baseline hormone value, and for time of sample (3 mo compared with 6 mo). $P \leq 0.05$ was considered statistically significant.

Results

Thyroid

T_3 (nmol/L) mean (95% CI)		Baseline	3 months	6 months
	Control (n=25)	1.72 (1.64, 1.80)	1.65 (1.55, 1.75)	1.66 (1.55, 1.76)
	ISP56 (n=24)	1.83 (1.70, 1.96)	1.79 (1.65, 1.92)	1.79 (1.66, 1.92)
	ISP90 (n=22)	1.75 (1.66, 1.85)	1.66 (1.56, 1.76)	1.80 (1.67, 1.93)[(a)]
	(a): Significantly different from control, P=0.04			
T_4 (nmol/L) mean (95% CI)	Control (n=26)	93.3 (87.5, 99.0)	86.4 (79.3, 93.5)	89.4 (82.3, 96.5)
	ISP56 (n=24)	102.1 (94.7, 109.5)	100.9 (93.0, 108.9)[(b)]	104.3 (97.2, 111.5)[(b)]
	ISP90 (n=22)	97.2 (88.9, 105.5)	92.9 (84.3, 101.5)	98.4 (90.9, 105.9)
	(b): Averages of the differences at 3 or 6 months are significantly different from those of the control, P=0.02			
FTI (%) mean (95% CI)		Baseline	3 months	6 months
	Control (n=26)	2.29 (2.14, 2.44)	2.13 (1.95, 2.31)	2.24 (2.06, 2.42)
	ISP56 (n=24)	2.44 (2.28, 2.60)	2.40 (2.24, 2.57)[(c)]	2.52 (2.36, 2.67)[(c)]
	ISP90 (n=22)	2.36 (2.19, 2.53)	2.27 (2.07, 2.46)	2.42 (2.25, 2.59)
	(c): Averages of the differences at 3 or 6 months are significantly different from those of the control, P=0.03			
TSH (mU/mL) Mean (95% CI)		Baseline	3 months	6 months
	Control (n=24)	2.25 (1.82, 2.79)	2.23 (1.87, 2.65)	2.00 (1.65, 2.43)
	ISP56 (n=24)	2.22 (1.94, 2.54)	2.13 (1.88, 2.41)	2.05 (1.83, 2.29)
	ISP90 (n=22)	2.04 (1.68, 2.49)	2.39 (1.89, 3.03)[(d)]	2.22 (1.79, 2.75)[(d)]
	(d): Averages of the differences at 3 or 6 months are significantly different from those of the control, P=0.01			

Risk of Bias appraisal — **Tier: 1**

Bias domain	Question	Score	Judgement
Selection	Was the administered dose adequately randomised?	+	No information on randomisation method
	Was allocation to study groups adequately concealed?	+	It is not entirely clear how blinding was performed. Participants were supplied with foods and it appears as if blinding was done correctly
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Dietary background was standardised by giving dietary advice to subjects (all participants followed a basal diet for ≥14 days before baseline and throughout study). No significant difference at baseline
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Random effect models were used
Performance	Did deviations from the study protocol impact the results?	+	No deviations reported
	Were the research personnel and human subjects blinded to the study group during the study?	+	Blinded, but no further details
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Attrition is explained and in acceptable range

Bias domain	Question	Score	Judgement
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Analyses were conducted blinded
	Key question B: Can we be confident in the outcome assessment?	++	Detailed description and appropriate QC procedure
Selective reporting	Were all measured outcomes reported?	+	All measures reported

Pop et al., 2008 – Ref ID: 3288

Study characteristics and population	**Study design**	Double-blind, randomized, placebo-controlled trial
	N	36 enrolled; 30 completed the study and were included in the analysis (18 soy isoflavones; 12 placebo)
	Location	North Carolina, USA
	Sampling time frame	Not reported
	Menopausal status	Postmenopausal
	Age at baseline [mean ± SD]	Isoflavones: 56.78 ± 1.25 Placebo: 53.50 ± 1.06
	Age at menopause	Not reported
	Time since menopause:	Not reported
	Inclusion criteria	Postmenopausal status defined as LMP > 12 months before enrolment and FSH > 30 mIU/mL (later amended to 27 mIU/mL as the laboratory method used for the analysis changed) • BMI < 35 kg/m^2 • Healthy status verified by by medical history, physical examination by a licensed medical doctor, screening laboratory tests, chest x-ray and electrocardiogram and evidence of negative mammogram and Pap smear reports dated within one year from the enrolment.
	Exclusion criteria	Women taking hormone/estrogen therapy or SERMS within three months of enrolment • high risk of breast cancer (five year risk of 1.9% or higher) as assessed by NCI's Breast Cancer Risk Assessment Tool (http://bcra.nci.nih.gov) • Use of oral antibiotics within the three months prior to enrolment • use of supplements containing isoflavones within one month prior to enrolment • current tobacco use, or routine alcohol ingestion > 2 drinks/day.
	Funding source	Work funded by the NCI (NCI-N01-CN-75035) and by grants from the NIH to the UNC Clinical Nutrition Research Unit (DK56350), the UNC General Clinical Research Center (RR00046) and the Center for Environmental Health and Susceptibility (ES10126).
	Authors conflicts of interest	One of the author served on advisory boards for Dupont, Solae and Hershey Foods and received grant support from Mead Johnson and from the Egg Nutrition Center. Solae is a producer of soybean derivatives, and makes soy isoflavones. A subsidiary of Dupont supplied the tested isoflavone mixture to the National Cancer Institute who then provided it to the authors. There were no other potential conflicts with the work described
	Clinical trial registration and/or acronym	NCT00099008
Intervention/ exposure	**Intervention**	Isoflavones (PTI G-2535 Unconjugated Isoflavones–70) providing 900 mg/day
		Placebo capsules identical in size and colour, and containing excipients from the active formulation.
	Duration	84 days (treatment) – 112 days (follow up)
Statistical analysis	**Statistical analysis**	Descriptive and inferential statistical analyses were performed in order to look for trends that would point to differences in estrogenic effects, clinical laboratory tests, blood pressure, genotoxicity and apoptosis. Non-stratified (Active versus Placebo) and stratified (active equol-producers versus active non-equol producers) data were studied. Descriptive statistics (n, mean, standard error) were

		calculated for the measurements collected at day 1 and day 84, for the control and treatment groups and for the treated equol producer and non-equol producer groups. A permutation test was used to detect any differences in the mean change from day 1 to day 84 in the continuous response variables between groups (treated versus placebo and treated producers versus treated non-producers) and for the compared mean between groups at day 1 and day 84. A Fisher's exact test was used to detect any differences in the proportion of subjects that experienced side effects between the two groups. A two-sided significance level of 0.05 was used for all statistical tests.
Results	**Thyroid**	
	T4, TSH, T3 uptake, and FTI measured on days 1, 28, 84 and 112.	No significant change in the mean difference at day 84 from day 1, when comparing placebo with active groups.

Risk of Bias appraisal			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	-	There is insufficient information on randomisation method, however, subjects where stratified by equol producer status
	Was allocation to study groups adequately concealed?	+	No information given, but study is described as double blind
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Study assessed equol producer status and other parameters, subjects where randomised according to producer status. However, non-equal distribution of equol producers (4/18 in active, 2/12 in intervention group)
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Adjusted for producer status, however not clear whether other confounders were included. Groups were different in some aspects (eg age and BMI) and ethnic composition..
Performance	Did deviations from the study protocol impact the results?	+	No information given
	Were the research personnel and human subjects blinded to the study group during the study?	-	Active and placebo were identical, study described as double blind
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	loss of 6 subjects - not clear in which groups
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	No information on blinding given
	Key question B: Can we be confident in the outcome assessment?	+	Standard method
Selective reporting	Were all measured outcomes reported?	-	

Powles et al., 2008 – Ref ID: 16435

Study characteristics and population	**Study design**	randomized, double-blind trial
	N	401 allocated to treatment (199 RCE; 202 placebo); pre-menopausal women included in 1st/2nd/3rd year analysis: 260/243/222 (RCE: 133/123/111; placebo: 127/120/111) post-menopausal women included in 1st/2nd/3rd year analysis: 47/44/11 (RCE: 22/22/8; placebo: 25/22/11)
	Location	Royal Marsden Hospital (RMH) and the University Hospital of South Manchester (UHSM), UK.
	Sampling time frame	Not reported

	Menopausal status	Premenopausal: LMP within three months of randomization; or LMP >3 months before randomization, but FSH < 35 mLU/mL, LH ≤ 40 mLU/mL or both; or hysterectomy and FSH < 35 mLU/mL or any period after randomization. Postmenopausal: LMP > 1 year before randomization; LMP >1 year before randomization and FSH < 45 mLU/mL, LH > 40 mLU/mL or both; post hysterectomy and bi-lateral oophorectomy or hysterectomy and FSH > 35 mLU/mL, LH >40 mLU/mL or both. Peri-menopausal: participants who did not fit into either of the categories above.
	Age at baseline [median (range)]	45 (36-69)
	Age at menopause	Not reported
	Time since menopause:	Not reported
	Inclusion criteria	Healthy women aged between 35 and 70 years inclusive of at least one first-degree relative with breast cancer.
	Exclusion criteria	pregnant or lactating women • women taking oral contraceptives or HRT • women with a previous history of non-invasive or invasive breast cancer or other malignancy, except basal cell carcinoma or cervical cancer *in situ* • women with significant vasomotor symptoms because of the possible need for HRT.
	Funding source	funding support from Breakthrough Breast Cancer
	Authors conflicts of interest	None declared
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Red clover isoflavones: 40 mg/day Placebo: tablets of identical appearance
	Duration	3 years
Statistical analysis	**Statistical analysis**	Changes from baseline were assessed using the Wilcoxon's test and differences between treatment arms by the Mann-Whitney test.
Results	**Mammary gland**	

Breast density (% change, 95%CI)		Baseline to 1st year	Baseline to 2nd year	Baseline to 3rd year
Pre-menopausal:	Placebo	-0.82 (-1.85, +0.21%)	-2.02% (-3.24, -0.80%)	-6.60% (-9.04, -4.16%)
	RCE	-0.63% (-1.91, +0.66%)	-2.01 (-3.56, -0.47%)	-3.03% (-5.53, -0.54%)
	P	0.6	1.0	0.2
Post-menopausal:	Placebo	+0.6% (-3.1, 4.2%)	-3.9% (-8.0, 0.3%)	-8.0% (-15.7, -0.2%)
	RCE	-1.5% (-4.0, 1.0%)	-5.8% (-9.1, -2.6%)	-6.9% (-11.6, -2.1%)
	P	0.8	0.9	0.7

	Uterus	
	Endometrial thickness (change, mm)	Only postmenopausal women (n=36) underwent annual transvaginal ultrasound. No significant differences between RCE and placebo (see Figure 2 of the original publication for mean and 95% CI).

Risk of Bias appraisal			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	++	Randomisation clearly described and well conducted.
	Was allocation to study groups adequately concealed?	++	Local pharmacies held the randomization lists

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Not all confounders addressed, but groups are similar. No equol producer status assessment.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Direct comparison, but groups are comparable
Performance	Did deviations from the study protocol impact the results?	+	None reported
	Were the research personnel and human subjects blinded to the study group during the study?	-	There is not much information about blinding and whether placebo and intervention were identical
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	low attrition
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	no information given, but study described as double blind
	Key question B: Can we be confident in the outcome assessment?	-	no information given on validation
Selective reporting	Were all measured outcomes reported?	+	Protocol not available

Quaas et al., 2013 – 16436

Study characteristics and population	**Study design**	Double-blind, randomized, placebo-controlled trial
	N	350 allocated to treatment (175 soy protein; 175 placebo); 224 included in the analysis (121 soy protein; 103 placebo)
	Location	California, USA
	Sampling time frame	April 2004 to March 2009
	Menopausal status	Postmenopausal
	Age at baseline [mean (SD)]	Placebo: 60.1 (6.6) ISP: 60.9 (7.0)
	Age at menopause	Not reported
	Time since menopause (years) [mean (SD)]	Placebo: 9.8 (6.8) ISP: 11.0 (8.0)
	Inclusion criteria	Absence of vaginal bleeding for at least 1 year • serum estradiol level lower than 20 pg/mL.
	Exclusion criteria	Clinical signs, symptoms, or personal history of cardiovascular disease • diabetes mellitus or fasting serum glucose level higher than 126 mg/dL • fasting triglycerides level higher than 500 mg/dL • systolic blood pressure of 160 mm Hg or higher and/or diastolic blood pressure of 110 mm Hg or higher • untreated thyroid disease; serum creatinine level higher than 2 mg/dL • life-threatening illness with a prognosis of less than 5 years • alcohol intake of more than five drinks per day or substance abuse • use of postmenopausal HT • soy, nut, or related food allergies.
	Funding source	Study was supported by National Institutes of Health grant U01AT-001653 from the National Center for Complementary and Alternative Medicine, the Office of Dietary Supplements, and the Office of Research on Women's Health. Solae LLC (St Louis, MO) provided the study products for free.

	Authors conflicts of interest	None reported.	
	Clinical trial registration and/or acronym	NCT00118846 (ClinicalTrials.gov) WISH (Women's Isoflavone Soy Health) trial	
Intervention/ exposure	**Intervention**	Placebo: milk protein	
		ISP: 25 g of soy protein providing 154 mg/day of total isoflavone conjugates plus aglycones (91 mg/day as aglycones).	
	Duration	3 years	
Statistical analysis	**Statistical analysis**	All were intent-to-treat analyses, wherein participants were analyzed according to their randomized intervention. Treatment group comparisons on demographic and other baseline characteristics used χ^2 tests for categorical variables and *t* tests for continuous variables. For each participant, the change in the Endometrial echocomplex (EEC) from baseline was computed at each EEC follow-up visit. Baseline EEC and baseline and on-trial blood levels of genistein, daidzein, and glycitein were compared between treatment groups using Wilcoxon rank sum tests. Because of multiple EEC measurements within participants during the trial, treatment groups were compared on the mean EEC change from baseline using generalized estimating equations, specifying an identity link and an exchangeable correlation structure among repeated measurements within each participant. A covariate specifying the randomization stratification factor of baseline CIMT was included in the generalized estimating equation model. Treatment group comparisons on the incidences of endometrial sampling, endometrial hyperplasia, and endometrial cancer used Fisher's exact test. Statistical testing was conducted at a two-sided 0.05 significance level	
Results	**Uterus**		
	Endometrial echocomplex (mm): change from baseline [mean (SE)] P = 0.88	Placebo (n=103)	-0.95 (0.26)
		ISP (n=121)	-0.98 (0.20)
	Endometrial biopsy	Placebo (n=7/103; 6.8%)	Benign endometrial pathology, any type: 6/7 (85.7%)
			Benign, not otherwise specified:4/7 (57.1%)
			Atrophic: 2/7 (28.6%)
			Proliferative: 0/7 (0%)
			Endometrial hyperplasia/endometrial cancer: 1/7 (14.3)
		ISP (n=9/121; 7.4%)	Benign endometrial pathology, any type: 9/9 (100%)
			Benign, not otherwise specified:5/9 (55.6%)
			Atrophic: 3/9 (33.3%)
			Proliferative: 1/9 (11.1%)
			Endometrial hyperplasia/endometrial cancer: 0/9 (0)

Risk of Bias appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Well randomised, although stratified for different endpoint (CIMT)
	Was allocation to study groups adequately concealed?	+	Researchers were properly blinded
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	The study is designed for atherosclerosis and therefore not stratified for this endpoint. However, groups are comparable.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Direct comparison, but groups are comparable

Bias domain	Question	Score	Judgement
Performance	Did deviations from the study protocol impact the results?	+	None reported
	Were the research personnel and human subjects blinded to the study group during the study?	+	Products were indistinguishable, blinded
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	None reported
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Study researchers were blinded
	Key question B: Can we be confident in the outcome assessment?	+	Detailed description, but no quality control/validation data
Selective reporting	Were all measured outcomes reported?	+	No protocol available

Ryan-Borchers et al., 2008 – Ref ID: 3185

Study characteristics and population	**Study design**	Nested double blind, placebo-controlled study.
	N	77 included in the analysis (27 control cow milk; 26 soymilk; 24 isoflavone supplement);
	Location	Idaho and Washington states, USA
	Sampling time frame	Not reported
	Menopausal status	Postmenopausal women between 50 and 65 years of age
	Age at baseline [mean ± SEM]	Control: 55.7 ± 0.8 Soymilk: 55.8 ± 0.9 Supplement: 54.8 ± 0.7
	Age at menopause	Not reported
	Time since menopause (years since LMP) [mean ± SEM]	Control: 10.1 ± 1.7 Soymilk: 7.7 ± 1.3 Supplement: 6.0± 1.0
	Inclusion criteria	Women free of major health conditions who had not menstruated for more than 1 year.
	Exclusion criteria	Presence of legume allergies • history of smoking • kidney stones • thyroid medication use • antibiotic therapy within the past 6 months • use of HRT
	Funding source	Funding for this study was provided, in part, by a grant from the Washington State Attorney General Office.
	Authors conflicts of interest	None reported
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Control: 706 mL cow milk/day and placebo supplement
		Soy milk: 706 mL soymilk/day and placebo supplement (providing 71.6 ± 3.1 mg isoflavones/day)
		Supplement: 706 mL cow milk and placebo supplement (providing 70 mg isoflavones/day)
	Duration	16-week
Statistical analysis	**Statistical analysis**	Part of a larger research project designed to primarily investigate the effects of soy isoflavones on cognitive function. Sample sizes were determined on the basis of expected changes in cognition, rather than thyroid function. Data were analyzed by ANOVA using a general linear model. Protected LSD was used for multiple mean comparisons. The statistical model included block, subject, treatment (control, soymilk, or supplement), period (week 0 and week 16), and the interaction of treatment by period. Treatment block was not found to be significant and, therefore, removed from the model. Statistical significance was set at P < 05.

Results	Thyroid			
	TSH mU/L [mean ± SEM]		Baseline	16 weeks
		Control (n=27)	2.3 ± 0.3	2.3 ± 0.4
		Soymilk (n=26)	2.9 ± 0.3	3.0 ± 0.3
		Supplement (n=24)	2.5 ± 0.3	2.5 ± 0.3

Risk of Bias appraisal			Tier: 2
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Study is described as blinded.
	Was allocation to study groups adequately concealed?	+	Study is described as blinded.
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	-	Study design reduced exposure to background diet; considerable differences btw groups
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Study design reduced exposure to background diet (4-week adjustment diet to minimise intake of foods containing isoflavones) however considerable differences btw groups
Performance	Did deviations from the study protocol impact the results?	+	Not reported
	Were the research personnel and human subjects blinded to the study group during the study?	-	This is not clear, however, soy and cow's milk are different in appearance and taste and it is unlikely that blinding was successful.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	No information given
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	No information given - but laboratory assay.
	Key question B: Can we be confident in the outcome assessment?	+	No information on QC and validation data.
Selective reporting	Were all measured outcomes reported?	+	All information given

Sammartino et al., 2003 – Ref ID: 15955

Study characteristics and population		
	Study design	Randomised, open clinical trial
	N	70 allocated to treatment (genistein, n=35, placebo, n=35); 63 completed study (genistein, n=32, placebo, n=31);
	Location	Single site, Italy
	Sampling time frame	Not reported
	Menopausal status	Post-menopausal
	Age at baseline [mean ± SD]	Genistein: 51.9 ± 1.85 Placebo: 51.6 ± 1.65
	Age at menopause	Not reported
	Time since menopause, months: [mean ± SD]	Genistein: 17.6 ± 3.1 Placebo: 17.0 ± 3.4
	Inclusion criteria	Menopausal status confirmed by FSH > 40 IU/L, 17β-oestradiol < 20 pg/ml) • at least seven moderate-to-severe hot flushes (including night sweats) in 24 hours during the 2 weeks of the pre-study period
	Exclusion criteria	Neoplastic, metabolic and infectious diseases • concomitant use of any drugs • BMI > 30 kg/m^2 • past or concomitant use of HRT or any other drug used for the treatment of climacteric symptoms • endometrial thickness > 5 mm or presence of endometrial abnormalities.
	Funding source	Not reported.

	Authors conflicts of interest	Not reported.
	Clinical trial registration and/or acronym	Not reported.
Intervention/ exposure	**Intervention**	Genistein: 36 mg/day Control: calcium supplements
	Duration	12 cycles of 28 days (approx. 1 year)
Statistical analysis	**Statistical analysis**	Difference between groups at baseline evaluated by Student's t test for unpaired data. A Shapiro-Wilk W test was performed to evaluate distribution of data for endometrial thickness. As data showed a non-normal distribution, differences between groups and within each group were evaluated by the Mann-Whitney U test. Statistical significance was set at $p < 0.05$.

Results

Uterus				
Endometrial thickness mean (mm) (±SD) (see also Figure 1 in the original publication for more details)		Baseline	6-cycles	12-cycles
	Control	3.1 (2.3-4.2)	3.0 (2.1-4.0)	2.9 (2.0-7.0)
	Genistein	3.2 (2.3-4.5)	3.1 (2.4-4.1)	2.9 (2.1-6.8)

Risk of Bias appraisal:		**Tier: 3**	
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	Computer-generated randomisation list
	Was allocation to study groups adequately concealed?	--	Open study
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	This was not taken into account. No information given.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Participants were comparable in BMI, Age and time since menopause. Background diet was not recorded and there was no information whether soy-consumption was assessed.
Performance	Did deviations from the study protocol impact the results?	+	No information given, but only 5 subjects dropped out due to lack of compliance, suggesting no deviation.
	Were the research personnel and human subjects blinded to the study group during the study?	--	Open study.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Only 3/4 (Intervention vs Control) participants dropped out.
Information/detection	Were the outcome assessors blinded to study group or exposure level?	++	Open study
	Key question B: Can we be confident in the outcome assessment?	-	Method is well described, but no information on reproducibility/reliability.
Selective reporting	Were all measured outcomes reported?	+	Data are given as mean and range.

Unfer et al., 2004 – Ref ID: 3106

Study characteristics and population	**Study Design**	Randomised, double-blind, placebo-controlled trial
	N	N= 376 allocated to treatment (soy isoflavones, N=179; placebo, N=197); N included in the analysis (N soy protein; N placebo)
	Location	Italy
	Sampling time frame	November 1996 – December 2002
	Menopausal status	Post-menopausal

	Age at baseline [mean ± SD]	Placebo: 50 ± 3.9; Isoflavones: 49.3 ± 4.3
	Age at menopause [mean ± SD]	Placebo: 49.8 ± 6.3 Isoflavones: 50.2 ± 6.5
	Time since menopause (years): [mean ± SD]	Placebo: 5.8 ± 4.5 Isoflavones: 5.6 ± 4.3
	Inclusion criteria	Intact uterus, absence of menses for at least 12 months, FSH ≥ 30 IU/L, body weight range within 20% of normal range
	Exclusion criteria	Use of medications containing oestrogens, progestins or androgens within 8 weeks of the beginning of the study • presence of endometrial hyperplasia
	Funding source	Not reported
	Authors conflicts of interest	Not reported
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Soy isoflavones: 100 mg/day
	Duration	5 years
Statistical analysis	**Statistical analysis**	Data were expressed as means ± SD and percentages- The between-group differences were measured by means of one-way analysis of variance or Student's *t* test as appropriate. Fisher's exact test was used post hoc to determine significant differences. P < 0.05 was considered statistically significant.
Results	**Uterus**	

		Baseline	30-month	60-month
Endometrial biopsies [n, (%)]	Placebo	N=197	N = 193	N= 165
	Unassessable	49 (24.8%)	48 (24.9 %)	41 (24.8 %)
	Inactive:	73 (37.0 %)	69 (35.7 %)	60 (36.36 %)
	Atrophic:	71 (36.0 %)	76 (39.4 %)	64 (38.8 %)
	Proliferative:	3 (1.5%)	0	0
	Simple hyperplasia	0	0	0
	Complex hyperplasia	0	0	0
	Atypical hyperplasia	0	0	0
	Isoflavones	N=179	N=176	N=154
	Unassessable	48 (26.8 %)	45 (25.5%)	30 (19.5%)
	Inactive:	67 (37.4 %)	71 (40.3 %)	70 (45.4 %)
	Atrophic:	62 (34.6 %)	60 (34.1 %)	43 (27.9 %)
	Proliferative:	2 (1.1 %)	0	5 (3.2 %)
	Simple hyperplasia	0	0	5 (3.2%)
	Complex hyperplasia	0	0	1 (0.6%)
	Atypical hyperplasia	0	0	0

Risk of Bias appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	-	Authors state allocation was random - no more information
	Was allocation to study groups adequately concealed?	-	Soy was given as tablet, no information about placebo (except for identical looking) but described as blinded.

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Background diet was not taken into consideration, but no statistically significant differences between groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Were not taken into consideration
Performance	Did deviations from the study protocol impact the results?	+	Not described
	Were the research personnel and human subjects blinded to the study group during the study?	-	Study was described as blinded - no further information
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Loss of subjects is mentioned but not explained.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	The same pathologist who was blinded to the patient's protocol regimen interpreted biopsies and classified them according to standard criteria.
	Key question B: Can we be confident in the outcome assessment?	+	Assessed according to then valid standards - no information on QC or validation
Selective reporting	Were all measured outcomes reported?	+	All outcomes reported and measured

Upmalis et al., 2000 – Ref ID: 16165

Study characteristics and population	**Study design**	Double-blind, randomized, placebo-controlled trial
	N	N allocated to treatment (N soy protein; N placebo); N included in the analysis (N soy protein; N placebo)
	Location	Multicentre (15 sites), USA
	Sampling time frame	Not reported
	Menopausal status	Post-menopausal
	Age at baseline [mean (SD)]	Placebo: 54.4 (4.0) Isoflavones: 54.8 (4.9)
	Age at menopause	Not reported
	Time since menopause:	Not reported
	Inclusion criteria	Age ≥ 50 years • good overall health • body weight within ± 35% range for BMI • FSH ≥ 40 • E2 ≤ 25 pg/mL • 5 or more vasomotor symptoms per day • no menses for at least 6 months • HRT discontinued for at least 60 days before study entry.
	Exclusion criteria	History of breast cancer, hyperplasia, endometrial carcinoma or cervical neoplasia • positive pregnancy test • undiagnosed abnormal vaginal bleeding • bilateral oophorectomy or hysterectomy • thromboembolic disorders • history of cardiovascular disease • liver disease • history of chronic alcoholism • medication hypersensitivity • allergy to dietary supplements ingredients • uncontrolled addiction or severe depression • acute systemic infection • abnormal laboratory values.
	Funding source	Not reported
	Authors conflicts of interest	Not reported
	Clinical trial registration and/or acronym	Not reported
Intervention/ exposure	**Intervention**	Placebo
		Soy: soy extract providing ~ 50 mg/day of genistin and daidzin
	Duration	12 weeks

Statistical analysis	**Statistical analysis**	Not reported for safety endpoints.		
Results	**Uterus**			
	Endometrial thickness (mm) [mean (SD)]		Baseline	Post-treatment
		Placebo	3.7 (2.7)	3.6
		Soy	3.5 (1.9)	3.6

Risk of Bias appraisal:			**Tier: 2**
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	+	No information on randomisation method, but groups were similar
	Was allocation to study groups adequately concealed?	+	No information given, but described as blinded
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No information on background diet, but subjects given advice on low PE intake. No equol producer status assessment, but groups broadly comparable.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Not included, but groups were comparable
Performance	Did deviations from the study protocol impact the results?	+	none reported - but study amendments are mentioned in methodology
	Were the research personnel and human subjects blinded to the study group during the study?	-	No information given
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Low attrition for safety, but not efficacy
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	no information given
	Key question B: Can we be confident in the outcome assessment?	-	no information given about method
Selective reporting	Were all measured outcomes reported?	+	protocol not available

Verheus et al., 2008 – Ref ID 3127

Study characteristics and population	**Study design**	Double-blind, randomized, placebo-controlled trial
	N	202 allocated to treatment (100 soy protein; 102 placebo); 126 included in the analysis (70 soy protein; 56 placebo)
	Location	Netherlands
	Sampling time frame	March and September 2000
	Menopausal status	Postmenopausal women
	Age at baseline [mean (SD)]:	Isoflavones: 66.3(4.3) Placebo: 65.3(4.0)
	Age at menopause [mean (SD)]:	Isoflavones: 48.3(5.5) Placebo: 49.3(3 .6)
	Time since menopause: [years (SD)]	Isoflavones: 18.1 (7.0) Placebo: 16.1 (5.8)
	Inclusion criteria	Described in other publication (Kok et al., 2004). Healthy post-menopausal women aged 60–75 years enrolled in national screening program for breast cancer
	Exclusion criteria	Liver or renal disease • history of thromboembolism • history or presence of malignancy (except non-melanoma skin cancer) • current users of HRT or used HRT in the past 6 months • lactose intolerance • known allergy for milk protein or soy • endometrium thickness more than 4 mm.
	Funding source	Grant support: Netherlands Organisation for Scientific Research and Netherlands Organisation for Health Research and Development grants 2200.0048 and 014-91-069.

	Authors conflicts of interest	No potential conflicts of interest were disclosed.
	Clinical trial registration and/or acronym	None reported
Intervention/ exposure	**Intervention**	Isoflavones: 99 mg/day isoflavones aglycones Placebo: 36.5 g of identical-looking and identical-testing milk powder
	Duration	12 months
Statistical analysis	**Statistical analysis**	Both measures of mammographic density (dense area and % density) were log10 transformed to normalize their distributions. These transformed values were used in linear regression analyses. For ease of interpretation, geometric means and 95% confidence intervals (95% CI) are presented. The non-dense area was normally distributed. The difference in baseline-to-final visit change in mammographic measures between the soy and the placebo groups was determined using linear regression. The models included change as dependent variable and group allocation and baseline measurement of the breast measure under study as independent variables. The intention-to-treat analyses of the original study consisted of 175 women. Because mammographic data were unavailable for 49 women, the intention-to-treat analyses included 126 women and will therefore be referred to as modified intention-to-treat analyses. Original per-protocol analyses included 153women. Modified per-protocol analyses included 112 women who finished the trial and of whom mammograms were available. To determine the potential effect of the time interval between finishing the trial and the day of second mammography, subgroup analyses were done using the median time period as cut-off point. The same procedure was used for analyses with subgroups of high and low values of mammographic measures at baseline, as women with high baseline values may experience larger decrease. Finally, subjects were stratified according to equol metabolizer status. As not all women in the placebo group participated in a post-trial soy challenge, metabolizer status could be assessed in only 38 of the 56 women in the placebo group of the modified intention-to-treat analyses. Differences in effect between subgroups were tested by adding interaction terms between subgroup and intervention group to the regression models also containing the individual terms. Two-sided P values of <0.05 were considered statistically significant.

Results

Mammary gland

		Baseline	Post-treatment
Mammographic density % density (ITT analysis, n=126)	Placebo:	15.4%	10.8%
	Isoflavones	10.6%	8.1%
	Difference in change (95% CI)	-0.44 (-3.61 to 2.72) *P*=0.78	
% density (modified per-protocol analysis, n=112)	Placebo:	15.1%	10.7%
	Isoflavones	10.3%	7.9%
	Difference in change (95% CI)	0.91 (-4.36 to 2.54) P=0.60	
% density (stratified for equol-producing capacity, n=108)		Baseline	Post-treatment
Equol producers (n=31)	Placebo:	12.2%	9.0%
	Isoflavones	11.8%	8.3%
	Difference in change (95% CI)	-0.00 (-0.09 to 0.09) *P*=0.94	
Equol non-producers (n=77)	Placebo:	15.5%	10.2%
	Isoflavones	10.0%	7.8%
	Difference in change (95% CI)	-0.01 (-0.04 to 0.03) *P*=0.79	

Risk of Bias appraisal			Tier: 1
Bias domain	**Question**	**Score**	**Judgement**
Selection	Was the administered dose adequately randomised?	++	Detailed description (in paper referred to)
	Was allocation to study groups adequately concealed?	++	Blinding performed by group not involved in trial
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Equol levels were measured to determine which women were able to metabolize this compound. Analysis stratified by this variable.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	Statistical models included potential confounders
Performance	Did deviations from the study protocol impact the results?	+	No deviation reported
	Were the research personnel and human subjects blinded to the study group during the study?	++	Placebo and isoflavones intervention were reported to be identical-looking and identical-tasting powders
Attrition/exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	--	Subjects with implants and too large breasts had to be excluded - loss of >20%
Information/detection	Were the outcome assessors blinded to study group or exposure level?	+	No information given, but study design suggests blinding.
	Key question B: Can we be confident in the outcome assessment?	++	Well described, including QC and validation procedures
Selective reporting	Were all measured outcomes reported?	+	All reported

Appendix B – Summary of animal studies included in the systematic review

Adaikan et al., 2009 – Ref ID: 14737

Animal model	**Species**	Rabbit	
	Strain (source)	New Zealand White	
	Number of animals	Total: 24 OVX + 6 intact controls	
	Age (weight):	6-month old	
	Diet:	Standard rabbit diet (Glen Forest Stock Feeders, Glen Forest, Australia)	
	Dosing method:	gavage	
	Funding source	Research grant no. R-174-000-075-213 from the National Medical Research Council, Singapore.	
	Authors' conflicts of interest	Nothing to disclose	
Dosing	**Intervention**	SHAM: vehicle (water)	
		OVX control: vehicle (water)	
		E2: 100 mg/kg E2 valerate	
		RCE: 6.68 mg/kg red clover extract (estimated equivalent to 100 µg/kg daidzein)	
		DAI: 100 µg/kg daidzein	
	Start of intervention since OVX	4 weeks	
	Duration	12 weeks	
Statistical analysis	**Statistical analysis**	The results were expressed as median values with range and analysed by one-way analysis of variance and nonparametric and two independent samples tests. The statistical significance in results was based on comparison of data with operated and normal control rabbits (P<.05).	
Results	**Uterus:**		
	Weight (g) Median (min-max)	SHAM	11.03 (8.58-13.50)
		OVX control	2.60 (1.90-6.53)
		E2	14.66[(a)] (9.76-20.88)
		RCE	4.39 (1.03-12.46)
		DAI	6.03 (4.49-9.72)
		a: significantly increased versus OVX control (p < 0.05)	

Risk of Bias Appraisal		**Tier:**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	In general, body weights were not influenced by treatment.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	Animals were maintained in steel cages. No specification of the standard rabbit diet. No information on soy based ingredients
	Were experimental conditions identical across study groups?	++	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	There was no loss of animals during the study.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	The study was not performed blinded.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	

Bias domain	Question	Score	Judgement
Information/ detection	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Alves et al., 2008 – Ref ID: 14764

Animal model	**Species**	Rat	
	Strain (source)	Wistar (supplied by the animal facility from the Experimental Surgery Laboratory of the Health Sciences Center, Federal University of Rio de Janeiro, UFRJ).	
	Number of animals	Total: 32 ; 8/group	
	Age (weight):	3-month (200-300 g)	
	Diet:	commercial animal feed for experimental animals (Guabi Nutrilabor, Mogiana Alimentos S.A. Campinas, São Paulo, Brazil).	
	Dosing method:	gavage	
	Funding source	Not reported	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	SHAM: sham operated (n=7):	
		OVX control: tap water	
		E2: estradiol valerate, 0.029 mg/kg (equivalent to 2 mg/day in adults);	
		RCE: 1.15 mg/kg bw/day of isoflavones (equivalent to 80 mg in adults) from a *T. pratense* extract (9.7% isoflavones)	
		CR: 0.058 mg/kg of deoxyactein (equivalent to 4 mg/day in adults) from a *C. racemosa* extract	
	Start of intervention since OVX	21 days	
	Duration	28 days	
Statistical analysis	**Statistical analysis**	For comparison of the percentage of immunohistochemical markers (ER and Ki67) among the four experimental groups, the Kruskal–Wallis ANOVA nonparametric test was used. To identify the groups that presented statistically significant differences, the Kruskal–Wallis test for multiple comparisons was used. The significance level was set as $p < 0.05$.	
Results	**Uterus:**		
	Ki-67 protein expression in endometrial receptors (%) Mean ± SD; Median (min-max)	OVX control	7.3 ± 0.3; 7.2 (7-7.8)
		E2	15.5 ± 0.3 15.5 (14.9-15.9)
		RCE	4.3 ± 0.2; 4.3 (4 - 4.6)
		CR	3.9 ± 0.2 3.9 (3.6-4.1)
		OVX control different from all other groups ($p < 0.0001$) E2 different from RCE and CR groups CR group different from RCE	
	Histology	OVX control	Atrophic endometrium
		E2	Proliferative endometrium, glandular growth with various mitosis sites.
		RCE	Atrophic endometrium
		CR	Atrophic endometrium
Risk of Bias Appraisal			**Tier: 1**

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	basal diet not specified so soy isoflavone exposure is likely sham control used
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Baeza et al., 2009 – Ref ID: 1251

Animal model	**Species**	Rats
	Strain (source)	Wistar albino from Harlan Iberica (Barcelona, Spain).
	Number of animals	Total: 85
	Age (weight):	12 months (not reported)
	Diet:	Not reported
	Dosing method:	Soy extract in drinking water, r, pre-diluted in ethanol (final ethanol concentration in the bottle was 1%)
	Funding source	Grants from the Spanish Ministry of Science and Technology (MCYT; No. BFU 2005-06777), the IMSERSO of the Spanish Ministry for Social Affaire and Labor (GHESME) and RETICEF (RD06/0013/0002 and RD06/0013/0003) from ISCIII
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	Control (Intact):
		Control OVX
		PO: 300 mg/kg/day of soy extract in drinking water
		Oestrogen (Positive control): 125 µg/week of oestradiol valerianate s.c. injection
		GH: recombinant Growth Hormone 2 mg/kg/day s.c. (not relevant to this risk assessment)
		MEL: melatonin 1 mg/kg/day in drinking water (not relevant to this risk assessment)
	Start of intervention since OVX	9.5 months
	Duration	10 weeks,
Statistical analysis	**Statistical analysis**	Data were expressed as the mean±S.D. of the values. The normality of data distribution was tested by the Kolmogorov–Smirnov test. The data were statistically evaluated by the two-way ANOVA and Student's t-tests. The post hoc comparisons used were the Bonferroni test (when equal variances are assumed) or the Tamhane's T2 test (when equal variances are not assumed). The minimum significance level was $p < 0.05$.

Results	**Uterus: weight**	
	Absolute weight (g); mean (± SD)	This parameter was significantly reduced in control OVX animals compared to the intact ones (0.7 ± 0.2 g for intact animals and 0.25 ± 0.03 g for OVX animals, $p < 0.001$). Moreover, when hormone treatments were administered, only oestradiol was able to restore uterine weight (0.94 ± 0.19 g, $p < 0.05$), in comparison with the weight of the OVX control rats.

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Intact controls were not sham-operated
	Did researchers adjust or control for other exposures that are anticipated to bias results?	--	Housing of the animals was not further specified. No information on cages, diet. Moreover, no details about the analyses of the treatment. How did the authors monitor the treatment with 300 mg soy extract/kg/day. Limited reporting of the study
	Were experimental conditions identical across study groups?	+	Housing condition were not very well described, but there is no reason to assume there were differences among the groups.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	Loss of animals were not reported. Each group consisted out of 8 (controls) of 7 (Soy extract) animals.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	The weight of the uterus was most probably not assessed blinded, but this will not influence the outcome of the study.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Bahr et al., 2009 – Ref ID: 10960

Animal model	**Species**	Rats
	Strain (source)	Sprague-Dawley (Harlan, Indianapolis)
	Number of animals	Total: 56; 5-9/group
	Age (weight):	3-month
	Diet:	Casein diet (or soy protein diet)
	Dosing method:	Dietary administration
	Funding source	National Institutes of Health Grant AG 17521 and National Institute of Environmental Health Science Grant ES07326
	Authors' conflicts of interest	Not reported

Dosing	**Intervention**	SHAM: Intact, control diet
		OVX control
		Low-SPI: soy protein isolate at 100 g/kg diet
		High-SPI: soy protein isolate at 200 g/kg diet
		Low-Ex: ethanolic extract of SPI at 17.2 g/kg diet
		High-EX: ethanolic extract of SPI at 34.4 g/kg diet
		E2: OVX, 17β-oestradiol, silastic implant s.c. delivering physiological concentrations of blood E2
	Start of intervention since OVX	1 week (immediately after for E2)
	Duration	12 weeks
Statistical analysis	**Statistical analysis**	All data analysed using the non-parametric Kruskall-Wallis test, followed by Steel multiple comparison tests.
Results	**Uterus:**	
	Histological findings:	Histological features in the SPI and EX groups were not different from those in the OVX control group, with epithelial cells being low columnar to cuboidal in shape in all rats. Uteri of intact group had normal histology, with cystic dilation of uterine glands and small lesions of stratified glandular epithelium (squamous metaplasia) seen occasionally. The E2 group showed similar features to those of intact rats in the estrous phase except for extensive squamous metaplasia of the glandular epithelium in 8/9 rats.
	PCNA	Positive cells for PCNA and stroma were significantly reduced in number in the OVX and in SPI and EX groups compared with intact control. In the intact group, immunoreaction of PCNA was observed in nuclei of glandular and luminal epithelial cells and stroma cells. For more details see Fig. 4 in the original publication.
	Relative wet weight (mean mg/g body weight)	Animals in the SPI and EX groups were not different from OVX control (< 0.5 mean uterine wet weight/body weight). Uterine weight was significantly increased in the OVX animals + E2 (~ 2.5 mean uterine wet weight/body weight). For more details see Fig. 3 in the original publication.

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Soy-free diet and sham control group
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Blinding was not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Bitto et al., 2009 – Ref ID: 14841

Animal model	**Species**	rat
	Strain (source)	Wistar Kyoto (Charles River, Italy) The same experiment was also conducted in a group of spontaneously hypertensive obese rats (SHROB), a genetically modified strain which was not considered relevant for this risk assessment
	Number of animals	Total, n=40;10/group
	Age (weight):	6-month (250 g)
	Diet:	low soy protein content (less than 8%) laboratory food (Mucedola, Italy)
	Dosing method:	gavage
	Funding source	The work has been supported only by Departmental funding.
	Authors conflicts of interest	Nothing to declare
Dosing	**Intervention**	OVX control: vehicle (5 g carboxymethylcellulose, 5 ml benzyl alcohol, 9 g NaCl, 4 ml Tween 80 in 1000 ml distilled water)
		OVX GEN: genistein, 4.8 mg/kg bw/day
		Intact Control
		Intact GEN
	Start of intervention since OVX	4 weeks
	Duration	4 weeks
Statistical analysis	**Statistical analysis**	Data are expressed as means ± S.D. Comparison between the means of the two groups was performed using two-tail ANOVA followed by Bonferroni's test and considered significant at the P < 0.05 level.
Results	**Uterus:**	
	Weight (g)	No statistically significant difference between OVX control and OVX animals treated with genistein. For more detail see Figure 2 in the original publication.

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	low soy protein-cntg diet used (<8%) no sham control used
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Breitman et al., 2003 – Ref ID: 5800

Animal model	**Species**	Rats	
	Strain (source)	Sprague-Dawley from Charles River Canada (Montreal, Quebec)	
	Number of animals	Total 50 (sham, n=10; OVX , n=40)	
	Age (weight):	90-day (not reported)	
	Diet:	AIN 93G containing 0.2% Ca (Dyets, Inc., Bethlehem, PA) devoid of isoflavones	
	Dosing method:	Dietary administration	
	Funding source	Dairy Farmer's of Canada for graduate student funding and the J.P. Bickell Foundation for project funding.	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	SHAM: isoflavone-free diet	
		OVX control: isoflavone-free diet	
		High-Ca: OVX, 2.5% Calcium	
		IF: OVX, isoflavone extract, 1.6 g/diet	
		IF + High-Ca: OVX, isoflavone extract, 1.6 g/diet + 2.5% Ca	
	Start of intervention since OVX	1 week	
	Duration	8-week	
Statistical analysis	**Statistical analysis**	Kruskal–Wallis one-way ANOVA on ranks followed by Tukey's post hoc test was used to detect differences among groups. Differences were considered statistically significant if P < 0.05.	
Results	**Uterus**		
	Relative weight (g/kg bw) Mean ± SD	SHAM	1.65 ± 0.27[a]
		OVX	0.21 ± 0.05
		High-Ca	0.28 ±0.04
		IF	0.19 ± 0.04
		IF HighCa	0.23 ± 0.09
		(e): different from control (P < 0.05)	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Body weight was measured each week. Age of the rats was similar among all groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	No information about the cages used. An AIN diet was used for all groups.
	Were experimental conditions identical across study groups?	++	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	No information about blinding.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Burdette et al., 2001 – Ref ID: 11684

Animal model	**Species**	Rats	
	Strain (source)	Sprague-Dawley, Harlan (Indianapolis, IN)	
	Number of animals	5-6 per group	
	Age (weight):	7-week (~ 230 g)	
	Diet:	Harlan/Teklad Global 16% protein rodent diet (Indianapolis, IN), without alfalfa or soybean meal	
	Dosing method:	Dietary administration	
	Funding source	Support for this research was provided by National Institutes of Health Grant P50 AT00155, the Office of Dietary Supplements (ODS), the National Institute of General Medicine (NIGMS), the Office for Research on Women's Health (ORWH) and the National Center for Complementary and Alternative Medicine (NCCAM).	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	Group I (n=5): Control (sesame oil and 0.1% CMC)	
		Group II (n=6): 17β-estradiol 50 µg/kg bw/day and 0.1% CMC	
		Group III (n=6): red clover extract 250 mg/kg bw/day and sesame oil	
		Group IV (n=6): red clover extract 500 mg/kg bw/day and sesame oil	
		Group V (n=6): red clover extract 750 mg/kg bw/day and sesame oil	
		Group VI (n=6): red clover extract 250 mg/kg bw/day plus 17β-oestradiol, 50 µg/kg bw/day	
		Group VII (n=6): red clover extract 500 mg/kg bw/day plus 17β-oestradiol, 50 µg/kg bw/day	
		Group VIII (n=6): red clover extract 750 mg/kg bw/day plus 17β-oestradiol, 50 µg/kg bw/day.	
	Start of intervention since OVX	Not reported	
	Duration	21 days	
Statistical analysis	**Statistical analysis**	Uterine and organ weights were analyzed by a multiple comparison analysis using a one-way ANOVA and the follow-up analysis was performed using Tukey's test. For estrogenic analysis, the three doses of red clover were compared with the vehicle and estradiol treatment groups. For the antiestrogenic analysis, the three groups treated with red clover plus estradiol were compared with the vehicle and estradiol treatment groups. Body weights were analyzed as a two-way ANOVA based on different days and different weights. Tukey's test was used as the follow-up test. Data are reported as the mean SD. Difference were considered significant at $P < 0.05$.	
Results	**Mammary gland**		
	Mammatrophic effects (examination of duct branching and alveolar structure)	Glands from rats treated with vehicle only displayed thin branches and little alveolar budding. The mammary glands of 17β-oestradiol-treated rats demonstrated extensive ductal branching and defined buds. Administration of the red clover extract for 21 d at all three tested doses did not stimulate the mammary glands. Rats that were treated with red clover plus 17β-oestradiol were comparable to the 17β-oestradiol treatment group; no obvious attenuation of alveolar budding or ductal branching resulted from the addition of red clover (data not shown).	
	Uterus		
	Uterine weight (g)	OVX control	0.10 ± 0.01
		17β-oestradiol, 50 µg/kg bw/day	0.39 ± 0.05[a]
		Red clover, 250	0.10 ± 0.02[b]
		Red clover, 500	0.11 ± 0.02[b]

		Red clover, 750	0.13 ± 0.00[b]
		Red clover, 750 + 17β-oestradiol, 50 µg/kg bw/day	0.38 ± 0.04[b,c]
		a:significant increase compared with vehicle b:significant difference compared with 17β-oestradiol treatment groups c: The Panel assumes this is a typographical mistake, since the values reported would seem different from controls and not from 17β-oestradiol treatment	

Risk of Bias Appraisal		**Tier: 2**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Body weight was measured during the study. Age of the animals was similar among the groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Feed was low in phytoestrogen. No sham-operated control. OVX control. No information about the cages used.
	Were experimental conditions identical across study groups?	+	Housing conditions among the groups were similar. Adequate vehicle controls, but no sham-operated controls.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	--	Endpoint mammary gland: Insufficient information, but the mammatrophic effects were evaluated by examination of duct branching and alveolar structure, which is a rather subjective analyses and should be evaluated blinded.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	-	
	Key question B: Can we be confident in the outcome assessment?	-	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Carbonel et al., 2011a – Ref ID: 14909

Animal model	**Species**	Rat	
	Strain (source)	Not specified, provided by the Center for Development of Experimental Models of the Federal University of Sa˜o Paulo (UNIFESPEPM).	
	Number of animals	Total: 50; 10/group	
	Age (weight):	3 months of age (180-210 g)	
	Diet:	soy-free diet	
	Dosing method:	gavage	
	Funding source	Fundação de Apoio ã Universidade Federal de São Paulo (FAP/UNIFESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasilia, Brazil	
	Authors' conflicts of interest	None reported	
Dosing	**Intervention**	OVX control: vehicle (propylene glycol)	
		SE-46: OVX, concentrated soy extract, 46 mg/kg bw/day	
		SE-120: OVX, concentrated soy extract, 120 mg/kg bw/day	
		E2: OVX, conjugated oestrogens, 50 µg/kg bw/day	
		SE-46 + E2	
	Start of intervention since OVX	28 days	
	Duration	21 days	
Statistical analysis	**Statistical analysis**	Data were initially submitted to analysis of variance. Whenever a significant difference was detected, the study was complemented with the Tukey-Kramer test for multiple comparisons. The significance level for the null hypothesis was set at 5% ($P \leq 0.05$).	
Results	**Uterus::**		
	PCNA % in vaginal epithelium	OVX control	4.30 ± 2.97[a]
		SE-46:	6.31 ± 3.21[a]
		SE-120	57.66 ±12.46
		E2	59.99 ± 18.67
		SE-46 + E2	63.21 ± 11.21
		a $P < 0.05$ compared with SE-120, E2 and SE-46 + E2	
	Ki-67 (%) in vaginal epithelium	OVX control	3.10 ± 2.52[a]
		SE-46:	4.15 ± 2.18[a]
		SE-120	39.48 ± 15.98
		E2	51.42 ± 12.34
		SE-46 + E2	49.55 ± 16.25
		(f): $P < 0.05$ compared with SE-120, E2 and SE-46 + E2	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age and weight of the animals was comparable among the groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	They used plastic cages, but diet was soy-free.
	Were experimental conditions identical across study groups?	++	Housing conditions were similar for all rats
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	Loss of animals was not reported
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information provided. Most probably not blinded.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	

Bias domain	Question	Score	Judgement
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Carbonel et al., 2011b – Ref ID: 12969

Animal model	**Species**	Rat	
	Strain (source)	Wistar EPM-1	
	Number of animals	Total: 40; 10/group	
	Age (weight):	90 days (250 g)	
	Diet:	Not specified	
	Dosing method:	gavage	
	Funding source	CNPq and CAPES	
	Authors' conflicts of interest	None reported	
Dosing	**Intervention**	OVX control: vehicle (propylene glycol)	
		GES-42: OVX,42 µg/g/day genistein	
		GES-125: OVX, 125 µg/g/day genistein	
		GES-250: OVX, 250 µg/g/day genistein	
	Start of intervention since OVX	28 days	
	Duration	30 days	
Statistical analysis	**Statistical analysis**	The data underwent analysis of variance (ANOVA), which was complemented by the Tukey-Kramer test for multiple comparisons. The level of significance of a null hypothesis was 5% ($p < 0.05$).	
Results	**Uterus::**		
	Histology:	OVX control	Slim and atrophic with few endometrial glands. The endometrium was covered by a simple cubic epithelium and by squamous epithelium in some areas. The lamina propria contained countless cells with round, markedly stained nuclei and very little cytoplasm.
		GEN-42	Histological features of the endometrium and myometrium very similar to those of OVX control.
		GEN-125	Enlarged uteri with thicker layers and covered by simple prismatic epithelium. Lamina propria contained cells with large, clear nuclei and pronounced nucleoli. A large concentration of endometrial glands constituted by large cubic cells was observed. In the endometrial stroma, countless eosinophils were noted.
		GEN-250	Thee uterus was larger than in other groups, with a viscous clear fluid, and covered by simple prismatic epithelium and, in some areas, non-keratinized stratified squamous epithelium, indicating the presence of squamous metaplasia. In most animals (n = 8), prominences were observed on the base of the superficial epithelium in the direction of the lamina propria. Endometrial glands were dilated and constituted by large cuboidal cells, and in some areas by non-keratinized stratified squamous epithelium, indicating squamous metaplasia. In the lamina propria, a large concentration of eosinophils was observed, some infiltrating the superficial or glandular epithelium.

	Morphometric analysis	OVX control	All measured parameters (endometrium area, number of glands/area, area of endometrial glands, eosinophils) did not differ from GEN-42
		GEN-42	All measured parameters did not differ from OVX-control
		GEN-125	All measured parameters significantly increased compared to control and GEN-42.
		GEN-250	Number of glands/area did not differ from GEN-125. All other measured parameters significantly increased compared to control and lower doses.
	Weight (g)	OVX control	2.7 ± 0.5[a]
		GES-42	3.2 ± 0.8[a]
		GES-125:	6.2 ± 0.2[b]
		GES-250	9.5 ± 1.3[c]
		a, b, c: statistical comparison P < 0.05 c>b>a	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	diet not specified so basal level of exposure to soy isoflavones is likely No sham control group
	Were experimental conditions identical across study groups?	+	
Attrition/exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/detection	Were the outcome assessors blinded to study group or exposure level?	+	blinding was not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Carbonel et al., 2015 – Ref ID: 19012

Animal model	**Species**	Rat
	Strain (source)	Not specified
	Number of animals	Total: 60 (10/group)
	Age (weight):	3 months (180-210 g)
	Diet:	Soy-free food (Formula Labina Especial brand for rodents)
	Dosing method:	gavage
	Funding source	FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo)
	Authors' conflicts of interest	None reported

Dosing	**Intervention**	OVX control: vehicle (propylene glycol)	
		GEN-50: genistein, 50 mg/kg bw/day	
		E2: 17β-oestradiol, 10 µg/kg bw/day	
		OVX control-LATE: vehicle (propylene glycol)	
		GEN-50-LATE: genistein, 50 mg/kg bw/day	
		E2-LATE: 17β-oestradiol, 10 µg/kg bw/day	
	Start of intervention since OVX	Immediately or after 30 days (late group)	
	Duration	30 days	
Statistical analysis	**Statistical analysis**	Values were reported as means ± standard deviation. One-way ANOVA was employed followed by the Tukey test. The level of significance was set at 5% ($p < 0.05$).	
Results	**Uterus:**		
	Ki-67 expression (Anti-Ki-67 antigen expression, % of positively marked cells)	Higher percentage of cell proliferation (Ki-67) in groups where the initiation of treatment was delayed, either with oestradiol or genistein (E2-LATE > E2 and GEN-50-LATE > GEN-50)	
	Histological analyis	OVX control	Endometrium covered by simple cubic epithelium and by squamous epithelium in some areas. The lamina propria contained numerous cells with round, markedly stained nuclei and very little cytoplasm.
		GEN-50	Enlarged uteri with thicker layers. The uteri were covered by a simple prismatic epithelium and the lamina propria contained cells with a large, clear nuclei and pronounced nucleoli. A large concentration of endometrial glands constituted by large cubic cells was observed and the endometrial stroma presented many eosinophils.
		E2	Uterus larger than the other groups and covered by a simple prismatic epithelium containing leukocytes infiltration.
		OVX control-LATE	Same as OVX control.
		GEN-50-LATE	Same as GEN-50
		E2-late	Uterus larger than the other groups and covered by a simple prismatic epithelium containing leukocytes infiltration. Some areas demonstrated non-keratinized, stratified, squamous epithelium and the endometrial glands were dilated and consisted of large cuboidal cells, indicating squamous metaplasia. In the lamina propria, a large concentration of eosinophils was observed, some of which infiltrated the superficial or glandular epithelium.
	Morphometric analysis	OVX control	The measured parameters (endometrial area, endometrial thickness, epithelial thickness, number of endometrial glands/area, number of blood vessels) did not differ from other OVX control group and the two GEN groups.
		GEN-50	The measured parameters did not differ from other GEN group and the two OVX control groups.
		E2	The measured parameters were higher than the other groups, but lower than the E2-late groups.
		OVX control-LATE	The measured parameters did not differ from other OVX control group and the two GEN groups.

		GEN-50-LATE	The measured parameters did not differ from other GEN group and the two OVX control groups
		E2-late	The measured parameters were higher than all the other groups.
Risk of Bias Appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Body weights were not recorded in this study
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	plastic cages were used for housing, which may contain BPA. The animals were fed specific soy-free diet.
	Were experimental conditions identical across study groups?	++	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	All assessments were performed by three experienced observers, who were blinded to the data of the animals. After completion of the study, the same observers re-examined the slides to ensure the reproducibility of the semi-quantitative assessment.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Castillo et al., 2006 – Ref ID: 14918

Animal model	**Species**	Rat
	Strain (source)	Wistar rats of were
	Number of animals	Total: 18; 6 animals/group + 6 (Young intact)
	Age (weight):	22 months (2 months intact control group)
	Diet:	Standard laboratory rat diet (A.04; Panlab, Barcelona, Spain)
	Dosing method:	Drinking water
	Funding source	Grants from C.A.M. (8.5/0062/2001) and G.H.E.S.M.E. 2002 project from I.M.S.E.R.S.O. (Ministerio de Trabajo y Asuntos Sociales). Ms. Castillo and Ms. Salazar were supported by grants of the Ministerio de Educacion, Cultura y Deportes.
	Authors' conflicts of interest	Not reported.
Dosing	**Intervention**	OVX control
		IF: commercial soy extract containing 9–11% of isoflavones
		E2: oestradiol valerianate (125 mg/week diluted in sunflower oil, sc).
	Start of intervention since OVX	10 months

	Duration	10 week	
Statistical analysis	**Statistical analysis**	Results are expressed as the mean ± SEM, from n = 6. The results are presented as the mean ± SEM. Mean comparison was done by the Kuskal–Wallis test followed by a Mann Whitney test; a confidence level of 95% (p < 0:05) was considered significant.	
Results	**Uterus:**		
	Relative uterine weight (g/100 g)	Young intact	0.182 ±0.02
		OVX control	0.039 ±0.004[a]
		IF	0.037 ±0.003[a]
		E2	0.251 ±0.03[b]
		a: different (p < 0.001) vs young and E2 b: different (p < 0.001) vs other groups	

Tier of Reliability		**Tier: 2**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	-	Insufficient information about body wts and controls were 2 months old female sham-operated rats, whereas the treated animals were 22 months of age.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	All animals were similarly treated and housed. Diet was standard lab, so not phytoestrogen free. No information about cages.
	Were experimental conditions identical across study groups?	+	Sham-operated controls were 20 months younger than the experimental animals.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of rats reported
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	There insufficient information provided, but study is most probably not performed blinded.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Catania et al., 2002 – Ref ID: 14919

Animal model	**Species**	
	Strain (source)	Sprague-Dawley
	Number of animals	6/group
	Age (weight):	Mature (250-280 g)
	Diet:	Not reported
	Dosing method:	gavage
	Funding source	Not reported
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	SHAM OVX: vehicle
		OVX control: vehicle
		IF: OVX, soy fraction (SOYPH) with isoflavones 5 mg/kg in vehicle (saline)
		E2: OVX, 17β-oestradiol s.c. 20 µg/100 µL cotton seed oil
		E2-vehicle: OVX, s.c. 20 µg/100 µL cotton seed oil

	Start of intervention since OVX	3 weeks
	Duration	4 weeks
Statistical analysis	**Statistical analysis**	One-way analysis of variance (ANOVA) with the Scheffé posthoc test for multiple comparisons. Data expressed as means ± SD. Statistical significance was set at P < 0.05.
Results	**Uterus:**	
	Uterine weight (% SHAM ovx animals)	No statistically significant difference between OVX control and animals treated with genistein (~60% reduction of uterine weight versus SHAM). E2 animals had significantly higher weight (~80% of uterine weight versus SHAM). Values extrapolated from Figure 1 in the original publication.

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	the basal diet was not specified so soy exposure cannot be ruled out sham control was used
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	blinding was not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Chen et al., 2009 – Ref ID: 4676

Animal model	**Species**	Mice
	Strain (source)	BALB/c (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan)
	Number of animals	
	Age (weight):	8-week old (at OVX)
	Diet:	Control: modified corn oil AIN-93 diet (soy free)
	Dosing method:	Dietary administration
	Funding source	grants from the National Science Council (NSC 92-2321-B-002-011; 93-2321-B-002-012).
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	SHAM: control diet
		OVX control: control diet
		E2: 17β-oestradiol supplemented, 2 mg/kg diet
		GEN: genistein, 0.2 g/kg diet
		Yam: 630 g TNG powder/kg diet
	Start of intervention since OVX	3 days

	Duration	12 weeks	
Statistical analysis	**Statistical analysis**	Values are presented as means with standard deviation. One-way ANOVA tests were used to determine the significance of differences among groups. The group-wise comparison was performed by Duncan's multiple range test. Effects were considered significant at P < 0.05.	
Results	**Uterus:**		
	Weight (mg)	SHAM	102 ± 52.6[a]
		OVX control	22.4 ± 15.1
		E2	139.5 ± 38.4[ab]
		GEN	21.5 ± 9.5
		YAM	22.0 ± 21.3
		a: p < 0.05 versus OX control b: p > 0.05 versus SHAM	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Body weight was measured twice weekly and feed intake was monitored every day. The OVX mice did not differ in weight gain with the sham operated group.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	No specifications of the cages used, but the diet was soy-free. All diets contained corn oil instead of soy oil to eliminate any additional dietary isoflavones
	Were experimental conditions identical across study groups?	++	
Attrition/exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	No loss of animals occurred during the study
Information/detection	Were the outcome assessors blinded to study group or exposure level?	++	The study was not performed blinded.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Cho et al, 2012 – Ref ID: 4121

Animal model	**Species**	Mice
	Strain (source)	ICR (from Samtako Korea, Osan, Korea).
	Number of animals	Total: 20, 5 aniamls/group
	Age (weight):	30 weeks
	Diet:	isocaloric cornstarch diet in distilled water
	Dosing method:	Dietary administration
	Funding source	Grant A050376 from the Korean Health 21 Research and Development Project, Ministry of Health and Welfare, Republic of Korea and by the Technology Development Program of the Ministry of Agriculture and Forestry (105054-03-2-HD110), and with support from the "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ008506)" Rural Development Administration, Republic of Korea
	Authors' conflicts of interest	None declared.

Dosing	**Intervention**	SHAM: sham-operated group
		OVX control:
		IPL-200: 200 mg/kg/day IPL, extracts
		IPL-500: 500 mg/kg/day IPL, extracts
	Start of intervention since OVX	Not specified
	Duration	4 weeks
Statistical analysis	**Statistical analysis**	All group comparisons were made using Student'st-test. Results presented as means standard errors of the mean (SEM), unless otherwise noted. $p < 0.05$ was considered statistically significant
Results	**Uterus:**	
	Weight (g)	No statistically significant difference between treated and OVX control (see Figure 2B in the original publication).

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No specifications of the cages used. The diet is most probably soy-free. Treatment was performed by gavage, once per day at 11.00 am.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Most probably soy-free diet. Sham operated controls and OVX controls.
	Were experimental conditions identical across study groups?	++	All animals were maintained of the same isocaloric cornstarch diet in distilled water.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals occurred
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	The study was most probably not performed blinded, but this will not have influenced the outcome of the study.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Dong et al., 2014 – Ref ID: 19076

Animal model	**Species**	Rat
	Strain (source)	Sprague Dawley (Experimental Animal Center of the Honk Kong Chinese University, Hong Kong, SAR)
	Number of animals	Total: 72
	Age (weight):	6-month
	Diet:	Moderate Calcium content (MCD;TD98005, 0.6% Ca and 0.65% P, from Harlan Teklad)
	Dosing method:	gavage
	Funding source	Research grant no. R-174-000-075-213 from the National Medical Research Council, Singapore.
	Authors' conflicts of interest	Nothing to disclose
Dosing	**Intervention**	SHAM: sham operated, vehicle
		OVX control: vehicle
		E2: 17β-oestradiol, injected i.p. at a weekly dose of 200 µg/kg in the MCD diet

		PR: *Puerariae radix* water extract, 300 mg/kg/day	
		WE: *Fructus Ligustri Lucidi* (FLL) water extract, 700 mg/kg/day	
		WE/PR: *Puerariae radix* water extract 300 mg/kg/day and *Fructus Ligustri Lucidi* (FLL) water extract 700 mg/kg/day	
	Start of intervention since OVX	2 weeks	
	Duration	12 weeks	
Statistical analysis	**Statistical analysis**	Data reported as mean (SEM). Analysis of the effects of the different treatments on OVX rats was performed with one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test as posttest, to compare the group means if the overall P < 0.05. Interaction analysis (by two-way ANOVA) was performed within four groups (OVX, PR, WE and WE/PR). The statistical significance in results was set at (P<0.05).	
Results	**Uterus:**		
	Uterine index (mg/kg)	SHAM	0.50 (0.04)[a]
		OVX control	0.12 (0.01)
		E2	0.25 (0.02)[a]
		PR	0.21 (0.02)[b]
		WE	0.13 (0.01)
		WE/PR	0.19 (0.01)
		a: significantly increased versus OVX control (p < 0.001) b: significantly increased versus OVX control (p < 0.05)	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	
	Were experimental conditions identical across study groups?	+	experimental design appears sound
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	no discussion of blinding
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Foth and Cline, 1998 – Ref ID: 15120

Animal model	**Species**	Monkey
	Strain (source)	Cynomolgus macaques (*Macaca fascicularis*)
	Number of animals	15/group
	Age (weight)	Adult, age not further specified
	Diet:	Base diet with casein as protein source for OVX control and E2 and a soy protein isolate for SPI and SPI + E2 groups.
	Dosing method:	Dietary administration

	Funding source	Supported in part by grant DAMD 17–94-J-4201 from the US Army Medical Research Acquisition Activity, Fort Detrick, MD, and by a supplement to grant RR008562–02 from the Office of Research on Women's Health, National Institutes of Health, Bethesda, MD.	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	Control: casein	
		SPI: dose equivalent to 148 mg/day for women on energy basis	
		E2: casein and oestradiol at a dose equivalent to 1 mg/day for women on energy basis	
		SPI + E2: dose equivalent to 148 mg/day and 1 mg/day respectively for women	
	Start of intervention since OVX	Not reported	
	Duration	6 months	
Statistical analysis	**Statistical analysis**	Means were compared by analysis of variance and Dunnett's test for comparisons with controls	
Results	**Mammary gland:**		
	Ki-67 (% of positive cells) in lobules and large ducts	E2 treated animals had a significant increase in Ki-67 labelling. No significant differences between other groups. For more details see Figure 4 in the original publication.	
	Histopathologic findings: mammary epithelial hyperplasia (grade)	OVX control	Grade 0 (none): 4/14 Grade 1 (mild): 7/14 Grade 2 (moderate): 3/14 Grade 3 (marked):0/14
		E2	Grade 0 (none): 0/13 Grade 1 (mild): 2/13 Grade 2 (moderate): 10/13 Grade 3 (marked):1/13
		SPI	Grade 0 (none):9/12 Grade 1 (mild): 2/12 Grade 2 (moderate): 1/12 Grade 3 (marked): 0/12
		E2 + SPI	Grade 0 (none):1/9 Grade 1 (mild): 2/9 Grade 2 (moderate): 2/9 Grade 3 (marked): 4/9
	Uterus:		
	Ki-67 (% of positive cells) in epithelium and stroma	E2 treated animals had a significant increase in Ki-67 labelling in both epithelium and stroma. No significant differences between other groups. For more details see Figure 2 in the original publication.	
	Endometrial thickness and (mm; mean) and endometrial gland area (%)	Both E2 and E2 + SPI treated animals had a significant increase in endometrial thickness and endometrial gland area. For more details see Figure 1 in the original publication.	
	Histopathologic findings: uterine histologic appearance	OVX control	Atrophic: 0/14 Simple glandular hyperplasia: 3/14 Cystic glandular hyperplasia: 0/14 Irregular glandular hyperplasia: 0/14
		E2	Atrophic: 0/13 Simple glandular hyperplasia: 8/13 Cystic glandular hyperplasia: 3/13 Irregular glandular hyperplasia: 2/13
		SPI	Atrophic: 9/12 Simple glandular hyperplasia: 3/12 Cystic glandular hyperplasia: 0/12 Irregular glandular hyperplasia: 0/12

		E2 + SPI	Atrophic: 0/9 Simple glandular hyperplasia: 4/9 Cystic glandular hyperplasia:4/9 Irregular glandular hyperplasia: 1/9
	Histopathologic findings: uterine epithelial hyperplasia (grade)	OVX control	Grade 0 (none): 11/14 Grade 1 (mild): 2/14 Grade 2 (moderate): 1/14 Grade 3 (marked):0/14
		E2	Grade 0 (none): 0/13 Grade 1 (mild): 2/13 Grade 2 (moderate): 10/13 Grade 3 (marked):1/13
		SPI	Grade 0 (none): 9/12 Grade 1 (mild): 2/12 Grade 2 (moderate): 1/12 Grade 3 (marked):0/12
		E2 + SPI	Grade 0 (none): 0/9 Grade 1 (mild): 4/9 Grade 2 (moderate): 4/9 Grade 3 (marked):1/9
	Uterine weight (g)	OVX control	1.81 ± 1.45
		E2	4.66 ± 1.03[a]
		SPI	2.30 ± 1.06
		E2 + SPI	5.09 ± 2.60[a]
		a: significant increase (P < 0.05) versus OVX control	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	The weight of the animals was not recorded during the study. Age of the animals was similar among the groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	The diet was atypical moderately atherogenic North American diet (40% energy from fat) The protein source was casein for controls and estrogen treated animals and for the isoflavones groups, the soy isolate provided the dietary protein requirement. Diets were otherwise equivalent, with the same energy, fat, fiber, methionine and vitamin E contents. The composition of the isoflavone soy isolate is not given.
	Were experimental conditions identical across study groups?	++	Housing conditions were similar among the groups. No sham operated group. Diet was similar among the groups except the addition of estrogen of isoflavones.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	Histopathological assessments of tissue sections were made by a pathologist blinded to the treatment groups.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	

Bias domain	Question	Score	Judgement
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Francisco et al., 2013 – Ref ID:454

Animal model	**Species**	Rat (*Rattus norvegicus albinus*)	
	Strain (source)	Strain not reported. Sourced from Center for Development of Experimental Models (CEDEME) of the Federal University of São Paulo (UNIFESPEPM	
	Number of animals	50 (10/group)	
	Age (weight):	90-day (180-230)	
	Diet:	Soy-free diet	
	Dosing method:	gavage	
	Funding source	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasilia-BR.	
	Authors' conflicts of interest	The Authors' report no conflict of interest	
Dosing	**Intervention**	OVX control: vehicle (propylene glycol)	
		SE-46: OVX, concentrated soy extract, 46 mg/kg bw/day	
		SE-120: OVX, concentrated soy extract, 120 mg/kg bw/day	
		E2: OVX, conjugated oestrogens, 50 µg/kg bw/day	
		SE120+E2: 120 mg/kg SE per day and 50 µg/kg CE per day	
	Start of intervention since OVX	28 days	
	Duration	21 days	
Statistical analysis	**Statistical analysis**	Data were initially submitted to analysis of variance (ANOVA). Whenever a significant difference was detected, the study was complemented with the test for multiple comparisons of Tukey–Kramer. The significance level for the null hypothesis was set at 5% ($p \leq 0.05$).	
Results	**Uterus:**		
	histological analysis	OVX control	The endometrium and myometrium were dense and thin. Glands were small and closed. The luminal epithelium was unorganized, without columnar morphology, and the nuclei randomly positioned in the cells. There was little, if any, interstitial space among the stromal cells.
		SE-46:	Not different from OVX control.
		SE-120	Endometrial glands and myometrium were increased in size. The endometrial glands had large lumina, many of which contained secretory products. Interstitial space appeared between stromal cells. Luminal epithelial cells presented a columnar aspect, with basally located nuclei. The endometrium was better developed than SE-46, with many endometrial glands, with simple cylindrical epithelium bearing a large amount of eosinophils in the endometrium and myometrium.

		E2	Endometrial glands and myometrium were increased in size. The endometrial glands had large lumina, many of which contained secretory products. Interstitial space appeared between stromal cells. Luminal epithelial cells presented a columnar aspect, with basally located nuclei. The endometrium was better developed than SE-46, with many endometrial glands, with simple cylindrical epithelium bearing a large amount of eosinophils in the endometrium and myometrium.
		SE-120+ E2	Endometrial glands and myometrium were increased in size. The endometrial glands had large lumina, many of which contained secretory products. Interstitial space appeared between stromal cells. Luminal epithelial cells presented a columnar aspect, with basally located nuclei. The endometrium was better developed than SE-46, with many endometrial glands, with simple cylindrical epithelium bearing a large amount of eosinophils in the endometrium and myometrium.
	Morphometric analysis	OVX control	All measured parameters (endometrium/ myometrium area, number of endometrial glands/area, eosinophils) did not differ from SE-46
		SE-46	All measured parameters did not differ from SE-46
		SE-120	All measured parameters significantly increased compared to control and SE-46
		E2	All measured parameters significantly increased compared to control and SE-46
		SE-120 + E2	All measured parameters significantly increased compared to control and SE-46
	Uterus: weight	OVX control	2.70±0.51
		SE-46	4.20±0.80
		SE-120	6.20±0.70[a]
		E2	7.70±0.90[a]
		SE-120+ E2	6.20±0.20[a]
		a: significant ($p < 0.05$) increase compared with control and SE-46	

Risk of Bias Appraisal		**Tier: 2**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	-	It was stated in the "Materials and Methods" that the low dose group received 46 mg/kg concentrated soy extract and the high dose 120 mg/kg. The Panel noted that according to the information reported in the publication the doses administered would be 19.6 and 51.1 mg/kg bw/day and not 21.3 and 42.6 mg/kg bw/day.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	No information about the plastic cages. A soy-free diet was used and it was a blinded randomized study.
	Were experimental conditions identical across study groups?	++	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	The study was performed with 10 animals/group. The uterus weight was given for 10 animals/group.

Bias domain	Question	Score	Judgement
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	It was a blinded study
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	+	The only risk of bias in this study is the unclear exposure of the high dose group in comparison with the low dose group
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Gallo et al., 2005 – Ref ID: 15145

Animal model	**Species**	Rats	
	Strain (source)	Sprague-Dawley from Harlan Nossan S.r.l. (Correzzana MI, Italy).	
	Number of animals	Total, 60; 12/group	
	Age (weight):	3-month old (237 g)	
	Diet:	0.4% calcium, 0.3% phosphorus, and 3 UI/g diet of vitamin D3 (Harlan Teklad, Udine, Italy);	
	Dosing method:	Gavage	
	Funding source	Not reported	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	SHAM: control diet	
		OVX control	
		SSE-50: OVX, standardised soy extract: 50 mg/kg bw/day	
		SSE-100: OVX, standardised soy extract: 100 mg/kg bw/day	
		E2: OVX, 17β-oestradiol(0.5 mg/kg/day	
	Start of intervention since OVX	1 day	
	Duration	6 weeks	
Statistical analysis	**Statistical analysis**		
Results	**Uterus:**		
	Histological findings: (n of findings/n animals)	SHAM	Hyperplasia: 2/11 Atrophy: 0/11[a] Stromal oedema: 0/11
		OVX control	Hyperplasia: 0/11 Atrophy: 10/10[b] Stromal oedema: 0/10
		SSE-50	Hyperplasia: 0/11 Atrophy: 10/11[b] Stromal oedema: 8/11 [a,b]
		SSE-100	Hyperplasia: 1/12 Atrophy: 10/12[,b] Stromal oedema: 10/12 [a,b]
		E2	Hyperplasia: 10/12 [a,b] Atrophy: 0/12[a] Stromal oedema: 10/12[a,b]
		a: significantly different from OVX control ($p < 0.001$) b: significantly different from SHAM control ($p < 0.001$)	

	Weight (g)	SHAM	0.51 ± 0.12[a]
		OVX control	0.12 ± 0.04[b]
		SSE-50	0.19 ± 0.15[b]
		SSE-100	0.18 ± 0.10[b]
		E2	0.45 ± 0.07
		a: significantly different from OVX control (p < 0.001) b: significantly different from SHAM control (p < 0.001)	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age and body weights were recorded and similar among the groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Animals received a standard commercial diet, not soy-protein free. No information about cages and housing
	Were experimental conditions identical across study groups?	++	Housing conditions of the animals was similar among the groups.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals was recorded.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information provided.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Gallo et al., 2006 – Ref ID: 15147

Animal model	**Species**	Rats
	Strain (source)	Sprague-Dawley from Harlan Nossan S.r.l. (Correzzana MI, Italy).
	Number of animals	Total, 40; 10/group
	Age (weight):	3-month old (not reported)
	Diet:	0.4% calcium, 0.3% phosphorus, and 3 UI/g diet of vitamin D3 (Harlan Teklad, Udine, Italy);
	Dosing method:	Gavage
	Funding source	Not reported
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	SHAM: control diet, vehicle (distilled water)
		OVX control, vehicle
		SSE-100: OVX, standardised soy extract: 100 mg/kg bw/day
		E2: OVX, 17β-oestradiol(0.5 mg/kg/day)
	Start of intervention since OVX	1 day
	Duration	6 weeks
Statistical analysis	**Statistical analysis**	All data analysed using the non-parametric Kruskall-Wallis test, followed by Steel multiple comparison tests.

Results	**Mammary gland:**		
	Histological findings:	SHAM	Presence of terminal ducts and alveolar buds.
		OVX control	Regressive process in the parenchymal structure. All animals had atrophic mammary glands with small ducts lined by a flattened atrophic epithelium and lacking significant lobulo-alveolar structures.
		SSE-100	Partial regression of the glandular atrophy with mammary gland showing limited increase in the number of small lobulo-alveolar units with a histological appearance between that of OVX and SHAM. No detail information provided
		E2	Stimulated mammary glands compared with OVX rats, with an average lobulo-alveolar development not different from SHAM control.
	Ki-67 proliferation marker in epithelium (E) and stroma (S) [median value (min-max]	SHAM	E: 2 (1-6)[a] S: 1 (0-12)
		OVX control	E: 1 (0-4)[a] S: 2 (1-2)
		SSE-100	E: 0 (0-2)[b] S: 2 (0-6)
		E2	E: 4 (1-6)[a] S: 5 (1-12)
		a: significantly different from OVX control ($p < 0.05$) b: significantly different from SHAM control ($p < 0.05$)	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No detail information provided
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Sham control was used. Insufficient detail was provided to evaluate effect from the basal diet (not specified if the diet was phytoestrogen free).
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Not deemed relevant for pathology assessment and immunostaining
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	Methods considered acceptable.
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Gallo et al., 2008 – Ref ID: 15146

Animal model	**Species**	Rats	
	Strain (source)	Sprague-Dawley from Harlan Nossan S.r.l. (Correzzana MI, Italy).	
	Number of animals	Total, 40; 10/group	
	Age (weight):	3-month old (not reported)	
	Diet:	Phytoestrogen free, semi-purified (Harlan, Italy);	
	Dosing method:	Gavage	
	Funding source	Not reported	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	SHAM: control diet, vehicle	
		OVX control, vehicle	
		SSE-100: OVX, standardised soy extract: 100 mg/kg bw/day	
		E2: OVX, 17β-oestradiol(0.5 mg/kg/day)	
	Start of intervention since OVX	1 day	
	Duration	6 weeks	
Statistical analysis	**Statistical analysis**	All data analysed using the non-parametric Kruskall-Wallis test, followed by Steel multiple comparison tests.	
Results	**Uterus:**		
	Histological findings:	SHAM	Normal histology: 9/10; hyperplasia: 1/10
		OVX control	All uteri described as showing atrophic epithelium.
		SSE-100	Histological features not significantly changed, 9/10 animals showing subatrophic/atrophic epithelium; endometrial hyperplasia in 1/10.
		E2	Epithelial hyperplasia in 9/10 animals, remaining animal showing normal appearance
	Ki-67 proliferation marker (% positive cells) in luminal (LE) and glandular epithelium (GE)	SHAM	LE: 29.6 ± 13.5%[a] GE: 18.76 ± 11.8%
		OVX control	LE: 12.7 ± 5.7% GE: 7.5 ± 4.8%
		SSE-100	LE: 5.7 ± 5.7% [a] GE: 8.2 ± 6.7%
		E2	LE: 24.0 ± 13% GE: 19.9± 9.4%[b]
		a: significantly different from OVX control ($p < 0.05$) b: significantly different from OVX control ($p < 0.01$)	
	Relative weight (mg/g body weight)	SHAM	1.9 ± 0.4[a]
		OVX control	0.6 ± 0.3
		SSE-100	0.5 ± 0.4
		E2	1.6 ± 0.2 [a]
		a: significantly different from OVX control ($p < 0.05$)	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Soy-free diet and sham control group
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	

Bias domain	Question	Score	Judgement
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Blinding to treatment group was used in immunohistochemistry analyses. Two readers read all slides and discrepancies evaluated separately.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Hertrampf et al., 2009 – Ref ID: 15219

Animal model	**Species**	Rats
	Strain (source)	Wistar from Janvier (Janvier, Le Genest St Isle, France)
	Number of animals	7/group
	Age (weight):	12-week (240-260 g)
	Diet:	Diet low in phytoestrogen content (IDD) (SSniff GmbH, Soest, Germany)
	Dosing method:	Dietary administration (two additional groups treated s.c. with E2 and genistein not considered)
	Funding source	Not reported
	Authors' conflicts of interest	None declared
Dosing	**Intervention**	OVX control: diet
		IRD: diet rich in isoflavone content (IRD) (Harlan Winkelmann, Borchen, Germany). Isoflavone content measured as 232 ± 10 g/g daidzein, 240 ± 36 g/g genistein
		GEN-700: an IDD enriched with genistein 700 mg/kg diet
	Start of intervention since OVX	14 days
	Duration	12 weeks
Statistical analysis	**Statistical analysis**	All data are expressed as arithmetic means with their standard deviations. Statistical significance of differences was calculated using one-way analysis of variance (ANOVA) followed by post hoc Tukey HSD test where appropriate. Statistical tests were used for comparisons between groups and statistical significance was established at $P < 0.05$.
Results	**Uterus:**	
	Relative wet weight (mg/kg bw)	No statistically significant difference between OVX control and IRD and GEN (~ 100 mg/kg bw in all the three groups). Statistically significant increase in the E2 group (~ 1 000 mg/kg bw) but to a lesser extent than SHAM group (~ 1 500 mg/kg bw). Genistein s.c. also significantly increased uterine weight (~ 500 mg/kg bw). Values extrapolated from Figure 1A in the original publication.
Risk of Bias Appraisal		**Tier: 1**

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	low isoflavone diet used sham controls used
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Hidaka et al., 2003 – Ref ID: 15224

Animal model	**Species**	Rat
	Strain (source)	Sprague Dawley (rom Seac Yoshitomi Ltd (Fukuoka Prefecture, Japan)
	Number of animals	Total: 30, 6/group
	Age (weight):	90-days
	Diet:	MF Powdered pellets (Oriental yeast)
	Dosing method:	Dietary
	Funding source	Contract/grant sponsor: Fujicco Co Ltd, Kobe, Japan
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	SHAM:
		OVX control
		P40: MF powdered pellets containing 0.22% (w/w) Fujiflavone P40 (Fujicco Co. Ltd, Kobe, Japan)
		E2: subcutaneously injected with 17β-oestradiol (in 20% polyethylene glycol 400) 5 days/week at a dose of 10 µg/kg bw
		OVX + PEG: subcutaneously injected with 20% polyethylene glycol 400, 5 days/week
	Start of intervention since OVX	Not specified
	Duration	7 weeks
Statistical analysis	**Statistical analysis**	Data obtained from 3–5 measurements and were expressed as the mean standard deviation. Statistical comparisons were made by ANOVA and Scheffé's tests using a statistical software program. The difference was considered significant when $p < 0.05$.

Results	**Uterus:**		
	Weight (mg)	SHAM	495 ± 35[a]
		OVX control	115 ± 9[b]
		P40	165 ± 9[a,b]
		E2	489 ± 10[a]
		OVX + PEG	110 ± 9[b]
		a: significant difference (P < 0.05) compared with OVX control; b: significant difference (P < 0.05) compared with SHAM control	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	sham control used dietary soy content not specified so basal level of exposure to soy isoflavones is likely
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Hong et al., 2009 – Ref ID: 9712

Animal model	**Species**	rats
	Strain (source)	Sprague-Dawley
	Number of animals	Total: 24; 6 animals/group
	Age (weight):	4-week
	Diet:	AIN-93M purified rodent diet
	Dosing method:	
	Funding source	
	Authors' conflicts of interest	
Dosing	**Intervention**	SHAM control
		OVX-control
		SP: isoflavones from soy pulp diet, 1.8 mg/kg diet
		FSP: isoflavones from fermented soy pulp diet, 1.8 mg/kg diet
	Start of intervention since OVX	1 week
	Duration	7 week
Statistical analysis	**Statistical analysis**	All analysis done in triplicate and data obtained analysed by ANOVA after estimation of the means and standard errors. Significant differences among treatment groups were determined by Duncan's multiple range test at p < 0.05.

Results	**Uterus:**		
	Weight (g)	SHAM	0.463 ± 0.025[a]
		OVX-control	0.050 ± 0.003
		SP	0.053 ± 0.003
		FSP	0.072 ± 0.010 [a]
		a: different from OVX control (p < 0.05)	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age and body wt was similar among the groups
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	All animals were maintained on a soybean oil containing diet, but the co-exposure would not cause bias. No plastic cages were used.
	Were experimental conditions identical across study groups?	++	Housing conditions were identical among the groups.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals was reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information provided.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Kakehashi et al., 2012 – Ref ID: 15337

Animal model	**Species**	rats	
	Strain (source)	Donryu (Japan SLC, Shizuoka, Japan)	
	Number of animals	Total: 68; Experiment 1: 10 animals	
	Age (weight):	5-week (200 g)	
	Diet:	NIH- 07PLD diet (phytoestrogen-low diet; Oriental Yeast, Tokyo, Japan). No phytoestrogens.	
	Dosing method:	Dietary administration	
	Funding source	Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare of Japan.	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	OVX Control	
		IA-rich extract: 0.6 % corresponding to 0.2 % isoflavones aglycones and corresponding to 150 mg/kg bw/day	
	Start of intervention since OVX	2-weeks	
	Duration	2 weeks	
Statistical analysis	**Statistical analysis**	Not specified for the endpoint uterine weight.	
Results	**Uterus:**		
	Relative weight	OVX Control	0.32 ± 0.04%
		IA-150	0.58 ± 0.13%[a]
		a: significantly different from OVX control (p < 0.005)	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**

Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	A rat strain susceptible to uterine tumors was used. A number of supporting molecular and histopathological studies increase confidence in endpoints evaluated
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	A low phytoestrogen diet was used
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	blinding not specifically addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	multiple overlapping endpoints evaluated
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Kang et al., 2015 – Ref ID: 19187

Animal model	**Species**	Rat
	Strain (source)	Sprague Dawley (OrientBio, Seungnam, Korea)
	Number of animals	8/group
	Age (weight):	6 weeks
	Diet:	pelleted diet (Superfeed Co., Seoul, Korea) containing soybean oil
	Dosing method:	gavage
	Funding source	National Research Foundation of Korea grant funded by the Korean government (MSIP; No. 2011-0030124).
	Authors' conflicts of interest	None declared
Dosing	**Intervention**	SHAM: sham operated, vehicle
		OVX-control: vehicle
		RCE-40: OVX, standardized red clover extract (8% isoflavones) at 40 mg/kg bw/day
		PCP-20: OVX, PCP preparation containing 0.90 mg/g of ellagic acid at 20 mg/kg bw/day
		Mix-30: OVX, RC:PCP 2:1 mixture (g/g), 30 mg/kg
		Mix-60: OVX, RC:PCP 2:1 mixture (g/g), 60 mg/kg
		Mix-120: OVX, RC:PCP 2:1 mixture (g/g), 120 mg/kg
	Start of intervention since OVX	28 days
	Duration	84 days
Statistical analysis	**Statistical analysis**	The data are presented as means ± standard deviation. Significant differences among means within a group were examined using a parametric method (one-way ANOVA) or non-parametric method (Kruskal-Wallis H test). Variance homogeneity was evaluated using the Levene test. If the Levene test indicated no significant deviation from variance homogeneity, the obtained data were analyzed with a one-way ANOVA followed by the Tukey test as a post hoc test. In cases where significant deviations from variance homogeneity were observed, the data were analyzed using the Kruskal-Wallis H test followed by Tamhane post hoc test.

Results	**Uterus:**		
	Morphometric analysis	SHAM	Total thickness (mm): 3.21 ± 0.65 Epithelial thickness (µm): 38.47 ± 5.18 Mucosa thickness (µm): 967.28 ± 220.32 Uterine glands (%): 28.78 ± 4.83
		OVX control	Total thickness (mm): 0.54 ± 0.11[a] Epithelial thickness (µm): 9.76 ± 2.26[b] Mucosa thickness (µm): 176.98 ± 21.58[a] Uterine glands (%):4.85 ± 2.17[b]
		RCE-40	Total thickness (mm): 0.89 ± 0.10[a,c] Epithelial thickness (µm): 16.90 ± 1.51[b,d] Mucosa thickness (µm):227.65 ± 31.82[a,c] Uterine glands (%): 9.28 ± 1.53[b,d]
		PCP-20	Total thickness (mm): 0.73 ± 0.09[a,e] Epithelial thickness (µm): 19.16 ± 1.72[b,d] Mucosa thickness (µm):261.87 ± 32.06[a,c] Uterine glands (%):11.03 ± 3.19[b,d]
		Mix-30	Total thickness (mm): 0.84 ± 0.15[a,e] Epithelial thickness (µm): 18.79 ± 2.93[b,d] Mucosa thickness (µm):286.24 ± 41.40[a,c] Uterine glands (%):11.34 ± 3.50[b,d]
		Mix-60	Total thickness (mm): 1.10 ± 0.10[a,c] Epithelial thickness (µm): 22.81 ± 2.02[b,d] Mucosa thickness (µm):420.77 ± 94.19[a,c] Uterine glands (%):17.67 ± 2.71[b,d]
		Mix-120:	Total thickness (mm): 1.45 ± 0.36[a,c] Epithelial thickness (µm): 24.28 ± 2.31[b,d] Mucosa thickness (µm):484.97 ± 56.84[a,c] Uterine glands (%):20.10 ± 3.13[bd]
		a: different from SHAM ($p < 0.01$, Tamhane test) b: different from SHAM ($p < 0.01$, Tukey test) c: different from OVX control ($p < 0.01$, Tamhane test) d: different from OVX control ($p < 0.01$, Tukey test) e: different from OVX control ($p < 0.05$, Tamhane test)	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	groups assigned "based on body weight"
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	
	Were experimental conditions identical across study groups?	+	vehicle and sham controls used
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	Bone was primary focus of the study
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Kawakita et al., 2009 – Ref ID: 15353

Animal model	**Species**	rats
	Strain (source)	Sprague–Dawley
	Number of animals	Total: 80; 20/group
	Age (weight):	90-day (210 g)
	Diet:	Food pellets (without any alfalfa or soybean components and with standard 0.44% calcium content which was slightly reduced in treated groups so to compensate with the calcium contained in the alkaline supplementation)
	Dosing method:	Dietary administration
	Funding source	The research costs were handled thanks to an unrestricted grant from Canova Foundation, Italy, a nonprofi t organization devoted to research
	Authors' conflicts of interest	None disclosed
Dosing	**Intervention**	SHAM: sham operated animals,
		OVX control: OVX, standard food
		RCE: Standardised red clover extract (40% isoflavones) at 6 mg/kg/day
		RCE+BP: Standardised red clover extract (40% isoflavones) at 6 mg/kg/day plus a modified alkaline supplementation through a nasogastric tube
	Start of intervention since OVX	
	Duration	3 months
Statistical analysis	**Statistical analysis**	All data were expressed as means ± standard deviation. Significance of the results was determined by one-way analysis of variance (ANOVA) and Bonferroni analysis was applied to test the differences between individual groups. A p value < 0.05 was considered statistically significant.
Results	**Uterus:**	
	Weight:	Significantly increased in RCE and RCE + BP groups (~ 200 mg) vs OVX control (~ 100 mg), however still ~ 50% lower than the SHAM group. Values extrapolated from Figure 1B in the original publication.

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	sham vs ovx used
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	neck bone reported - no change in long bone parameters noted in text

Bias domain	Question	Score	Judgement
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Kikuchi-Hayakawa et al., 1998 – Ref ID: 6198

Animal model	**Species**	Rats	
	Strain (source)	Wistar (CLEA Japan, Tokyo, Japan)	
	Number of animals	Total 20 (sham-operated, n=5; OVX, n=15)	
	Age (weight):	8-month old (333-339 g)	
	Diet:	Stock diet (MF, Oriental Yeast, Tokyo)	
	Dosing method:	Dietary administration	
	Funding source	Not reported	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	SHAM: control diet	
		OVX control: control diet	
		SM: unfermented soy milk containing 1691 µg/g isoflavones, mostly as glucosides	
		FSM: fermented soy milk containing 1725.7 µg/g isoflavones, mostly as aglycones	
	Start of intervention since OVX	1 week	
	Duration	6 weeks	
Statistical analysis	**Statistical analysis**	The results are expressed as means and SD. Means were compared by variance analysis and subsequent Tukey's HSD comparison after logarithmic transformation to stabilise the variance, if the variance was significantly different (Bartlett test). For non-parametric data, the Kruskal-Wallis test and subsequent non-parametric Tukey's test were done.	
Results	**Uterus:**		
	Weight (g)	SHAM	0.456 ± 0.067[a]
		OVX-Control	0.124 ± 0.024
		SM	0.135 ± 0.018
		FSM	0.139 ± 0.018
		a: weight in OVX-Control was 27% of that in SHAM.	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age and body wts of the rats were similar among the groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	The basal diet was not isoflavones free. No plastic cages.
	Were experimental conditions identical across study groups?	++	All animals were maintained on the basal diet with or without the soy milk or fermented soy milk.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals was reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	

Bias domain	Question	Score	Judgement
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Kishida et al., 2008 – Ref ID: 10162

Animal model	**Species**	Rats	
	Strain (source)	Sprague-Dawley (Nippon SLC, Shizuoka, Japan)	
	Number of animals:	6/group	
	Age (weight):	5-week	
	Diet:	Control diet containing: casein; corn oil; AIN-76 mineral mixture; AIN-76 mineral mixture containing choline bitartrate (20 g/100g); sucrose; α-corn starch	
	Dosing method:	Dietary administration	
	Funding source	Study partly supported by Uehara Memorial Foundation, the Fuji Foundation for Protein Research, and a Grant-in-Aid for Scientific research from the Ministry of Education, Science, Sports and Culture of Japan.	
	Authors' conflicts of interest	None declared	
Dosing	**Intervention**	OVX-control	
		IF-300: OVX, fermented soybean extract 300 mg/kg diet	
		Positive control: subcutaneous implantation of 17β-oestradiol-benzoate at 4.2 µg/rat/day (β-oestradiol 3-benzoate,0.25 mg/pellet, 60-day release	
	Start of intervention since OVX	Not specified	
	Duration	28-day	
Statistical analysis	**Statistical analysis**	Two-way ANOVA followed by Student's t test with Bonferroni corrections	
Results	**Uterus:**		
	weight	OVX control	0.08 ± 0.01
		IF-300	0.12 ± 0.02
		E2	0.32 ± 0.04[a]
		a: different from the OVX control group (P < 0.05)	
Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age and body wts were similar among the groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	All animals were maintained on the same AIN 76 diet, which contains soy protein. This would not appreciable bias results
	Were experimental conditions identical across study groups?	++	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported.

Bias domain	Question	Score	Judgement
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	All animals were maintained under the same conditions
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	.
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Lee et al., 2004 – Ref ID: 5420

Animal model	**Species**	Rat	
	Strain (source)	Sprague Dawley	
	Number of animals	Total: 24	
	Age (weight):	12-week	
	Diet:	Experimental diet, soy free	
	Dosing method:	Dietary administration	
	Funding source	Grant from Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (00-PJ1-PG4-PT04-0003)	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	SHAM: diet TD88190 Harlan Teklad	
		OVX control: diet TD88190 Harlan Teklad	
		IF: isoflavone extract at 6.25 g/kg diet	
		E2: 17β-oestradiol, 0.0039 g/kg diet	
	Start of intervention since OVX	not reported	
	Duration	16 week	
Statistical analysis	**Statistical analysis**	ANOVA; significant differences between groups determined by Duncan's multiple range test, with significance detected at either $p < 0.05$ or $p < 0.01$	
Results	**Uterus**		
	Relative weight (g/100g bw)	SHAM	0.209 ± 0.055
		OVX	0.024 ± 0.007[a]
		IF	0.035 ± 0.008[a]
		E2	0.106 ± 0.022[a,b]
		a: different from SHAM ($p < 0.01$) b: different from OVX control ($p < 0.01$)	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age and body wts of the rats was similar among the groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	The animals were maintained on an AIN diet. No information about the cages.
	Were experimental conditions identical across study groups?	++	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	

Bias domain	Question	Score	Judgement
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Legette et al., 2009 – Ref ID: 1203

Animal model	**Species**	rats	
	Strain (source)	Sprague Dawley (Harlan)	
	Number of animals	Total: 78; 15-16/group	
	Age (weight)	6 mo old)	
	Diet:	AIN-93M diet	
	Dosing method:	Diet	
	Funding source	Purdue University, University of Alabama Botanical Center for Age Related Diseases, and NIH grants P50 AT00477-01 and P50 AT000477-07S1.	
	Authors' conflicts of interest	C. M. Weaver is on the board of Pharmavite; S. Barnes has a U.S. patent on the use of conjugated isoflavones for prevention of osteoporosis	
Dosing	**Intervention**	SHAM: sham operated animals	
		OVX control	
		EQ-50: racemic equol, 50 mg /kg diet	
		EQ-100: racemic equol, 100 mg /kg diet	
		EQ-200: racemic equol, 200 mg /kg diet	
	Start of intervention since OVX	4 days	
	Duration	8 weeks	
Statistical analysis	**Statistical analysis**	In the case of non-normal distribution (reproductive tissue data), data underwent log transformation before analysis by 1-way ANOVA and Tukey's test. Significance was accepted at $P < 0.05$.	
Results	**Mammary gland:**		
	PCNA immunostaining (%)	SHAM	14.8 ± 15.3^{b}
		OVX-control	1.98 ± 2.11^{a}
		EQ-50	1.14 ± 1.66^{a}
		EQ-100	2.32 ± 3.72^{a}
		EQ-200	2.59 ± 4.70^{a}
		Values are means ± SD, n = 15–16. Means without a common letter differ, $P < 0.05$.	
	Gland proliferation (visual scoring)	SHAM	1.94 ± 0.570^{b}
		OVX-control	0.53 ± 0.740^{a}
		EQ-50	0.38 ± 0.50^{a}
		EQ-100	0.47 ± 0.510^{a}
		EQ-200	0.94 ± 0.570^{a}
		Values are means ± SD, n = 15–16. Means without a common letter differ, $P < 0.05$.	

Uterus:		
Epithelial PCNA immunostaining (%)	SHAM	37.6 ± 21.8[b]
	OVX-control	1.14 ±2.09[a]
	EQ-50	0.670 ± 1.15[a]
	EQ-100	8.95 ± 12.4[b]
	EQ-200	15.3 ± 18.0[b]
	Values are means ± SD, n = 15–16. Means without a common letter differ, P < 0.05.	
Stroma PCNA immunostaining (%)	SHAM	19.2 ± 21.1[b]
	OVX-control	0.650 ± 1.45[a]
	EQ-50	0.920 ± 1.92[a]
	EQ-100	2.67 ± 2.99[ab]
	EQ-200	4.18 ± 3.98 [b]
	Values are means ± SD, n = 15–16. Means without a common letter differ, P < 0.05.	
Epithelial proliferation (visual scoring)	SHAM	1.94 ± 0.770 [b]
	OVX-control	0.000 ± 0.000 [a]
	EQ-50	0.130 ± 0.340 [a]
	EQ-100	0.330 ± 0.490 [ac]
	EQ-200	0.750 ± 0.580[c]
	Values are means ± SD, n = 15–16. Means without a common letter differ, P < 0.05.	
Weight (g)	No statistically significant difference between OVX control and EQ-50 and EQ-100 (~ 0.150 g). Uterine weight was significantly increased in EQ-200 (~ 0.200 g, P < 0.05). Values extrapolated from Figure 1B of the original publication.	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	The female CD rats were 6 months of age, of which 62 were OVX and 16 were SHAM operated 2 days before shipment. Body weights and uterus weight were determined after 8 weeks of treatment. Dietary intake was similar among all OVX groups, which were pair-fed to the mean intake of the SHAM rats. Among the OVX groups, equol did not affect mammary epithelial proliferation as evident by PCNA immunostaining.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Plastic cages were used. Not further specified. AIN-93 diet not further specified, most probably Soya free.
	Were experimental conditions identical across study groups?	++	All animals received the same AIN-93M base diet and the OVX groups were pair-fed to the mean intake of the SHAM rats.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	None of the animals died during the study.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	--	The study was not performed in a blinded fashion.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	The immunostaining technique was not described in detail. Reference to a previous paper.
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Li and Yu, 2003 – Ref ID: 13554

Animal model	**Species**	Rat	
	Strain (source)	SD from Animal Center of National Birth Control Board	
	Number of animals	Total: 40, 10 animals/group	
	Age (weight):	(230 ± 20g)	
	Diet:	Not reported	
	Dosing method:	gavage	
	Funding source	Work supported by the National Natural Sciences Foundation of China (39830430) and the "985 Promotion Plan" of Peking University.	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	SHAM: sham-operated, vehicle	
		OVX-control: vehicle	
		E2: 10 µg/kg estradiol once every two days after ovariectomized;	
		GEN-45: genistein, dissolved in 0.5% CMC–Na and given daily by oral gavage at 45 mg/kg bw/day	
	Start of intervention since OVX	Not reported.	
	Duration	84	
Statistical analysis	**Statistical analysis**	One way analysis of variance (ANOVA) tests were used for comparisons between every two groups. All results are expressed as means ± S.D. Significance was considered at $p < 0.05$.	
Results	**Uterus:**		
	Histology:	SHAM: cubic or column-like epithelium OVX-control: Thin and flat after. GEN-45: cubic epithelium E2: squamous epithelium	
	Weight (mg)	SHAM	370±49
		OVX	108±23[a]
		E2	213±49[a,c]
		GEN-45	137±28[a,b]
		a: $p < 0.05$ compared with SHAM b: $p < 0.05$ compared with E2 c: $p < 0.01$ compared with OVX control	
	Weight index (mg/g)	SHAM	1.1882 ± 0.1370
		OVX	0.3664 ± 0.1289[a]
		E2	0.7236 ± 0.2692[a,c]
		GEN-45	0.4044 ±0.0893 [a,b]
		a: $p < 0.01$ compared with SHAM b: $p < 0.01$ compared with E2 c: $p < 0.01$ compared with OVX control	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	The age and the body wts were similar among the groups. Body wt was determined weekly
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	No data on housing conditions of the animals was provided. No data on the type of diet was given.
	Were experimental conditions identical across study groups?	+	General housing conditions seems similar among the groups. Vehicle controls were adequate and a sham-operated group was included.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals was reported

Bias domain	Question	Score	Judgement
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information is given.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Liu et al., 2007 – Ref ID: 10424

Animal model	**Species**	Rats	
	Strain (source)	Wistar from Tianjin Laboratory Animal Center (Tianjin, China)	
	Number of animals	Total: 50; 10/group	
	Age (weight):	12-week (233 ± 16 g)	
	Diet:	Not reported	
	Dosing method:	gavage	
	Funding source	Not reported	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	SHAM control: vehicle	
		OVX-control: vehicle	
		OVX-exercise: vehicle + daily running on a treadmill for 60 minutes/day at 20m/minute up a 5° slope	
		OVX-IF: isoflavones, 50 mg/kg bw/day	
		OVX-IF+ exercise: isoflavones, 50 mg/kg bw/day	
	Start of intervention since OVX	4 days	
	Duration	8-week	
Statistical analysis	**Statistical analysis**	Data were presented as means ± SEM. The significance of the differences was determined by two-way ANOVA. Differences were considered significant at the level of $p<0.05$, $p<0.01$ and $p<0.001$.	
Results	**Uterus**		
	Relative weight (g)	SHAM	0.169±0.041
		OVX control	0.028±0.003
		OVX-exe	0.037±0.006[a]
		OVX-IF	0.045±0.011[b]
		OVX-IF-exe	0.043±0.009[b]
		a: significantly different from OVX control ($p<0.05$) b: significantly different from OVX control ($p<0.01$)	
Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Insufficient data on the basal diet. No information on housing of the animals was provided.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	No information on the diet was provided.
	Were experimental conditions identical across study groups?	++	All animals were housed similarly.

Bias domain	Question	Score	Judgement
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals was reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information was provided
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Mathey et al., 2007 – Ref ID: 13554

Animal model	**Species**	Rat
	Strain (source)	Wistar from INRA Clermont-Ferrand/ Theix (St Genès-Champanelle, France)
	Number of animals	Total: 70 (10/group)
	Age (weight):	105-day old
	Diet:	soybean-protein-free powdered semi-purified diet
	Dosing method:	Dietary administration
	Funding source	Beghin-Meiji for supplying fructooligosaccharides (Actilight®) and financial support Work supported by the European thematic network Phytohealth.
	Authors' conflicts of interest	None reported
Dosing	**Intervention**	SHAM: intact ovaries, control diet
		OVX control: control diet
		GEN: genistein, 10 mg/kg bw/day
		DAI: daidzein, 10 mg/kg bw/day
		DAI-scFOS: daidzein, 10 mg/kg bw/day + short-chain FOS (short-chain fructooligosaccharides)
		DAI-lac: daidzein, 10 mg/kg bw/day + *Lactobacillus casei*
		EQ: equol, racemic, 10 mg/kg bw/day
	Start of intervention since OVX	Immediately after surgery
	Duration	90 days
Statistical analysis	**Statistical analysis**	Results expressed as means ± SEM. ANOVA was first performed to test for any significant differences among groups. When significant (P<0.05), the Student-Newman-Keul's multiple comparisons test was applied to determine the specific differences between means. Parametric ANOVA was performed when data were sampled from populations with equal variance. If not, nonparametric methods were selected. Then, a Kruskall-Wallis test was first carried out. If it indicated a significant difference among groups (P<0.05), the Mann-Whitney U test was used to determine specific differences. The level of significance was set at P<0.05 for all statistical tests.

Results	**Uterus:**		
	Relative weight (g/kg bw) Mean (SE)	SHAM	0.62[b] (0.04)
		OVX control	0.09[a] (0.003)
		GEN-10	0.10[a] (0.01)
		DAI-10	0.10[a] (0.01)
		DAI-scFOS	0.10[a] (0.005)
		DAI-lac	0.10[a] (0.004)
		EQ-10	0.11[a,b] (0.01)
		a: P<0.0001 mean values significantly different from SHAM b: P<0.01 mean values significantly different from OVX	
Tier of Reliability		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	semi-purified basal diet was used sham controls used
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Bias domain	**Question**	**Score**	**Judgement**
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Mosquette et al., 2006 – Ref ID: 1618

Animal model	**Species**	Rat
	Strain (source)	Wistar rats
	Number of animals	Total: 56; 8/group
	Age (weight):	3-month old (180-210 g)
	Diet:	Standard diet, soy free.
	Dosing method:	gavage
	Funding source	Support provided by CNPq, 041/2003 CAPES (Brasilia-Brazil), and FAPESP 2003/13407-0.
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	OVX control: vehicle (propylene glycol)
		IF-10: OVX,4.3 mg/kg bw/day isoflavones
		IF-50: OVX,21.3 mg/kg bw/day isoflavones
		IF-100: OVX,42.6 mg/kg bw/day isoflavones
		IF-300: OVX,127.8 mg/kg bw/day isoflavones
		IF-600: OVX,255.6 mg/kg bw/day isoflavones
		E2: OVX, CEE, 200 µg/kg bw/day
	Start of intervention since OVX	1 month
	Duration	21 days

Statistical analysis	**Statistical analysis**	Data from morphometric analyses are given as mean ± standard deviation (S.D.); the differences were analyzed using one-way ANOVA followed by the Turkey's post hoc test, with the level of significance set at P < 0.05.	
Results	**Uterus::**		
	Histology:	OVX control	Endometrium was dense and thin. Glands were small and closed. The luminal epithelium was unorganized, without columnar morphology and the nuclei randomly positioned in the cells. There was little, if any, interstitial space between the stromal cells. Myometrium showed a low number of collagen fibers among the uterine myocytes, which had elliptic, heterochromatic and small nuclei, compared to other groups.
		IF-10	Histological features of the endometrium and myometrium very similar to those of OVX control.
		IF-50	Histological features very similar to those of OVX control. Myometrium presented a large cytoplasm with euchromatic and large nuclei
		IF-100	Slightly proliferative endometrium, with many endometrial glands, with simple cylindrical epithelium bearing a large amount of eosinophils. Myometrium presented a large cytoplasm with euchromatic and large nuclei
		IF-300	Markedly proliferative endometrium, with many endometrial glands, with simple cylindrical epithelium bearing a large amount of eosinophils. Endometrial enlargement observed similar to E2. Myometrium was noticeably thicker and infiltrated with numerous leukocytes, mainly eosinophils and showed the greatest increase in the number of myocytes and a large amount of collagen fibers, similar to E2.
		IF-600	Markedly proliferative endometrium, with many endometrial glands, with simple cylindrical epithelium bearing a large amount of eosinophils. Myometrium presented a large cytoplasm with euchromatic and large nuclei
		E2	The size of endometrial epithelial and stromal cells was increased. The endometrial glands had large lumina, many of which contained secretory products. Interstitial space appeared between stromal cells. Luminal epithelial cells presented a columnar aspect, with basally located nuclei. Myometrium was noticeably thicker and infiltrated with numerous leukocytes, mainly eosinophils and showed the greatest increase in the number of myocytes and a large amount of collagen fibers
	Morphometric analysis	OVX control	All measured parameters did not differ from IF-10 and IF-50.
		IF-10	All measured parameters did not differ from OVX-control and IF-50.
		IF-50	All measured parameters did not differ from OVX control and IF-10.

		IF-100	Endometrial area and thickness index significantly increased compared to control and IF-10 and IF-50.
		IF-300	All measured parameters significantly increased compared to OVX-control, IF-10 and IF-50. Largest values of endometrial area and thickness index, number of endometrial glands and eosinophils, as well as of myometrial area.
		IF-600	All measured parameters significantly increased compared to OVX-control, IF-10 and IF-50. Thickness index significantly increased compared to control and lower doses.
		E2	Largest values of endometrial area and thickness index, number of endometrial glands and eosinophils, as well as of myometrial area.
	PCNA in endometrial cells (% positive nuclei)	OVX control	Luminal epithelium: 4.2 ± 2.8 Glandular epithelium: 7.1 ± 6.6 Stroma: 0.2 ± 0.3
		IF-10	Luminal epithelium: 5.7 ± 3.3 Glandular epithelium: 8.1 ± 6.6 Stroma: 1.1 ± 0.7
		IF-50	Luminal epithelium: 4.0 ± 2.1 Glandular epithelium: 5.3 ± 4.9 Stroma: 1.9 ± 2.9
		IF-100	Luminal epithelium: 59.9 ± 39.7[a] Glandular epithelium: 74.6 ± 23.4[a] Stroma: 15.1 ± 7.1[a]
		IF-300	Luminal epithelium: 74.9 ± 23.7[a] Glandular epithelium: 95.7 ± 24.9[a] Stroma: 20.4 ± 6.8[a]
		IF-600	Luminal epithelium: 65.8 ± 30.5[a] Glandular epithelium: 82.3 ± 28.6[a] Stroma: 26.7 ± 8.9[a]
		E2	Luminal epithelium: 79.3 ± 26.2[a] Glandular epithelium: 947.1 ± 29.3[a] Stroma: 36.2 ± 9.7[a]
		a: P < 0.01 compared to OVX control, IF-10, IF 50 (ANOVA and post hoc Turkey test)	
	Weight (g)	OVX control	2.7 ± 0.5[a]
		IF-10	3.2 ± 0.8[b]
		IF-50	4.2 ± 0.8[c]
		IF-100	6.2 ± 0.7[c]
		IF-300	7.7 ± 0.9
		IF-600	6.2 ± 0.2[c]
		E2	7.5 ± 0.1
		a P < 0.01 compared to IF-100, IF-300, E2 b P < 0.01 compared to IF-300, and E2 c P < 0.01 compared to IF-300, and E2	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	Type of cages used no specified. Diet was soy-bean free.
	Were experimental conditions identical across study groups?	++	Housing conditions were identical among the groups as was the vehicle used.

Bias domain	Question	Score	Judgement
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	All animals (n=8/group) were treated by gavage for 21 consecutive days and then sacrificed by decapitation.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	No information about blinded evaluation was reported, but this will not have influenced the outcome of the study.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Mu et al., 2009 – Ref ID: 15689

Animal model	**Species**	Mice
	Strain (source)	Kunming mice, from Animal Laboratories of Xi'an Jiaotong University, China
	Number of animals	50 total; 10 each group
	Age at ovariectomy:	2–3 months
	Diet:	Not reported
	Dosing method:	Oral administration
	Funding source	Shaanxi Natural Science Foundation (Grant no. 2003-B21)
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	Control1: SHAM operated animals, vehicle alone
		Control2: OVX animals, vehicle alone
		F1: OVX, formononetin, 50 mg/kg body weight
		F2: formononetin, 500 mg/kg body weight
		DES: OVX, stilbestrol 0.20 mg/kg body weight
	Start of intervention since OVX	Not reported
	Duration	6 months
Statistical analysis	**Statistical analysis**	All values are expressed as mean ± S.E. and evaluated by one-way ANOVA followed by Student's t-test to detect inter-group differences. Differences were considered to be statistically significant if po0.05.
Results	**Uterus:**	
	Weight	The uterine weight of the intact animals was heavier than the OVX animals treated by vehicle (p<0.01). In the OVX animals, the uterus weight of mice treated with DES was highly significantly heavier than those of vehicle- and formononetin-treated mice (p<0.01). However, uterus weight of mice in the group treated with a high dose of formononetin (0.5 g/kg day) was lower than those treated with a low dose of formononetin (0.5 g/ kg day). In the SHAM group and OVX group, both treated with vehicle, the uterus weight of mice in the SHAM group was heavier than in the OVX group, but had no differences between the uterus weights of mice treated with a high dose of formononetin
Risk of Bias Appraisal		**Tier: 1**

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Sham control used
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	The diet used is not specified. Basal levels of soy isoflavone exposure are likely
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Nguyen et al., 2013 – Ref ID: 4038

Animal model	**Species**	Mice
	Strain (source)	C57BL/6J (Winkelmann, Borchen)
	Number of animals	9-10/group
	Age (weight):	2-month old
	Diet:	Soy-free diet
	Dosing method:	Dietary administration
	Funding source	European Union-funded research project EUGeneHeart (LSHM-CT-2005-018833)
	Authors' conflicts of interest	None disclosed
Dosing	**Intervention**	Intact control: soy-free (SF) control diet
		OVX-control: SF control diet
		GEN-0.01: genistein-enriched diet (Ssniff, Soest), 0.032 mg/mouse/day (actual consumption)
		GEN-0.03: genistein-enriched diet (Ssniff, Soest), 0.098 mg/mouse/day (actual consumption)
		GEN-0.1: genistein-enriched diet (Ssniff, Soest), 0.36 mg/mouse/day (actual consumption)
		GEN-0.3: genistein-enriched diet (Ssniff, Soest), 0.99 mg/mouse/day (actual consumption)
		GEN-1: genistein-enriched diet (Ssniff, Soest), 3.18 mg/mouse/day (actual consumption)
		GEN-3: genistein-enriched diet (Ssniff, Soest), 8.58 mg/mouse/day (actual consumption)
		GEN-10: genistein-enriched diet (Ssniff, Soest), 28.2 mg/mouse/day (actual consumption)
	Start of intervention since OVX	Immediately after OVX
	Duration	3 months

Statistical analysis	**Statistical analysis**	Data are shown as the mean ± SEM. Comparisons between multiple groups were performed using analysis of variance with Tukey's post hoc test adjusting for multiple comparisons, considering P < 0.05 significant.
Results	**Uterus:**	
	Weight (mg)	Absolute weight was significantly increased only in the GEN 10 group (~ 70-80 mg) compared with OVX control (~ 20 mg). Values extrapolated from Figure 3(a) in the original publication.
	Uterine weight to body weight ration	Same pattern as above with uterus-to-bw ratio significantly increased only in the GEN 10 group (~ 4) compared with OVX control (~ 0.5). Values extrapolated from Figure 3(b) in the original publication.

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Removal of the ovaries caused an increase in body weight when compared to mice with intact ovaries (24% increase; P<0.05). The highest concentration of genistein (3 and 10 g/kg food) inhibited body weight gain. The highest dose group had a significantly lower body wt than the group of mice with intact ovaries (29% lower; P<0.001). OVX mice had a significant decrease in uterus weight compared with mice with intact ovaries. The top-dose genistein group had a significantly higher uterus weight than the intact ovaries/soy-free fed group. Although the dose of 3 g genistein/kg food led to a significant increase in uterus wt in comparison with all remaining genistein treated groups, the uterus wt in this group was significantly lower than that of mice with intact ovaries.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	No specifications of the cages used. But the basel diet was soy-bean free.
	Were experimental conditions identical across study groups?	++	The mice were exposed to the genistein via the diet. The experimental conditions for all mice were similar.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	Each group consisted of 9-10 animals. At the end of the study, measurements were performed on 9-10 animals/group. No loss of animals occurred.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	The weight of the uteri was not performed blinded, but this will not have influenced the outcome of the study.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	

Bias domain	Question	Score	Judgement
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Overk et al., 2008 – Ref ID: 4723

Animal model	**Species**	Rats
	Strain (source)	Sprague–Dawley (from Harlan, Indianapolis, IN).
	Number of animals	6-7/group
	Age (weight):	8 weeks (~ 200g)
	Diet:	Harland/Teklad purified diet AIN-93M certified isoflavone-free (Indianapolis, IN)
	Dosing method:	gavage
	Funding source	Grant P50 AT00155 provided jointly by the Office of Dietary Supplements (ODS), National Center for Complementary and Alternative Medicine (NCCAM), the Office for Research on Women's Health (ORWH), and the National Institute of General Medicine (NIGMS) of the National Institutes of Health (NIH)
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	OVX control: vehicle (1% CMC and corn oil)
		E2: OVX, 17β-oestradiol, 10 µg/rat/day, s.c.
		RCE-4: OVX, *T. pratense*, extract, 4 mg/kg bw/day
		RCE-40: OVX, *T. pratense*, extract, 40 mg/kg bw/day
		RCE-400: OVX, *T. pratense*, extract, 400 mg/kg bw/day
		HOP-4: OVX, *H. Lupulus* extract, 4 mg/kg bw/day
		HOP -40: OVX, *H. Lupulus* extract, 4 mg/kg bw/day
		HOP -400: OVX, *H. Lupulus* extract, 4 mg/kg bw/day
		ISOX: Isoxanthohumol, 0.4 mg/rat/day, s.c.
		OVX control: vehicle (oil), s.c.
		E2: OVX, 17β-oestradiol, 10 µg/rat/day in oil, i.p.
		8-PN-0.4: Synthetic 8-Prenylnaringenin, 0.4 mg/kg bw/day, i.p.
		8-PN-4: Synthetic 8-Prenylnaringenin, 4 mg/kg bw/day, i.p.
		8-PN-40: Synthetic 8-Prenylnaringenin, 40 mg/kg bw/day, i.p.
	Start of intervention since OVX	Not specified
	Duration	3 weeks
Statistical analysis	**Statistical analysis**	Data were analyzed using one-way ANOVA with the Dunnett post hoc test, and the confidence interval was set at 95%
Results	**Uterus:**	
	Weight (g)	Trend towards an increase in uterin weight was observed om animals treated with RCE however no statistically significant difference with OVX control. For more details, see Figure 2 in the original publication.

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	All animals had a similar age and a similar weight at the beginning of the study.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	No detailed information about the cages used, but thediet used was soybean oil free.
	Were experimental conditions identical across study groups?	+	All animals were housed similarly

Bias domain	Question	Score	Judgement
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Two rats of the high dose red clover group were euthanized due to gavage failure.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	The study was most probably not performed blinded
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Pan et al., 1999 – Ref ID: 12095

Animal model	**Species**	Rats	
	Strain (source)	Sprague-Dawley (Harlan)	
	Number of animals	15 young adult rats and 15 retired breeders	
	Age (weight):	2-3 months old (180-200 g) 8-10 months old (300-360 g)	
	Diet:	casein/lactalbumin-based diet	
	Dosing method:	Dietary administration	
	Funding source	Supported by a grant from Protein Technologieq Interndtional. Inc (St Louis, MO) and Grant P01-HL45666 from the National Heart, Lung, and Blood Institute (Bethesda, MD)	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	Young OVX: casein/lactalbumin-based diet, 25 g/day	
		Old OVX: casein/lactalbumin-based diet, 40 g/day	
		Young SPI: control diet with isoflavone equivalent to a woman's dose of 150 mg/day	
		Old SPI: control diet with isoflavone equivalent to a woman's dose of 150 mg/day	
		Young E2: control diet with E2 equivalent to a woman's dose of 2mg/day	
		Old E2: control diet with E2 equivalent to a woman's dose of 2mg/day	
	Start of intervention since OVX	3 days	
	Duration	8 weeks	
Statistical analysis	**Statistical analysis**	Between-group comparisons were done adjusting for multiple comparison by the Student-Newman-Keuls multiple range test. Because there were differences in the effects of treatment on some of the variables in the two age groups, the data were analysed separately for the young animals and retired breeders.	
Results	**Uterus:**		
	Weight (g)	Young OVX	0.1 06± 0.007
		Old OVX	0.1 56 ± 0.010
		Young SPI	0.106 + 0.012
		Old SPI	0.149 ± 0.006
		Young E2	0.383 [a] ± 0.01
		Old E2	0.428[a] ± 0.021
		a: significantly different from other groups	
Risk of Bias Appraisal			**Tier: 1**

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	no sham control group was used without comment (potentially problematic for CNS effects) soy-free basal diet was used
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Phrakonham et al., 2007 – Ref ID: 16809

Animal model	**Species**	Rats
	Strain (source)	Wistar rats supplied by INRA Clermont-Ferrand/Theix (St Gènes-Champanelle, France)
	Number of animals	Total: 40; 10 animals/group
	Age (weight):	105-day old
	Diet:	Soybean-protein free powdered semi-purified diet (INRA, Jouy-en-Josas, France)
	Dosing method:	Dietary administration
	Funding source	This work supported by grants from the Conseil Régional de Bourgogne and the Institut National de la Recherche Agronomique (France).
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	OVX control: control diet
		GEN-10: genistein, 10 mg/kg bw/day
		DAI-10: daidzein, 10 mg/kg bw/day
		EQ-10: racemic equol, 10 mg/kg bw/day
	Start of intervention since OVX	Immediately after surgery
	Duration	8 months
Statistical analysis	**Statistical analysis**	Data expressed as means ± SD. Differences between groups were analyzed by ANOVA followed by Newman–Keul's multiple comparison test or by the non-parametric Kruskall–Wallis test followed by the Mann–Whitney U-test when appropriate. Correlation studies between gene expressions and uterotrophic parameters were performed using Pearson's test. Significance was considered at $P < 0.05$

Results	**Uterus:**		
	Histopathology	OVX control	The parameters measured were uterine wall thickness; uterus section surface; vaginal wall thickness; vaginal epithelium thickness; % of cornified cells)
		GEN-10	None of the measured parameters was different from OVX control.
		DAI-10	None of the measured parameters was different from OVX control.
		EQ-10	Uterine wall thickness and uterus section surface singinficantly greater than control.
	Relative weight (mg/100 g bw)	OVX control	23.1 ± 2.7[b]
		GEN-10	26.1 ± 4.1[ab]
		DAI-10	27.4 ± 4.8[ab]
		EQ-10	30.2 ± 5.9[a]
		a, b: Different letters indicate significantly different values (P < 0.05)	

Risk of Bias Appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age of the animals was similar among the groups. Body weights were lower in the groups maintained on genistein, daidzein or equol.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	A soy protein-free semi purified diet was used. No information about the cages.
	Were experimental conditions identical across study groups?	++	Housing conditions were similar for all the groups.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animalsreported
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information provided. Most probably not blinded.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Rachoń et al., 2007a – Ref ID: 16809

Animal model	**Species**	Rat
	Strain (source)	Sprague–Dawley (from Gottingen's University Hospital)
	Number of animals	Total 80; 11-12/group
	Age (weight):	3 months at OVX (242 g)
	Diet:	soy-free food (Ssniff Spezialdiaten GmbH, Soest, Germany)
	Dosing method:	Dietary administration
	Funding source	European Commission Grants: EURISKED (contract no. EVK1-CT2002-00128) and CASCADE (contract no. FOODCT-2004-506319).

	Authors' conflicts of interest	None declared.	
Dosing	**Intervention**	OVX control: soy-free diet	
		E2-low: estradiol-3 benzoate, 4.3 mg/kg chow (actual consumption 0.07 mg/animal/day)	
		E2-high: estradiol-3 benzoate, 17.3 mg/kg chow (actual consumption 0.20 mg/animal/day)	
		DAI-low: daidzein, 250 mg/kg chow (actual consumption 4.47 mg/animal/day)	
		DAI-high: daidzein, 1 000 mg/kg chow (actual consumption 15.84 mg/animal/day)	
		PUE-low: puerarin, 600 mg/kg chow (actual consumption 10.49 mg/animal/day)	
		PUE-high: puerarin, 3 000 mg/kg chow (actual consumption 48.24 mg/animal/day)	
	Start of intervention since OVX	not specified	
	Duration	3 months	
Statistical analysis	**Statistical analysis**	Data presented as arithmetic means ± S.E.M. Relative changes of mRNA levels were analyzed in the real-time PCR experiments. The mean value of the absolute data measured in the control group was set 100% and all other values determined in the respective assay were expressed in relation to this average value. One-way ANOVA followed by Dunnett's post hoc test for multiple comparisons was performed to compare the differences between the studied groups. P values <0.05 were considered statistically significant.	
Results	**Uterus:**		
	Weight (mg)	OVX control	79.4 ± 2.4
		E2-low	503.1 ± 17.8[a]
		E2-high	537.5 ± 20.5[a]
		DAI-low	78.4 ± 2.4
		DAI-high	98.0 ± 4.0[a]
		PUE-low	80.3 ± 4.0
		PUE-high	138 ± 9[a]
		a: P < 0.05 with one way ANOVA followed by Dunnett's post hoc test	
	Uterine weight/body weight ratio (%)	OVX control	0.024 ± 0.004
		E2-low	0.184 ± 0.028[a]
		E2-high	0.233 ± 0.032[a]
		DAI-low	0.023 ± 0.003
		DAI-high	0.030 ± 0.005[a]
		PUE-low	0.024 ± 0.003
		PUE-high	0.047 ± 0.010[a]
		a: P < 0.05 with one way ANOVA followed by Dunnet's post hoc test	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	-	The rats were maintained on a soy-free diet. The makrolon plastic cages may contain Bisphenol A. There was no sham-operated control and no vehicle controls.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	
	Were experimental conditions identical across study groups?	++	Housing conditions were similar among the groups.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	No loss of animals reported. The number of animals/group was adequate.

Bias domain	Question	Score	Judgement
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Analyses were most probably not performed blinded. Insufficient information provided.
Information/ detection	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Rachoń et al., 2007b – Ref ID: 15876

Animal model	**Species**	Rat
	Strain (source)	Sprague–Dawley (from Gottingen's University Clinic)
	Number of animals	Total 60; 12/group
	Age (weight):	3 months at OVX (244 g)
	Diet:	soy-free food (Ssniff Spezialdiaten GmbH, Soest, Germany)
	Dosing method:	Dietary administration
	Funding source	European Commission Grants: EURISKED (contract no. EVK1-CT2002-00128) and CASCADE (contract no. FOODCT-2004-506319).
	Authors' conflicts of interest	None declared.
Dosing	**Intervention**	OVX control: soy-free diet
		E2-low: estradiol-3 benzoate, 4.3 mg/kg chow (actual consumption 0.07 mg/animal/day)
		E2-high: estradiol-3 benzoate, 17.3 mg/kg chow (actual consumption 0.20 mg/animal/day)
		EQ-low: daidzein, 50 mg/kg chow (actual consumption 0.92 mg/animal/day)
		EQ-high: daidzein, 400 mg/kg chow (actual consumption 6.54 mg/animal/day)
	Start of intervention since OVX	not specified
	Duration	3 months
Statistical analysis	**Statistical analysis**	Data presented as arithmetic means ± S.E.M. Relative changes of mRNA levels were analyzed in the real-time PCR experiments. The mean value of the absolute data measured in the control group was set 100% and all other values determined in the respective assay were expressed in relation to this average value. One-way ANOVA followed by Dunnett's post hoc test for multiple comparisons was performed to compare the differences between the studied groups. P values <0.05 were considered statistically significant.

Results	**Uterus:**		
	Histological analysis	OVX control	Endometrium composed of low cuboidal epithelium lining the uterine lumen and uterine stroma composed of unresponsive stromal cells.
		E2-low	Not different from OVX control
		E2-high	In 6/12 animals the endometrial epithelium was composed of tall columnar cells which presented increased mitotic activity and some differentiation in the stroma.
		EQ-low:	Endometrium composed of tall, pseudostratified columnar cells with high mitotic activity. Eosinophilic and neutrophilic infiltration of the endometrium was seen in 1/12 animals.
		EQ-high	Endometrium composed of tall, pseudostratified columnar cells with high mitotic activity. Signs of squamous metaplasia were also present and uterine stroma was composed of well-differentiated, responsive stromal cells. Eosinophilic and neutrophilic infiltration of the endometrium was seen in 3/12 animals.
	Morphometric analysis	OVX control	The measured parameters (epithelial height, thickness of the stroma, thickness of the myometrium) did not differ from the EQ-low group
		EQ-low	Same as OVX control
		EQ-high	The parameters measured were slightly, but significantly increased compared to OVX control.
		E2-low	The parameters measured were significantly increased compared to OVX control.
		E2-high	The parameters measured were significantly increased compared to OVX control.
	% PCNA positive cells (uterine stroma)	Animals treated with EQ-high had a signifcantly higher percentage (~ 3.5%) of PCNA-positive cells in the uterine stroma compared to controls (~ 2.5%, same as EQ-low).. In both groups treated with E2 the percentages were also significantly increased compared to OVX control (~ 3.5% and ~ 4.5% for E2-low and E2-high, respectively). Values extrapolated from Figure 2a in the original publication.	
	% PCNA positive cells (uterine epithelium)	Animals treated with EQ-high had a signifcantly lower percentage (~ 5 %) of PCNA-positive cells in the uterine epithelium compared to controls (~ 7%, same as EQ-low). In both groups treated with E2 the percentages were also significantly decreased compared to OVX control (~ 3% and ~ 2.5% for E2-low and E2-high, respectively). Values extrapolated from Figure 2b in the original publication.	
	Weight (mg)	OVX control	79.5 ± 8.1
		E2-low	503.0 ± 61.6[a]
		E2-high	538.0 ± 71.0[a]
		EQ-low:	77.6 ± 9.5
		EQ-high	107.0 ± 21.1[a]
		a: P < 0.05 with one way ANOVA followed by Dunnett's post hoc test	
	Uterine weight/body weight ratio (%)	OVX control	0.023 ± 0.005
		E2-low	0.185 ± 0.028[a]
		E2-high	0.232 ± 0.031[a]
		EQ-low	0.021 ± 0.003
		EQ-high	0.034 ± 0.008[a]
		a: P < 0.05 with one way ANOVA followed by Dunnet's post hoc test	
Risk of Bias Appraisal			**Tier: 1**

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No sham-operated control
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	soy-free basal diet used
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Rachoń et al., 2008 – Ref ID: 643

Animal model	**Species**	Rat
	Strain (source)	Sprague–Dawley (from Gottingen's University Clinic)
	Number of animals	Total 60; 12/group
	Age (weight):	3 months at OVX (244 g)
	Diet:	soy-free food (Ssniff Spezialdiaten GmbH, Soest, Germany)
	Dosing method:	Dietary administration
	Funding source	European Commission Grants: EURISKED (contract no. EVK1-CT2002-00128) and CASCADE (contract no. FOODCT-2004-506319).
	Authors' conflicts of interest	None declared.
Dosing	**Intervention**	OVX control: soy-free diet
		E2-low: estradiol-3 benzoate, 4.3 mg/kg chow (actual consumption 0.07 mg/animal/day)
		E2-high: estradiol-3 benzoate, 17.3 mg/kg chow (actual consumption 0.20 mg/animal/day)
		EQ-low: daidzein, 50 mg/kg chow (actual consumption 0.92 mg/animal/day)
		EQ-high: daidzein, 400 mg/kg chow (actual consumption 6.54 mg/animal/day)
	Start of intervention since OVX	not specified
	Duration	3 months
Statistical analysis	**Statistical analysis**	Data presented as arithmetic means ± S.E.M. One-way ANOVA followed by Dunnett's post hoc test for multiple comparisons was performed to compare the differences between treatment groups with controls. P values <0.05 were considered statistically significant. The study had at least 80% power to detect differences in the studied parameters of 1 SD between values from the treatment groups and control at the two-tailed P less than 0.05 level.

Results	**Mammary gland:**		
	Histological analysis	OVX control	Terminal ducts, n: 48 ± 2
			Type I lobules, n: 6.5 ± 0.4
			Type II lobules, n: 39 ± 2
		EQ-low:	Terminal ducts, n: 51 ± 4
			Type I lobules, n: 6.5 ± 0.4
			Type II lobules, n: 41 ± 3
		EQ-high	Terminal ducts, n: 63 ± 3[a]
			Type I lobules, n: 7.0 ± 0.4
			Type II lobules, n: 52 ± 3[a]
		E2-low	Terminal ducts, n: 78 ± 3 [a]
			Type I lobules, n: 12.0 ± 0.6[a]
			Type II lobules, n: 56 ± 3[a]
		E2-high	Terminal ducts, n: 96 ± 3[a]
			Type I lobules, n: 21.9 ± 1.0[a]
			Type II lobules, n: 69 ± 6[a]
		a: P < 0.05 with one way ANOVA followed by Dunnet's post hoc test	
	PCNA positive cells (%)	OVX control	Terminal ducts, n: 2.0 ± 0.1
			Type I lobules, n: 2.2± 0.2
			Type II lobules, n: 2.0 ± 0.1
		EQ-low:	Terminal ducts, n: 2.2 ± 0.1
			Type I lobules, n: 2.2± 0.1
			Type II lobules, n: 2.1 ± 0.1
		EQ-high	Terminal ducts, n: 2.6 ± 0.1[a]
			Type I lobules, n: 2.4± 0.1
			Type II lobules, n: 2.7 ± 0.2[a]
		E2-low	Terminal ducts, n: 3.6 ± 0.1[a]
			Type I lobules, n: 4.6± 0.1[a]
			Type II lobules, n: 3.9 ± 0.1[a]
		E2-high	Terminal ducts, n: 5.0 ± 0.1[a]
			Type I lobules, n: 9.3 ± 0.4[a]
			Type II lobules, n: 5.1 ± 0.04[a]
		a: P < 0.05 with one way ANOVA followed by Dunnet's post hoc test	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Food consumption was determined. Body weight was not reported.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Animals were house din Macrolon cages which could contain BPA. They were maintained on a soy-free diet.
	Were experimental conditions identical across study groups?	++	Housing conditions were similar among the groups.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported.

Bias domain	Question	Score	Judgement
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	No information about blinding. The mammary gland histology is a subjective measure and the differences between the groups were very small.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	The analyses are rather subjective, although consistently among the groups. Also the PCNA determinations were determined by counting the positive cells by light microscopy without blinding of the slides.
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Rimoldi et al., 2007 – Ref ID: 4863

Animal model	**Species**	rats
	Strain (source)	Sprague-Dawley
	Number of animals	11/12 animals group
	Age (weight):	3 months
	Diet:	Soy-free food during and after breeding, after OVX soy-free pelleted chow supplemented with potato proteins (D-59494; Ssniff Spezialdiäten GmbH, Soest, Germany).
	Dosing method:	Dietary administration
	Funding source	Partly by the European Union (EURISKED contract EVK1-CT-2002-00128 and CASCADE contract Food-CT-2004- 506319).
	Authors' conflicts of interest	None declared.
Dosing	**Intervention**	OVX control: same diet as above
		GEN-low: genistein, 5.4 mg/kg bw/day
		GEN-high: genistein 53 mg/kg bw/day
		E2-low: estradiolbenzoate 0.17 mg/kg bw/day
		E2-high: estradiolbenzoate 0.7 mg/kg bw/day
	Start of intervention since OVX	Not reported
	Duration	3 months
Statistical analysis	**Statistical analysis**	The number of PCNA and PR positive cells per slide in relation to the total number of alveolar and terminal bud epithelial cells was set by the authors. The percentages of related values were used to calculate means. Similarly, the authors used individual uterine weights to calculate means and to perform statistical analysis of the data by analysis of variance followed by Newman-Keuls post hoc test. Morphologic features were statistically analysed with contingency tables compared with controls.
Results	**Mammary gland:**	
	Immunostaining for PCNA (% PCNA-positive epithelial cells)	Percentage of PCNA-positive epithelial cells was significantly increased in both GEN groups (~ 2.5% and ~3% for GEN-low and GEN-high, respectively) versus control (~ 1.5%). The effect was lower than the one observed with E2 (~4% and ~7% for E2-low and E2-high, respectively). Values extrapolated from Figure 6 C in the original publication.

	Histology: Luminal formation Secretion	OVX control	Absent: 6/12 Incipient: 6/12 Clear: 0/12
		GEN-low	Absent: 6/12 Incipient: 6/12 Clear: 0/12
		GEN-high	Absent: 6/12 Incipient: 6/12 Clear: 0/12
		E2-low	Absent: 0/12 Incipient: 0/12 Clear: 11/11*
		E2-high	Absent: 0/12 Incipient: 0/12 Clear: 11/11*
	Secretion	OVX control	0/12
		GEN-low	0/12
		GEN-high	0/12
		E2-low	0/11
		E2-high	9/11*
		* $p < 0.05$ compared with control.	
	Uterus:		
	Histology:	OVX control	Hyperplastic/hypertrophic glands: 0/12 Squamous metaplasia: 0/12 Cystic glands:0/12 Pyometra: 0/12
		GEN-low	Hyperplastic/hypertrophic glands: 0/12 Squamous metaplasia:0/12 Cystic glands:0/12 Pyometra:0/12
		GEN-high	Hyperplastic/hypertrophic glands:0/12 Squamous metaplasia:0/12 Cystic glands:0/12 Pyometra:0/12
		E2-low	Hyperplastic/hypertrophic glands: 6/11 Squamous metaplasia:0/12 Cystic glands: 0/12 Pyometra: 0/12
		E2-high	Hyperplastic/hypertrophic glands:10/10 Squamous metaplasia:9/11 Cystic glands: 4/10 Pyometra: 5/11
	Uterine wet weight (mg)	OVX control	91.6
		GEN-low	87.92
		GEN-high	196.8*
		E2-low	478.4*
		E2-high	548.5*
		* $p < 0.05$ compared with control.	
Risk of Bias Appraisal			**Tier: 1**

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	The E2 low and GEN high animals gained less weight and the E2 high gained no weight as compared to the initial body weight. The GEN high group gained less weight than the controls. The difference with the controls was much more pronounced in the groups treated with E2 low and E2 high.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	The control basal diet was soy-free. The type of cages were not specified.
	Were experimental conditions identical across study groups?	+	The housing conditions of the animals of all groups was similar.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals during the study were reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	The study was not performed blinded.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	All analyses during the study were assessed under similar conditions.
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Santell et al., 1996 – Ref ID: 15958

Animal model	**Species**	rats
	Strain (source)	Sprague Dawley (Harlan, Indianapolis)
	Number of animals	Experiment 1: 42 animals (6/group) + 8 intact + 6 OVX control Experiment 2:
	Age (weight):	56 days
	Diet:	AIN-76 or AIN-93G
	Dosing method:	Dietary administration
	Funding source	Not reported
	Authors' conflicts of interest	Not reported
Dosing	**Intervention Experiment 1**	INTACT control: modified AIN-76 diet
		OVX Control: modified AIN-76 diet
		GEN-150: 150 µg/g genistein
		GEN-375: 375 µg/g genistein
		GEN-750: 750 µg/g genistein
		E2-0.5: 0.5 µg/g estradiol
		E2-1.0: 1.0 µg/g estradiol
		E2-1.5: 1.5 µg/g estradiol
	Intervention Experiment 2	Baseline control: killed before treatment(n=10):
		OVX Control: modified AIN-76 diet
		GEN-750: genistein, 750 µg/g
		E2-1.0: 1.0 µg/g estradiol
		E2-1.0+GEN-150: 1.0 µg/g estradiol +150 µg/g genistein
		E2-1.0+GEN-375: 1.0 µg/g estradiol + 375 µg/g genistein
		E2-1.0+GEN-750: 1.0 µg/g estradiol + 750 µg/g genistein
	Start of intervention since OVX	14 days

	Duration	Experiment 1: 5 days		
		Experiment 2: 21 days		
Statistical analysis	**Statistical analysis**	Fur uterine weight: ANOVA performed on log-transformed data followed by multiple means comparison using the least significant difference method. For mammatrophic effects: data were ranked and analyzed with Kruskal-Wallis nonparametric ANOVA. Raw mean and rank mean scores are included in the table. When a significant treatment effect was found, the rank means were compared with the least significant difference method.		
Results	**Mammary gland:**			
	Histology: Mean rank score for lobulo alveolar(Lob/av) and ductal structure (Duct)	Baseline	35.65^{c}	34.35^{c}
		OVX control	11.75^{ab}	22.83^{abc}
		GEN-750	34.42^{c}	27.08^{bc}
		E2-1.0	7.83^{a}	11.83^{a}
		E2-1.0+GEN-150	16.58^{ab}	13.42^{a}
		E2-1.0+GEN-375	18.75^{b}	19.17^{ab}
		E2-1.0+GEN-750	31.25^{c}	28.58^{bc}
		Values in a column with different superscripts are significantly different ($p < 0.05$)		
	Uterus:			
	Experiment 1: Uterine weight (mg) Wet/Dry	INTACT control	386.6 ± 41^{e}	73.7 ± 6.5^{d}
		OVX Control	76.5 ± 3.2^{a}	20.7 ± 1.1^{a}
		GEN-150	92.4 ± 2.6^{a}	28.0 ± 2.8^{b}
		GEN-375	135.6 ± 9.8^{b}	30.0 ± 3.6^{bc}
		GEN-750	189.3 ± 26.6^{c}	39.0 ± 5.6^{bc}
		E2-0.5	122.1 ± 6.6^{b}	17.6 ± 1.7^{a}
		E2-1.0	194.8 ± 8.8^{c}	40.4 ± 6.0^{c}
		E2-1.5	255.0 ± 8.8^{d}	54.0 ± 2.2^{d}
		Values in a column with different superscripts are significantly different ($p < 0.05$)		
	Experiment 2: Uterine weight (mg) Wet/Dry	Baseline	130.7 ± 5.5^{b}	
		OVX control	96.5 ± 3.9^{a}	
		GEN-750	343.6 ± 24.3^{d}	
		E2-1.0	220.1 ± 17.3^{c}	
		E2-1.0+GEN-150	241.3 ± 24.9^{c}	
		E2-1.0+GEN-375	312.4 ± 13.4^{d}	
		E2-1.0+GEN-750	305.6 ± 24.3^{d}	
		Values in a column with different superscripts are significantly different ($p < 0.05$)		

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	serum concentrations of total and aglycone genistein reported
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	soy-free basal diet used E2 positive control group included Gen + E2 group used to test antagonism sham control not used
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	

Bias domain	Question	Score	Judgement
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Scott et al., 2008 – Ref ID: 4730

Animal model	**Species**	Monkey	
	Strain (source)	Cynomolgus macaques (*Macaca fascicularis*) from the Institut Bongor, Indonesia	
	Number of animals	Total 161; 15/group	
	Age (weight)	Adult, age not further specified	
	Diet:	Base diet with casein as protein source for OVX control and E2 and a soy protein isolate for SPI and SPI + E2 groups.	
	Dosing method:	Dietary administration	
	Funding source	National Cancer Institute, NIH, under contract NO1-CO-12400, National Center for Complementary and Alternative Medicine, NIH RO1 AT00639	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	SPI-: control, soy protein isolate depleted of isoflavones (n=26 without prior use of oral contraceptive, OC-; n=26 with prior use of oral contraceptive, OC+)	
		SPI+: dose equivalent to 129 mg/day for women on energy basis (n=28 without prior use of oral contraceptive, OC-; n=27 with prior use of oral contraceptive, OC+)	
		CEE: alcohol washed soy protein isolate plus conjugated equine oestrogens (CEE, Premarin) at a dose comparable to 0.625 mg/day for women on energy basis (n=29 without prior use of oral contraceptive, OC-; n=25 with prior use of oral contraceptive, OC+)	
	Start of intervention since OVX	Not reported	
	Duration	3 years	
Statistical analysis	**Statistical analysis**	Statistical significance was determined using factorial two-way ANOVA by treatment for log-transformed variables in which oestrogen metabolite concentrations could be detected. The analyses included all treatment groups and effects of past exposure to oral contraceptives was tested for interaction in order to exclude it as a potential confounding effect on the endpoints examined.	
Results	**Mammary gland:**		
	Mammary expression of Ki-67 mRNA (geometric means with 95% CI)	SPI-	0.019 (0.014-0.026)
		SPI+	0.011 (0.008-0.015)[a]
		CEE	0.041 (0.030-0.055)[b]
		a: significantly decreased (P < 0.01) versus SPI- group b: significantly increased (P < 0.0001) versus SPI- group.	
	Lobular area (%)	SPI-	0.64 (0.49-0.84)
		SPI+	0.56 (0.43-0.73)
		CEE	1.64 (1.26-2.14)[a]
		a: significantly increased (P < 0.0001) versus SPI- group.	
	Uterus:		
	Average thickness (mm)	SPI-	0.89 (0.78-1.0)
		SPI+	0.83 (0.72-0.94)
		CEE	2.7 (2.4-3.1)[a]
		a: significantly increased (P < 0.0001) versus SPI- group.	
	Epithelial area (%)	SPI-	7.3 (6.5-8.2)
		SPI+	6.9 (6.1-7.7)
		CEE	16.1 (14.3-18.0)[a]
		a: significantly increased (P < 0.0001) versus SPI- group.	

	Glandular area (%)	SPI-	9.3 (8.1-10.5)
		SPI+	8.7 (7.7-9.9)
		CEE	28.4 (15.0-32.2)
		a: significantly increased (P < 0.0001) versus SPI- group.	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Body weights were not recorded.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	The control diets were soy protein/isoflavone free.
	Were experimental conditions identical across study groups?	+	Housing conditions of the animals were comparable.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	All measurements were made blinded to treatment group.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	+	Effect of isoflavone diet was only reported in a table. Not all the findings have been discussed in detail.
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Su et al., 2013 – Ref ID: 12777

Animal model	**Species**	Rats
	Strain (source)	Sprague-Dawley (SD) rats
	Number of animals	Total: 40.; bilaterally ovariectomized (n = 30)
	Age (weight) at ovariectomy:	3 months (280–300 g)
	Diet:	Not specified
	Dosing method:	daily oral administration
	Funding source	Research grant DOH94-TD-1010 from the Department of Health, Executive Yuan, and NSC 96-2320-B-242-004 and NSC 97-2320-B-242-004 from the National Science Council, Executive Yuan, Taiwan
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	Sham-operated (n = 10): control diet
		Control: OVX, control diet
		BCA: OVX, Biochanin A, 25 mg/kg bw/Day
		E2: intraperitoneal injection of E2 (23 μg/kg bw/day) (Sigma-Aldrich) on 3 consecutive days per week
	Start of intervention since OVX	Not reported
	Duration	14 weeks

Statistical analysis	**Statistical analysis**	The results of the in vivo and in vitro data are presented as the mean ± standard deviation (SD). Differences among the groups (Sham, OVX, OVX+E2, and OVX+BCA) were analyzed statistically using one-way analysis of variance (ANOVA), followed by Fisher's test. A P value of <0.05 was considered statistically significant	
Results	**Uterus:**		
	Weight (g)	SHAM	0.45 ± 2.1[a]
		OVX	0.27 ± 2.6
		OVX + E2	0.41 ± 3.4[a]
		OVX + BCA	0.29 ± 2.1
		a: P < 0.05, when compared with the OVX group.	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Sham control used. Diet not specified so basal level of soy isoflavone exposure is likely
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	

Tansey et al., 1998 – Ref ID: 2803

Animal model	**Species**	Rats
	Strain (source)	Harlan Sprague-Dawley (Charles River Laboratories, Raleigh, NC)
	Number of animals	Total: 127; 8-12/group
	Age (weight):	40-day old, OVX after adjustment period
	Diet:	Casein diet or soy protein diet (low SBE, equivalent to 11.6 mg isoflavones/1800 cal or High-SBE diet equivalent to 117.8 mg isoflavones /1800 cal)
	Dosing method:	Dietary administration
	Funding source	BSGM Venture Grant, NIH Training grant #RR07009-19 and by an Administrative Supplement from the Office of Research on Women's Health to NCRR Grant RR08562
	Authors' conflicts of interest	Not reported

Dosing	**Intervention**	SHAM: Intact, control diet (n=8)
		SHAM: Intact, Low-SBE (n=10)
		SHAM: Intact, High-SBE (n=10)
		OVX control: casein diet (n=11)
		Low-SBE: OVX, 11.6 mg isoflavones/1800 cal (n=11)
		High-SBE: OVX, 117.8 mg isoflavones/1800 cal (n=11)
		Low-E2: OVX, CEE (Premarin) at 0.313 mg CEE/1800 cal (n=11)
		High-E2: OVX, CEE (Premarin) at 0.625 mg CEE/1800 cal (n=12)
		Low-SBE+Low-E2: OVX, isoflavones and CEE (n=11)
		Low-SBE+High-E2: OVX, isoflavones and CEE (n=11)
		High-SBE+Low-E2: OVX, isoflavones and CEE (n=11)
		High-SBE+High-E2: OVX, isoflavones and CEE (n=11)
	Start of intervention since OVX	Immediately after
	Duration	2 months
Statistical analysis	**Statistical analysis**	ANOVA, P < 0.05 as level of significance.
Results	**Uterus:**	
	Histological findings: Luminal epithelial height (microns)	Low-SBE and High-SBE were not significantly different from OVX control. Addition of E2 resulted in an increased luminal epithelial height. For more details see Figure 6 in the original publication.
	PCNA staining of endometrial surface cells and glands	Both OVX control and High-SBE showed low levels of proliferation whereas addition of E2 to the three diets resulted in a significant increase of proliferation. For more details see Figure 3 in the original publication.
	Relative wet weight (mg/g body weight)	Animals in the Low-SBE and high-SBE groups were no different from OVX control fed casein diet. Addition of E2 to the diets, at both doses significantly increased uterine weight. For more details see Figure 2B in the original publication.

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Blinding was not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Teixeira et al., 2014 – Ref ID:16112

Animal model	**Species**	Rats	
	Strain (source)	Wistar	
	Number of animals:	5/group	
	Age (weight):	3-month (231 g)	
	Diet:	Not reported	
	Dosing method:	gavage	
	Funding source	FAPESP 2011/1898-0 and 2011/11900-0.	
	Authors' conflicts of interest	None reported	
Dosing	**Intervention**	SHAM: vehicle (propylene glycol)	
		OVX-control: vehicle	
		IF-50: OVX, soy extract equivalent to 50 mg genistein/kg bw/day.	
		E2: OVX, 10 µg/kg/day diethylstilbestrol (dissolved in 0.1 ml corn oil and was administered via s.c. injection behind the neck cuff)	
	Start of intervention since OVX	7 days	
	Duration	30 days	
Statistical analysis	**Statistical analysis**	The groups were compared using one-way analysis of variance (ANOVA), followed by the Tukey – Kramer test. A p value < 0.05 was considered statistically significant.	
Results	**Uterus: histomorphometry**		
	Endometrial thickness (µm; mean ±SEM)	SHAM	654.1 ± 96.3[a]
		OVX control	400.7 ± 33.8
		IF-50	380.3 ± 21.4
		E2	584.7 ± 63.9 [a]
	Miometrial thickness (µm; mean ±SEM)	SHAM	281.1 ± 30.8 [a]
		OVX control	400.7 ± 33.8[b]
		IF-50	380.3 ± 21.4
		E2	584.7 ± 63.9
	Glandular area (mm^2; mean ±SEM)	SHAM	72.5 ± 28.4 [a]
		OVX control	26.6 ± 3.8
		IF-50	24.9 ± 6.8
		E2	69.4 ± 23.0 [a]
		a: SHAM and E2 > OVX control and IF-50 b: E2 > SHAM > OVX control and IF-50	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age and body wt of the rats were similar amon the groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	The basal diet was not minimized in phytoestrogens. Type of cages was not reported. Sham-operated control, but no information about vehicle treatment of the controls.
	Were experimental conditions identical across study groups?	++	Housing conditions of the rats was similar among the groups.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported.

Bias domain	Question	Score	Judgement
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information provided.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Uesugi et al., 2001 – Ref ID: 14256

Animal model	**Species**	Rats	
	Strain (source)	Sprague–Dawley (conventional) from Japan SLC (Hamamatsu).	
	Number of animals	5-6/group	
	Age (weight):	10 weeks at OVX ()	
	Diet:	Commercial food (Casein diet, CLEA Japan, Inc., Tokyo) containing 0.8% Ca and 0.7% P 1 week after OVX: commercial calcium-deficient diet (powder) containing 0.004% Ca and 0.3% P (Diet 11- Ca, Japan CLEA, Tokyo), soy-free	
	Dosing method:	gavage	
	Funding source	Not reported	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	SHAM: intact ovaries, sham-operated, vehicle (water containing 1% hydroxypropyl cellulose)	
		OVX control: vehicle	
		GEN-50: genistin, 50 mg/kg bw/day	
		GEN-100: genistin, 1000 mg/kg bw/day	
		DAI-25: daidzin, 25 mg/kg bw/day	
		DAI-50: daidzin, 50 mg/kg bw/day	
		GLY-25: glycitin, 25 mg/kg bw/day	
		GLY-50: glycitin, 50 mg/kg bw/day	
		E2: estrone, 7.5 µg/kg/d in sesame oil, s.c.	
	Start of intervention since OVX	Immediately after	
	Duration	4 weeks	
Statistical analysis	**Statistical analysis**	Data expressed as means and S.E.M. for each of the groups and compared using analysis of variance and Student's t-test or Dunnet's multiple range test. p values of less than 0.05 were considered to indicate significant differences.	
Results	**Uterus:**		
	Relative weight (mg/100 bw)	SHAM	172.0 ± 77
		OVX	41.6 ± 2.0[a]
		GEN-50	42.0 ± 6.9
		GEN-100	71.9 ± 9.2[b]
		DAI-25	48.0 ± 9.3[a]
		DAI-50	73.1 ± 7.7[b]
		GLY-25	42.3 ± 9.0
		GLY-50	73.0 ± 7.9[b]
		E2	128.3 ± 7.7[c]
		a: $p < 0.01$ compared with SHAM b: $p < 0.01$ compared with OVX control c: $p < 0.001$ compared with OVX control	
Risk of Bias Appraisal			**Tier: 1**

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Casein diet used; sham controls used
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Vera et al., 2006 – Ref ID: 10284

Animal model	**Species**	Rat
	Strain (source)	SHR (Harlan Laboratories, Barcelona, Spain)
	Number of animals	8/group
	Age (weight):	23-week
	Diet:	soy-free chow(AIN 76)
	Dosing method:	gavage
	Funding source	This work was supported by grants from Comisión Interministerial de Ciencia y Tecnología SAF 2001-2953, SAF2004-06762, and AGL2004-06685-C04-1/ALI (Acción Movilizadora de Alimentos Funcionales). This work was funded in part by the Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III (Red HERACLES RD06/0009)
	Authors' conflicts of interest	None reported
Dosing	**Intervention**	SHAM: vehicle for genistein, 1% w/v methylcellulose
		OVX control: vehicle,
		GEN-10: genistein, 10 mg/kg bw/day
		E2: 17β-oestradiol, 2 mg/kg/wk, s.c.
	Start of intervention since OVX	3 weeks
	Duration	35 days
Statistical analysis	**Statistical analysis**	Values are expressed as mean ± SEM of 8 rats. Statistical analysis was performed by a one-way analysis of variance followed by a Newman-Keuls test. Because most of the parameters analyzed could not be measured at baseline (except body weight and blood pressure, which were not statistically significant among groups), all statistical comparisons were performed at the end of the study period. $P < 0.05$ was considered statistically significant.

Results	**Uterus:**		
	Weight (mg)	SHAM	234 ± 11
		OVX-control	54 ± 3[a]
		GEN	57 ± 3[a]
		E2	170 ± 13[ab]
		a: P < 0.05 compared with SHAM b: P < 0.05 compared with OVX placebo	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age and wt of the animals was similar among the groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	The animals were maintained on a soy-free chow diet (AIN-76). Controls were treated with vehicle
	Were experimental conditions identical across study groups?	++	Housing conditions were similar among the groups.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	N loss of animals was reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information provided.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Wang et al., 2003 – Ref ID: 11449

Animal model	**Species**	mice
	Strain (source)	ddY strain from Shizuoka (Shizuoka, Japan)
	Number of animals	8/group
	Age (weight):	Eight-week
	Diet:	AIN-93G formulation with corn oil instead of soybean oil
	Dosing method:	Dietary administration
	Funding source	Work supported by a Science and Technology Agency Fellowship Award (Japan), and by Grant-in-Aid 13680175 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	SHAM: sham-operated, control diet
		OVX control: OVX, control diet
		PR5: OVX, 5% PR diet
		PR10: OVX, 10% PR diet
		PR20: OVX, 20% PR diet
		E2: OVX, 17β-estradiol, 0.03μg/day injected s.c. using a miniosmotic pump
	Start of intervention since OVX	immediately
	Duration	28 days

Statistical analysis	**Statistical analysis**	Data presented as means ± SEM. The significance of the differences was determined by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. Differences were considered significant at the level of P < 0.05.
Results	**Uterus:**	
	Weight (mg):	No statistically significant difference between OVX control and animals treated with *Pueraria radix*. Uterine weight in animals treated with E2 was not different from SHAM control.

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age and weight of the animals was similar among the groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Control diet was phytoestrogen free (AIN 93A). No information about the cages. Sham-operated control group and OVX control group. Insufficient information about the levels of isoflavones (daizein and genistein).
	Were experimental conditions identical across study groups?	++	Housing conditions were similar among the groups
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Insufficient information about blinding, but for weighing of the uteri, this is not very important since it is not a subjective measure.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Wang et al., 2008 – Ref ID: 1354

Animal model	**Species**	Rat
	Strain (source)	Sprague–Dawley (from the laboratory animal centre of FMMU, Xi'an China)
	Number of animals	Total: 48; 8/group
	Age (weight):	9-week old (235 ± 5.6)
	Diet:	pelleted commercial diet (containing 0.97% calcium, 0.85% phosphorus and 1.05IU/g of Vitamin D3).
	Dosing method:	
	Funding source	No financial support to declare
	Authors' conflicts of interest	None declared
Dosing	**Intervention**	SHAM: sham operation, intact ovaries
		OVX-control: vehicle
		GEN-9: genistein, 9mg/kg bw/day
		NO-GEN-4.5: NO-releasing prodrug of genistein, 4.5 mg/kg bw/day
		NO-GEN-9: NO-releasing prodrug of genistein, 9 mg/kg bw/day
		NO-GEN-18: NO-releasing prodrug of genistein, 18 mg/kg bw/day

	Start of intervention since OVX	1 week
	Duration	12 weeks
Statistical analysis	**Statistical analysis**	Student-t test was used to determine the statistical difference between SH group and OVX group. Statistical differences between OVX group and treatment groups were analyzed using one-way analysis of variance (ANOVA) followed by LSD post hoc test. Statistical significance was set at P <0.05.
Results	**Uterus:**	
	Weight (g)	Uterine weight was significantly increased in the GEN-9 and NO-GEN-4.5 groups (23.4% and 26.6% heavier than OVX control, respectively). No such effect was observed at the two higher doses of NO-GEN. For more details see Figure 5B in the original publication.

Risk of Bias Appraisal		**Tier:**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Types of cages and soy-free diet was not specified
	Were experimental conditions identical across study groups?	+	
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No deaths were reported. All animals were necropsied 12 weeks after start of the treatment
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	The weight of the uteri was most probably not determined blinded, but this will not influence the outcome of the study.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	The weight of the uteri of all animals were determined at the end of the study.
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Wood et al., 2004 – Ref ID: 16271

Animal model	**Species**	Monkey
	Strain (source)	Cynomolgus macaques (*Macaca fascicularis*)
	Number of animals	Total 189; 15/group
	Age (weight)	Adult, age not further specified
	Diet:	Base diet with casein as protein source for OVX control and E2 and a soy protein isolate for SPI and SPI + E2 groups.
	Dosing method:	Dietary administration
	Funding source	Work supported by Program Project Grant HL-45666 from the National Institutes of Health/National Heart, Lung, and Blood Institute, Bethesda, Maryland, NIH/National Center for Complementary and Alternative Medicine R01-AT00639, and NIH/National Center for Research Resources T32 RR 07009.

	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	Control: soy protein isolated depleted of isoflavones	
		SPI: dose equivalent to 129 mg/day for women on energy basis	
		CEE: alcohol washed soy protein isolate plus conjugated equine oestrogens (CEE, Premarin) at a dose comparable to 0.625 mg/day for women on energy basis	
	Start of intervention since OVX	Not reported	
	Duration	3 years	
Statistical analysis	**Statistical analysis**	Means were compared by analysis of variance and Dunnett's test for comparisons with controls	
Results	**Mammary gland:**		
	Ki-67 (% of positive cells) in lobules and ducts	No statistically significant difference between OVX control and SPI group. CEE treated animals had a significant increase in Ki-67 labelling. For more details see Figure 1 of the original publication.	
	Mammary gland thickness (mm), mammary gland epithelial area (% total area)	SPI treated animals had a significant decrease in mammary gland thickness; E2 treated animals had a significant increase in mammary gland epithelial area. For more details see Figure 3 of the original publication.	
	Histopathologic findings	SPI-	Mild to moderate lobular enlargement: 2/57 Small focal papillary ductal hyperplasia: 3/57
		SPI+	Mild to moderate lobular enlargement: 0/60 Small focal papillary ductal hyperplasia: 0/60
		SPI + E2	Mild to moderate lobular enlargement: 31/63 Small focal papillary ductal hyperplasia: 1/63
	Uterus:		
	Ki-67 (% of positive cells) in superficial and basal glands	No statistically significant difference between OVX control and SPI group. E2 treated animals had a significant increase in Ki-67 labelling in both superficial and basal glands. For more details see Figure 2 of the original publication.	
	Endometrial thickness and (mm) and endometrium epithelial gland area (% total area)	No statistically significant difference between OVX control and SPI group. E2 treated animals had a significant increase in endometrial thickness epithelial area. For more details see Figure 3 of the original publication.	
	Histopathological findings: uterine epithelial hyperplasia (grade)	SPI-	Minimal or mild: 57/57 Moderate to marked: 0/57
		SPI+	Minimal or mild: 1/60 Moderate to marked: 0/60
		SPI+ E2	Minimal or mild: 32/62 Moderate to marked: 26/62
Risk of Bias Appraisal			**Tier: 1**

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age was similar among the animals. Body weight was recorded during the study and was similar among the groups.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	The isolated soy proteins used contained on average 1.1 mg genistein; 0.365 mg daidzein an d0.08 mg glycitein/g SPI, whereas the the alcohol extracted soy protein contained 0.04 mg genistein; 0.01 mg daidzein and 0.01 mg glycitein/g isolate (expressed in aglycon units).
	Were experimental conditions identical across study groups?	++	Housing conditions were similar among the groups.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	The pathologists evaluated the slides blinded to treatment groups..
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Wood et al., 2006 – Ref ID: 1714

Animal model	**Species**	Monkey *Macaca fascicularis*
	Strain (source)	All animals were originally imported from the Institut Pertanian Bogor in Bogor, Indonesia.
	Number of animals	30 total 10/group
	Age (weight):	18.4 ± 0.5 years (
	Diet:	Diets provided 0 (Control), 0.297 (Isoflavones), and 0.566 mg (racemic equol) isoflavonoids/kcal and were otherwise identical in macronutrients, cholesterol, calcium, and phosphorus.
	Dosing method:	Dietary intake,
	Funding source	Supported by grants from the National Institutes of Health (NCCAM R01-AT00639, NCRR T32 RR 07009, NCI P30-CA71789) and the American Cancer Society (IRG-93-035-09)
	Authors' conflicts of interest	Not reported.
Dosing	**Intervention**	Control diet
		Isoflavones (IF): 537 mg/ 1800 kcal of isoflavones (genistein, daidzein, glycitein) in a concentrate equivalent to 35.7 mg/kg bw/day
		Equol (EQ): 1020 mg/1800 kcal of purified racemic equol equivalent to 68 mg/kg bw/day
	Start of intervention since OVX	4.5 years.
	Duration	28-33 days

Statistical analysis	**Statistical analysis**	A general linear model was used to determine group means and test for significant group differences following treatment. All measurements were made blinded to treatment group. For uterine measures, endometrial thickness was unmeasurable (no defined lumen) in four samples (3 Con, 1 EQ) and uterine area in 3 samples (1 Con, 1 IF, 1 EQ). For breast measures, lobular epithelium was absent in one sample (Con) and ductal epithelium in nine samples (4 Con, 3 IF, 2 EQ), reducing sample size for these particular endpoints. All variables were evaluated for their distribution and equality of variances between groups, and log10 transformations were performed where appropriate to improve normality and homogeneity of variance. For log-transformed data, reported values were retransformed to the original scale using the inverse log. Data are reported either as mean (± standard error) for untransformed data or mean (90% confidence interval) for retransformed data. Data were analyzed using the SAS statistical package (version 8; SAS Institute, Cary, NC). A two-tailed significance level of 0.05 was chosen for all comparisons	
Results	**Mammary gland:**		
	Histology:	All breast biopsies exhibited diffuse glandular atrophy, and no proliferative lesions were noted.	
	MKi-67 breast epithelium (% of positive cells) – lobules	Control:	7.1 (4.8–10.5)
		IF:	6.3 (4.2–9.1)
		Equol:	2.8 (1.7–4.2)
		Historical control:	2.3 (1.3–3.7)[b]
		Historical E2:	10.7 (7.5–15.3)
	MKi-67 breast epithelium (% of positive cells) – Ducts	Control:	1.9 (0.9–3.3)
		IF:	1.0 (0.5–1.9)
		Equol:	0.9 (0.4–1.6)
		Historical control:	0.1 (0.0–0.5)
		Historical E2:	4.0 (2.7–5.6)[b]
	Uterus:		
	Histology:	Histologic evaluation of uteri revealed diffuse atrophy in all groups, characterized by simple, well-spaced glands, cuboidal epithelium, and densely packed stromal cells. Typical oestrogenic effects would include a thickened endometrium, more columnar epithelial cells, increased glandular complexity, and stromal oedema. No such changes were seen, and no hyperplastic lesions were noted in any of the groups.	
	MKi-67 uterine epithelium (% of positive cells) – Superficial cells	Control:	3.2 (2.2–4.8)[b]
		IF:	2.1 (1.6–2.9)[b]
		Equol:	4.1 (2.8–6.1)
		Historical control:	12.2 (8.0–18.6)
		Historical E2:	39.8 (30.6–51.7)[a,b]
	MKi-67 uterine epithelium (% of positive cells) – Basal cells	Control:	0.0 (0.0–0.3)
		IF:	0.1 (0.0–0.4)
		Equol:	0.0 (0.0–0.3)
		Historical control:	0.3 (0.0–0.8)
		Historical E2:	2.7 (1.9–4.7)[a,b]
	Endometrial thickness (mm):	Control:	1.0 (0.9–1.2)
		IF:	1.0 (0.9–1.1)
		Equol:	0.9 (0.8–1.1)
		Historical control:	0.8 (0.7–0.9)
		Historical E2:	2.1 (1.9–2.4)[a,b]

	Weight (g)	Control:	1.1 (1.0–1.3)[b]
		IF:	1.2 (1.1–1.4)
		Equol:	1.1 (1.0–1.3)[b]
		Historical control:	1.6 (1.4–1.8)
		Historical E2:	4.6 (4.1–5.1)[a,b]
	Uterine weight (% body wt x 10^2)	Control:	3.1 (2.8–3.5)[b]
		IF:	3.4 (3.0–3.8)
		Equol:	3.2 (2.8–3.6)
		Historical control:	4.4 (3.9–5.0)
		Historical E2:	12.6 (11.3–14.0)[a,b]
	a: significantly different from control ($p < 0.05$) b: significantly different from historical control ($p < 0.05$)		

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	The age and body weights of the animals were accounted for.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	It is not specified whether the basal diet was soy-free. However, all groups were maintained on the same basal diet. Moreover, the doses are expressed as aglycone units as calorically scaled human equivalents.
	Were experimental conditions identical across study groups?	++	Housing conditions were for all groups similar.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals during the study.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	The study was most probably not performed blinded, but this will not have influenced the outcome of the study.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	Applicable to all the endpoints considered in this study.
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Wood et al., 2008 – Ref ID: 16269

Animal model	**Species**	Monkey *Macaca fascicularis*
	Strain (source)	All animals were originally imported from the Institut Pertanian Bogor in Bogor, Indonesia.
	Number of animals	Total: 63; 20-22 animals/group
	Age (weight):	21.8 ± 0.5 years
	Diet:	Baseline casein/lactalbumin-based (isoflavone-free).
	Dosing method:	Dietary intake,
	Funding source	Work supported by grants from the National Institutes of Health (NIH) National Center for Research Resources (NCRR) (K01 07009, T32 00019) and National Heart, Lung, and Blood Institute (NHBLI) (P01 45666).

	Authors' conflicts of interest	Not reported.
Dosing	**Intervention**	OVX Control: control diet
		E2: micronized oestradiol at 66 mg/kg bw/day (1.0 mg per 1800 kcal)
		Equol (EQ): racemic equol at 7.2mg/kg bw/day (105 mg per 1800 kcal)
	Start of intervention since OVX	4.5 years
	Duration	8 months
Statistical analysis	**Statistical analysis**	The power calculation for this study was based on breast epithelial proliferation determined by Ki67 labeling. The minimum difference between Control and E2 means was estimated to be 5.0% with a common intragroup s.d. of 4.0%. The sample size in each group providing an 80% chance at a 0.05 significance level to detect a statistically significant difference was 16 after adjusting for multiple comparisons. All data were evaluated for normal distribution and homogeneity of variances among groups. A general linear model was used to determine mean values and calculate group differences for BW, age, serum E2 and EQ, epithelial area, and qRT-PCR expression data. A two-tailed Fisher's exact test was used to evaluate treatment group differences in lesion type prevalence. Five baseline biopsy samples (two Control, one E2, two EQ) lacked epithelial tissue on histology and were thus excluded from analysis. Immunolabeling between normal epithelium and CCLs (CCC and CCH) was evaluated within each treatment group using a nonparametric Kruskal–Wallis test followed by two-sided Wilcoxon rank-sum pairwise analysis; for this comparison, the average values for ductal and lobular cells were used for normal epithelium. All pairwise P-values were adjusted for the number of pairwise tests using a Bonferroni correction. A two-tailed significance level of 0.05 was chosen for all comparisons.
Results	**Mammary gland:**	
	Histology:	Mammary glands from OVX control and EQ animals were diffusely atrophic with small scattered lobular units. In contrast, E2 treatment resulted in increased lobular size on qualitative assessment of whole mounts and greater epithelial density on histomorphometry (P= 0.02 compared to OVX Control).
	Prevalence of Mammary epithelial lesions (% n animals)	Overall prevalence of columnar cell change (CCC) was 41% (26/63), distributed as follows: control, 8; E2, 12; equol: 6. A total of 13 cases of columnar cell hyperplasia (CCH) were found so distributed: control, 3 (1 without atypia); E2, 9 (6 without atypia); EQ, 1 (without atypia). Six cases of atypical ductal hyperplasia (ADH) were identified, distributed as follows: control, 1; E2, 4; EQ, 1. In the E2 group, three out of the four cases exhibited characteristics of DCIS. Three cases of atypical lobular hyperplasia (ALH) were found distributed as follows: control, 2; E2, 1; EQ,0. Prevalence of total CCH lesions and CCH with atypia was higher in E2 animals compared with OVX control. Animals in the EQ group were not different from control. Values extrapolated from Figure 3 in the original publication.
	Ki-67 breast epithelium (% of positive cells) – lobules	Ki-67 was not increased in CCC or CCH lesions compared to normal ductal epithelium. Animals in the EQ group were not different from control group. The % of positive cells was significantly increased in the lobules of the E2 treated group. For more details see Figure 4 in the original publication.
Risk of Bias Appraisal		**Tier: 1**

Bias domain	Question	Score	Judgement
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Baseline values were given and followed in time (body weight and serum E2 and serum EQ).
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	The animals were maintained on a isoflavone-free diet.
	Were experimental conditions identical across study groups?	++	Animals were all housed similarly.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals was reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	++	Histomorphology was performed by digitizing the H&E stained slides using a Labophot 3 light microscope and Infinity 3 digital camera.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Wuttke et al., 2006 – Ref ID: 16299

Animal model	**Species**	Rats
	Strain (source)	Sprague–Dawley
	Number of animals	10-12/group
	Age (weight):	
	Diet:	Soy-free
	Dosing method:	Dietary administration
	Funding source	Work partly funded by the EU-project EURISKED (Grant #EVK1-CT-2002-00128), CASCADE (Grant #Food-CT-2004-506319), (E)UROESTRO(E)S (Grant #QLK6-CT-2000-00565), German Research Society (Grant #Wu 60/12-2) and by Bionorica AG, Neumarkt, Germany
	Authors' conflicts of interest	Not reported
Dosing	**Intervention**	OVX control
		E2: OVX, estradiol 17ß benzoate, daily exposure of 0.19 mg (free base)
		GEN: OVX, genistein 53 mg/day
		CR: OVX, *C. racemosa* extract BNO1055, 133 mg/day of extract
	Start of intervention since OVX	Immediately after
	Duration	3 months

Statistical analysis	**Statistical analysis**	Statistical analysis was done by ANOVA with Dunnett's Multiple Comparison Test against control. For the PCNA expression multiple t-test to compare between groups was used. Significance was considered when $p < 0.05$.
Results	**Mammary gland**	
	Histology	OVX: scarcity of luminal and alveolar structures
		E2: Many more luminal and alveolar structures in all animals. Secretory material in the lumina and acini was observed frequently.
		GEN: Slight increase of luminal and alveolar structures
		CR: No effect compared to OVX control. Acini were scarce and no luminal formation was observed indicating that these mammary glands remained as atrophic as in OVX control.
	PCNA positive cells (% of total cell number)	Percent of PCNA positive cells was significantly increased in GEN group (~ 3%) compared with OVX control (~ 1.5%), but to a lesser extent than E2 (~ 4%). Values extrapolated from Figure 4 of the original publication.
	Uterus	
	Weight (g)	Uterine weight was significantly increased in the GEN group (~ 175 mg) compared with OVX control (~ 100 mg) but to a much less exent than E2 (~ 450 mg). Values extrapolated from Figure 2a of the original publication.

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	No sham-operated controls. The animals were kept under soy-free conditions. No information about body weight of the animals. No further details about the source of genistein.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Animals were housed under soy-free conditions, but no further details about the feed and cages.
	Were experimental conditions identical across study groups?	++	Housing conditions of the animals was similar among the groups.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	Loss of animals was not reported
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Insufficient information provided. Most probably is the study performed without blinding.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	-	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Yamaguchi et al., 2001 – Ref ID: 16313

Animal model	**Species**	Rats
	Strain (source)	Wistar Imamichi from the Institute for Animal Reproduction (Ibaraki)
	Number of animals	6-10/group
	Age (weight):	Not reported (150 – 180 g)
	Diet:	Not specified
	Dosing method:	Dietary administration

	Funding source	Study partly supported by the Sasakawa Scientific Research Grant from The Japan Science Society and the US-Japan Cooperative Research Grant from the Japan Society for Promotion of Science.	
	Authors' conflicts of interest	Not reported	
Dosing	**Intervention**	Group I: Intact rats (oestrus)	
		Group II: OVX control	
		Group III: OVX, 17β-oestradiol dipropionate (EDP) (300 g/kg, s.c.) injected once a week for 3 weeks (totally 4 times in 4 weeks)	
		Group IV: OVX,soy isoflavones (IF): 67 mg/kg bw/day	
		Group V: OVX, soy isoflavones (IF): 157 mg/kg bw/day	
	Start of intervention since OVX	Immediately after	
	Duration	1 month	
Statistical analysis	**Statistical analysis**	Values were expressed as the mean ± S.E.M. When the dose-response curves were compared among the groups, the statistical significance was assessed by an analysis of variance (ANOVA) for repeated measures followed by the Tukey test. The differences were considered significant for P < 0.05.	
Results	**Uterus:**		
	Wet weight (mg/animal)	Intact control:	420 ± 39[a]
		OVX control:	112 ± 23
		E2:	417 ± 37[a]
		IF-67:	130 ± 10
		IF-157:	317 ± 32[a]
		a: significantly different from OVX control	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	The age and body weight of the animals were recorded. The study is designed to study the effects of phytoestrogens on the vasodilating responses of the thoracic aorta. Effect on uterine weight was only determined to control for the OVX and the effects of oestradiol.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	-	Insufficient information provided about the basal diet (phytoestrogen free?), the cages etc. Intact controls were not sham-operated. No vehicle controls for the estradiol treated animals.
	Were experimental conditions identical across study groups?	++	Housing conditions of the rats were similar among the groups.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Insufficient information provided but most probably the study was not performed in a blinded fashion. Most determinations were not subjective.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Yoneda et al., 2011 – Ref ID: 16333

Animal model	**Species**	Rats	
	Strain (source)	Sprague-Dawley	
	Number of animals	Total: 130; 5-15 animals/group	
	Age (weight):	180-270g	
	Diet:	control AIN-93G diet, which contained no soy oil or soy protein	
	Dosing method:	gavage	
	Funding source	None reported	
	Authors' conflicts of interest	None reported	
Dosing	**Intervention**	SHAM: sham operated, intact ovaries, vehicle (1% hydroxypropyl methylcellulose)	
		OVX control: vehicle	
		E2: conjugated equine estrogens (CEE; Premarin), 6 mg/kg bw/day	
		SE5-OH: fermented soy germ product, 2 000 mg/kg bw/day providing *S*-equol at 11.7 mg/kg bw/day	
		S-EQ: purified *S*-equol, at 11.7 mg/kg bw/day	
	Start of intervention since OVX	3 days	
	Duration	38	
Statistical analysis	**Statistical analysis**	All values are presented as mean ± SEM. Significant differences between the control group and treatment groups were analyzed by a one-way analysis of variance with a Dunnett multiple comparison test. Student's unpaired t test was used for comparison of sham and control groups. For all tests, the significance level was accepted at $P < 0.05$.	
Results	**Uterus**		
	Histology	SHAM	Epithelial cells with typical elongated cell bodies and elongated diffuse nuclei. Epithelial cells from rats in the control group were more cuboidal in appearance, with smaller, staining nuclei
		OVX control	Clear evidence of uterine atrophy
		E2	Epithelial cells with typical elongated cell bodies and elongated diffuse nuclei. Epithelial cells from rats in the control group were more cuboidal in appearance, with smaller, staining nuclei
		SE5-OH	Epithelial cells from rats in the SE5-OH and S-equol groups displayed more cuboidal morphology with denser, smaller nuclei
		S-EQ	Epithelial cells from rats in the SE5-OH and S-equol groups displayed more cuboidal morphology with denser, smaller nuclei
	Morphometric analysis	SHAM	The measured parameters (epithelial height, stromal expansion, myometrial thickness, stromal eosinophilia) were all significantly higher than OVX control.
		OVX control	All the measured parameters were significantly reduced compared to SHAM.
		E2	All the measured parameters were significantly reduced compared to OVX control.
		SE5-OH	None of the measured parameters was different from control.
		S-EQ	None of the measured parameters was different from control.

	Weight (g)	SHAM	0.55 ± 0.03
		OVX control	0.11 ± 0.00[a]
		E2	0.52 ± 0.04[b]
		SE5-OH	0.12 ± 0.01
		S-EQ	0.13 ± 0.01
		a: P < 0.01 versus SHAM by Student's t test. b: P < 0.01 versus OVX control by Dunnett multiple comparison tests.	
	Relative weight (g/ kg bw)	SHAM	1.69 ± 0.10
		OVX control	0.30 ± 0.01[a]
		E2	1.90 ± 0.19[b]
		SE5-OH	0.35 ± 0.01
		S-EQ	0.37 ± 0.01
		a: P < 0.01 versus SHAM by Student's t test. b: P < 0.01 versus OVX control by Dunnett multiple comparison tests.	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Age and body weight of the animals was similar among the groups at the start of the study and body weight and food intake were recorded during the study.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	The animals were maintained on an AIN-93G diet which contained no soy oil or soy protein. No information about the cages provided. The composition of the SE5-OH diet is given in detail
	Were experimental conditions identical across study groups?	++	Housing conditions of the various groups was similar.
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported.
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	-	Study was most probably not conducted blinded. Insufficient information provided.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Zhang et al., 2009 – Ref ID: 4561

Animal model	**Species**	Mice
	Strain (source)	C57BL/6J mice from Guangzhou University of Traditional Chinese Medicine, Guangzhou, China
	Number of animals	Total: 56; 10-12 animals/group
	Age (weight):	12-week
	Diet:	Phytoestrogen-free diet used as the control diet in this study and prepared according to the AIN-93M formulation where corn oil was used instead of soybean oil.
	Dosing method:	Dietary administration
	Funding source	Supported by the Areas of Excellence Scheme Established under the University Grants Committee of the Hong Kong Special Administrative Region, China (AOE/P-10/01), the Central Allocation Grant from the Research Committee of The Hong Kong Polytechnic University (GU 135, I-BB8N), and the Shenzhen-Hong Kong Innovation Circle Funding Scheme (2006).

	Authors' conflicts of interest	None disclosed	
Dosing	**Intervention**	SHAM: control diet	
		OVX-control: control diet	
		IF: OVX, Novasoy®, 2500 mg/kg diet	
		GEN: OVX, genistein 500 mg/kg diet	
		E2: OVX, 17β-oestradiol, 2 mg/kg	
	Start of intervention since OVX	2 weeks	
	Duration	7 weeks	
Statistical analysis	**Statistical analysis**	The data from these experiments were reported as mean ± SEM for each group. If variances associated with each experimental mean were unequal (Bartlett's test for homogeneity of variances), the data were log-transformed before analysis. Inter-group differences were analyzed by 1-way ANOVA and only followed by Tukey's multiple comparison test as a post test to compare the group means if the overall P-value was < 0.05. Differences of P < 0.05 were considered significant.	
Results	**Uterus:**		
	Relative weight (mg/g)	SHAM	2.64 ± 0.57[a]
		OVX	0.51 ± 0.02[b]
		IF	0.64 ± 0.03[b]
		GEN	0.62 ± 0.03[b]
		E2	4.65 ± 0.42[a]
		means ± SEM, n = 9–10 superscripts without a common letter differ, P < 0.05	

Risk of Bias Appraisal		**Tier: 1**	
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	+	Study designed to assess bone parameters
	Did researchers adjust or control for other exposures that are anticipated to bias results?	+	Simple diet substitution
	Were experimental conditions identical across study groups?	+	Diets carefully balanced
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	+	
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	Blinding not addressed
	Were confounding variables assessed consistently across groups using valid and reliable measures?	+	several bone parameters and genes were analysed no effect on uterine weight
	Key question B: Can we be confident in the outcome assessment?	+	
Selective reporting	Were all measured outcomes reported?	+	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	+	

Zhao et al., 2011 – Ref ID: 9236

Animal model	**Species**	Mice	
	Strain (source)	129/C57BL/6	
	Number of animals	5-7/group	
	Age (weight):	3-month	
	Diet:	Control diet (phytoestrogen free) prepared from Teklad Global 16% Protein Rodent diet (Harlan laboratories)	
	Dosing method:	Dietary administration	
	Funding source	Funded by grants from the Alzheimer's Association and National Institute on Aging. Also supported by the Bensussen Translational Research Fund and USC Memory and Aging Center.	
	Authors' conflicts of interest	None disclosed	
Dosing	**Intervention**	SHAM: control diet	
		OVX-control: control diet	
		IF: OVX, commercial soy extract providing 100 mg/kg diet of isoflavones	
		β -SERM: OVX, phyto-β-SERM diet, providing equal parts of genistein, daidzein and equol at 100 mg/kg diet	
	Start of intervention since OVX	Not reported	
	Duration	9 months	
Statistical analysis	**Statistical analysis**	Data presented as group means ± SEM. Statistically significant differences between groups were determined buy one-way analysis of variance followed by Student-Newman-Keuls multiple comparison *post hoc* test.	
Results	**Uterus:**		
	Ki-67 uterine gene expression average Δ (Ct)	SHAM	Not available
		OVX control	9.80 ± 0.42
		IF	9.17 ± 1.01
		β -SERM	10.39 ± 2.32
		No significant differences	
	PCNA uterine gene expression average Δ (Ct)	SHAM	Not available
		OVX control	5.34 ± 0.59
		IF	4.65 ± 0.65
		β -SERM	5.90 ± 0.65
		No significant differences	
	Weight (g)	SHAM	100 ± 11.11
		OVX control	47.07 ± 18.29[a]
		IF	45.61 ± 18.47[a]
		β -SERM	53.49 ± 9.00[a]
		a: P < 0.05 compared with SHAM	

Risk of Bias Appraisal			**Tier: 1**
Bias domain	**Question**	**Score**	**Judgement**
Confounding	Key question A: Did the study design or analysis account for important confounding and modifying variables assessed consistently across groups using valid and reliable measures?	++	Food intake and body wt gain were determined during the study.
	Did researchers adjust or control for other exposures that are anticipated to bias results?	++	The diet was soy-free. The study included a SHAM-operated group.
	Were experimental conditions identical across study groups?	++	Housing conditions were similar among the groups
Attrition/ exclusion	Were outcome data incomplete due to attrition or exclusion from analysis?	++	No loss of animals reported.

Bias domain	Question	Score	Judgement
Information/ detection	Were the outcome assessors blinded to study group or exposure level?	+	There is no evidence that the study was performed blinded.
	Were confounding variables assessed consistently across groups using valid and reliable measures?	++	
	Key question B: Can we be confident in the outcome assessment?	++	
Selective reporting	Were all measured outcomes reported?	++	
Repeated measurements	Key question C: Are the repeated measurements (if any) on the experimental units treated appropriately?	++	

Appendix C – Other adverse events related to breast, uterus and thyroid reported in the clinical studies included in the review

Table C1: Adverse events related to mammary gland

Ref. ID[a] Author, year	Design	Duration (months)	Dosing (mg/day)[b]	Active group (N)/ control group (N)[c]	Age at baseline	Adverse event reported	Description
Soy isoflavones							
16411 Levis et al., 2011	RCT, DB	24	200[d]	ISO: 122/99; placebo: 126/83	ISO: 53 (3.3); Placebo: 52 (3.3)	Abnormal mammogram results	This adverse event was reported by nine women (7.4 %) in the isoflavones group and by six women (4.8 %) in the placebo group
						Breast cancer	This adverse event was reported by one woman (0,8 %) in the isoflavones group and by one woman (0.8 %) in the placebo group
14960 Chilibeck et al., 2013	RCT, DB	24	105[e]	ISO: 90/76; exercise (Ex): 86/77; Ex + ISO: 87/72; placebo: 88/73	ISO: 56.7 (6.5); Ex: 55.3 (6.3); Ex + ISO: 55.8 (5.0); placebo: 56.4 (7.1)	Abnormal mammogram results	Abnormal mammogram requiring biopsy (benign outcome) reported by one participant in the exercise group
						Mastectomy	Mastectomy of both breasts was reported by one participant in the exercise group
						Breast cyst	This adverse event was reported by one participant in the ISO group
						Calcification of breast tissue	This adverse event was reported by one participant in the Ex + ISO group and by one participant in the placebo group
						Ruptured breast implant	This adverse event was reported by one participant in the placebo group
3071 Colacurci et al., 2013	RCT, DB	12	60	ISO : 65/62; Placebo : 65/62	ISO : 55.3 ± 7.6; Placebo : 55.7 ± 7.7	Mastodynia and mammary tension	No difference in reporting of the two events was observed between the groups
Soy protein							
1103 Carmignani et al., 2010	RCT, DB	4	90[f]	ISO: 20; HT:20 Placebo: 20	ISO: 52.9 ± 3.5; HT: 53.3 ± 4.5; Placebo: 50.9 ± 3,4	Mastalgia	Two cases in the isoflavones group (10 %); none in the placebo group (0 %), three cases in the HT group (15 %).
Genistein							
2282 Morabito et al., 2002	RCT, DB	12	54	GEN:30; HT: 30; Placebo: 30	GEN: 52 ± 3; HT: 52 ± 5; Placebo: 51 ± 4	Breast tenderness; sore	Reported by 1/30 women in the placebo group, 3/30 in the GEN group and 2/30 in the HT group.

Ref. ID[(a)] Author, year	Design	Duration (months)	Dosing (mg/day)[(b)]	Active group (N)/ control group (N)[(c)]	Age at baseline	Adverse event reported	Description
15095 Evans et al., 2011	RCT, DB	3	30	GEN:41/32; Placebo: 42/36	GEN: 53.39 ± 5.05; Placebo: 53.50 ± 4.44	Breast tenderness; sore	Reported by 2/41 women in the GEN group and by 3/42 in the placebo group.

(a): Refers to the Ref. ID number in Distiller
(b): Unless otherwise specified, doses are expressed as mg isoflavones/day.
(c): Allocated to treatment/completed.
(d): The authors report that each daily dose contained 91 mg genistein and 103 mg daidzein (ratio GEN:DAI = 0.9:1).
(e): The authors describe intervention as a supplement containing 55 mg isoflavones (approximately 35 mg expressed as aglycone weight) three times per day (ratio GEN:DAI:GLY= 1:1:0.2).
(f): Authors describe intervention as 20 g portions of a food powder containing 12 g of soy protein and a total of 45 mg (26.5 mg aglycones) isoflavones (ratio GEN:DAI:GLY = 1.9:1:0.4)

Table C2: Adverse events related to uterus

Ref. ID[(a)] Author, year	Design	Duration (months)	Dosing (mg/day)[(b)]	Active group (N)/ control group (N)[(c)]	Age at baseline	Adverse event reported	Description
Soy isoflavones							
16411 Levis et al., 2011	RCT, DB	24	200	ISO: 122/99; placebo: 126/83	ISO: 53 (3.3); Placebo: 52 (3.3)	Vaginal/uterine bleeding	This adverse event was reported by 17 women (13.9%) in the isoflavones group and in 18 women (14.3) in the placebo group
14960 Chilibeck et al., 2013	RCT, DB	24	105	ISO: 90/76; exercise: 86/77; Ex + ISO: 87/72; placebo: 88/73	ISO: 56.7 (6.5); Ex: 55.3 (6.3); Ex + ISO: 55.8 (5.0); Placebo: 56.4 (7.1)	Hysterectomy	This adverse event was reported by one participant in the exercise group
						Abnormal Pap test	This adverse event was reported by one participant in the ISO group and by one participant in the placebo group
						Abnormal polyp	This adverse event was reported by one participant in the placebo group
2414 Han et al., 2002	RCT, DB	4	100	ISO: 40/40; placebo: 40/40	ISO: 48 ± 1.1, placebo: 49 ± 1.3	Vaginal/uterine bleeding	This adverse event was reported by 17 women (13.9 %) in the isoflavones group and by 18 women (14.3) in the placebo group
Soy protein							
1103 Carmignani et al., 2010	RCT, DB	4	90	ISO: 20; HT: 20; placebo: 20	ISO: 52.9 ± 3.5; HT: 53.3 ± 4.5; placebo: 50.9 ± 3,4	Vaginal/uterine bleeding	One case in the isoflavones group (5 %), one case in the placebo group (5 %), five cases in the HT group (20 %)
Daidzein-rich isoflavones							
3110 Penotti et al., 2003	RCT, DB	6	72	ISO: 28/22; placebo: 34/27	ISO: 52.5 (2.5) range 49–58; placebo: 52.5 (2.3) range 49–57	Vaginal/uterine bleeding	No cases reported in either groups

Ref. ID[(a)] Author, year	Design	Duration (months)	Dosing (mg/day)[(b)]	Active group (N)/ control group (N)[(c)]	Age at baseline	Adverse event reported	Description
Glycitin-rich isoflavones							
1639 Nikander et al., 2005	C-O, RCT, DB	3	114	ISO: 32/28; placebo: 30/28	54 ± 6	Vaginal/uterine bleeding	Scanty bleeding in four women during the ISO treatment period; three women had bleeding during both treatment periods; none reported bleeding only during the placebo treatment period
Genistein							
2282 Morabito et al., 2002	RCT, DB	12	54	GEN:30; HT: 30; placebo: 30	GEN: 52 ± 3; HT: 52 ± 5; placebo: 51 ± 4	Vaginal/uterine bleeding	Reported by one woman in the placebo group, one in the GEN group and six in the HT group.
15431 Lappe et al., 2013	RCT, DB	6	30	GEN: 35/30; placebo: 35/28	GEN: 54.8 ± 2.5; placebo: 54.7 ± 2.3	Vaginal/uterine bleeding	Vaginal bleeding and spotting were reported by four subjects in the placebo group and by two subjects in the genistein group
15095 Evans et al., 2011	RCT, DB	3	30	GEN:41/32; placebo: 42/36	GEN: 53.39 ± 5.05; placebo: 53.50 ± 4.44	Vaginal/uterine bleeding	Reported by one woman in the GEN group and by none in the placebo group.

RCT: randomised controlled trial; DB: double-blind; HT: hormone therapy; –: information not available
(a): Refers to the Ref. ID number in Distiller
(b): Unless otherwise specified, doses are expressed as mg isoflavones/day.
(c): Allocated to treatment/completed.

Table C3: Adverse events related to thyroid

RefID[(a)] Author, year	Design	Duration (months)	Dosing (mg/day)[(b)]	Active group (N)/ control group (N)[(c)]	Age at baseline	Adverse event reported	Description
Soy isoflavones							
3288 Pop et al., 2008	RCT, DB	3	900	ISO: 18; placebo: 12	ISO: 56.8; placebo: 53.5	Grade I asymptomatic elevation of TSH	This event was reported in 1/12 in the placebo group, on day 28. The value returned to normal at day 84. The same subject had a decreased T_4 at 84. Normal thyroid indices when followed up
						Isolated decreases in T_4	This event was reported in 1/18 in the isoflavones group and 3/12 in the placebo group. By the end of the study the values had returned to normal in all four subjects

RCT: randomised controlled trial; DB: double-blind; –: information not available
(a): Refers to the Ref. ID number in Distiller
(b): Unless otherwise specified, doses are expressed as mg isoflavones/day.
(c): Allocated to treatment/completed

Annex A – Protocol for risk assessment for peri- and post-menopausal women taking food supplements containing isolated isoflavones

The method followed to perform this risk assessment is detailed in the protocol in Annex A to this scientific opinion. The document can be found at: http://www.efsa.europa.eu/en/efsajournal/doc/4246ax1.pdf

Annex B – List of studies excluded from the systematic review after screening of full text.

Annex B can be found at: http://www.efsa.europa.eu/en/efsajournal/doc/4246ax2.xlsx

This Excel file was adapted from the output generated from the Distiller SR database (https://distillercer.com/) documenting the screening process performed for the systematic review conducted within this risk assessment.

The first worksheet contains all the references included in the systematic review after the initial screening phase based on Title and Abstract.

The second worksheet contains a list of the human studies excluded after full text screening and the reason for their exclusion.

The third worksheet contains a list of the animal studies excluded after full text screening and the reason for their exclusion.

Screening was done in parallel by two screeners working independently. For a reference to be excluded it was sufficient that one of the exclusion criteria was identified by both screeners (criteria identified by only one screener are not provided in the list). The list provided contains the criteria that have been selected by both screeners for excluding the studies after checking the full text, when available.