ANSES Opinion
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The Director General

Maisons-Alfort, 8 January 2025

OPINION
of the French Agency for Food, Environmental and Occupational Health & Safety

on the development of long-term oral TRVs for isoflavones

ANSES undertakes independent and pluralistic scientific expert assessments.
ANSES primarily ensures environmental, occupational and food safety as well as assessing the potential health risks they may entail.
It also contributes to the protection of the health and welfare of animals, the protection of plant health and the evaluation of the nutritional characteristics of food.
It provides the competent authorities with all necessary information concerning these risks as well as the requisite expertise and scientific and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).
Its opinions are published on its website. This opinion is a translation of the original French version. In the event of any discrepancy or ambiguity the French language text dated 8 January 2025 shall prevail.

On 29 October 2022, ANSES received a formal request from the Directorate General for Health (DGS) and the Directorate General for Food (DGAL) to conduct the following expert appraisal: assessment of the health risk associated with the consumption of foods containing isoflavones. ANSES also assesses isoflavone levels in foods "as consumed", mainly for its total diet studies (TDSs), including TDS3, which is currently under way.

1. BACKGROUND AND PURPOSE OF THE REQUEST

Phyto-oestrogens occur naturally in certain plants. Their name comes from their oestrogenic activity, which stems from their structural similarity to 17 β-oestradiol and/or their ability to bind to oestrogen receptors. Phyto-oestrogens fall into two main groups:

- flavonoids, including coumestans and isoflavones, which are found mainly in pulses (beans, peas, broad beans), sometimes at physiologically active levels, especially in soy and soy-derived products,
- non-flavonoids, including enterolignans and their precursors (lignans), which are found in most fibre-rich plant-based food products (fruit, vegetables, cereals and oilseeds) and have a more moderate oestrogenic activity.

On 29 October 2022, ANSES received a formal request from the Directorate General for Health (DGS) and the Directorate General for Food (DGAL) to assess the health risk associated with the consumption of foods containing isoflavones. As part of this work, the Agency was asked to propose a long-term oral toxicity reference value (TRV) for isoflavones, taking the different population categories into account.

ANSES also assesses isoflavone levels in foods "as consumed", mainly for its total diet studies (TDSs) (ANSES, 2011; ANSES, 2016). These studies, conducted by ANSES for the whole of France using a standardised method recommended by the World Health Organization (WHO), are designed to monitor population exposure to chemical agents found in food. Quantitative health risk assessments are conducted where possible. In the absence of an TRV, the estimated intakes of isoflavones, equol and coumestrol for the general population, taken from the TDS2, were compared with the maximum intake limit of 1 mg.kg $bw^{-1}.d^{-1}$ for these substances, proposed by the Agency in 2005 (AFSSA, 2005). For 99.9% of adults and 99.5% of children, these intakes were below the limit. However, among consumers of soy-based products (soy beverages, soy desserts, tofu, etc.), they sometimes exceeded this maximum limit. In fact, while it seemed possible to rule out a health risk for the general population, this may not be the case for this category of consumers (ANSES, 2011).

In 2016, during the Infant TDS (iTDS), the highest concentrations of isoflavones were also found in products containing soy. However, the absence of toxicological benchmarks for children under 3 years of age meant that it was not possible to draw any conclusions about the health risk associated with exposure to these substances, with the exception of genistein. For the latter, according to the data, a risk could not be ruled out for children consuming soy-based products, on the basis of a LOAEL[1] of 35 mg.kg $bw^{-1}.d^{-1}$ (NCTR, 2005; Rozman *et al*., 2006), although this did not allow an TRV to be established (ANSES, 2016). As a provisional measure, a critical margin of exposure of 300 was selected (10 and 10 for inter- and intra-individual variability, respectively, and 3 because the point of departure was a LOAEL). However, in 14 children aged between 1 and 36 months who were soy consumers, the average exposure to genistein was 0.88 mg.kg $bw^{-1}.d^{-1}$, corresponding to a margin of safety of 40[2]. Isoflavones are also being investigated in the TDS3 study currently under way.

Assessing the risks of exposure to isoflavones therefore requires long-term oral TRVs for these substances to be available, taking the different population categories into account. This is particularly true for the adult population, menopausal women, pregnant and breastfeeding women, and children.

2. ORGANISATION OF THE EXPERT APPRAISAL

The expert appraisal was carried out in accordance with French Standard NF X 50-110 "Quality in Expert Appraisals – General requirements of Competence for Expert Appraisals (May 2003)".

[1] Lowest Observed Adverse Effect Level = minimum dose that has been observed to cause a harmful effect.
[2] The margin of safety is calculated by dividing the LOAEL (here 35 mg.kg $bw^{-1}.d^{-1}$) by the exposure of the population in question (here 0.88 mg.kg $bw^{-1}.d^{-1}$).

The expert appraisal fell within the sphere of competence of the Expert Committee on "Health reference values" (HRV Committee).

The methodological and scientific aspects of the rapporteurs' expert appraisal work were regularly submitted to the HRV Committee for its opinion and discussion, and were also presented to the Working Group on "Endocrine disruptors" (WG ED). This report takes into account the comments and additional information provided by the members of the HRV Committee and/or the WG ED.

This work was therefore conducted by a group of experts with complementary skills.

The work was adopted by the HRV Committee at its meeting on 28 June 2024.

ANSES analyses interests declared by experts before they are appointed and throughout their work, in order to prevent risks of conflicts of interest in relation to the points addressed in expert appraisals.

The experts' declarations of interests are made public via the website: https://dpi.sante.gouv.fr/.

3. ANALYSIS AND CONCLUSIONS OF THE HRV COMMITTEE

Isoflavones are non-steroidal polyphenolic compounds with a variety of biological activities, but they all share oestrogenic activities. These stem from their structural similarity to a greater or lesser degree to 17 β-oestradiol and their ability to activate oestrogen receptors, particularly the nuclear receptors α and β, and the membrane receptor. Oestrogenic effects have been noted *in vitro* at isoflavone concentrations 10 to 1000 times higher than those of oestradiol, and also *in vivo*.

Isoflavones can exist in free form (i.e. aglycones), which cause biological effects, and bound form (i.e. glycoside conjugated forms), which have no biological activity. There are also acetylated or malonyl derivatives.

Among all the isoflavones, genistein and daidzein are the most studied aglycones. Only these compounds have therefore been addressed in this expert appraisal.

3.1. Summary of the toxicological data

The data described in this section come from summary reports identified in the literature (AFSSA, 2005; EFSA 2015; ANSES 2016; VKM 2017; NCM 2020; SCCS 2022), supplemented by a literature search carried out over the period 2021-2024. The studies cited and outlined in these summaries are often lacking in detail and were themselves taken from EFSA's 2015 summary. The studies cited in these reports will therefore only be detailed briefly, unless they were selected as key studies for developing an TRV. Recent data identified for the period 2021-2024 will be detailed in this section.

Data referring to any potentially beneficial effects of isoflavones are not covered in this section and will not be addressed in this opinion.

Only statistically significant results are described in this section.

In the literature data, the doses administered to humans or animals were most often expressed as "total isoflavones". It should be noted that, depending on the study, this term indicates a dose of either isoflavones (without knowing precisely which ones it relates to) or genistein. As this definition varies, it will be specified for each study described.

3.1.1. Toxicokinetics

The glycoside forms (genistin and daidzin), as found in food, are not absorbed or only poorly absorbed by the gastrointestinal tract when consumed. These O-glycosylated forms must be hydrolysed by the bacterial enzymes of the intestinal microflora to yield aglycone compounds (genistein and daidzein) that can be absorbed through the intestinal barrier. They are then rapidly and almost completely absorbed by the gastrointestinal tract, in both humans and animals. These aglycone compounds are then metabolised in intestinal and liver cells by cytochrome P450 monooxygenases (phase I enzymes). Phase II enzymes, such as uridine diphosphate glucuronyltransferase (UDPGT) or sulphotransferases, then bind glucuronide and/or sulphate groups to the hydroxyl functional groups (either native or formed by the phase I enzymes). It should be noted that glycoside compounds can also undergo metabolism and complete degradation by the microbial flora in the intestinal tract. Faecal elimination is a minor route.

For genistein, a few studies describe pharmacokinetic (PK) models in rats (Hakami *et al*., 2021; Zager *et al*., 2007; Schlosser *et al.*, 2006) but no human model was identified in the literature. For daidzein, only one human physiologically-based pharmacokinetic (PBPK) model was identified (Wang *et al*., 2022), whose value lies in the possibility of estimating the rate of conversion of daidzein to equol by human intestinal flora.

3.1.2. Acute toxicity

3.1.2.1. Human data

No data on acute toxicity in humans were found in the literature.

3.1.2.2. Animal data

Genistein

In its 2015 report, EFSA cited two acute toxicity studies (McClain *et al*., 2005; 2006b cited in EFSA, 2015), one conducted on Beagle dogs and the other on two strains of Wistar rats. In the first experiment by McClain *et al.*, dogs were orally exposed to genistein for 4 weeks at doses of 0, 50, 150 and 500 mg.kg $bw^{-1}.d^{-1}$. Organ weights and haematological and biochemical parameters were measured. A toxicokinetic analysis was also carried out, with measurement of genistein levels in the blood and liver. All organs were removed for histopathological analysis. The results showed that administering genistein to dogs for a period of 4 weeks did not cause any adverse effects. The authors concluded that the no observed adverse effect level (NOAEL) for genistein was higher than 500 mg.kg $bw^{-1}.d^{-1}$ (McClain *et al*., 2005 cited in EFSA, 2015).

In the second study, a first experiment was carried out on Wistar Han-Ibm rats (males and females) fed a diet without genistein and a second on Wistar Crl:(WI)BR rats (males and females) fed a standard animal diet. In the first experiment, the animals were exposed to a single dose of 2000 mg.kg bw^{-1} of genistein (99.5% purity) by gavage and observed for 2 weeks (mortality and clinical signs). All the rats survived the treatment. After euthanasia, necropsy revealed no effects, and no change in body weight or organ weight (liver and kidneys). No histopathological studies were carried out.

In the second experiment, conducted according to the same protocol, the results were similar: all the Wistar Crl:(WI)BR rats survived. Lethargy was observed in all males and in one female one day after exposure; alopecia 14 and 15 days after exposure was reported at the dose of 2000 mg.kg bw^{-1} of genistein in females. The authors of the study concluded that genistein had low acute toxicity, with an LD_{50} higher than 2000 mg.kg bw^{-1} (McClain *et al*., 2006b cited in EFSA, 2015).

Daidzein

In an acute oral toxicity study, rats (n = 3/group) were given daidzein by gavage at 0, 300, 2000 and 5000 mg.kg $bw^{-1}.d^{-1}$ for the first 24 hours. The animals tolerated the highest dose without any toxicologically significant changes. On the basis of this study and observations, the authors estimated that the maximum tolerated dose was higher than 5000 mg.kg $bw^{-1}.d^{-1}$ and that daidzein had low acute toxicity in rats (Laddha *et al.,* 2020).

3.1.3. Repeated and chronic toxicity

3.1.3.1. Human data

Several summary reports have described the epidemiological data studying the association between exposure to isoflavones administered in different forms (soy extracts, soy protein, mixed isoflavones, genistein, daidzein, red clover extract) and the effects observed in men, peri- and post-menopausal women, pre-menopausal women, children and adolescents (EFSA, 2015; VKM, 2017; NCM, 2020). Only the conclusions of these summary reports are described in this opinion.

In its 2015 report, EFSA conducted a risk assessment focusing on the population of peri- and post-menopausal women, and considering the following target organs of interest: breast, uterus and thyroid. EFSA concluded that it was not possible to derive a single health-based guidance value or a safe intake level for food supplements containing isoflavones.

In its 2017 report, the Norwegian Scientific Committee for Food Safety (VKM) concluded that isoflavone supplementation of 40 or 80 $mg.d^{-1}$, taken for one to three months, may represent a risk of adverse effects on hormone levels in pre-menopausal women, men, adolescent boys and girls, or on the menstrual cycle in adolescent girls. The VKM stressed that there were not sufficient data to be able to draw any conclusions about potential adverse effects in children aged from 10 to 14 years.

In its 2020 report, the Nordic Council of Ministers (NCM) concluded that there was no identified risk to pregnant women (or their unborn children) on the basis of the HBGV established from animal experiments. For girls and boys (from 4 to 10 years of age), on the other hand, the NCM identified a potential risk for children on the basis of the HBGV established from animal experiments.

No recent data were identified that would enable one or more studies based on human data showing a dose-response relationship to be selected for developing an HBGV.

3.1.3.2. Animal data

In a repeated-dose toxicity study (OECD 407), Sprague-Dawley (SD) rats (n = 10/dose) received oral doses of daidzein of 0, 25, 50 and 100 mg.kg $bw^{-1}.d^{-1}$ for 28 days (Laddha *et al.*, 2020). The body weight and feed and water consumption of rats treated with daidzein were no different from those of controls. No differences were observed in haematological or biochemical parameters compared with controls. Electrolyte levels (sodium, potassium, phosphates) were also found to be normal compared with the control group. There were no changes in kidney function (24-hour urine collection in a metabolic cage). There were no significant differences in absolute and relative organ weights, and histological examination was normal after treatment. According to the authors, the NOAEL can be considered to be higher than 100 mg.kg $bw^{-1}.d^{-1}$.

In another study, Culçu *et al.* divided 28 female SD rats into four groups (n = 7/group) and exposed them orally for 90 days: group 1 was fed standard rat food (8 $g.d^{-1}$); group 2 received a soy-free infant formula (8 $g.d^{-1}$); group 3 a soy-based formula at 1.12 g/100 mL (8 $g.d^{-1}$; Similac®) and group 4 a soy-based formula containing 2.64 g/100 mL (8 $g.d^{-1}$; Modilac®). The authors concluded that infant formulas with a high soy content induced hyperthyroidism, with elevated serum levels of T3, T4 and TSH[3] (Culçu *et al.*, 2021).

3.1.4.Effects on reproduction and development

Several summary reports and reviews, including some older ones, reported a negative impact of isoflavones on male fertility, as well as effects on the female reproductive system (menopause) (AFSSA, 2005; VKM 2017).

The data from these summary reports have not been detailed. Only studies of interest identified in these reports or in the most recent data are described.

3.1.4.1. Human data

Most of the studies agree that regular consumption of soy-derived products has little or no harmful effect on male fertility (Messina *et al.,* 2022). However, these studies mainly only examined soy consumption in adulthood (40-70 $mg.d^{-1}$). The review by Messina *et al.* pointed out that consumption of isoflavones in Asian countries was estimated to be 30-50 $mg.d^{-1}$, while that in Western countries (USA - Europe) was less than 3 $mg.d^{-1}$ (Messina *et al.,* 2022).

In a cross-sectional study conducted in China on 161 adult men aged between 19 and 51 years with no prior medical history, no association was observed between urinary isoflavone concentrations and the semen parameters assessed (sperm count, concentration and motility) (Yang *et al.*, 2022). In contrast, higher urinary concentrations of genistein, glycitein and dihydrodaidzein were associated with lower plasma concentrations of testosterone.

In a recent American study (Mitsunami *et al*., 2023), the isoflavone consumption of 660 women attending a fertility centre was estimated using a dietary questionnaire covering the previous

[3] T3 = triiodothyronine, T4 = tetraiodothyronine or thyroxine, TSH = thyroid-stimulating hormone

3 months (median daily consumption estimated at 1.78 $mg.d^{-1}$). No association was observed between isoflavone consumption and ovarian reserve or plasma antimüllerian hormone levels.

In a prospective mother-infant cohort study, Chin *et al*. studied the association between feeding practices (soy-based milk, cow's milk or breast milk) and longitudinally measured reproductive hormones and the development of hormone-responsive tissues measured by sonography and clinical examination in infant boys. The authors did not observe any impact on the anatomy of these infant boys, but did note faster penis growth in infants fed soy milk than in those fed breast milk (observations up to 28 weeks of age) (Chin *et al.,* 2021).

Cited in the abovementioned NCM report, a UK study of 367 pregnant women found no effect of isoflavone consumption on the age of menarche in their daughters (Marks *et al*., 2017 cited in NCM, 2020). A meta-analysis conducted in 2010 and then updated in 2021, assessing the effect of isoflavones *in utero* on the reproductive hormone profile in boys, did not find any significant differences in concentrations of testosterone (free or total), sex hormone-binding globulin[4] (SHBG), oestrone and oestradiol (Reed *et al.*, 2021).

In the work of Upson *et al.* on a cohort of 1696 African-American women, negative associations were identified between high consumption of soy and soy-based products from early childhood and a higher incidence of uterine fibroids when women entered the pre-menopause (Upson *et al.*, 2015 cited in VKM, 2017), larger uterine fibroids (Upson *et al*., 2016a cited in VKM, 2017), heavier menstrual bleeding (Upson *et al*., 2016b cited in VKM, 2017) and the need for oral contraception to relieve menstrual pain (Upson *et al*., 2019 cited in VKM, 2017). However, intake estimates were semi-quantitative. Furthermore, a dietary bias linked to the ethnic origin of the individuals cannot be ruled out (Upson *et al*., 2016a cited in VKM, 2017).

The conclusions regarding the impact of phyto-oestrogens on the age at puberty in women are fairly contradictory. Some studies have shown an association between precocious puberty and high consumption of soy-based products, including infant formulas, in early childhood (D'Aloisio *et al*., 2013; Cheng *et al*., 2010 & 2012; Kim *et al.*, 2010 & 2011; Valladares *et al*., 2012), while others found no such advancement of puberty (Segovia-Siapco *et al*., 2014; Oliveira *et al.*, 2021; Testa *et al.*, 2018).

A retrospective case-control study conducted in Brazil on 161 children (84 with a diagnosis of precocious puberty and 77 control children) showed that most of the children with precocious puberty had been fed with soy-based formulas, whereas the control group consisted mainly of children who had been exclusively breastfed (Felicio *et al.*, 2021). Conversely, in their meta-analysis of six studies (3 on the frequency of precocious puberty and 3 on the average age of menarche), Oliveira *et al.* did not identify any link between soy intake in early childhood and an earlier onset of puberty (OR = 0.51; $CI_{95\%}$ = 0.09 to 2.94; p = 0.969) or mean age of menarche (OR = 0.14; $CI_{95\%}$ = -0.16 to 0.45; p = 0.186) (Oliveira *et al.,*2021).

3.1.4.2. Animal data

In the study by Nagao *et al.*, male and female SD rats (Crj:CD, IGS) were exposed by gavage to genistein doses of 0, 12.5, 25, 50 and 100 $mg.kg\ bw^{-1}.d^{-1}$ from postnatal day (PND) 1 to PND5. Examinations were carried out periodically on PND1, PND6, PND14 and PND21 and

[4] This is the protein that transports testosterone and oestradiol in the blood

then at 5, 7 and 9 weeks of age. The reported results showed histopathological changes in the ovaries and uteri of females at the highest dose of 100 mg.kg $bw^{-1}.d^{-1}$, and no reprotoxic effects in males (Nagao *et al.*, 2001). In both males and females, body weight at the dose of 100 mg.kg $bw^{-1}.d^{-1}$ was significantly lower than in the controls at each measurement time, and at the dose of 50 mg.kg $bw^{-1}.d^{-1}$ at 5, 7 and 9 weeks. In females, body weight was lower at all the doses at 9 weeks. Treatment had no effect on vaginal opening or preputial separation at any of the doses tested and any of the measurement times. Male fertility was not affected by exposure, and serum testosterone concentrations and sperm quality were unchanged. There were no histological alterations in the gonads of male rats. Female fertility was affected at all doses. Irregularity of the oestrous cycle and histopathological changes in the ovaries (atrophy, absence of corpora lutea and atretic follicles) and uterus (hypertrophy/hyperplasia) were observed at the dose of 100 mg.kg $bw^{-1}.d^{-1}$. This study identified a LOAEL of 12.5 mg.kg $bw^{-1}.d^{-1}$ associated with a functional effect on fertility (reduced fertility index) in females.

A 2008 report by the National Toxicology Program (NTP) described a multi-arm multigenerational study combining a reproduction study and a long-term toxicity study in NCTR CD (Sprague-Dawley) rats. Groups of 35 pairs of animals (for the F0, F1, F3 and F4 generations) or 40 pairs of animals (for the F2 generation) were exposed to genistein administered at doses of 0, 5, 100 and 500 ppm in the diet[5], i.e. 0.3 ± 0.03, 5.9 ± 0.5 and 28.9 ± 2.5 mg.kg $bw^{-1}.d^{-1}$ in males (F0), 0.3 ± 0.02, 6.9 ± 0.3 and 34.6 ± 1.6 mg.kg $bw^{-1}.d^{-1}$ in males (F1 and F2) and 0.5 ± 0.02, 10.0 ± 0.5 and 50.6 ± 2.4 mg.kg $bw^{-1}.d^{-1}$ in females (excluding the lactation period). The doses administered to females (F0-F2 generation) during lactation were 0.7 ± 0.04, 14.6 ± 0.9 and 78.0 ± 3.9 mg.kg $bw^{-1}.d^{-1}$.

In the multigenerational reproductive toxicology study, males and females were exposed to genistein for the duration indicated in the experimental design below:

- F0 exposed from PND42 to PND140 (98 days),
- F1 from conception to PND140 (161 days),
- F2 from conception to PND140 (161 days),
- F3 from conception to PND21, then given feed without the test substance from PND21 to PND140 (161 days in total, 42 days of feed with the test substance),
- F4 not exposed; given feed without the test substance from conception to PND140 (161 days in total),
- F5 not exposed; given feed without the test substance from conception to PND21 (42 days in total).

Twenty-five rats per sex from each generation (F0 to F4) were randomly selected for lifetime studies and scheduled for necropsy at PND140. Body weights and clinical findings for F0 animals were recorded weekly until the animals were euthanised. For generations F1 to F4, body weights and clinical findings were recorded weekly from PND21 until the end of the study. In addition, pup body weights were measured on PND2, PND4, PND7 and PND14. Necropsies were performed on all F0 to F4 generation animals, as well as on any that died or were found moribund before the end of the study.

The results showed a significant reduction in body weight in exposed females, greater at 500 ppm, in the generations continuously exposed to genistein (F0 to F2). Male body weight during the post-weaning period was lower in the F1 groups receiving 100 and 500 ppm. The

[5] 5K96 soy- and alfalfa-free diet, Purina Mills Inc.

birth weights of pups were lower in F1 males exposed to 100 ppm and in all the exposed groups of the F5 generation (males and females).

No effect of genistein on follicle number or ovarian histology was noted, although disruption of the oestrous cycle, including extended cycles, was observed in F1 and F2 animals exposed continuously to 500 ppm shortly after vaginal opening, but not after these animals had delivered and nursed their litters.

With regard to the measurement of anogenital distance (AGD), male pups (PND2) in the 500 ppm group of the F1 generation had decreased mean AGDs compared with controls in this generation, while females (PND2) had smaller AGDs compared with controls from the F1 (500 ppm), F2 (500 ppm) and F3 (100 ppm) generations.

The time of vaginal opening was accelerated in female pups of the F1 to F2 generations exposed to 500 ppm of genistein, and in the F3 generation exposed to 5 ppm. Body weight at vaginal opening was lower in female pups of the F1 to F3 generations following exposure to the 500 ppm dose, and in the F1 generation exposed to 5 ppm. This effect was not observed in the unexposed F4 generation.

In males, a delay in the time of testicular descent was observed only in the F3 generation exposed to genistein at 500 ppm from conception to weaning.

Concerning the effects on organ weights in the F0 to F5 generations, in both sexes they were limited overall to a single generation. Among these effects, a significant increase in pituitary gland weight was observed in F2 males exposed to 500 pm, without microscopic lesions. The only change induced by genistein on the weight of the reproductive system in both sexes concerned the F0 generation exposed at the end of puberty and in adulthood (increase in testicular weight at 500 ppm).

No exposure-related histological lesions were found in microscopic analyses of tissues from female rats. Exposure-related lesions in male rats were confined to the mammary gland and kidneys. An increased incidence of renal tubular mineralisation, or nephrocalcinosis, was observed in males exposed to 100 or 500 ppm; this increase was confined to the continuously exposed F1 and F2 generations.

While the genistein-induced nephrocalcinosis in males was of minimal to mild severity and did not appear to impact the animals' longevity or fertility, this endpoint, together with hyperplasia of the mammary gland in males, was observed in males of the F1 and F2 generations following exposure to genistein at 100 ppm or more.

This study identified a NOAEL of 5.9 mg.kg $bw^{-1}.d^{-1}$ (100 ppm) and a LOAEL of 35 mg.kg $bw^{-1}.d^{-1}$ (500 ppm) associated with mammary gland alveolar/ductal hyperplasia in males of the F0 generation, as well as a NOAEL of 0.35 mg.kg $bw^{-1}.d^{-1}$ (5 ppm) and a LOAEL from 7 mg.kg $bw^{-1}.d^{-1}$ (100 ppm) associated with mammary gland alveolar and ductal hyperplasia in males of the F1 and F2 generations.

Eustache *et al*. exposed pregnant Wistar Han rats (n = 10/dose) daily by gavage to genistein doses of 0, 1 and 10 mg.kg $bw^{-1}.d^{-1}$ from gestational day (GD) 1 to PND21 (weaning); the pups were then exposed by gavage to the same doses daily until PND80 (Eustache *et al.*, 2009). At PND25, the results showed no effect on AGD and genital organ development, except at the highest dose. At PDN85, the weight of the animals was increased at the dose of 1 mg.kg $bw^{-1}.d^{-1}$. There was no effect on the relative weight of the testes, seminal vesicles or ventral

prostate at either dose. The relative epididymal weight was reduced at the doses of 1 and 10 mg.kg $bw^{-1}.d^{-1}$. Relative liver weight was increased at 10 mg.kg $bw^{-1}.d^{-1}$. Sperm motility parameters were altered at 1 and 10 mg.kg $bw^{-1}.d^{-1}$, except for straightness of trajectory, which was only reduced at 1 mg.kg $bw^{-1}.d^{-1}$. The sperm concentration in the cauda epididymis was reduced at 10 mg.kg $bw^{-1}.d^{-1}$. The treatment had no effect on mating index, fertility index, post-implantation losses, average pup weight or sex ratio at birth. The number of litters was reduced at the doses of 1 and 10 mg.kg $bw^{-1}.d^{-1}$.

This study identified a LOAEL of 1 mg.kg $bw^{-1}.d^{-1}$ associated with a decrease in relative epididymal weight in males and a decrease in litter size in females.

In the study by Li *et al.*, female C57BL/6J mice were exposed at PND21 to genistein concentrations of 0, 5, 100 and 500 ppm (i.e. according to the authors, 0, 0.5, 10, 50 mg.kg $bw^{-1}.d^{-1}$). An analysis of the impact of genistein exposure on the rate of embryo implantation was also conducted by observing cycles in mice aged 5 to 8 weeks (i.e. the period of mammary maturation), focusing on the first 10 days following vaginal opening. The results showed decreased age at vaginal opening at the 100 and 500 ppm doses, an increase in the length of the oestrus phase at 500 ppm, an increase in the number of days spent in oestrus at all doses, a decrease in the number of animals with a normal oestrus cycle at 100 and 500 ppm, a significant increase in corpora lutea (ovulation indicators) at 5 ppm and accelerated mammary gland development at 100 and 500 ppm. The authors concluded that there was a precocious puberty effect associated with genistein exposure, with no repercussions on the rate of embryo implantation in mice (Li *et al.*, 2014). This study identified a LOAEL of 10 mg.kg $bw^{-1}.d^{-1}$ (100 ppm) associated with decreased age at vaginal opening, an increase in the length of oestrous cycles and accelerated mammary gland development in female mice.

In the study by Abo-Elsoud *et al*., male rabbits (strain unspecified, n = 7/dose) were exposed for 12 weeks to 0, 5 or 20 mg.kg $bw^{-1}.d^{-1}$ of soy isoflavones (ratio of 1 genistein to 5.7 daidzein) (Abo-Elsoud *et al.*, 2019). The results showed a drop in libido (increased reaction time), a dose-dependent decrease in sperm concentration, a decrease in serum testosterone (although this had no effect on litter size, weight or viability) and an increase in T3 compared with controls. Treatment had no effect on litter size, viability or weight at birth. The results showed no difference in the percentage of abnormal spermatozoa. This study identified a LOAEL of 5 mg.kg $bw^{-1}.d^{-1}$ associated with a significant decrease in sperm concentrations and blood testosterone levels, as well as a significant increase in T3 for a mixture of soy isoflavones.

In a second study, Eustache *et al*. exposed rats daily by gavage to a single dose of 1 mg.kg $bw^{-1}.d^{-1}$ of genistein, using two exposure schedules (Eustache *et al*., 2020):

- Exposure of females (F1 from unexposed pairs) from GD1 to PND21 (weaning). An F2 generation was produced and followed through to PND100;
- Exposure of male rats (F1 from unexposed pairs) from PND21 to PND100. These male rats were mated with unexposed females to give an F2 generation that was followed until PND100.

Genistein reduced epididymal sperm content in F1 animals exposed from the prepubertal period to adulthood (numerical values not provided in the article). The expression of genes involved in steroidogenesis was affected during both exposure windows, particularly exposure during gestation/lactation.

This study identified a LOAEL of 1 mg.kg $bw^{-1}.d^{-1}$ associated with a decrease in epididymal sperm content in males (F1 males exposed from PND21 to PND100).

Maraudino *et al*. exposed male and female CD-1 mice from PND1 to PND8 to a single oral dose of 50 mg.kg $bw^{-1}.d^{-1}$ of genistein diluted in sesame oil (Marraudino *et al*., 2021). The animals were then observed at PND12, PND22, PND30 and PND60. Observations included time to vaginal opening, oestrus cycle and mammary gland analysis (whole mount, at PND22, PND30 and PND60), uterus and testicle weights, body weight, food consumption, circulating hormone concentrations (leptin, progesterone and testosterone) and immunohistochemical labelling of kisspeptin, pro-opiomelanocortin (POMC) and orexin on brain sections. Exposure to genistein was associated with earlier vaginal opening and an altered oestrus cycle. Females exposed to genistein spent more time in oestrus and dioestrus than unexposed females, with a significant reduction in the pro-oestrus phase. A difference in absolute uterus weights was observed only on PND22. There was no statistically significant difference in testicle weights on PND22 and PND30 between exposed and control males. A reduction in the testicle weights of exposed individuals was observed on PND60. In females, exposure to genistein increased circulating progesterone and leptin levels on PND60, but not on PND30. A decrease in circulating testosterone levels was observed in males on PND60, but not on PND30. Regarding the effects on mammary gland development, exposure to genistein had no effect on terminal end buds, irrespective of the period of development in question. Similarly, exposure did not alter the overall architecture of the mammary gland on PND60.

In two studies carried out by the same team, prepubertal male Wistar rats were exposed by gavage to soy isoflavones (Glycine max L., soy extract standardised to 40% isoflavones, diluted in corn oil; composition not specified) at doses of 0, 0.5, 5 or 50 mg.kg $bw^{-1}.d^{-1}$ from PND23 to PND60 (Oliveira *et al*., 2022; Dal Forno *et al*., 2023). The control group received only corn oil. The results of the study by Oliveira *et al.* showed that exposure to isoflavones in prepubertal male rats had no effect on the animals' growth. An increase in the age at puberty was observed in male rats at all the doses tested. According to the authors, these results suggest that consumption of isoflavones during the prepubertal period disrupts the hypothalamic-pituitary-testicular axis in male rats, causing hypergonadotropic hypogonadism and altered expression levels of key genes regulating this axis.

The Oliviera *et al*. study identified a LOAEL of 0.5 mg.kg $bw^{-1}.d^{-1}$ associated with a delayed age of balanopreputial separation (delayed puberty) and a significant reduction in serum testosterone levels (Oliviera *et al.*, 2022).

The results obtained in the study by Dal Forno *et al.* showed that serum TSH concentrations were increased in the groups treated with 0.5 and 5 mg.kg $bw^{-1}.d^{-1}$, while no variations were observed in T3 and T4. According to the authors, these results suggest that consumption of soy isoflavones during the prepubertal period may induce subclinical hypothyroidism in male rats, with alterations in the regulation of the hypothalamic-pituitary-thyroid axis, modulation of thyroid hormone synthesis and peripheral alterations in thyroid hormone target organs.

In a study by Caceres *et al.*, male rats were orally exposed to isoflavones for 5 months. The animals were divided into three groups: a control group, a group with low isoflavone supplementation (17 mg.kg $bw^{-1}.d^{-1}$ of genistein and 12 mg.kg $bw^{-1}.d^{-1}$ of daidzein), and a group with high isoflavone supplementation (170 mg.kg $bw^{-1}.d^{-1}$ of genistein and 120 mg.kg $bw^{-1}.d^{-1}$ of daidzein) (Caceres *et al.*, 2023). After 12 weeks, there was a significant loss of body weight

in the low isoflavone supplementation group compared with the control group, while the high isoflavone supplementation group showed a significant gain in body weight.

Relative testicular weight was significantly reduced from week 12 onwards in rats receiving diets low or high in isoflavones. At the end of treatment, administration of a diet rich in isoflavones led to a significant reduction in the diameter of the seminiferous tubules compared with the control group and the low isoflavone supplementation group.

Forty per cent of the rats on a diet low in isoflavones and 80% of the rats on a diet high in isoflavones showed degeneration of the seminiferous tubules. From week 16 of treatment, sperm motility and sperm count were significantly reduced in the low and high isoflavone supplementation groups compared with the control group. Plasma progesterone concentrations were also significantly lower in the low and high isoflavone supplementation groups. Testicular testosterone concentrations were lower in both supplemented groups than in the control group. Oestradiol levels in both isoflavone groups were significantly higher from week 4 to week 20 than in the control group. These differences in testicular concentrations of testosterone and oestradiol between the control and treated groups were also reflected in the testosterone to oestradiol ratio (T/E2), with a reduction in the T/E2 ratio of the treated groups from week 8 until the end of the experiment, indicating a hormonal imbalance. The authors concluded that in adult male rats, a long-term diet with low or high doses of isoflavones compromises testicular functionality by causing an imbalance in hormonal homeostasis that leads to a reduction in sperm quality. This study identified a LOAEL of 0.56 mg.kg $bw^{-1}.d^{-1}$ associated with a significant decrease in circulating testosterone from the first week of exposure and dihydrotestosterone from the second week, and a semi-quantitative decrease (histology) in epididymal sperm.

3.1.5.Genotoxicity

The data identified in the literature on the genotoxicity of isoflavones mainly concern genistein and daidzein (SCCS, 2022). For both these substances, the gene mutation test on prokaryotic cells (Ames test) was negative. However, other *in vitro* tests considering various effects (chromosomal breakage or adduct formation) have been positive for these two compounds. Lastly, *in vivo* tests for mutagenesis or DNA damage (chromosomal aberrations, micronuclei) have been negative for genistein, but a sister chromatid exchange test was positive for daidzein and a DNA damage test (comet assay) was positive for genistein in a sensitised context (exposure to $NaNO_2$). Given the known mechanistic elements (possible interaction with the topoisomerase II-DNA complex), the genotoxic potential of isoflavones cannot be ruled out.

3.1.6.Carcinogenicity

3.1.6.1. In humans

The various summary reports agree on the ambiguity of conclusions in studies on the links between exposure to isoflavones and the risk of cancer (AFSSA, 2005; EFSA, 2015; ANSES, 2016; VKM, 2017; NCM, 2020; SCCS, 2022). These studies have focused mainly on the risk of hormone-dependent cancers, particularly breast cancer (in women), due to the oestrogenic properties of isoflavones. Although a large number of studies have highlighted protective associations with regard to the occurrence of breast cancer, the results remain heterogeneous, mainly depending on the woman's menopausal status. Some studies show protective associations exclusively in non-menopausal women or exclusively in menopausal women.

Other studies show no association regardless of status or, conversely, an increased risk. Studies focusing on prostate cancer, which are rarer than those on breast cancer, have equally divergent conclusions. For other tumour sites (endometrium, ovary, testis, thyroid and colon), there are not enough data available to be able to draw any conclusions on the presence or absence of associations.

Several recent meta-analyses (2021 to 2024) suggest a reduced risk of occurrence of breast cancer (Shin *et al.*, 2023; Yang *et al.*, 2023; Liu *et al.*, 2022; Boutas *et al.*, 2022), lung cancer in non-smokers (Chei *et al.*, 2022) and stomach cancer in consumers of non-fermented soy products (Wang *et al.*, 2021). Other meta-analyses conclude that there is an increased risk of stomach cancer (Kim *et al*., 2023), particularly among consumers of fermented soy-based products (Wang *et al*., 2021), and prostate cancer (Liu *et al.*, 2022).

Regarding studies published in recent years (2021 to 2024), a prospective cohort study showed that dietary intake of phyto-oestrogens was associated with a reduced risk of occurrence of ovarian cancer (Song *et al.*, 2024). Another prospective cohort study showed no association with the risk of breast cancer, whether or not fermented soy-based products were consumed (Shirabe *et al.*, 2021). However, this same study did highlight a reduced risk of occurrence of non-localised (metastasised) breast cancer in the event of high consumption of fermented products (Shirabe *et al.*, 2021). Note that one prospective cohort study showed no association with the risk of liver cancer (Abe *et al.*, 2021). Case-control studies in the general population have shown a reduction in the risk of oesophageal cancer (Sun *et al.*, 2021) and a reduction in breast cancer, but only in pre-menopausal women (Feng *et al.*, 2021). A case-control study nested within a prospective cohort showed a reduced risk of lung cancer in non-smokers (Li *et al*., 2022). Lastly, cross-sectional studies have shown associations with an excess risk of hormone-dependent cancers affecting the breast, endometrium and prostate (Liu *et al*., 2023; Lee *et al*., 2022).

It should be noted that the majority of studies on the carcinogenicity of isoflavones have been based on indirect assessments of exposure estimated using retrospective dietary questionnaires. These estimates may therefore be subject to numerous biases (measurement, confounding, recall). Some studies were based on ad hoc measurements of internal exposure by assaying one or more isoflavones in urine. These ad hoc (non-repeated) measurements may also not be a good indicator of exposure, given the short half-lives of isoflavones.

3.1.6.2. In animals

In the previously described 2008 NTP study in which male and female SD rats were exposed to genistein for two years, in female rats of the F1C generation, there were observed increases in the incidence of mammary gland adenomas and adenocarcinomas (combined tumours, $p = 0.037$) at the dose of 500 ppm (16/40 vs 9/44 in controls) but not at the 5 ppm and 100 ppm doses, in the incidence of pituitary adenomas alone (pars distalis) ($p = 0.004$) at the 500 ppm dose (38/47.9 vs 46/47.2 in controls) and in the incidence of combined pituitary adenomas and carcinomas (pars distalis) ($p = 0.004$) at the 500 ppm dose (38/47.9 vs 46/47.2 in controls), but not at the 5 and 100 ppm doses. There was no statistically significant increase in the incidence of tumours in male rats at the doses tested in the study of the F1C generation. Moreover, the observations did not show any increase in the incidence of tumours at the other sites analysed.

3.1.7.Sensitive population groups

Isoflavones have beneficial or adverse effects depending on the individuals concerned. This depends, among other things, on age, sex, menopausal status, period of exposure to isoflavones and tumour status (Hooper *et al*., 2010; EFSA, 2015; Wei *et al*., 2020; Lu *et al.*, 2022).

Based on the literature, the following sensitive populations were defined:

- young children, particularly young girls, in whom an early and prolonged exposure to isoflavones can lead to precocious puberty later in life (Testa *et al*., 2018; Segovia-Siapco *et al*., 2014),
- pre-menopausal women, who are more sensitive to the proliferative effect of isoflavones on mammary cells due to the density of oestrogen receptors. They should limit their consumption of products containing isoflavones (Petrakis *et al*., 1996; McMichael-Phillips *et al*., 1998; Hargreaves *et al*., 1999; Ollberding *et al.*, 2012),
- women with oestrogen-sensitive proliferative cells in the mammary gland. These women have an increased risk of developing breast cancer following isoflavone consumption, which should therefore be limited (Grace *et al*., 2004; Shike *et al*., 2014; Khan *et al*., 2012; Allred *et al*., 2001; Ju *et al*., 2001).
- pregnant women are not a sensitive population as such, but in view of certain experimental studies indicating a possible adverse effect on offspring, they may be considered as a sensitive population for the protection of their unborn children.

3.2. Identification of TRVs and intake limits

- **TRVs**

In 2015, EFSA conducted a health risk assessment for (peri)menopausal women, focusing on three target organs (mammary gland, uterus and thyroid) (EFSA, 2015). EFSA only took into account human studies conducted on (peri)menopausal women or animal studies on ovariectomised animals. In assessing the effects of isoflavones on these three target organs, EFSA concluded that it was not possible to extrapolate observations directly from any one organ to the others, given the differences in functions, receptor density, proportions of oestrogen α and β receptors and the effects of receptor activation. EFSA was unable to derive an TRV or a maximum intake limit for food supplements containing isoflavones, in the absence of any evidence of adverse effects on the mammary gland, uterus and thyroid, and limited evidence for breast cancer. EFSA specified that the conclusions of its assessment could not be extrapolated to other population groups and other situations in the general population.

In 2016, although no TRV had been established for isoflavones, as part of its infant TDS, ANSES calculated margins of exposure solely for genistein and for information only, based on a LOAEL of 35 mg.kg $bw^{-1}.d^{-1}$ from a multigenerational study (NCTR, 2005; Rozman *et al.*, 2006). This study showed that genistein exerted oestrogenic or anti-androgenic reprotoxic effects in SD rats. This study, assessed by the National Toxicology Program – Centre for the Evaluation of Risks to Human Reproduction (NTP-CERH), identified a LOAEL of 35 mg.kg $bw^{-1}.d^{-1}$ in male newborns (delayed testicular descent, decreased AGD). As a provisional measure, a critical margin of exposure of 300 was selected (10 to take account of inter-species variability, 10 for inter-individual variability and 3 because the point of departure was a LOAEL),

leading to a recommended maximum intake limit of 0.117 mg.kg $bw^{-1}.d^{-1}$ for children under 3 years of age (ANSES, 2016).

In 2020, the NCM developed two HBGVs for genistein based on animal studies. These values were of 0.09 mg.kg $bw^{-1}.d^{-1}$ for pregnant women and 0.07 mg.kg $bw^{-1}.d^{-1}$ for children over 3 years of age (NCM, 2020) (Table 1).

Table 1: HBGVs for genistein developed by the NCM

RV	**Organisation**	Nordic Council of Ministers	
	Year	2020	
	Name	HBGV	
	Value	**0.09 mg.kg $bw^{-1}.d^{-1}$**	**0.07 mg.kg $bw^{-1}.d^{-1}$**
Target population		**Pregnant women**	**Children over 3 years of age**
Critical effect		Effects on development	Advancement of puberty
Key study	**Reference**	NTP, 2008	Li *et al.,* 2014
	Species	Sprague-Dawley rats	C57BL/6J mice
	Exposure (duration, route)	Multigenerational (3 generations) Oral route	from PND21 to the age of 5, 6, 7 or 10 weeks Oral route (food)
Point of departure (PoD)		NOAEL = 100 ppm = 8.9 mg.kg $bw^{-1}.d^{-1}$*	LOAEL = 100 ppm = 20 mg.kg $bw^{-1}.d^{-1}$*
Time adjustment		/	/
Allometric adjustment		/	/
Uncertainty factors (UFs)		100 (UF_A: 10; UF_H: 10)	300 (UF_A: 10; UF_H: 10; $UF_{L/B}$: 3)

* use of a conversion factor recommended by EFSA of 0.2 for subacute exposure in mice. Thus, 1 $mg.kg^{-1}$ in the diet corresponds to 0.2 mg.kg $bw^{-1}.d^{-1}$.

- **Phyto-oestrogen intake limits**

Several countries have proposed maximum intake limits for phyto-oestrogens, some based on data on genistein. These include the Italian authorities in 2002, AFSSA[6] in 2005, the Food Safety Commission of Japan in 2006 and the Norwegian Scientific Committee for Food Safety in 2017 (VKM, 2017).

In 2002, the Italian health authorities advised the general public to keep their daily intake of phyto-oestrogens in food supplement form to below 80 $mg.d^{-1}$, expressed as the total quantity of isoflavone isomers[7] (Morandi *et al.*, 2005). This represents a maximum daily phyto-oestrogen intake of around 1 mg.kg $bw^{-1}.d^{-1}$ for a 70 kg adult.

In 2005, AFSSA proposed a maximum intake limit of 1 mg.kg $bw^{-1}.d^{-1}$ of isoflavones in aglycone equivalent (AFSSA, 2005). This maximum limit was based on effects mainly observed in rodents with weight loss following exposure to 50 mg.kg $bw^{-1}.d^{-1}$ of genistein (Slikker *et al.,* 2001 cited in AFSSA 2005), sometimes linked to a reduction in food consumption following exposure to soy protein in the form of flour, concentrate or isolate (11-39 mg.kg $bw^{-1}.d^{-1}$) (Rackis, 1979 cited in AFSSA 2005). However, the study by Okazaki *et al.* did not confirm these results, even at the highest dose of genistein tested (1000 mg.kg $bw^{-1}.d^{-1}$) for 28

[6] The French Food Safety Agency, which became ANSES in 2010.
[7] daidzin, glycitin, genistin, 6″-O-acetyldaidzin, 6″-O-acetylglycitin, 6″-O-acetylgenistin, 6″-O-malonyldaidzin, 6″-O-malonylglycitin, 6″-O-malonylgenistin, daidzein and genistein

days (NOAEL = 120 mg.kg $bw^{-1}.d^{-1}$) (Okazaki *et al.,* 2002). The NOAEL of 120 mg.kg $bw^{-1}.d^{-1}$ observed in rats corresponds to a plasma concentration above 5 µM[8]. This plasma concentration can be reached in humans with genistein doses of the order of 1.5 to 2 mg.kg $bw^{-1}.d^{-1}$.

In 2006, the Food Safety Commission of Japan set dietary intake limits for foods for specified health use containing soy isoflavones as an ingredient with presumed health benefits for adults (pre- and post-menopausal women and men), with an intake limit of 70-75 $mg.d^{-1}$ of soy isoflavones. For foetuses (pregnant and potentially pregnant women), infants and young children, it was not possible to recommend an intake limit (Food Safety Commission of Japan, 2006).

In 2017, the VKM assessed the risks of consuming 40 or 80 $mg.d^{-1}$ of soy isoflavones added to food supplements and other foods. For children aged from 10 to 13 years inclusive, there were not sufficient data to be able to draw any conclusions about potential adverse effects of isoflavones. Consumption of 40 or 80 $mg.d^{-1}$ of isoflavones for one to three months by adolescents of both sexes (14 to 17 years inclusive), women and men may represent a risk of negative effects on hormone levels, including menstrual function in girls and women. In (peri)menopausal women, these doses of isoflavones over several months and for up to several years do not appear to cause any adverse effects (VKM, 2017).

3.3. Proposed long-term oral TRV for the general population

3.3.1. Choice of the critical effect

The NTP study showed that the effects transposable to humans following long-term exposure and appearing at the lowest dose in male rats were limited to the mammary gland, with an increase in the incidence of mammary gland alveolar/ductal hyperplasia (NTP, 2008). The pattern (see report) of induction of hyperplasia across generations, with the strongest effects seen in the 100 and 500 ppm groups of the continuously exposed F1 and F2 generations, indicated that both developmental and postweaning exposures contribute to this effect. Late exposure in puberty and adulthood, as in the F0 generation, produced lesser effects.

The HRV Committee therefore selected the increased incidence of mammary gland alveolar/ductal hyperplasia in male rats as the critical effect.

3.3.2. Analysis of the existing long-term TRVs

In the absence of an TRV for the general population, the experts of the HRV Committee proposed developing this value for this target population.

3.3.3. Development of the TRV for the general population

3.3.3.1. Choice of the key study

Among the animal studies available, the NTP's multigenerational reproduction and development study goes into great depth in terms of duration, number of animals and pathological observations, following long-term exposure to genistein. **The NTP's study (NTP, 2008) was therefore selected as the key study.**

[8] "*In the absence of any kinetics data specific to this study, an estimate based on extrapolation of 'ingested-circulating' data would lead to plasma circulating levels of genistein higher than 5 µM in rats*" (AFSSA, 2005).

3.3.3.2. Choice of the point of departure

In the selected key study, male and female NCTR CD (Sprague-Dawley) rats (for the F0, F1, F2, F3 and F4 generations) were exposed to genistein administered in soy-free feed at doses of 0, 5, 100 and 500 ppm (i.e. 0.3 ± 0.03; 5.9 ± 0.5; 28.9 ± 2.5 mg.kg $bw^{-1}.d^{-1}$). Males of the F0 generation were exposed via feed for 98 days. In this study, exposure of male rats of the F0 generation led to an increase in the incidence of mammary gland alveolar/ductal hyperplasia at the dose of 500 ppm (28.9 mg.kg $bw^{-1}.d^{-1}$). This resulted in the dose of 100 ppm (5.9 mg.kg $bw^{-1}.d^{-1}$) being identified as a NOAEL.

This NOAEL of 5.9 mg.kg $bw^{-1}.d^{-1}$ was selected as the point of departure.

3.3.3.3. Allometric adjustment

An allometric adjustment was performed to reduce the uncertainty regarding inter-species variability. A human equivalent dose (HED) was calculated, using the following equation[9]:

Human equivalent dose = Animal dose x (Animal weight / Human weight)$^{1/4}$

The average weight of the rats was 0.426 kg. The average human weight used for the calculation was 70 kg.

i.e. a $NOAEL_{HED}$ = 5.9 x $(0.426/70)^{0.25}$ = 1.65 mg.kg $bw^{-1}.d^{-1}$

This $NOAEL_{HED}$ of 1.65 mg.kg $bw^{-1}.d^{-1}$ was selected as the PoD.

3.3.3.4. Choice of uncertainty factors

The TRV was calculated from the NTP's 2008 study using the following uncertainty factors (UFs) (ANSES, pending publication):

- Inter-species variability (UF_A): 2.5 to take account of the toxicodynamic component, as a dose adjustment was made;
- Inter-individual variability (UF_H): 10 by default;
- Subchronic to chronic transposition (UF_S): √10, as the exposure period of 98 days corresponded to a subchronic duration (exposure of male animals of the F0 generation from PND42 to PND140);
- Use of a point of departure ($UF_{B/L}$): 1, as the PoD was a NOAEL;
- inadequacy of the data (UF_D): 1, as multiple studies on genistein toxicity are available.

An overall uncertainty factor of 79 was therefore used for developing the TRV.

3.3.3.5. Proposed long-term oral TRV for the general population and confidence level

TRV = $NOAEL_{HED}$/UF = 1.65/79 = **0.02 mg.kg $bw^{-1}.d^{-1}$**

The overall confidence level for this TRV was estimated at 3/5; this corresponds to a **moderate confidence level**.

[9] This equation is taken from the recommendations of the US EPA (US EPA, 2006).

3.4. Proposed long-term oral TRV for pregnant women (unborn children), women of childbearing age and prepubertal children

3.4.1. Choice of the critical effect

Several studies conducted on rodents showed effects on reproduction (Nagao *et al*., 2001; NTP, 2008; Caceres *et al*., 2020; Oliviera *et al*., 2022; DelForno *et al*., 2022; Abo Elsoud *et al*., 2019; Eustache *et al.*, 2020 and 2009; Maraudino *et al.*, 2020).

Among the effects identified were delayed puberty in male rats, decreased age at vaginal opening, an increase in the length of oestrous cycles and accelerated mammary gland development in female mice, mammary gland alveolar/ductal hyperplasia in males, functional effects on fertility (decrease in fertility index) in females, a decrease in relative epididymal weight in male rats exposed *in utero* and after birth and a statistically significant decrease in litter size (resulting from mating male F1 generation rats with unexposed females). Among these effects, the decrease in relative epididymal weight in male rats and the statistically significant decrease in litter size were observed at the lowest doses identified in the studies deemed to be of good quality, for all effects combined.

The HRV Committee therefore considered all the effects on reproduction (decrease in relative epididymal weight in male rats and statistically significant decrease in litter size) to be critical effects.

3.4.2. Analysis of the existing long-term TRVs

In the NCM's 2020 report, two HBGVs of 0.09 and 0.07 mg.kg $bw^{-1}.d^{-1}$ were developed based, respectively, on developmental effects observed in female rats (NTP, 2008) and precocious puberty effects in female mice (Li *et al.*, 2014). These two HBGVs were not adopted by the HRV Committee, which considered that data from other studies identified other critical effects with lower PoDs. Although of good methodological quality, there was nothing in the study by Li *et al*. to enable the exposure doses of the animals expressed in mg.kg $bw^{-1}.d^{-1}$ to be recalculated and confirmed. This uncertainty meant that this HBGV could not be used.

As a consequence, given these limitations, the HRV Committee did not retain the existing HBGVs and proposed developing a long-term oral HBGV.

3.4.3. Development of the long-term oral TRV for pregnant women (unborn children), women of childbearing age and prepubertal children

3.4.3.1. Choice of the key study

Eustache *et al*. exposed pregnant Wistar Han rats daily by gavage to genistein doses of 1 and 10 mg.kg $bw^{-1}.d^{-1}$ from GD1 to PND21 (weaning). The pups were then exposed by gavage daily until PND80. This study reported a decrease in relative epididymal weight in male rats exposed *in utero* and after birth, and a statistically significant decrease in litter size (resulting from mating male F1 generation rats with unexposed females). These effects are considered harmful and can be transposed to humans. These effects were observed at the lowest doses identified in the studies deemed to be of good quality, for all effects combined.

The 2009 study by Eustache *et al.*, deemed to be of good quality, was therefore selected as the key study.

3.4.3.2. Choice of the point of departure

In the key study selected (Eustache *et al.*, 2009), the developmental effects on offspring exposed *in utero* and postnatally were those that appeared at the lowest dose. **The HRV Committee therefore selected as its point of departure a LOAEL of 1 mg.kg bw^{-1}.d^{-1} associated with a decrease in relative epididymal weight in male rats exposed *in utero* and after birth, and a statistically significant decrease in litter size resulting from mating F1 generation males with unexposed females.**

3.4.3.3. Allometric adjustment

An allometric adjustment was performed to reduce the uncertainty regarding inter-species variability. An HED was calculated with the equation given in Section 3.3.3.3., using an average body weight of 0.25 kg for rats and 70 kg for humans.

i.e. a $LOAEL_{HED}$ = 1 x $(0.25/70)^{0.25}$ = 0.24 mg.kg bw^{-1}.d^{-1}

This $LOAEL_{HED}$ of 0.24 mg.kg bw-1.d-1 was selected as the PoD.

3.4.3.4. Choice of uncertainty factors

The TRV was calculated from the 2009 study by Eustache *et al*. using the following uncertainty factors (UFs) (ANSES, pending publication):

- Inter-species variability (UF_A): 2.5 to take account of the toxicodynamic component, as a dose adjustment was made;
- Inter-individual variability (UF_H): $\sqrt{10}$, as the TRV had already been developed for a sensitive population;
- Subchronic to chronic transposition (UF_S): 1, as the critical effects resulted from *in utero* exposure;
- Use of a point of departure ($UF_{B/L}$): $\sqrt{10}$, as the PoD used was a LOAEL;
- Inadequacy of the data (UF_D): 1, as multiple studies on genistein toxicity are available.

An overall uncertainty factor of 25 was therefore used for developing the TRV.

3.4.3.5. Proposed long-term oral TRV for pregnant women (unborn children), women of childbearing age and prepubertal children, and confidence level

TRV = $LOAEL_{HED}$/UF = 0.24/25 = **0.01 mg.kg bw^{-1}.d^{-1}**

The overall confidence level for this TRV was estimated at 2.8/5; this corresponds to a **moderate confidence level**.

3.5. HRV Committee conclusion and recommendations

Two long-term oral TRVs were developed for genistein:

- one for the general population,
- one for pregnant women (unborn children), women of childbearing age and prepubertal children.

Although the calculation of these TRVs was based on data specific to the effects of genistein, the values can be extended to the sum of isoflavones expressed in mg.kg bw^{-1}.d^{-1} of aglycone equivalent (free or conjugated).

The long-term oral TRV was based on the increased incidence of mammary gland alveolar/ductal hyperplasia in male rats. A moderate confidence level was assigned to this TRV.

A long-term oral TRV for pregnant women (unborn children), women of childbearing age and prepubertal children has been proposed for genistein based on a decrease in relative epididymal weight in male rats exposed *in utero* and after birth, and a statistically significant decrease in litter size resulting from mating F1 generation males with unexposed females. A moderate confidence level was assigned to this TRV.

Table 2: Long-term oral TRVs for genistein for the general population and pregnant women (unborn children), women of childbearing age and prepubertal children

RV	**Organisation**	ANSES	
	Year	2024	
	Name	Long-term oral TRV	
	Value	0.02 mg.kg $bw^{-1}.d^{-1}$	0.01 mg.kg $bw^{-1}.d^{-1}$
Target population		General population	Pregnant women (unborn children), women of childbearing age and prepubertal children
Critical effect		Increased incidence of mammary gland alveolar/ductal hyperplasia in male rats of the F0 generation	Decrease in relative epididymal weight in male rats and decrease in litter size
Key study	**Reference**	NTP, 2008	Eustache *et al*., 2009
	Species	NCTR CD (Sprague-Dawley) rats	Wistar Han rats
	Exposure (duration, route)	From postnatal day 42 to postnatal day 140 (F0)	From gestation to postnatal day 80
Point of departure (PoD)		NOAEL = 5.9 mg.kg $bw^{-1}.d^{-1}$	LOAEL = 1 mg.kg $bw^{-1}.d^{-1}$
Time adjustment		None	None
Allometric adjustment		$NOAEL_{HED}$ = 1.65 mg.kg $bw^{-1}.d^{-1}$	$LOAEL_{HED}$ = 0.24 mg.kg $bw^{-1}.d^{-1}$
Uncertainty factors (UFs)		79 (UF_A: 2.5; UF_H: 10; UF_L: 1; UF_S: $\sqrt{10}$; UF_D: 1)	25 (UF_A: 2.5; UF_H: $\sqrt{10}$; UF_L: $\sqrt{10}$; UF_S: 1; UF_D: 1)
Confidence level		Moderate	Moderate

PND: postnatal day, NOAEL: no observed adverse effect level (maximum dose not leading to an observed harmful effect), LOAEL: lowest observed adverse effect level (minimum dose leading to an observed harmful effect), HED: human equivalent dose, $UF_{A\text{-}TD}$: toxicodynamic component of the inter-species uncertainty factor, UF_H: intra-species or inter-individual uncertainty factor, $UF_{B/L}$: uncertainty factor related to the point of departure, UFs: uncertainty factor related to the duration of the key study, UF_D: uncertainty factor related to the lack of data.

According to both *in vitro* and *in vivo* experimental studies, the oestrogenic potential of genistein is greater than that of daidzein. However, daidzein is transformed into equol by the intestinal microbiota, and the oestrogenic potential of equol is similar to that of genistein (Shor *et al*., 2002; Setchell *et al*., 2002). Thus, for equol-producing individuals, genistein and daidzein ultimately exert oestrogenic effects of comparable intensity. Given these equivalences and the fact that *in vivo* toxicological data relating to endocrine disruption almost exclusively concern the effects of genistein, calculation of these TRVs was based on data specific to the effects of genistein. These TRVs can be extended to the sum of isoflavones (aglycones and glucuronides) expressed in mg.kg $bw^{-1}.d^{-1}$ of aglycone equivalent (free or conjugated).

It is important to bear in mind that these two proposed TRVs were determined according to a methodology validated for chemical agents to which exposure is undesirable (pesticides, hazardous products, feedstocks, etc.). However, this formal request concerns a group of compounds occurring naturally in food, and for which there is a large volume of human data testing their possible beneficial rather than adverse effects.

4. AGENCY CONCLUSIONS AND RECOMMENDATIONS

The French Agency for Food, Environmental and Occupational Health & Safety endorses the two proposed health-based guidance values (TRVs) developed for long-term oral exposure to genistein; one for the general population and the other for women of childbearing age, prepubertal children and pregnant women. Although based on data specific to genistein, the Agency considers that these two TRVs can be extended to the sum of isoflavones expressed in $mg.kg.bw^{-1}.d^{-1}$ of aglycone equivalent. Due to an absence of data, it was not possible to propose an TRV for post-menopausal women.

The Agency reiterates that an TRV is a toxicological indicator for qualifying or quantifying a risk to human health.

The general approach used to define TRVs is therefore based on the choice of a critical effect corresponding to the most sensitive harmful effect duly documented, i.e. appearing at the lowest dose, independently of the underlying biological mechanisms. The critical effects selected for the two TRVs in this opinion, identified from the scientific studies currently available, are reprotoxic effects that may result from an endocrine mode of action. In this respect, the Agency stresses that genistein and daidzein have been examined under the Cosmetics Regulation, mainly due to concerns about their endocrine-disrupting properties (SCCS, 2022). These concerns persist following this assessment.

The results obtained support the need for a broader methodological debate on the development of TRVs for substances with endocrine activity and the use of such toxicological benchmarks in quantitative approaches to health risks. To achieve this, ANSES will undertake a consultation with its European counterparts in order to share practices for determining TRVs and conducting risk assessments for endocrine disruptors or substances with endocrine activity.

In the meantime, ANSES considers that these TRVs can already be used to assess the risks associated with situations of enforced exposure to isoflavones, for example in cases where only a single menu is offered in collective catering. These TRVs were used for the expert appraisal on assessment of the health risk associated with the consumption of foods containing isoflavones (Request 2022-SA-0221).

Lastly, the Agency stresses that in addition to a risk assessment based on an TRV, the choice of risk management measures in a given exposure context can also include other parameters, such as any requirements identified for the target populations, whether this concerns substances or their vectors, in cases where their nutritional or physiological benefits cannot be substituted.

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KEY WORDS

Valeur toxicologique de référence, VTR, voie orale, long terme, isoflavones, daïdzéine, génistéine, équol, toxicité chronique.

Key words

Health-based reference value, TRV, oral route, long term, isoflavones, daidzein, genistein, equol, chronic toxicity.