



DRAFT NTP BRIEF ON SOY INFANT FORMULA

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*NTP Center for the Evaluation of
Risks to Human Reproduction*



National Institute of Environmental Health Sciences
National Institutes of Health
U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

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PREFACE

Soy infant formula contains soy protein isolate and is fed to infants as a supplement to or a replacement for human milk, or as an alternative to cow milk formula. Soy protein isolate contains isoflavones with estrogenic activity called “phytoestrogens,” a subset of plant-derived compounds with biological activity similar to the female hormone estrogen, which occur naturally in some legumes. Isoflavones are found in many soy-based food products in addition to soy infant formula, such as tofu, soy milk, and in some over-the-counter dietary supplements. Soy infant formula was selected for expert panel evaluation because of: (1) the availability of developmental toxicity studies in laboratory animals exposed to soy or genistein (the most abundant isoflavone found in soy infant formula), as well as a number of studies on human infants fed soy infant formula, (2) the availability of information on isoflavone exposures in infants fed soy infant formula, and (3) public concern for effects of soy infant formula on infant or child development.

The current NTP-CERHR assessment of soy infant formula is a follow-up to a previous evaluation initiated in 2004 that did not result in the publication of any NTP Monographs. In the previous evaluation, CERHR convened an expert panel on March 15–17, 2006, to conduct two evaluations, one on the potential developmental and reproductive toxicities of soy infant formula, and a separate evaluation for genistein. The expert panel reports were released for public comment on May 5, 2006 (71 FR 28368). On November 8, 2006 (71 FR 65537), CERHR released draft NTP Briefs on Soy Infant Formula and Genistein that provided the NTP’s interpretation of the potential for these compounds to cause adverse reproductive and/or developmental effects in exposed humans. However, these draft NTP Briefs were not finalized nor were NTP Monographs published. In 2008 CERHR renewed efforts to complete the evaluation of soy infant formula and genistein. In updating the literature review, CERHR determined that an updated evaluation by an expert panel was needed because of the number of new publications related to human exposure, health assessment of infants fed soy infant formula, or developmental toxicity in laboratory animal models that had been published since finalization of the 2006 expert panel reports. The intent to conduct an updated evaluation was announced on October 2, 2008 (73 FR 57360). The current evaluation focuses only on soy infant formula and the potential developmental toxicity of its major isoflavone components, i.e., genistein, daidzein (and its estrogenic metabolite, equol), and glycitein. CERHR narrowed the scope of the current evaluation to include only developmental toxicity because the assessment of reproductive effects of genistein following exposure to adults was not considered relevant to the consideration of soy formula use in infants during the 2006 evaluation.

An expert panel met at a public meeting on December 16-18, 2009 to complete the updated evaluation of soy infant formula and their final report was released for comments on January 15, 2010 (75 FR 2545). The final Expert Panel Report on Soy Infant Formula presented conclusions on: (1) the strength of the scientific evidence that soy infant formula or its isoflavone constituents are developmental toxicants based on data from *in vitro*, animal, or human studies; (2) the extent of isoflavone exposures in infants fed soy infant formula; (3) the assessment of the scientific evidence that adverse developmental health effects may be associated with such exposures; and (4) their assessment of data gaps in the soy infant formula

and isoflavone literature to identify research and testing priorities to reduce uncertainties and increase confidence in future evaluations.

The NTP Brief on Soy Infant Formula presents the NTP's opinion on the potential for exposure to soy infant formula to cause adverse developmental effects in humans. The NTP Brief is intended to provide clear, balanced, scientifically sound information. It is based on information about soy infant formula provided in the expert panel report, public comments, additional scientific information made available since the expert panel meeting, and peer reviewer critiques of the draft NTP Brief.

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WHAT IS SOY INFANT FORMULA

Soy infant formula is fed to infants as a supplement to or a replacement for human milk, or as an alternative to cow milk formula. In the United States, the Food and Drug Administration (FDA) regulates the nutrient composition of soy infant formula as well as other infant formula types such as cow milk formula. Infant formulas must comply with the Infant Formula Act of 1980 and subsequent amendments passed in 1986 (FDA 2000). The specified nutrient levels are based on the recommendations of the Committee on Nutrition of the American Academy of Pediatrics and are reviewed periodically as new information becomes available. In the United States, a relatively small number of companies market soy infant formula (see Expert Panel Report, Table 4). The primary ingredients in soy infant formula include corn syrup, soy protein isolate, vegetable oils, sugar, vitamins, minerals, and other nutrients. Soy protein isolate is made from soybeans and is present in infant formulas at 14–16% by weight. In addition, the formulas are fortified with nutrients such as iron, calcium, phosphorous, magnesium, zinc, manganese, copper, iodine, sodium selenate, potassium, chloride, choline, inositol, and vitamins A, C, D, E, K, and B (B1, B2, B6, B12, niacin, folic acid, pantothenic acid, and biotin). Contaminants of soy protein include phytates (1.5%), which bind minerals and niacin, and protease inhibitors, which have antitrypsin, antichymotrypsin, and antielastin properties. Formulas are fortified with minerals to compensate for phytate binding and heated to inactivate protease inhibitors. Aluminum from mineral salts is found in soy infant formulas at concentrations of 600–1300 ng/mL, levels that exceed aluminum concentrations in human milk, 4–65 ng/mL (Bhatia and Greer 2008). The typical reconstitution of powdered formula is the addition of 8.7–9.3 g powdered formula to 2 fluid ounces of water (Drugstore.com 2004). Soy infant formulas are also available as concentrated liquids (generally 1 part soy infant concentrate to 2 parts water) and as ready-to-feed formulations.

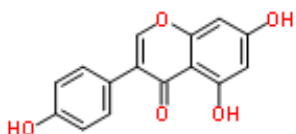
Soy protein isolate contains isoflavones with estrogenic activity called “phytoestrogens,” a subset of plant-derived compounds with biological activity similar to the female hormone estrogen that occurs naturally in some legumes. Phytoestrogens are found in many soy-based food products in addition to soy infant formula, such as tofu and soy milk, and in some over-the-counter dietary supplements. In soy infant formula, nearly all the phytoestrogens are bound to sugar molecules and these phytoestrogen-sugar complexes (“glucosides”) are not generally considered hormonally active. There are three major glucosides found in soy infant formula: genistin, daidzin, and glycitin (Figure 1). Before isoflavone glucosides can be absorbed into the systemic circulation, they are typically first hydrolyzed to their sugar-free forms (“aglycones”). In addition, several studies show that isoflavones can also be absorbed as glucosides (Allred *et al.* 2005; Hosoda *et al.* 2008; Kwon *et al.* 2007; Steensma *et al.* 2006). The sugar-free forms of these phytoestrogens are the biologically active forms and are called genistein, daidzein, and glycitein, respectively. Daidzein also produces an estrogenic metabolite called equol in some people. Glycosidase activity occurs in food products (by endogenous enzymes or those added during processing), in the cells of the gastrointestinal mucosa, or in colon microbes, and isoflavones can be measured in blood within an hour of soy ingestion

(Kano *et al.* 2006; Larkin *et al.* 2008). Aglycones undergo passive diffusion across the small and large intestinal brush border (Larkin *et al.* 2008). Once absorbed, the body then binds, i.e. conjugates, the free phytoestrogens to another molecule such as glucuronic acid. As much as 97-99% of the phytoestrogens in human blood are bound, or conjugated, to another molecule. The relative amounts of phytoestrogens in soy infant formula are genistin > daidzin > glycitein, which also corresponds to their relative estrogenic potency based on *in vitro* estrogen-receptor activities of the sugar-free forms of these phytoestrogens (UK-Committee-on-Toxicity 2003).

Figure 1. Chemical Structures of Isoflavones Associated with Soy Infant Formula

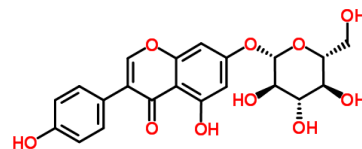
Genistein

$C_{15}H_{10}O_5$
MW: 270.24
CASRN: 446-72-0



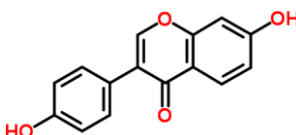
Genistin

$C_{21}H_{20}O_{10}$
MW: 432.37
CASRN: 529-59-9



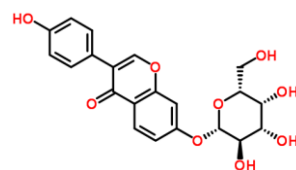
Daidzein

$C_{15}H_{10}O_4$
MW: 254.24
CASRN: 486-66-8



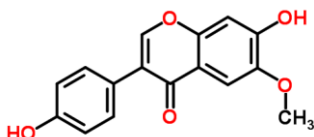
Daidzin

$C_{21}H_{20}O_9$
MW: 416.37
CASRN: 552-66-9



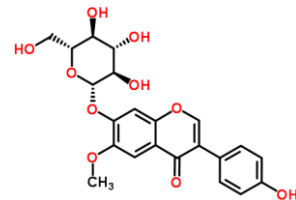
Glycitein

$C_{16}H_{12}O_5$
MW: 284.26
CASRN: 40957-83-3



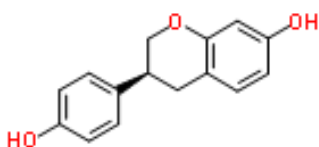
Glycitin

$C_{22}H_{22}O_{10}$
MW: 446.41
CASRN: 40246-10-4



Equol

$C_{15}H_{14}O_3$
MW: 242.27
CASRN: 531-95-3



USE OF SOY INFANT FORMULA AND EXPOSURE TO ISOFLAVONES IN INFANTS AND ADULTS

Usage

Sales of soy infant formula represented ~12% of the United States infant formula market based on 2009 dollar sales (personal communication with Robert Rankin, Manager of Regulatory and Technical Affairs at the International Formula Council, October 13, 2009). The use of soy infant formula in the United States has decreased by almost half between 1999 and 2009, from 22.5% to 12.7%, calculated based on total formula sold corrected for differences in formula cost.¹ The usage and sales of soy infant formula vary worldwide, ranging from 2 to 7% of infant formula sales in the United Kingdom, Italy, and France, and 13% in New Zealand (Agostoni *et al.* 2006; Turck 2007), to 31.5% in Israel (Berger-Achituv *et al.* 2005).

Recent data from the Infant Feeding Practices Study II (IFPS II), a longitudinal mail survey of mothers of infants conducted by the FDA in 2005–2007, indicated that ~57 to 71% of infants were fed infant formula (of any kind) during the first 10 months of life (Grummer-Strawn *et al.* 2008). However, many aspects of infant formula use from this study are unknown, including what percent of infants were exclusively fed infant formula compared to what percent were fed a mixture of infant formula and breast milk. It is also unknown what proportion of formula-fed infants were exclusively fed soy infant formula, although it is not likely a large percentage. For example, in one prospective cohort study where parents chose the feeding method, only 23% of infants included in the “soy infant formula” group were exclusively fed soy infant formula from birth to 4 months of age (Gilchrist *et al.* 2009). In a study of Israeli infants (3-24 months old), only 21.4, 16, and 18.5% of infants included in the “soy” group were exclusively fed soy infant formula the first year of life, the second year of life, or the first two years of life, respectively (Zung *et al.* 2008). Another study of feeding patterns in Israeli infants reported that of the formula-fed infants, 9% were started with a soy infant formula, but 50% were switched to a cow milk-based formula at some time (Nevo *et al.* 2007). This study also found that the type of formula used was changed for 47% of the formula-fed infants during the first 6 months of life, and that 12% had more than two changes.

Commonly cited reasons for using soy infant formula are to feed infants who are allergic to dairy products or are intolerant of lactose, galactose, or cow-milk protein (Essex 1996; Tuohy 2003). In May 2008, the American Academy of Pediatrics (AAP) released an updated policy statement on the use of soy protein-based formulas (Bhatia and Greer 2008). The overall conclusion of the AAP was that although isolated soy protein-based formulas may be used to provide nutrition for normal growth and development in term infants, there are very limited

¹ Public comment from the International Formula Council (IFC), received December 3, 2009 (available at <http://cerhr.niehs.nih.gov/chemicals/genistein-soy/SoyFormulaUpdt/SoyFormula-mtg.html>) and personal communication with Dr. Haley Curtis Stevens, IFC.

indications for their use in place of cow milk-based formula. The only circumstances under which the AAP recommends the use of soy infant formula are instances where the family prefers a vegetarian diet or for the management of infants with galactosemia or primary lactase deficiency (rare). Soy infant formula is not currently recommended for preterm infants by the AAP or the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) Committee on Nutrition (Agostoni *et al.* 2006).

Specific conclusions in the 2008 AAP report are:

- Lactose free and reduced lactose-containing cow milk formulas are now available and could be used for circumstances in which elimination or a reduction in lactose in the diet, respectively, is required. Because primary or congenital lactase deficiency is rare, very few individuals would require a total restriction of lactose. Lactose intolerance is more likely to be dose dependent. Thus, the use of soy protein-based lactose-free formulas for this indication should be restricted.
- The routine use of isolated soy protein-based formula has no proven value in the prevention or management of infantile colic or fussiness.
- Isolated soy protein-based formula has no advantage over cow milk protein-based formula as a supplement for the breastfed infant, unless the infant has one of the indications noted above.
- Soy protein-based formulas are not designed for or recommended for preterm infants. Serum phosphorus concentrations are lower, and alkaline phosphatase concentrations are higher in preterm infants fed soy protein-based formula compared to preterm infants fed cow milk-based formula. As anticipated from these observations, the degree of osteopenia is increased in infants with low birth weight receiving soy protein-based formulas. The cow milk protein-based formulas designed for preterm infants are clearly superior to soy protein-based formula for preterm infants.
- For infants with documented cow milk protein allergy, extensively hydrolyzed protein formula should be considered, because 10% to 14% of these infants will also have a soy protein allergy.
- Infants with documented cow milk protein-induced enteropathy or enterocolitis frequently are as sensitive to soy protein and should not be given isolated soy protein-based formula. They should be provided formula derived from hydrolyzed protein or synthetic amino acids.
- The routine use of isolated soy protein-based formula has no proven value in the prevention of atopic disease [hypersensitivity reactions, allergic hypersensitivity affecting parts of the body not in direct contact with the allergen] in healthy or high-risk infants.

Additional Sources of Soy Intake by Infants

A number of studies have reported on the use of soy foods in the context of infant feeding and feeding transitions during the first years of life. Data from IFPS II indicated that ~6% of infants consume soy foods by 1 year of age (Grummer-Strawn *et al.* 2008). A survey of the isoflavone content of infant cereals in New Zealand led the authors to conclude that supplementation of the diet of a 4-month old infant fed soy infant formula with a single serving of cereal can increase isoflavone intake by more than 25%, depending on the brand used (Irvine *et al.* 1998). Infants may also be exposed to soy flour and soy oil by the use of soy-containing fortified spreads as a complementary food to address growth and nutritional issues in countries with high incidence of childhood malnutrition, such as Malawi (Lin *et al.* 2008; Phuka *et al.* 2008).

The consumption of soy milk by children is currently being assessed in the 2008 Feeding Infants and Toddlers Study (FITS), a survey of the eating habits and nutrient intakes of > 3,000 children from 4 to 24 months of age² sponsored by Nestle Nutrition Institute. Based on survey data collected in 2002, soy milk was reported as one of the more frequently consumed beverages in children 15-18 months of age, but not in younger infants or older toddlers 19-24 months of age (Skinner *et al.* 2004). A 2006 presentation from the Executive Director of the Soyfoods Association of North America, Nancy Chapman³, cited 2002 FITS data to report that out of 600 toddlers surveyed, almost 4% consumed soy milk at least once a day. Overall, soy milk is one of the fastest growing markets in the soy food industry (United Soybean Board 2009). However, it is unclear whether this growth trend extends to infants and toddlers.

Daily Intake and Biological-Based Indicators of Exposure

A number of studies in the United States and abroad have measured total isoflavone levels in infant formulas (see Expert Panel Report, Table 9). For infant formulas manufactured in the United States, the range of total isoflavone levels reported in reconstituted or “ready-to-feed” formulas was 20.9–47 mg/L formula (Franke *et al.* 1998; Setchell *et al.* 1998). The range of total isoflavones content in soy infant formula samples collected in the United States and other countries is 10-47 mg/L (Genovese and Lajolo 2002; Setchell *et al.* 1998). Genistein is the predominant isoflavone found in soy infant formula (~58-67%), followed by daidzein (~29-34%) and glycitein (~5-8%). The isoflavone content in soy infant formula appears to be much less variable than the isoflavone content of soy beans or other soy products (e.g. soy supplements or soy protein isolates) (see Expert Panel Report, Section 1.2.2.4).

Infants fed soy infant formula have higher daily intakes of genistein and other isoflavones than other populations (Table 1). However, differences in methods used to select representative

² Preliminary findings from the 2008 FITS are available at <http://medical.gerber.com/starthealthystayhealthy/FITSStudy.aspx>. The 2008 survey was sponsored by Nestlé Nutrition and conducted by Mathematica as a followup to the FITS 2002 study.

³ Presentation available at http://www.soyfoods.org/wp/wp-content/uploads/2006/12/soymilk_in_school_meals.pdf.

samples and calculate intake estimates limit the ability to compare intake estimates across studies, especially for dietary surveys. In addition, isoflavone intake appears to be highly variable in soy-consuming adult populations. Recognizing these caveats, the relative ranking of total isoflavone intake appears to be infants exclusively fed soy infant formula > vegan adults > Japanese adults consuming a traditional diet > vegetarian adults > omnivores consuming Western diets.

Table 1. Comparison of Estimated Intake of Genistein and Total Isoflavones in Infants Fed Soy Infant Formula to Other Populations

Population, diet	Daily Intake (mg/kg bw/day)*		Reference
	Total Isoflavone	Genistein	
Infants			
United States, soy infant formula	2.3 – 9.3	1.3 – 6.2	Table 26 of expert panel report
United States, cow milk formula	0.0002 - 0.0158		(Knight <i>et al.</i> 1998; Kuhnle <i>et al.</i> 2008)
United States, breast milk	0.0002 - 0.0063		(Friar and Walker 1998; Setchell <i>et al.</i> 1998)
Adults*			
United States, omnivore	0.0097 ^a – 0.096 ^b	0.005 ^a – 0.056 ^b	^a (Haytowitz 2009); ^b (Tseng <i>et al.</i> 2008);
United States, vegetarian	0.21	0.14	(Kirk <i>et al.</i> 1999)
European, omnivore	0.007 – 0.009	0.004 – 0.005	(Mulligan <i>et al.</i> 2007)
European, vegetarian	0.100 – 0.112	0.057 – 0.062	
United Kingdom, vegan	1.07	–	(Friar and Walker 1998)
Japanese, traditional diet	0.67 ^b	0.077 ^a – 0.43 ^b	^a (Fukutake <i>et al.</i> 1996); ^b (Arai <i>et al.</i> 2000)

*Daily intakes for adults were based on mg/day estimates presented in Table 25 of the expert panel divided by 70 kg body weight.

Infants fed soy infant formula also have higher blood-based levels of genistein and daidzein compared to other populations such as vegans and Asian populations consuming a traditional diet high in soy foods (Table 2). The latest findings for the United States, reported by Cao *et al.* (2009), were that concentrations of total genistein in whole blood samples from infants fed soy infant formula were 1455 ng/ml at the 75th percentile and 2763.8 ng/ml at the 95th percentile (personal communication with Dr. Yang Cao, NIEHS); both of these values are higher than the maximum total genistein concentrations available for any other population. The geometric mean of total genistein measured in these infants was 757 ng/ml, a value that is 53.3- and 70.1-times higher than the corresponding levels detected in infants fed cow milk formula or breast milk, respectively (Table 2). Average blood levels of total genistein in the soy infant formula-fed infants were ~160-times higher than the mean levels of total genistein in omnivorous adults in the United States (4.7 ng/ml) reported by Valentin-Blasini (2003); a similar pattern was observed for urinary concentrations of genistein and daidzein (Cao *et al.* 2009; U.S. Centers for Disease Control and Prevention 2008). It is not known for infants how long it takes to achieve maximum blood concentrations of genistein and daidzein. In adults, it is ~5.7 and 6.2 hours, respectively (Cassidy *et al.* 2006), thus the blood levels of isoflavones sampled at least one hour

after feeding as reported in Cao *et al.* (2009) may not represent the maximum concentration for each infant.

Table 2. Average Blood-Based Levels of Genistein and Daidzein in Infants and Adult Populations

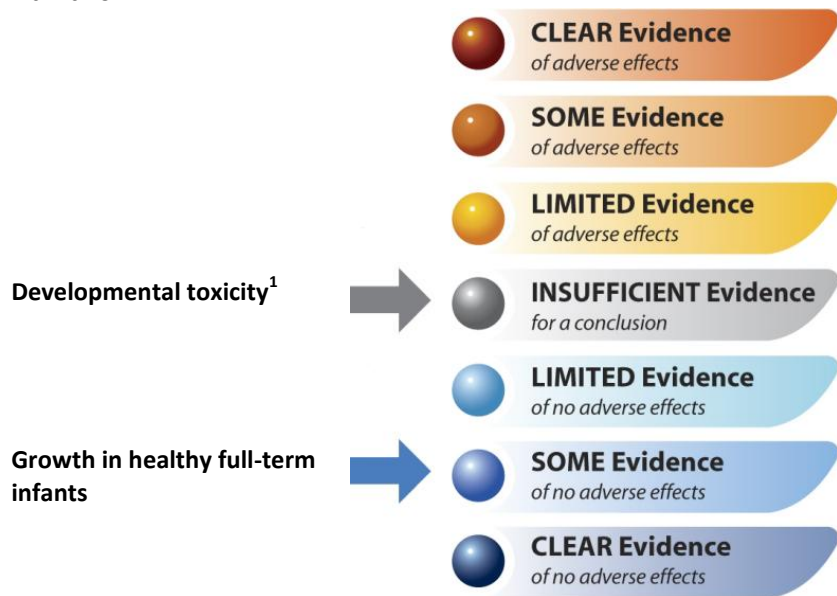
Population, diet	Sample	Average Total Isoflavone Concentration, ng/ml		Reference
		Genistein	Daidzein	
US infants, soy infant formula	Whole blood	757 1455, 75 th percentile	256 519, 75 th percentile	(Cao <i>et al.</i> 2009)
US infants, soy infant formula	Plasma	684	295	(Setchell <i>et al.</i> 1997)
US infants, cow milk formula	Whole blood	14.2	5.5	(Cao <i>et al.</i> 2009)
US infants, cow milk formula	Plasma	3.16	2.06	(Setchell <i>et al.</i> 1997)
US infants, breastfed	Whole blood	10.8	5.3	(Cao <i>et al.</i> 2009)
US infants, breastfed	Plasma	2.77	1.49	(Setchell <i>et al.</i> 1997)
US adults, omnivores	Serum	4.7 (<LOD – 203, range)	3.9 (<LOD – 162, range)	(Valentin-Blasini <i>et al.</i> 2003)
Japanese men, traditional diet	Plasma	105.2 (24 – 325, range)	71.3 (14.8 – 234.9, range)	(Adlercreutz <i>et al.</i> 1994)
Finnish women, vegetarians	Plasma	4.6	4.7	(Adlercreutz <i>et al.</i> 1994)
UK adults, vegans/vegetarians	Plasma	40	20	(Peeters <i>et al.</i> 2007)

CAN SOY INFANT FORMULA OR ITS ISOFLAVONE CONTENTS ADVERSELY AFFECT HUMAN DEVELOPMENT?⁴

Possibly. Appropriate levels of sex hormones are essential for normal development and function of the reproductive system. Because soy infant formula contains compounds with estrogen-like activity, concern has been expressed that feeding soy infant formula might adversely affect development of the reproductive system. There are presently not enough data from studies in humans to confirm or refute this possibility (Figure 2). Likewise, data from the studies in laboratory rodents and primates are not sufficient to permit a firm conclusion regarding the developmental toxicity of soy infant formula (Figure 3). However, blood levels of total genistein in infants fed soy infant formula can exceed blood levels in rats administered genistein in the diet or in mice treated by subcutaneous injection (sc injection) at dose levels that induce adverse developmental effects. Because of the high blood levels of isoflavones in infants fed soy infant formula and the lack of robust studies on the human health effects of soy infant formula, the possibility that soy infant formula may adversely affect human development cannot be dismissed.

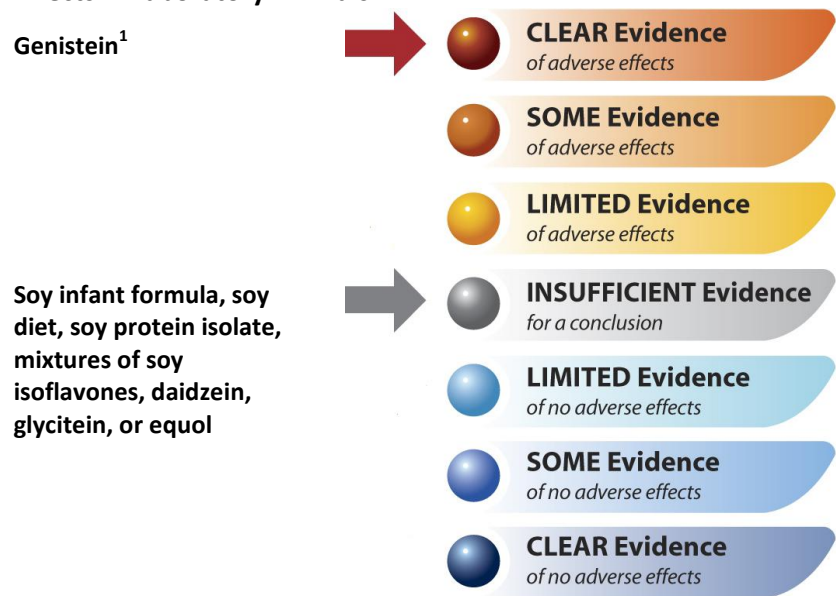
⁴ Answers to this and subsequent questions may be: *Yes, Probably, Possibly, Probably Not, No, or Unknown*

Figure 2. The Weight of Evidence that Soy Infant Formula or its Isoflavone Contents Causes Adverse Developmental Effects in Humans



¹Based on consideration of the following endpoints: bone mineral density, allergy/immunology, thyroid function, reproductive endpoints, cholesterol, diabetes mellitus, and cognitive function

Figure 3. The Weight of Evidence that Soy Infant Formula, Other Soy Products, or Individual Isoflavones Cause Adverse Developmental Effects in Laboratory Animals



¹Manifested as: decreased age at vaginal opening; abnormal estrous cyclicity; decreased fertility, implants, and litter size; and histopathology of the female reproductive tract.

Supporting Evidence

Human Studies

There is a relatively large literature describing growth or other health parameters in infants fed soy infant formula. These studies provide sufficient evidence to conclude that use of soy infant formula does not impair growth during infancy in healthy full-term infants. However, this literature is considered insufficient to reach a conclusion on whether the use of soy infant formula adversely affects human development with respect to effects on bone mineral density, allergy/immunology, thyroid function, reproductive system endpoints, cholesterol, diabetes mellitus, and cognitive function (Figure 2). Commonly encountered limitations of these studies include: inadequate sample size, short-duration of follow-up, unspecified method of assignment to feeding groups, the use of self-selected breast- and formula-feeding mothers, changes in feeding methods (i.e., formula-type and/or breast milk), lack of information regarding the introduction of solid foods, and inadequate consideration of potential confounding variables. When the expert panel reviewed this literature, only 28 of the ~80 published human studies on soy infant formula were considered to have utility for the NTP-CERHR evaluation process (see Expert Panel Report, Table 153).

A number of critical research needs were also identified during the course of the evaluation based on case reports, pilot studies in humans, or findings in laboratory animals. In particular, there is a need to (1) assess the potential impacts of soy infant formula use on reproductive tissues or function during infancy, childhood, and later in life and (2) monitor soy infant formula fed-infants who have congenital hypothyroidism for possible decreases in the effectiveness of thyroid hormone replacement therapy, i.e., *L*-thyroxin. A discussion of the findings, conclusions, and research recommendations regarding effects of soy infant formula on growth and the gastrointestinal system, reproductive system and breast tissue, and thyroid function are described below.

Growth and Gastrointestinal Effects

Although the NTP considered the human studies insufficient to assess whether the use of soy infant formula adversely affects development, the NTP concurs with the expert panel that there is sufficient evidence to conclude that use of soy infant formula does not negatively impact growth in healthy, full-term infants. Of the 28 human studies considered by the expert panel to have utility for the NTP-CERHR, 13 assessed growth outcomes and 11 of these studies reported no decreases in growth measurements (Chan *et al.* 1987; Hillman 1988; Hillman *et al.* 1988; Jung and Carr 1977; Köhler *et al.* 1984; Kulkarni *et al.* 1984; Lasekan *et al.* 1999; Mimouni *et al.* 1993; Sellars *et al.* 1971; Steichen and Tsang 1987; Venkataraman *et al.* 1992). Two of the 13 studies reported significant decreases in growth measurements in infants fed soy formula when compared to infants fed casein- and rice-based hydrolyzed formulas (Agostoni *et al.* 2007) or compared to infants fed a milk-based formula (Cherry *et al.* 1968). In addition to these “limited” utility studies, there were a large number of “no utility” studies of small sample size included in the expert panel report that consistently reported similar growth trajectories of

anthropometric measurements among the different infant feeding groups. Based on this overall pattern of response, the NTP concludes there is “some evidence of no adverse effects” on growth in healthy full-term infants (Figure 2).

It is worth noting that although all of the studies of gastrointestinal effects reviewed by the expert panel were classified as having “no utility,” extensive reviews by the AAP and ESPGHAN have noted the possibility of adverse effects in a subset of infants with documented cow milk protein allergy (Agostoni *et al.* 2006; Bhatia and Greer 2008). Infants with documented cow milk protein-induced enteropathy or enterocolitis frequently are sensitive to soy protein and should not be given soy protein formulas. Instead, the recommendation is to provide formula derived from hydrolyzed protein or synthetic amino acids (Agostoni *et al.* 2007).

Reproductive System

The NTP considered the existing literature in humans “insufficient” for assessing impacts on the reproductive system from the use of soy infant formula (Figure 2); only three studies were considered by the expert panel to be of sufficient utility for assessing these types of effects (Boucher *et al.* 2008; Freni-Titulaer *et al.* 1986; Strom *et al.* 2001). The most comprehensive assessment of reproductive function of men and women following use of soy infant formula did not report significant impacts, but it also lacked sufficient power for several endpoints (i.e., cancer, reproductive organ disorders, hormonal disorders, libido dysfunction, sexual orientation, and birth defects in the offspring) to rule out increased risks (Strom *et al.* 2001). Two significant findings were reported in this study related to menstrual cycling in adult women who were fed soy formula during infancy. One was that women who had been given soy infant formula reported having longer menstrual periods (adjusted mean difference of 0.37 days; 95% CI, 0.06-0.68, P=0.02) and a soy infant formula-associated increase in the risk of experiencing extreme menstrual discomfort (unadjusted RR, 1.77; 95% CI, 1.04-3.00, P=0.04). However, these findings would not be considered statistically significant if a multiple comparison adjustment were applied to account for the number of hypothesis. The remaining two studies of “limited” utility dealt exclusively with an association of soy infant formula consumption and effects on the breast, i.e., premature thelarche (Freni-Titulaer *et al.* 1986) or risk of breast cancer in adulthood (Boucher *et al.* 2008). These two studies are discussed below in the context of other findings on the breast related to the use of soy infant formula.

Subsequent to the expert panel evaluation, a study was published that reported a 25% higher early uterine fibroid diagnosis (diagnosis by the age of 35) for women who reported being fed soy formula during infancy (relative risk = 1.25, 95% confidence interval of 0.97 – 1.61) (D'Aloisio *et al.* in press). There was also a higher risk of a similar magnitude in association with being fed soy formula within the first two months of life (adjusted RR = 1.25; 95% CI: 0.90, 1.73). These findings were based on assessment of 19,972 non-Hispanic white women ages 35 to 59 at enrollment in the NIEHS Sister Study. The most common signs of fibroids are longer menstrual periods, heavy bleeding, and pelvic pain (Mayo Clinic), all of which were evaluated to some degree in the Strom *et al.* (2001) study. indications of heavy bleeding were not observed in that study based on self-reported assessment of menstrual flow (heavy/extremely heavy,

clots versus extremely light/light/average, normal), but a significant association was reported between use of soy infant formula and longer menstrual periods (discussed above) based on assessment of the number of days requiring pads or tampons. With respect to pelvic pain, the other significant finding from Strom *et al.* (2001) was an higher reporting of extreme menstrual discomfort. The finding of higher risk of early uterine fibroid diagnosis associated with use of soy infant formula is also broadly consistent with reports that *in utero* exposure to the synthetic estrogen diethylstilbestrol is also associated with fibroid diagnosis (Baird and Newbold 2005; D'Aloisio *et al.* in press) as well as histopathological findings reported in the uterus of adult mice treated with genistein as neonates (Newbold *et al.* 2001). One limitation to the D'Aloisio *et al.* (in press) study is the use of a self-administered family history questionnaire and dichotomous response (“ever” or “none” on soy infant formula feeding; “yes” or “no” on soy infant formula feeding \leq 2 months of age) for assessing exposure to soy infant formula. The NTP agrees with the author’s interpretation that the association with early diagnosis of uterine fibroids is interesting and needs to be replicated. Another observation from the NIEHS Sister Study, currently available only in abstract form, that is more difficult to interpret are findings that use of soy infant formula was associated with both higher odds of very early menarche (<11 yrs) and late menarche. (D'Aloisio *et al.* 2009).

In addition to the three studies considered of “limited” utility described above (Boucher *et al.* 2008; Freni-Titulaer *et al.* 1986; Strom *et al.* 2001), the expert panel evaluated four other studies of infants fed soy infant formula that included assessment of reproductive system development; however, these studies were considered to have “no utility” for the evaluation (Bernbaum *et al.* 2008; Giampietro *et al.* 2004 Zung, 2008 #2434; Gilchrist *et al.* 2009). The expert panel spent a considerable amount of time discussing the outcomes from two of these studies. One was a pilot study to identify estrogen responsive endpoints in infants (Bernbaum *et al.* 2008), and the other was an interim analysis from an ongoing prospective cohort design study (Gilchrist *et al.* 2009).

The pilot study by Bernbaum *et al.* (2008) was conducted as part of the Study of Estrogen Activity and Development (SEAD), a series of mostly cross-sectional pilot studies designed to establish methods for future larger studies evaluating the estrogenic effects of soy infant formulas (or any putative estrogenic exposure) on the developing infant (<http://www.niehs.nih.gov/research/atniehs/labs/epi/studies/sead/index.cfm>)⁵. SEAD had a

⁵ Analysis of isoflavones in the blood, urine, and saliva from these children based on feeding regimen are presented in Cao *et al.* (2009). Other data from this pilot study have only appeared in abstract form and include characterization of sex hormones (*Pediatric Academic Societies*, 2007 meeting), thyroid hormones (*International Society for Environmental Epidemiology*, 2009 meeting), and ultrasound evaluation of breast, testes, ovary, thyroid, and uterus (*Pediatric Academic Societies*, 2006 meeting). Abstracts from the Pediatric Academic Societies meetings that mention the SEAD study and have not yet been presented in peer-reviewed publications are available at http://www.pas-meeting.org/2009Baltimore/abstract_archives.asp.

[8406.2] Umbach, D., Phillips, T., Davis, H., Archer, J., Ragan, B., Bernbaum, J., Rogan, W. (2007) Relationship of Endogenous Sex Hormones and Gonadotropins to Soy infant formula Diet in Infants;
[2757.8] Estroff, J., Parad, R., Stroehla, B., Umbach, D., Walter Rogan, W. (2006) Developing Methods for Studying Estrogen-like Effects of Soy Isoflavones in Infants 3: Ultrasound

mixed, cross-sectional study design that included equal numbers of infants fed soy infant formula, cow milk formula, or breast milk. The pilot study evaluated breast and genital development in infants during the first 6 months of life, i.e., breast buds, breast adipose tissue, testicular volume and position, vaginal discharge, and cell maturation. Of these measurements, the authors considered measurement of breast buds and vaginal wall cell maturation to be the most valuable for evaluating exposures to compounds with estrogenic-like activity in humans. Breast bud diameter was maximal in the week after birth and smaller in older infants, both boys and girls, at 2 weeks to 6 months. The maturation index of vaginal wall cells was maximal in 1 week old infants and lowest at 1 month. Breast bud diameter and vaginal wall cell maturation index were considered the most estrogen-sensitive endpoints because they displayed a pattern of reversion during the period when infants would be withdrawing from the high maternal estrogen exposures that occur during pregnancy. While the authors very clearly described this study as a pilot and of too small a size to make reliable inferences about feeding regimens, the trajectory of maturation index appeared to differ in the infants fed soy infant formula ($p = 0.07$), such that these infants tended to have a higher maturation index at 3 to 6 months compared to infants fed breastmilk or a cow milk-based formula. Vaginal cell maturation indices are used as a measure of estrogen effects in adult women and have also been used in the diagnosis and evaluation of treatment for precocious puberty in girls [reviewed in Berbaum *et al.* (2008)]. The expert panel considered this pilot study of “no utility” for the evaluation given the variability observed and because the sample size was very small (once gender and age were considered) and thus underpowered statistically to detect any relevant associations. Based on the results of the pilot studies, a prospective study of infants fed soy infant formula, cow milk formula, or breast milk ($n=300$; 50 boys and 50 girls in each feeding group) has been planned and will include assessment of the endpoints evaluated in the pilot studies as well as others that allow testing of additional hypotheses, e.g., altered response to vaccination, changes in play behavior, or language acquisition in toddlers. Recruitment for this prospective study, which will be carried out at the Children’s Hospital of Philadelphia, is expected to begin in spring 2010.

The study by Gilchrist *et al.* (2009) was an interim report from a prospective, longitudinal study in children aged 2-3 months through 6 years who were breast-fed, cow milk formula-fed, or soy infant formula-fed as infants being conducted by the Arkansas Children’s Nutrition Center (ACNC). The completed study will include assessments of growth, development, body composition, endocrine status, metabolism, organ development, brain development, cognitive function, language acquisition, and psychological development at 3, 6, 9, 12, and 18 months and at 2, 3, 4, 5, and 6 years. The interim examination of the data published by Gilchrist *et al.* (2009) summarized differences in hormone-sensitive organ size at 4 months of age in infants fed soy infant formula (SF) ($n=39$, 19 males and 20 females), milk formula (MF) ($n=41$, 18 males and 23 females), or breast milk (BF) ($n=40$, 20 males and 20 females) (Gilchrist *et al.* 2009). A major limitation in the study is the amount of cross-feeding that occurred in the cohort.⁶ All

⁶ The Berbaum *et al.* (2008) study appears to have required stricter criteria for feeding regimen eligibility compared to Gilchrist *et al.* (2009). In Berbaum *et al.* (2008), breast milk and cow’s milk regimens prohibited use of

breastfed infants were stated to be exclusively fed breast milk the entire study time. Only 23% of infants in the SF group were exclusively fed soy infant formula from birth, 45% were switched to exclusive soy infant formula feeding within 4 weeks, and 32% were switched to soy infant formula between 4 and 8 weeks. Thus, the length of soy infant formula exposure varied from 2 to 4 months. Fifty-four percent of the infants in the MF group were stated to be exclusively fed milk formula from birth, 41% switched from breast milk to cow's milk formula within 4 weeks, and 5% switched between 4 and 8 weeks. At age 4 months, anthropometric measures (weight, length, and head circumference) were assessed using standardized methods, and body composition was assessed by air displacement plethysmography. Breast buds, uterus, ovaries, prostate and testicular volumes were measured by ultrasonography.

Gilchrist *et al.* (2009) concluded that the results did not support major diet-related differences in reproductive organ size as measured by ultrasound in infants at age 4 months, although there was some evidence that ovarian development might be advanced in milk formula-fed infants and that testicular development might be slower in both milk formula and soy infant formula infants as compared with infants fed breast milk. The direction of effect on testicular volume was opposite of that reported by Tan *et al.* (2006) in a study of marmoset monkeys with seven sets of co-twins where one twin from each set was fed a cow milk-based formula as the control and the other twin was fed soy infant formula milk for 5-6 weeks during infancy (infants also nursed during this period).

With respect to future consideration of the cohort described in Gilchrist *et al.* (2009), the expert panel noted the benefit of longitudinal data in characterizing differences in developmental endpoints across the exposure groups as a valuable study design feature. However, when exposure is mixed due to the cross-feeding across groups, the effects may be attenuated or exaggerated which makes the results thus far of no utility. Given that the report by Gilchrist *et al.* (2009) is an interim report from an ongoing prospective study, the expert panel noted that the completed study would have greater value if continued recruitment did not permit such extensive dietary transitions or data are collected prior to these transitions.

Effects on the Breasts

Seven studies evaluated by the expert panel included some assessment of the breast, either breast bud size in infants, age at breast development in girls, or risk of breast cancer in adulthood. Some of these studies were small in sample size or had other experimental features that resulted in their classification as "no utility" by the expert panel. However, the NTP considered findings from all of the studies for any overall pattern of response on breast development (Table 3) given that understanding possible effects on breast tissue, especially breast cancer risk, is of particular interest in the context of soy use, such as based on

soy foods in baby's lifetime; however, infants in the soy infant formula group were allowed breast milk or cow milk while the baby was in the hospital just after birth. In older infants, ≥ 3 months, soy infant formula regimen must have been fed exclusively and continuously for at least two-thirds of the child's lifetime, including 2 weeks before the study examination.

geographical differences in dietary ingestion of soy, e.g., Western versus Asian diets, or use of soy supplements.

One study assessed the association between use of soy infant formula and breast cancer in adulthood. Boucher *et al.* (2008) compared women with and without breast cancer and reported reduced, but non-significant, associations between soy infant formula intake and breast cancer: soy infant formula only during first 4 months of life: OR = 0.42, 95% CI = 0.13 – 1.40; soy infant formula only during 5-12 months of age: OR = 0.59, 95% CI = 0.18 – 1.90). Although non-significant, this pattern is consistent with conclusions from meta-analyses of limited human data and the animal model data (discussed below) that provide some support for a potential modestly protective effect for some soy or soy isoflavone exposures, e.g., childhood/adolescent exposure might have a small reduction in risk.

Other studies assessed breast bud development in infants or indication of premature thelarche, defined as breast development before the age of 8 without evidence of sexual hair development, estrogenization of vaginal mucosa, acceleration of linear growth, rapid bone maturation, adult body odor, or behavioral changes typical of puberty. One study of “limited utility” based on retrospective patient recall reported that use of soy infant formula may be associated with premature thelarche, or the start of breast development, before age 8 in girls, without other indications of sexual maturation (130 subjects from 552 potentially eligible girls) (Freni-Titulaer *et al.* 1986). Age-matched controls were recruited and parents were interviewed with regard to family history and possible exposures including the use of soy infant formula. Multivariate analysis did not show a significant relationship between premature thelarche and soy infant formula feeding except when the analysis was restricted to girls with onset of premature thelarche before 2 years of age (OR 2.7, 95% CI 1.1–6.8). Other significant factors included maternal ovarian cysts (OR 6.8, 95% CI 1.4–33.0) and consumption of chicken (OR 4.9, 95% CI 1.1–21.9). Consumption of corn was protective (OR 0.2, 95% CI 0.0–0.9). All other studies reporting on breast development in infants or young children were considered of “no utility” by the expert panel. The clinical or pathophysiological outcomes of premature thelarche are not clear. For example, a study by de Vries *et al.* (2009) suggests that premature thelarche does not predict precocious puberty. In this study, breast development and puberty were followed in 139 girls diagnosed with premature thelarche; it regressed in 50.8%, persisted in 36.3%, progressed in 3.2%, and had a cyclic course in 9.7%. With respect to age at diagnosis, progressive or cyclic course was more commonly found among girls presenting after 2 years (52.6%) compared with girls presenting at birth (13.0%) or at 1 to 24 months (3.8%). Precocious puberty occurred in 13% of girls and was not related to age at premature thelarche or clinical course.

The only other study reporting an association between soy infant formula and breast development reported an increased prevalence of breast buds in females during the second year of life (but not during the first year) (Zung *et al.* 2008), a finding that was interpreted by the authors as suggesting that soy phytoestrogens may have a “preserving” effect on breast tissue in infants. The authors also suggested that the lack of association during the first year

could be a function of the high plasma levels of endogenous estrogens that infants have at that time, potentially masking any estrogenic effects of soy phytoestrogens. Giampietro *et al.* (2004) also looked at female infants during this age range, but reported no difference in breast bud prevalence in children ages 7-96 months. Gilchrist *et al.* (2009) also reported no differences in breast bud volume at 4 months of age in girls or boys in relation to feeding regimen and there were no apparent differences in pattern of breast bud development in girls or boys based on feeding regimen in the pilot data presented in Bernbaum *et al.* (2008). However, infants in both of these studies were assessed at ≤ 6 months which would limit the ability to identify any effect consistent with the “preserving” effect reported in Zung *et al.* (2008).

Table 3. Summary of Epidemiological Findings of Breast-Related Measures in Association with Use of Soy Infant Formula

Breast-related Endpoint and Reference	Study Design	Sample Size	Major Findings	Expert Panel's Utility Category
<i>breast cancer</i> in adulthood (Boucher <i>et al.</i> 2008)	population-based case-control design; association of breast cancer with type of milk consumed during infancy	adults with breast cancer (N=372) and controls without breast cancer matched within 5-year age groups (N=356)	non-significant suggestions of reduced risk: soy infant formula only during first 4 months of life: OR = 0.42, 95% CI = 0.13 – 1.40; soy infant formula only during 5-12 months of age: OR = 0.59, 95% CI = 0.18 – 1.90 (multivariate analysis to control for possible confounding factors)	limited utility
<i>breast development</i> , age when started to wear a bra (Strom <i>et al.</i> 2001)	retrospective cohort study of adults who participated as infants in a non-randomized controlled feeding study	adults fed SF (N= 127) or CM (N=268) during infancy	no difference in unadjusted or adjusted means (SF = 12.3 years versus MF = 12.3 years; multivariate analysis to control for possible confounding factors)	limited utility
<i>breast development</i> , premature thelarche (Freni-Titulaer <i>et al.</i> 1986)	age-matched pair case-control study	girls with premature thelarche and age-matched controls (N=120 for each group in final analysis)	premature thelarche before 2 years of age and consumption of SF (OR 2.7, 95% CI 1.1–6.8; multivariate analysis to control for possible confounding factors)	limited utility
<i>breast development</i> , breast bud diameter and palpable buds in infants from birth to 6 months (Bernbaum <i>et al.</i> 2008)	mixed cross-sectional (pilot study to identify techniques for assessing infants' responses to withdrawal from maternal estrogen)	37 male and 35 female infants <48 hr to 6 months; one-third of the children of each sex and age interval were fed BM, MF, or SF	↓breast bud size and ↓ proportion of children with palpable buds during the 6-month period of assessment in both boys and girls; no obvious difference in pattern for infants in the SF group (statistical analyses not conducted to determine effects of feeding regimen)	no utility
<i>breast development</i> , presence or absence of breast buds in children ages 7-96 months (Giampietro <i>et al.</i> 2004)	retrospective study	48 children (27 boys and 21 girls) exclusively fed SF for at least 6 months (range of 6-82 months; median 12 months)	none of the girls demonstrated clinical signs of precocious puberty and none of the males showed gynecomastia (univariate analysis)	no utility
<i>breast development</i> , breast bud volume in 4-month old infants (Gilchrist <i>et al.</i> 2009)	prospective longitudinal cohort study (interim analysis)	20 boys and 20 girls in BM group; 18 boys and 23 girls in MF group; 19 boys and 20 girls in SF group	no effect on breast bud volume in boys or girls (univariate analysis)	no utility
<i>breast development</i> , prevalence of breast buds in female infants ages 3-24 months (Zung <i>et al.</i> 2008)	cross-sectional	<u>Both years</u> : 92 in SF group and 602 in a combined "milk" group of infants fed MF or BM. <u>First year</u> : 42 in SF, 370 in "milk". <u>Second year</u> : 50 in SF, 232 in "milk"	breast buds more prevalent in 2 nd year of life in infants fed SF vs. "milk" (OR 2.45 95% CI 1.11-5.39), no differences in 1 st year of life. No differences in infants exclusively fed soy infant formula compared to those that had mixed feeding (univariate analysis)	no utility

soy infant formula (SF); milk formula (MF); breast milk(BM)

Thyroid

Although the expert panel considered the evidence insufficient to reach a conclusion on whether use of soy infant formula produces or does not produce adverse effects on thyroid function, they identified continued observational studies of thyroid function in infants fed soy infant formulas as a research need. This recommendation was based on case-studies for a special cohort of infants and children with congenital hypothyroidism (CH) fed soy infant formula who demonstrated a delay of thyroid stimulating hormone (TSH) levels returning to normal after adequate treatment; these children may need increased doses of levothyroxine (also called L-thyroxin) and closer follow-up. This conclusion is consistent with the recommendation of the New Zealand Ministry of Health that clinicians treating infants with hypothyroidism who consume soy-based infant formula closely monitor the doses of thyroxin required to maintain a euthyroid state (New Zealand Ministry of Health 1998). In addition, the New Zealand Ministry of Health recommends that clinicians treating children for medical conditions who consume a soy-based infant formula be assessed for thyroid function if there are concerns for unsatisfactory growth and development.

In the 1950s and 1960s, cases of altered thyroid function, mostly goiter, were reported in infants fed soy infant formula at a time when the formula contained soy flour. The cases of goiter in infants were consistent with reports from the 1930s that rats fed soybeans developed goiters (reviewed in (Fitzpatrick 2000; McCarrison 1933; Sharpless 1938; UK-Committee-on-Toxicity 2003; Wilgus *et al.* 1941). The problem of goiter in infants fed soy infant formula was eliminated in 1959 by adding more iodine to the formulas and in the mid-1960s by replacing the high-fiber soy flour with soy protein isolate. Although the early reports of goiter in infants fed soy infant formula have mostly ceased since manufacturers began supplementing soy infant formula with iodine,⁷ there have been reports that use of soy infant formula in infants with congenital hypothyroidism may decrease the effectiveness of thyroid hormone replacement therapy, i.e., L-thyroxin (Chorazy *et al.* 1995; Conrad *et al.* 2004; Jabbar *et al.* 1997). This effect has been attributed to fecal wastage with decreased enterohepatic circulation (Chorazy *et al.* 1995; Jabbar *et al.* 1997; Shepard 1960).

Laboratory Animal Studies

Only two studies have assessed the effects of direct ingestion of soy infant formula in laboratory animals during infancy. Thus, there is insufficient evidence to reach a conclusion on whether use of soy infant formula causes, or does not cause, developmental toxicity in animal models. The weight-of-evidence determinations presented in [Figure 3](#) also include conclusions based on animal studies administering (1) the individual isoflavones found in soy infant formula, namely genistein; (2) diets with high isoflavone content compared to soy-free or low soy diets; and (3) soy protein isolate or mixtures of isoflavones (i.e., genistein and daidzein).

⁷ In 1998, the New Zealand Ministry of Health noted one case report (Labib *et al.* 1989) on thyroid abnormalities associated with soy-based infant formula since iodine supplementation.

Weight of Evidence Conclusions Based on Animal Studies of Genistein, Daidzein, Equol, and Glycitein

The expert panel reviewed more than 120 laboratory animal studies involving treatment with genistein or other individual isoflavones in its evaluation of soy infant formula. Of these, 74 were considered to be of “limited” or “high” utility (see Expert Panel Report, Tables 154–156). Seventy of these studies involved treatment with genistein. Based on these studies, exposure to genistein produced *clear evidence* of adverse effects on the female reproductive system following treatment during development (Figure 3). Studies that demonstrated clear evidence of developmental toxicity for genistein involved treatment only during the period of lactation in rodents (PND1–21) as well as multigenerational studies that included exposure during gestation, lactation, and post-weaning. A study of neonatal mice treated orally with genistin, the glucoside form of genistein that predominates in soy infant formula, also supports *clear evidence* of adverse effects on development of the female reproductive tract.

In contrast, only a very small number of studies have been published on the other isoflavones associated with soy infant formula, daidzein and its estrogenic metabolite equol, and no studies have evaluated the effects of developmental exposure to glycitein. Detection of typical estrogenic effects in these studies was mixed. For example, two of the four studies considered of “limited” utility by the expert panel evaluated age at vaginal opening in rats treated with equol (Bateman and Patisaul 2009) or daidzein (Kouki *et al.* 2003) and neither reported the classic estrogenic effect of earlier age at opening. As part of a study that was primarily designed to assess the impact of *in utero* treatment of genistein and daidzein on uterine HOX10 gene expression, Akbas *et al.* (2007) evaluated uterotrophic response to these isoflavones in adult mice and did not detect an increase in uterine weight in mice treated with a single dose of 2 mg/kg of daidzein. Kouki *et al.* (2003) reported no effect on estrous cyclicity in rats treated by sc injection with ~19 mg daidzein/kg bw/day on PND1-5. In contrast, treatment with the same dose levels of genistein caused the predicted estrogenic effect in all of these studies. However, two of the four studies did report effects that were consistent with an estrogenic effect. Bateman and Patisaul (2009) reported that sc injection of 10 mg equol/kg bw/day on PND0-3 (day of birth, PND=0) in rats induced abnormal estrus cycles beginning at week 5 following vaginal opening. Genistein and estradiol benzoate also induced abnormal estrous cycles in this study. Kouki *et al.* (2003) reported a significant decrease in ovarian weight on PND60 in rats treated by sc injection with ~19 mg daidzein/kg bw/day on PND1-5; this same effect was observed in animals treated with estradiol or genistein. Based on the small number of studies and the inconsistent findings, the evidence is *insufficient* to determine whether daidzein or equol produces or does not produce developmental toxicity in laboratory animals.

“Clear Evidence” of Adverse Effects of Genistein/Genistin in Studies Where Treatment Occurred During Lactation

Genistein induced adverse effects on the female reproductive tract when administered via sc injection during the period of lactation. Many of these studies were conducted by the same research group and used an experimental design where CD-1 mice were treated on PND1–5 with genistein, typically by sc injection, and the reproductive system was assessed during late postnatal life or adulthood (Jefferson *et al.* 2009b; Jefferson *et al.* 2005; Newbold *et al.* 2001; Padilla-Banks *et al.* 2006). In young animals, neonatal treatment with 50 mg genistein/kg bw/day on PND1–5 led to a higher incidence of multi-oocyte follicles on PND4-6 (Jefferson *et al.* 2006) and PND19 (Jefferson *et al.* 2002) compared to age-matched controls. In adulthood, the effects of neonatal exposure to 50 mg genistein/kg bw/day were manifest as a lower number of live pups per litter (Padilla-Banks *et al.* 2006), a lower number of implantation sites and corpora lutea (Jefferson *et al.* 2005), and a higher incidence of histomorphological changes of the reproductive tract (i.e., cystic ovaries, progressive proliferative lesions of the oviduct, cystic endometrial hyperplasia, and uterine carcinoma) (Newbold *et al.* 2001) relative to control females. In addition, the reproductive performance of the neonatally-treated mice was tested during adulthood and there was a significant negative trend for the number of dams with litters at PND1–5 dose levels of 0, 0.5, 5, or 50 mg genistein/kg bw/day (Jefferson *et al.* 2005). In this study, there were no live litters produced by female mice treated with 50 mg genistein/kg bw/day as neonates and a reduction in the litter size in the females exposed to 0.5 and 5 mg genistein/kg bw/day on PND1-5. Because the effects were more pronounced in animals at 6 months of age than at 2 or 4 months of age, the authors suggested that reproductive senescence may occur earlier in these animals as a result of the neonatal treatment (Jefferson *et al.* 2005). Finally, an alteration in the distribution of females in various stages of the estrous cycle was observed in animals exposed to ≥ 0.5 mg genistein/kg bw/day on PND1-5 (Jefferson *et al.* 2005).

Similar effects on female reproductive tract development were observed with oral treatment with genistin, the glycosylated form of genistin, directly to mouse neonates on PND1–5 (Jefferson *et al.* 2009a). The effects of neonatal genistin exposure (expressed as aglycone equivalents) were manifested as a reduction in the number of live pups per dam at 37.5 mg/kg bw/day, altered estrous cyclicity at ≥ 25 mg/kg bw/day, impaired fertility (based on a reduction in the number of plug positive dams delivering pups), and a higher incidence of multi-oocyte follicles at PND19 at ≥ 12.5 mg/kg bw/day. Interestingly, neonatal treatment with genistin administered orally on PND1–5 elicited a greater uterotrophic response on PND5 compared to oral administration of the comparable dose level of genistein. Genistin, expressed in aglycone equivalents, significantly increased uterine wet weight on PND5 following treatment on PND1–5 with 25 and 37.5 mg/kg bw/day relative to controls, whereas genistein did not produce any uterotrophic response at 37.5 mg/kg bw/day. In addition, although genistein induced a significant uterotrophic response at a higher dose level (75 mg/kg bw/day), the magnitude of the response was smaller than that produced by genistin at lower administered dose levels.

The reason for the greater potency of genistin in the neonatal uterotrophic assay is not entirely clear, but this finding is consistent with the much higher maximum blood levels of total genistein detected in the mice after treatment with 60 mg genistin/kg bw/day (37.5 mg genistein/kg bw/day when expressed as aglycone equivalents) or 37.5 mg/kg bw/day genistein (5189 versus 270.2 ng/ml, respectively). The level of the biologically active unconjugated aglycone form of genistein was similarly elevated. Blood levels of total genistein following this oral treatment with genistin were also higher than those reported by this research group in mice that were treated with 50 mg/kg bw/day genistein by sc injection on PND1–5, the dose level and route of administration that caused many of the effects described above. This treatment resulted in a maximum serum concentration⁸ of total genistein of 1350 ng/ml, of which ~46% (621 ng/ml), was present as unconjugated genistein (Doerge *et al.* 2002). By way of comparison, blood levels of total genistin in infants fed soy infant formula at higher percentiles fall within the range of these values (Table 5). The findings of higher blood levels following genistin treatment are supported by a rat study by Kwon *et al.* (2007), which reported that genistin is more bioavailable than genistein possibly because it can be absorbed after hydrolysis to genistein, as well as absorbed in its intact form by passive transport across the membrane of the small intestine and via a sodium-dependent glucose transporter (SGLT1) in the small intestine brush border membrane.

Adverse effects on female reproductive development were also observed in rats exposed to genistein via sc injection or orally as neonates. These effects included earlier onset of vaginal opening and altered estrous cycling in Long Evans rats treated with 10 mg/kg bw/day by sc injection on PND0–3 (day of birth, PND0) (Bateman and Patisaul 2009); earlier onset of vaginal opening, altered estrous cyclicity, and a decrease in the number of corpora lutea in Wistar rats treated with 19 mg/kg bw/day on PND1–5 by sc injection (Kouki *et al.* 2003); and decreased fertility, polyovular follicles in weanling females, and decreased number of implants per litter in Sprague Dawley rats treated orally with genistein at dose levels of 12.5 to 100 mg/kg bw on PND1–5 (Nagao *et al.* 2001).

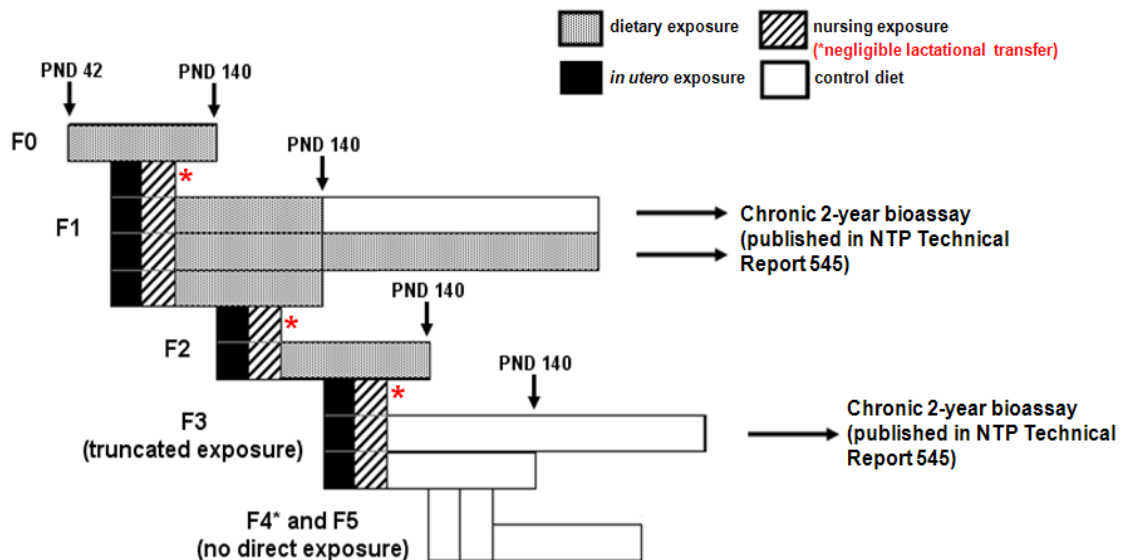
With respect to sexual maturation, an earlier onset of vaginal opening was observed in rodents exposed directly to genistein during the period of lactation. This effect was seen in CD-1 mice treated by sc injection on PND15–18 with 10 mg/kg bw/day (3.1 day advance) (Nikaido *et al.* 2005) and rats treated by sc injection as neonates with 10 mg/kg bw/day (~2-day advance) (Bateman and Patisaul 2009) or ~19 mg/kg bw/day (7 day advance) (Kouki *et al.* 2003). A 4-day earlier onset of vaginal opening was also reported in a study where rats were treated by sc injection with 2 mg genistein/kg bw/day on PND1–6, followed by oral treatment with 40 mg/kg bw/day on PND7–21 (Lewis *et al.* 2003). An exception to this pattern was a delay in vaginal opening reported by Jefferson *et al.* (2009a) in CD-1 mice treated orally with 37.5 mg/kg bw genistin on PND1–5; 50% of these females exhibited a 2-day delay and some did not have complete vaginal opening even 5 days after the last of the control animals.

⁸ Sample collected 30 minutes following dose administration

“Clear Evidence” of Adverse Effects of Genistein in Studies with Gestational, Lactational, and Post-Weaning Treatment

Clear evidence of adverse effects on the female reproductive tract was also observed in the NTP multigenerational reproductive toxicity study presented in NTP Technical Report 539 (NTP 2008a) where animals were fed dietary genistein at dose levels of 0, 5, 100, and 500 ppm. Additional data that assist in interpreting some of the effects observed in the multigenerational study are reported in NTP Technical Report 545, a chronic 2-year bioassay of genistein at these same dose levels where animals were treated from conception through weaning, 20 weeks of age, or until the end of the 2-year period (NTP 2008b). The study designs for these NTP Technical Reports are presented in Figure 4.

Figure 4. Study Designs of NTP Multigenerational Study (Technical Report 539) and Chronic Two-Year Bioassay (Technical Report 545)



*F₄ generation was mated as F₀ to F₃ to produce F₅ litters

A number of effects related to growth and reproductive and developmental parameters were observed at 500 ppm (~35 mg/kg bw/day in males and ~51 mg/kg bw/day in females during the entire feeding period):

- **Reduced litter size:** Litter size of the 500 ppm group in the F₂ generation was significantly smaller compared to controls and the litter sizes in the F₁, F₂, and F₃ generations showed negative exposure concentration trends. These trends appeared to be largely determined by the 12% to 31% reduction in litter size in the 500 ppm group of those generations. No other impacts on fertility and no histopathologic lesions were observed in females.

- Accelerated vaginal opening: Females exposed to 500 ppm showed an accelerated time of vaginal opening (approximately 3 days) in the F₁ and F₂ generations, while the 5 ppm group showed an earlier time of vaginal opening (1.3 days) in the F₃ generation. Other studies administering genistein via the diet during gestation, lactation, or/and postnatal life also observed a younger age at vaginal opening (Casanova *et al.* 1999; Delclos *et al.* 2001; You *et al.* 2002a).
- Altered estrous cyclicity: When examined shortly after vaginal opening, estrous cycles of 500 ppm females in the F₁ and F₂ generations were significantly longer (approximately 3 days and 1 day, respectively) than those of their respective control groups. Other estrous cycle disturbances were confined to the 500 ppm group of the F₁ generation and included reduced time in proestrus and an increase in the number and percentage of aberrant cycles, with the exception of decreased time in diestrus for 100 ppm females in the F₄ generation. When the estrous cycles of animals were examined prior to termination from PND130 – 140, the only significant effects were a decreased time in estrus and increased time in diestrus in 5 ppm females of the F₂ generation, and an increased number of abnormal cycles in 500 ppm females of the F₃ generation.

Alterations in estrous cyclicity were also observed in the NTP 2-year chronic bioassay presented in NTP Technical Report 545 (NTP 2008b). In this study, animals were either (1) exposed from conception through 2 years, designated F₁ continuous, or F₁C; (2) exposed from conception through 20 weeks followed by control diet to 2 years, designated F₁ truncated at PND140 or F₁T140; or (3) exposed from conception through weaning followed by control diet to 2 years, designated F₃ truncated at PND21, or F₃T21. Estrous cycles were monitored starting at 5 months of age (~PND150) to provide an estimate of when the animals began to show aberrant cycles, a condition known to precede reproductive senescence. An earlier onset of aberrant estrous cycles was observed at 500 ppm in the F₁C, F₁T140, and F₃T21 (with some evidence for effects at 5 or 100 ppm that were considered “marginal”). In all cases, the prevalent stage that caused the judgment of aberrant cycling was estrus, which appeared consistent with an acceleration of the senescence pattern typical of the Sprague-Dawley rat. While aberrant estrous cycles were not observed in PND130-140 rats in the NTP multigenerational study, those females delivered and nursed litters shortly before evaluation, which may have had an impact on the observed cycle effects. The interpretation of earlier onset of reproductive senescence is consistent with the finding by Jefferson *et al.* (2005) related to the number of plug-positive mice that produced litters following treatment with genistein by sc injection on PND1-5 (Jefferson *et al.* 2005). One hundred percent of plug-positive mice in the control group delivered litters when assessed at 2, 4, or 6 months of age, while the percentages decreased at these time points in animals treated with 0.5 mg/kg bw/day (100, 100, and 60%) or 5 mg/kg bw/day (75, 88, and 40%). Mouse dams exposed to the highest dose (50 mg/kg bw/day) on PND1-5 did not produce litters even at 2 months of age.

- Decreased body weight: While pup birth weights were not significantly affected by genistein in the F1 through F4 generations (with the exception of 100 ppm males in the F1 generation), both sexes in all generations showed depressed body weight gains during the pre-weaning period in the 500 ppm groups. Male pup pre-weaning body weight gains were also depressed in the 5 and 100 ppm groups in the F1 generation. In the postweaning period, exposure to 500 ppm genistein reduced body weights predominantly in females of generations in which rats were ingesting the compound throughout adulthood (F0 through F2). In the F1 generation, postweaning body weights were reduced in all 100 and 500 ppm groups, with a more pronounced effect in the females. In the unexposed F4 generation, female post-weaning body weight was also depressed, although to a lesser extent than in the earlier generations. Significant decreases in postweaning body weight in males were confined to the F1 generation and were not seen in the similarly exposed F2 generation. In the unexposed F5 generation, pup birth weights in all exposed groups of both sexes were significantly lower than those in the controls, although this was interpreted as more likely a chance observation rather than a carryover effect from exposures in earlier generations. Other studies administering genistein via the diet during gestation, lactation or/and postnatal life also observed transient or permanent decreases in body weight (Awoniyi *et al.* 1998; Casanova *et al.* 1999; Delclos *et al.* 2001; Ferguson *et al.* 2009; Flynn *et al.* 2000; Masutomi *et al.* 2003; You *et al.* 2002a).
- Decreased anogenital distance: Male and female pups exposed to 500 ppm in the F₁ generation had slightly reduced anogenital distances relative to controls when analyzed with body weight as a covariate. Female pups also had reduced anogenital distances in the F₂ (500 ppm) and F₃ (100 ppm) generations, although the statistical significance was dependent on the analysis method applied.
- Increased time to testicular descent: Increased time to testicular descent was observed in 500 ppm males of the F₃ generation, although no other effects of genistein on male sexual development were reported.

Given the experimental design of multigenerational studies, it is impossible to determine whether the observed effects could be attributed to exposure during the period of lactation only. Exposures through placental transfer, lactational exposure, and feed ingestion could all have contributed to the reported findings. Studies conducted in conjunction with the NTP multigenerational study showed that genistein readily crosses the placenta; however, there was only limited lactational transfer via milk during nursing (Doerge *et al.* 2001). Specific findings were that fetal serum concentrations of total genistein were ~13- to 28-fold lower than maternal concentrations following treatment of Sprague-Dawley rat dams with a single gavage dose of 20, 34, or 75 mg/kg bw genistein on GD 20 or 21 (Doerge *et al.* 2001). However, the percent of genistein present as aglycone was greater in the fetuses at all dose levels (27 to 34%) compared to dams (8 to 18%), which resulted in blood levels of the biologically active genistein aglycone that were more similar between the fetus and dam as compared to the levels of total genistein. In contrast, there was limited transfer of genistein from dams to rat pups during

lactation. Doerge *et al.* (2006) fed rat dams 500 ppm genistein (~51 mg/kg bw/day) in the diet starting immediately after parturition and assessed internal exposures to genistein in the pups during the early postnatal period when pups were exclusively nursing. The average serum levels of genistein measured on PND10 from dams were ~2.6 times higher than milk levels of genistein collected on PND7 (1.22 µM or 329.7 ng/ml compared to 0.47 µM or 127.0 ng/ml, respectively). On a daily intake basis, the estimated dose of genistein to dams from the feed was ~100 higher than to the neonates from milk (51 versus 0.51 mg/kg bw/day). Serum levels in the pups were ~30 times lower than in dams, 0.039 µM compared to 1.22 µM. The limited lactational transfer of genistein suggests that effects observed in the F₃ generation (treatment from conception to PND21) were induced by *in utero* exposure or indicate a very sensitive response to neonatal exposure. With respect to support for sensitivity of response from lactational exposure, the body weight gain in pups from PND7-10 was significantly lower for pups of genistein-fed dams (1.26 g) compared to pups from control dams (1.46 g) in the lactational transfer study (Doerge *et al.* 2006).

“Insufficient Evidence” for a Conclusion Based on Animal Studies of Soy Infant Formula

Only three publications report on the developmental effects of exposure to soy infant formula. One study in rats initiated treatment after the period of lactation and had several technical limitations that led the expert panel to consider it of “no utility” for the evaluation (Ashby *et al.* 2000). Two other publications reported data based on the same group of male marmosets treated during infancy and assessed either as juveniles (Sharpe *et al.* 2002) or adults (Tan *et al.* 2006), and both of these studies were considered of “limited” utility by the expert panel. While there were permanent effects on testicular cell populations (discussed further below), there were no obvious effects on reproductive function, i.e. fertility or permanent changes in testosterone levels. Overall, the evidence is insufficient to determine whether soy infant formula causes or does not cause developmental toxicity, due to the small number of studies, the limitations in their experimental designs, and failure to detect adverse functional effects.

Two studies reported the effects of feeding soy infant formula (versus standard cow milk formula) directly to infant marmosets (non-human primates) during the period of lactation (from PND4 or PND5 to PND35 to PND45; n=13 twin sets, plus four singletons) (Sharpe *et al.* 2002). Upon completion of treatment, the soy infant formula-fed males had significantly lower plasma testosterone levels than their cow milk formula-fed co-twins. Histopathological analysis on the testes of a subset of the co-twins on PND35 to PND45 revealed an increase in Leydig cell abundance per testes in the soy infant formula-fed marmosets compared to their cow milk formula-fed co-twin, in the absence of a significant change in testicular weight. A follow up study was conducted on the remaining animals when they were sexually mature (80 weeks of age or older; n=7 co-twin sets) (Tan *et al.* 2006). The males fed soy infant formula as infants had significantly heavier testes and an increase in the number of Leydig cells and Sertoli cells per testis as compared to cow milk formula-fed controls in the absence of a significant effect on timing of puberty, adult plasma testosterone levels, or fertility. The authors’ suggest that the increase in testes weight was likely due to an increase in testicular cell populations. Tan *et al.*

(2006) also state that the permanent change in Leydig and Sertoli cell populations may be due to compensation for Leydig cell failure following soy infant formula exposure during lactation. Since the animals were also allowed to nurse from their mothers, the authors suggest these studies may actually underestimate the effects of soy infant formula on human testicular development. In addition, the small number of animals studied and the lack of information on normal variability in the endpoints limit the utility of these studies.

“Insufficient Evidence” for a Conclusion Based on Animal Studies of Soy Protein Isolate, Soy-Based Diets, or Mixtures of Isoflavones

Twenty-eight studies involving administration of soy protein isolate, soy-based diets, or mixtures of isoflavones to experimental animals were also judged by the expert panel to have utility in their evaluation. However, the heterogeneity of this literature in terms of administered form of soy, amount of isoflavones, and differences in the experimental protocols hinders a clear interpretation of the toxicity literature. As a result, the NTP concurs with the expert panel that although some of the studies have identified potential developmental effects, these studies have yet to be replicated and overall provide insufficient evidence to conclude that soy isoflavone mixtures, including soy-based diets, produce or do not produce developmental toxicity in experimental animals.

Most of the developmental studies performed in rodents examined the effects of dietary soy products or soy-isoflavone preparations added to soy-free diets, and it is not clear to what extent these treatments are appropriate models for soy infant formula. In addition, the dietary interventions used in the experimental animal studies differ from one another, which can complicate interpretation of the literature. For example, one research group used a soy-based diet containing 102 mg genistein and 87 mg daidzin/kg diet (Masutomi *et al.* 2004) while another researcher used a phytoestrogen-free casein-based diet (AIN-93g) supplemented with soy protein isolate containing 286 mg genistein and 226 mg daidzein/kg diet (Ronis *et al.* 2009). There is also a paucity of dose-response studies of dietary soy product or soy-isoflavone preparations; for example, only one study evaluated by the expert panel utilized a soy-free diet supplemented with an isoflavone mixture giving rise to five different isoflavone intake levels (McVey *et al.* 2004a, b).

A generally consistent pattern of increased testicular weight was observed in rats and mice treated with soy diet or isoflavone supplements during gestation and lactation or continuous exposure, similar to the effect described above in marmosets treated with soy infant formula during infancy. Increased testicular weights were observed in 5/8 studies (Akingbemi *et al.* 2007; Mäkelä *et al.* 1995; McVey *et al.* 2004b; Odum *et al.* 2001; Ruhlen *et al.* 2008), while one study in rats reported a decrease (Atanassova *et al.* 2000) and two studies in rabbits observed no effect on testicular weight (Cardoso and Bao 2007, 2008). In particular, Akingbemi *et al.* (2007) reported an increase in testes weights (absolute and relative) on PND28 rats with exposure to a soy-based diet supplemented with 5-1000 ppm and 50-1000 ppm isoflavones, respectively. At PND90, absolute testes weights were decreased by the 50-1000 ppm isoflavone

supplementation concurrent with an increase in serum testosterone levels at 1000 ppm isoflavone supplementation, relative to controls. McVey *et al.* (2004b) reported an increase in absolute testes weights at PND28, but not at PND120, in male rats continually exposed to soy-based diets containing from 36.1 to 1047 ppm isoflavones. Makela *et al.* (1995) observed increased testes weights in rats with continual exposure to a soybean diet at 12 months of age, but not at 2 months of age. Increased testes weights in rats were also observed by Ruhlen *et al.* (2008) at PND90 and Odum *et al.* (2001) at PND68 and PND128 with continual exposure to soy-based diets relative to soy-free diets. In contrast, Atanassova *et al.* (2000) reported decreased testes weights in soy-diet control males relative to soy-free diet fed males. Interestingly, there was a decrease in spermatocyte nuclear volume per Sertoli Cell on PND18 and PND25 as well as a decrease in Sertoli Cell nuclear volume per testes at PND18 in soy-diet control males relative to soy-free diet males (Atanassova *et al.* 2000).

There was less consistent data on timing of puberty and growth in rats and mice following exposure during gestation and lactation or continuous exposure to soy diet or supplements. Two of four studies reported a decrease in the age of vaginal opening of 5.9 days (Guerrero-Bosagna *et al.* 2008) or 1 day (Hakkak *et al.* 2000), and the remaining two studies reported an increase in age at vaginal opening (Odum *et al.* 2001; Ruhlen *et al.* 2008). Inconsistent effects were also reported for growth in rodents treated during development. Studies reported increases in body weight (Masutomi *et al.* 2004); both increases and decreases in body weight, depending on time at assessment (Akingbemi *et al.* 2007; Mardon *et al.* 2008; Odum *et al.* 2001; Ruhlen *et al.* 2008); decreases in body weight (Atanassova *et al.* 2000; Gorski *et al.* 2006; Lephart *et al.* 2001; Lund *et al.* 2001); or no effect on body weight (McVey *et al.* 2004b; Pastuszewska *et al.* 2008).

Timing of Exposure and Effects on the Mammary Gland

Female

Timing of exposure during development appears to be important in determining the impact of soy isoflavones on mammary gland developmental pace and susceptibility to cancer risk. In general, there appears to be a lack of consensus in whether or not there is a “protective” effect or increased risk for hyperplasia/tumors following genistein treatment during the period of lactation. In an evaluation of three studies in rodents, (Cabanés *et al.* 2004; Hilakivi-Clarke *et al.* 1999b; Padilla-Banks *et al.* 2006), the common theme observed in the treated animals was that terminal end buds (TEBs) were in greater number earlier in development and lower in number later in development when compared to controls, suggesting precocious development of the mammary epithelium. All of these studies utilized a sc injection of genistein directly to the pups at dose levels ranging from 0.7 – 50 mg/kg bw/day, and varied slightly in the timing of exposure, but all studies included at least 5 days of the nursing period. TEBs are considered to be very susceptible to chemical carcinogens, thus a decrease in the abundance of TEBs is an indicator of decreased cancer susceptibility (Russo *et al.* 1990). One of the three studies (Hilakivi-Clarke *et al.* 1999b) reported decreased multiplicity of tumors, but not incidence, in genistein-dosed rat offspring exposed to a chemical carcinogen, when compared to controls.

Another study in rats (Cabanes *et al.* 2004) reported development of lobulo-alveolar structures, often correlated with decreased sensitivity to a carcinogen. However, a study in mice (Padilla-Banks *et al.* 2006) and a fourth study in rats (Foster *et al.* 2004) each observed hyperplasia and preneoplastic lesions in female offspring allowed to age normally following genistein exposure via sc injection to the pups during lactation. Some of these changes were similar to the types of changes normally observed in lactating animals (e.g., secretory changes in epithelial cells and lobular expansion) in addition to findings of increased incidences of atypical epithelial hyperplasia, microcalcifications, and *in situ* carcinoma (rats only) as compared to controls.

In contrast, exposure to genistein only during the period of gestation has been associated with effects on the pup mammary gland that are consistent with an increased susceptibility to mammary gland carcinogenesis. An increase in TEBs in female mice was observed on PND35 and 45 following administration of genistein (~0.7 to 0.8 mg/kg bw/day) to the dam via sc injection on GD 15-20 (Hilakivi-Clarke *et al.* 1998). In another publication, this research group reported an increased incidence of mammary gland tumors in rats following dimethylbenzanthracene (DMBA) treatment on PND50, following gestational exposure on GD15-20 (~0.1 or ~1.5 mg/kg bw/day via sc injection to dams, but not ~0.5 mg/kg bw/day)(Hilakivi-Clarke *et al.* 1999a).

The NTP conducted a 2-year cancer bioassay of genistein that included a group of rats exposed via diet beginning with conception throughout life (National Toxicology Program (NTP) 2008) (Figure 4). There was some evidence of carcinogenicity based on an increased incidence of mammary gland adenoma or adenocarcinoma (combined) and pituitary gland neoplasms in females. The effects of genistein on the mammary gland were less clear with shorter periods of exposure, and equivocal evidence of mammary gland adenomas or adenocarcinomas was reported for females exposed from conception to weaning or conception to PND140. In addition, there were conflicting results from two studies with dietary exposure to genistein: one study using only prenatal exposure reported an increase in the number of TEBs at 8 weeks of age and a higher incidence of chemically-induced mammary tumors, but no changes in latency to tumors or multiplicity (Hilakivi-Clarke *et al.* 2002), and another study (Fritz *et al.* 1998) reported a persistent decrease in TEBs leading to a reduced tumor multiplicity and no change in tumor latency following gestational and lactational genistein exposures (incidence was not reported). Two common threads were apparent: developmental timing of genistein exposure was related to TEB versus mature duct end numbers and the level of TEBs present at the time of carcinogen exposure was related to number of tumors.

Exposure to dietary soy protein isolate appears to have a protective effect on female mammary gland development based on three rodent studies evaluating the effects reported for the abundance of TEBs or response to a chemical carcinogen challenge. In two studies, soy protein isolate was administered in diet to rats from pre-conception (Hakkak *et al.* 2000) and/or during pregnancy, lactation, and throughout life of the F1 female offspring (Simmen *et al.* 2005). In both of these experiments, F1 rats exposed to soy protein isolate displayed a longer latency to develop mammary gland tumors and a lower incidence of females with at least one mammary

gland tumor following exposure to a chemical carcinogen on PND50. Thomsen *et al.* (2006) administered a soy protein isolate in diet to mice during lactation or during lactation and throughout adulthood. They reported exposure to soy protein isolate during lactation increased the number of TEBs immediately after weaning (PND28) compared to controls. On PND42-43, the female rats continually exposed to soy protein isolate had a lower number of TEBs and on PNDs 70-73, there was no treatment difference in the number of TEBs. The authors speculated that treatment enhanced normal development and that the effects of treatment on tumor susceptibility may depend on the timing of exposure, such that a protective effect may be expected if carcinogenic insult is initiated late in puberty, i.e., PND42–43, versus at an earlier point in development.

Male

One of the most consistent findings of the NTP studies was morphological changes in the mammary gland of male rats (Latendresse *et al.* 2009; NTP 2008a). In the NTP perinatal dose selection study for genistein that tested dose levels of 5, 25, 100, 250, 625, and 1,250 ppm, an increased incidence of mammary gland hypertrophy was observed in males at ≥ 25 ppm and hyperplasia at ≥ 250 ppm. In a multigenerational evaluation of 0, 5, 100, or 500 ppm genistein (Latendresse *et al.* 2009), the incidence of mammary gland alveolar/ductal hyperplasia was significantly higher in 500 ppm males in the F₀ through F₂ generations and in 100 ppm males in the F₁ and F₂ generations. In the F₃ generation, a significant, positive, linear, exposure-concentration trend in the incidences of mammary gland hyperplasia occurred, but no exposed group differed significantly from controls in pairwise comparisons. Both developmental and adult exposures contributed to the maintenance of these effects. More dramatic effects of genistein on the incidences of male mammary gland hyperplasia were observed in the continuously exposed F₁ and F₂ generations as compared to the late adolescent and adult exposures of the F₀ generation and the pre-weaning-only exposure of the F₃ generation. Mammary gland hyperplasia was absent in males not directly or indirectly exposed to genistein (F₄ generation)(Latendresse *et al.* 2009; 2008a).

Mammary gland hyperplasia was also observed in the NTP 2-year chronic study at a lower incidence compared to the multigenerational study. In the 500 ppm dose group of the chronic study, the proportion of male mammary glands having hyperplasia (ductal and alveolar combined) was 19% of the F₁C (exposed conception to 2 yr) and 20% of the F₁T140 (exposed conception to 140d) (Latendresse *et al.* 2009). In the multigenerational study, the incidence of mammary gland hyperplasia at 500 ppm was 60% in the F₁ males and 72% in the F₂ males (Latendresse *et al.* 2009). There was no clear evidence of progression of male mammary gland hyperplasia to neoplasia in the chronic study; i.e., there was “no evidence” of carcinogenicity activity in males of any generation for mammary gland or other tissue. Based on these data, Latendresse *et al.* (2009) concluded that the decline in incidence of mammary hyperplasia observed in the NTP chronic study was most likely due to regression of hyperplasia and glandular involution. Three other studies of dietary exposure during gestation and lactation or continuous exposure in male rats have reported an increase in mammary gland branching and

epithelial cell proliferation (You *et al.* 2002a); an increase in mammary gland branching, TEBs, and lateral buds in male rats (You *et al.* 2002b); and an increase in size and tissue density of the mammary glands (Wang *et al.* 2006).

Consideration of Equol Production

One important factor in interpreting the isoflavone literature is consideration of species differences in the ability to produce equol, an estrogenic metabolite of daidzein. It is generally accepted that a greater proportion of rodents and monkeys metabolize daidzein to equol compared to humans or pigs (Gu *et al.* 2006). The metabolic profile of daidzein varies in humans with 30 to 50% of individuals being classified as equol producers, and some individuals producing little or no equol, presumably due to differences in microbial factors, dietary consumption, lifestyles, or genetic factors (Atkinson *et al.* 2008a). Human infants are generally considered less able to produce equol compared to adults due to immaturity in gut microflora and/or underdeveloped metabolic capacity (Setchell *et al.* 1997). The expert panel considered the issue of equol production and concluded that rodent and monkey models receiving soy infant formula or other isoflavone mixture that included daidzein were relevant for humans because: (1) daidzein has estrogenic activity of its own and (2) some portion of human infants produce equol. The NTP concurs with this conclusion but recognizes that additional *in vivo* studies specifically designed to address the interactions between various soy isoflavones would be useful.

Equol is an estrogenic metabolite of daidzein with *in vitro*-based estimates of estrogenic potency that are generally intermediate between daidzein and genistein, e.g., [Table 4](#). Overall, equol elicits estrogenic responses based on *in vivo* studies using classic measures of estrogenicity, although some studies suggest that equol may not be exerting these effects with a potency predicted from the *in vitro* studies (Bateman and Patisaul 2009; Breinholt *et al.* 2000; Medlock *et al.* 1995; Nielsen *et al.* 2009; Rachon *et al.* 2007; Selvaraj *et al.* 2004); see also Expert Panel Report, Section 2.2.9.2. For example, neonatal treatment with 10 mg genistein/kg bw/day by sc injection caused a ~2-day advancement in the day of vaginal opening, while there was no effect in animals treated with the same dose level of equol (Bateman and Patisaul 2009). However, the estrous cycles of these animals were significantly altered and less than 30% of females in both groups displayed regular estrous cycles (most animals were in persistent estrus or diestrus) by 10 weeks of age. Kouki *et al.* (2003) found less indication for estrogenic activity of daidzein compared to genistein in a study that compared the effects of neonatal treatment with ~ 19 mg/kg bw/day of either isoflavone (by sc injection). Estrogenic responses reported for genistein, but not detected for daidzein, included earlier onset of vaginal opening, persistent or prolonged estrous, loss of corpora lutea, and reduced lordosis quotient in female rats. Allred *et al.* (2005) reported that a smaller percentage of equol is circulating in the unconjugated form compared to genistein following oral exposure and suggested this may contribute to a reduced *in vivo* potency relative to *in vitro* predictions. In this study, the percentage of genistein present as aglycone (9%) was higher than the percentage of equol present as aglycone (1%) following ingestion of a soy flour diet in female Balb/c mice.

Table 4. Comparison of *In Vitro* Measures of Isoflavone Estrogenicity (Choi *et al.* 2008)

Compound	Relative binding affinity (%) ^a			Relative estrogenic activities		
	ER α	ER β	β/α	ER binding ^b	Yeast transactivation ^b	E-screen ^b
E ₂	100	100	1	++++	++++	++++
Genistein	2.07	14.8	7.1	+++	++++	+++
Daidzein	0.55	0.46	0.8	++	++	++
Equol	1.70	4.45	2.6	++	+++	++++
Glycitein	0.32	0.44	1.4	++	not determined	++

^aRelative binding affinity = (IC50 of E₂) ÷ (IC50 of test compound) × 100.

^bBased on comparisons to E₂ alone: ++++ (≥ 100%), +++ (66% – 99%), ++ (33% – 66%), + (1% - 33%); potency estimates for ER binding were based on binding data for at least one ER type.

From Table 1 in Choi *et al.* (2008)

Assessment of other non-estrogenic endpoints leads to similar conclusions. Studies, mostly *in vitro*, have also examined effects of soy isoflavones on endpoints such as: effects on bone, cardiovascular/lipid regulation, cell growth, inflammation, immunity, and neurology (Expert Panel Report, Table 78). Of the 77 studies that presented data on these endpoints, the majority reported a similar pattern of relative ranking of genistein ≥ equol > daidzein based on magnitude of effect or relative potency. Across these studies, genistein was more potent than equol or daidzein in 60 of approximately 117 endpoints examined. The relative effects for all three isoflavones were similar in another 52 of these endpoints. Daidzein or equol caused a greater effect as compared to genistein for only five endpoints. It is worth noting that 16 of these studies also reported that genistein inhibited tyrosine kinase activity, while inhibition of this enzyme by daidzein was not observed. The tyrosine kinase activity data suggest that the effects of genistein could be due in part to a non-estrogen receptor mode of action. In all cases where an effect was observed, the isoflavones acted in the same direction (e.g., genistein and daidzein both inhibited bone resorption (Blair *et al.* 1996)). Collectively, these data do not support the notion that daidzein or equol markedly “offset” genistein activity.

It is generally accepted that a greater proportion of rodents and monkeys metabolize daidzein to equol compared to humans or pigs (Gu *et al.* 2006). The metabolic profile of daidzein varies in humans with some individuals producing little or no equol, presumably due to differences in microbial factors, dietary consumption, lifestyles, or genetic factors (Atkinson *et al.* 2008a). Human infants are generally considered less able to produce equol compared to adults due to immaturity in gut microflora and/or underdeveloped metabolic capacity (Setchell *et al.* 1997). The species differences in daidzein metabolism are not considered a significant factor in rodent studies where only genistein was administered and animals were fed a soy-free- or low-phytoestrogen diet. However, it can complicate the interpretation of studies that include daidzein for reaching conclusions on potential effects in human infants fed soy infant formula. One concern is that use of rodents or monkeys as animal models may “overestimate” the potential health risk to human infants fed soy infant formula. A negating effect of daidzein and/or equol on estrogenic effects of genistein is not generally predicted unless perhaps the binding of less potent isoflavone, i.e., daidzein, to estrogen receptors limits the access of genistein to those receptors. However, this would only make sense conceptually if the relative

concentrations of the weak binders were much higher than concentrations of genistein, and they are not.

Based on detection frequency, the percentage of infants with detectable levels of equol in urine or plasma is similar to the percentage of adults considered to be “equol producers.” Equol was detected in the urine of 25% of 4-6 month old infants (Hoey *et al.* 2004) and in the plasma of 4 of 7 (57%) 4-month old infants fed soy infant formula (Setchell *et al.* 1997), values that are comparable to the frequently cited range of 30-50% of adults considered to be equol producers (Atkinson *et al.* 2008a; Atkinson *et al.* 2008b; Bolca *et al.* 2007; Hall *et al.* 2007; Setchell *et al.* 2003). In a larger sample, Cao *et al.* (2009) were not able to detect equol in the blood (n= 27) or saliva (n=120) of infants aged 0 to 12 months on a soy infant formula diet for at least two weeks, although it was detectable in the urine of a small proportion, 6 of 124 (5%), of infants. One reason why equol might not have been detected in the Cao *et al.* (2009) study is because of the relatively high limit of detection. The mean plasma concentration of equol measured in soy formula fed infants by Setchell *et al.* (1997) was ~ 2 ng/ml (range across infants in all feeding groups was <LOD to ~5.5 ng/ml) while the limit of detection in whole blood for equol in Cao *et al.* (2009) was 12 ng/ml.

Both Setchell *et al.* (1997) and Cao *et al.* (2009) reported detecting equol in a greater proportion of infants fed cow milk-based formula compared to other feeding methods. In Setchell *et al.* (1997), 100% of infants fed a cow milk-based formula had detectable plasma levels of equol with a peak level up to 2 orders of magnitude higher than in infants fed soy-based formula. In contrast, equol was only detected in 4 of 7 (57%) infants fed soy infant formula and 1 of 7 (14%) breastfed infants. In Cao *et al.* (2009) equol was also detected in a higher percentage, 22%, of infants fed a cow milk-based formula compared to those fed soy infant formula (5%) or breast milk (2%), although the geometric means of urinary equol in the infants were comparable between feeding regimens (soy infant formula, cow milk formula, and breast milk were 2.3 ng/ml, 2.4 ng/ml, and 1.7 ng/ml equol, respectively). The finding of equol being more readily detected in infants fed a cow milk-based formula is not unexpected given that cows can produce equol from either the formononetin found in red clover or daidzein found in soy (King *et al.* 1998). There are also data suggesting that equol concentrations may be higher in organic milk products presumably because organic dairy cows eat more forage legumes compared to conventionally raised cows (Hoikkala *et al.* 2007).

Of the infants who do produce equol, they do not seem to produce equol to the same extent as adults. This conclusion is based on the most recent CDC data from NHANES. The geometric mean (10th – 90th percentile) of equol detected in urine for people aged 6 years and older was 8.77 µg/L (<LOD – 38.5) (U.S. Centers for Disease Control and Prevention 2008). This value is approximately 3.7 to 5.2-fold higher than urinary concentrations of equol measured in infants by the CDC and reported in Cao *et al.* (2009), which included infants fed soy infant formula who were exposed to higher daidzein levels than older children and adults.

Limitations of Studies that Only Administer Genistein

A major limitation in extrapolating the results of the genistein-only studies in laboratory animals that presented evidence of development toxicity to humans fed soy infant formula is the uncertainty on whether another component of soy infant formula, either isoflavone or other, could act to dampen the effects of genistein. As discussed above, a “negating” effect of daidzein or equol on genistein would not be predicted given that they all exhibit estrogenic activity; the prediction would be an exacerbation of estrogenic response. However, to date, these predictions have not been tested for the endpoints described above that present “clear evidence” of adverse effect for genistein, i.e., decreased in litter size, altered estrous cyclicity, etc.

In addition, it is also theoretically possible that non-isoflavone components of soy infant formula may alter the biological activity of the soy isoflavones. However, assessing such an interaction is complicated from an experimental design perspective. Treatment of infant animals with soy infant formula in an “off the shelf” preparation administered in an amount relevant for humans is quite challenging from a logistical perspective. Oral treatment with soy infant formula at levels that are comparable to intakes for human infants on a body weight-corrected basis would require that neonatal rodents be treated more than 15 times a day. In addition, neonatal animals need to nurse and interact with their mothers along with ingesting soy infant formula; therefore it is unlikely that sufficient soy infant formula could be administered to a laboratory animal at the concentration and volume (corrected for body weight) that is administered to a human infant. For example, the marmoset monkeys discussed in Sharpe *et al.* (2002) and Tan *et al.* (2006) were only fed soy infant formula 3 or 4 times a day during an 8-hour period on the weekdays and 1 or 2 times a day during a 2-hour period on weekends. At other times, the infant marmosets were with their mothers and free to nurse. On a volume-ingested basis corrected for body weight, the marmosets consumed approximately half the volume of 1-month old human infants exclusively fed soy infant formula, ~0.1 L/kg bw versus ~0.2 L/kg bw. The estimated intake of total isoflavones in the marmosets, 1.6–3.5 mg/kg bw/day, was approximately 20 to 85% of the estimated intake in human infants at 1-month old.

Although it may not be possible to administer infant laboratory animals a soy formula preparation that directly models human infant exposure, the NTP believes that utilization of the genistein/genistin-only studies in laboratory animals would be enhanced if the adverse findings (e.g., decreased litter size, altered estrous cyclicity, early onset of vaginal opening) were also observed following co-treatment with other soy isoflavones such as daidzein.

SHOULD FEEDING INFANTS SOY INFANT FORMULA CAUSE CONCERN

Possibly. Infants fed soy infant formula are reported to consume as much as 6.2 mg/kg bw/day of total genistein, thus a 5 kg infant would consume ~30 mg/day of total genistein. Blood levels of total genistein in infants fed a soy infant formula diet can exceed those reported in young rats or mice treated with genistein during development at dose levels that produced adverse effects, i.e., early onset of sexual maturation, altered estrous cyclicity and decreased litter size (Table 5). While these types of adverse effects have not been reported in humans during 60 years of soy infant formula usage, adequate studies of the reproductive system have also not been conducted on girls or women following use of soy infant formula during infancy. Thus, the data in humans are not sufficient to dismiss the possibility of subtle or long-term adverse health effects in these infants.

In a study of 27 infants fed soy infant formula, the median serum level of total genistein was 890 ng/ml, with serum levels of total genistein reaching 1455 ng/ml at the 75th percentile (Cao *et al.* 2009) and 2763.8 at the 95th percentile (personal communication with Dr. Yang Cao, NIEHS). These blood levels in infants can exceed maximum concentrations of total genistein associated with dose levels of genistein that caused adverse developmental effects in rodents. Specifically, the maximum blood level of total genistein measured in female mice following daily sc injection of 50 mg/kg bw/day genistein on PND1-5 was 1837 ng/ml or 6.8 μ M (Doerge *et al.* 2002). A number of adverse effects on the female reproductive tract were reported in other studies that used this treatment protocol, including increased incidence of multi-oocyte follicles (Jefferson *et al.* 2006; Jefferson *et al.* 2002), lower number of live pups per litter (Jefferson *et al.* 2005; Padilla-Banks *et al.* 2006), lower number of implantation sites and corpora lutea (Jefferson *et al.* 2005), and higher incidence of histomorphological changes of the reproductive tract (i.e., cystic ovaries, progressive proliferative lesions of the oviduct, cystic endometrial hyperplasia, and uterine carcinoma) (Newbold *et al.* 2001). Similarly, blood levels of total genistein measured in human infants fed soy infant formula can exceed levels of total genistein measured in the NTP multigenerational study in rats on PND21 and PND140 following dietary treatment with 500 ppm (~35–51 mg/kg bw/day) of genistein (Chang *et al.* 2000). Effects observed at the 500 ppm dose level included reduced litter size, decreased body weight, accelerated vaginal opening, altered estrous cyclicity, delayed testicular descent, and mammary gland hyperplasia in males (NTP 2008a) (Table 5).

Comparisons based on blood levels of unconjugated genistein between humans and rodents are more difficult because only total genistein was measured in the infants fed soy formula (Cao *et al.* 2009). However, in adults approximately 1-3% of total genistein is present in the unconjugated form (Setchell *et al.* 2001). If this range is applied to the blood levels of total genistein measured in infants fed soy formula, then the estimated levels of unconjugated genistein at the 50th percentile would be 8.9–26.7 ng/ml (based on total genistein of 891 ng/ml) and at the 95th percentile the levels would be 27.6–82.9 ng/ml (based on a total genistein of 2763.8 ng/ml). These estimates of unconjugated genistein in infant blood are similar to the estimated levels of unconjugated genistein in the F1 rats on PND21 or PND140 in the NTP

multigenerational study at a dietary dose level of 500 ppm where adverse effects were reported ([Table 5](#)). The estimated levels of genistein aglycone in human infants are ~7-times lower than those estimated for the C_{max} for total genistein following sc injection in mice of 50 mg/kg bw/day on PND1-5.

Table 5. Summary of Blood Levels of Genistein in Human Infants Fed Soy Infant Formula and Laboratory Animals Treated with Genistein/Genistin, and Associated Effects Observed in Laboratory Animals

Blood genistein		Description of exposure studies	Associated effects observed in laboratory animals
Total genistein, ng/ml	Aglycone, ng/ml (%)		
5189, C _{max}	1513, C _{max} (29%)	Female mice on PND5 following oral treatment with 37.5 mg/kg bw/day genistin (expressed in aglycone equivalents) on PND1-5 (Jefferson et al. 2009a)	Abnormal estrus cyclicity, decrease in litter size, altered ovarian differentiation, delayed vaginal opening, delayed parturition (Jefferson et al. 2009a)
3563	35.6 – 106.9 (1-3%)*	Infants fed soy infant formula, 99 th percentile (personal communication, Dr. Yang Cao, NIEHS)	
2764	27.6 – 82.9 (1-3%)*	Infants fed soy infant formula, 95 th percentile (personal communication, Dr. Yang Cao, NIEHS)	
2145 (female, PND140) 1620 (male, PND140)	21.5– 107.3 (female) 16.2 – 81 (male) (1-5%)	Rats treated with genistein via the dam during gestation and lactation and directly through the diet after weaning with 500 ppm genistein (average dose of ~35 mg/kg bw/day in males to 51 mg/kg bw/day in females during the entire feeding period) (Chang et al. 2000)	Reduced litter size, decreased body weight, accelerated vaginal opening, altered estrous cyclicity, delayed testicular descent, and mammary gland hyperplasia in males (NTP 2008a)
1837, C _{max}	575, C _{max} (31.3%)	Female mice on PND5 following sc injection of 50 mg/kg bw/day genistein on PND1-5 (Doerge et al. 2002)	Increased incidence of multi-oocyte follicles (Jefferson et al. 2006; Jefferson et al. 2002), lower number of live pups per litter (Jefferson et al. 2005; Padilla-Banks et al. 2006), lower number of implantation sites and corpora lutea (Jefferson et al. 2005), higher incidence of histomorphological changes of the reproductive tract (i.e., cystic ovaries, progressive proliferative lesions of the oviduct, cystic endometrial hyperplasia, and uterine carcinoma) (Newbold et al. 2001)
1455	14.6 – 43.7 (1-3%)*	Infants fed soy infant formula, 75 th percentile (Cao et al. 2009)	
891	8.9 – 26.7 (1-3%)*	Infants fed soy infant formula, median (Cao et al. 2009)	
757	7.6 – 22.7 (1-3%)*	Infants fed soy infant formula, geometric mean (Cao et al. 2009)	
505 (female, PND21) 564 (male, PND21)	5.1 – 25.3 (female) 5.6 – 28.2 (male) (1-5%)	Rats treated with genistein via the dam during gestation and lactation and directly through the diet after weaning with 500 ppm genistein (Chang et al. 2000). Average dose of ~35 mg/kg bw/day in males to 51 mg/kg bw/day in females during the entire feeding period) (NTP 2008a)	Reduced litter size, decreased body weight, accelerated vaginal opening, altered estrous cyclicity, delayed testicular descent, and mammary gland hyperplasia in males (NTP 2008a)

*The fraction of total genistein present as aglycone has not been established for human infants. The estimated range of 1 – 3% is based on data from adults (**Setchell et al. 2001**).

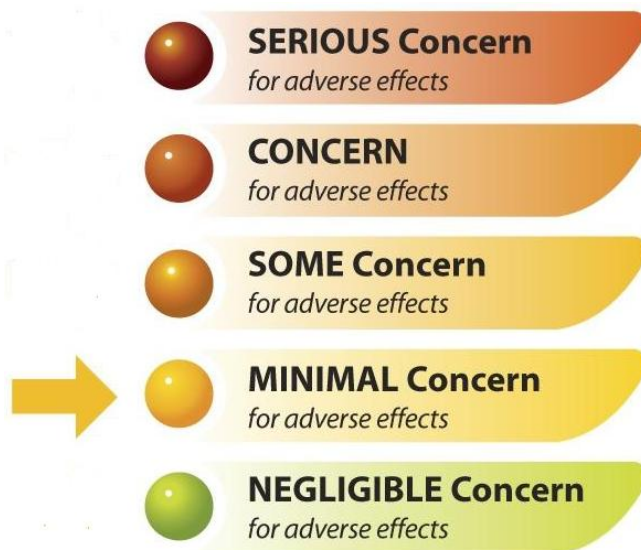
The NTP concurs with the conclusion of the CERHR Expert Panel on Soy Infant Formula that there is *minimal concern* for adverse effects on development in infants who consume soy infant formula.

This level of concern represents a “2” on the five-level scale of concern used by the NTP (Figure 5). It is based primarily on findings from studies in laboratory animals exposed to genistein, the primary isoflavone in soy infant formula. The existing epidemiological literature on soy infant formula exposure is insufficient to reach a conclusion on whether soy infant formula does or does not cause adverse effects on development in humans. There is “clear evidence” for adverse effects of genistein on reproductive development and function in female rats and mice manifested as accelerated puberty (i.e., decreased age at vaginal opening), abnormal estrous cyclicity, cellular changes to the female reproductive tract, and decreased fecundity (i.e., decreased fertility, implants, and litter size). Also, Infants fed soy infant formula can have blood levels of total genistein that exceed those measured in neonatal or weanling rodents following treatment with genistein at dose levels that induced adverse effects in the animals. However, the NTP accepts the conclusions of the expert panel that the current literature in laboratory animals is limited in its utility for reaching conclusions for infants fed soy infant formula. The NTP agrees with the expert panel that the individual isoflavone studies of genistein, or its glucoside genistin, in laboratory animals would benefit from data on the effects of mixtures of isoflavones and/or other components present in soy infant formula because these mixture studies would better

replicate human infant exposures. In addition, a limitation of many of the studies that observed adverse effects in rodents is that exposure occurred during the period of gestation, lactation, and beyond weaning, which made it difficult to distinguish the effects of isoflavones that might have occurred as a result of exposure during lactation alone. A better approximation of human exposure of infants fed soy infant formula would be data from animals

exposed during lactation only. Thus, the NTP is initiating a series of studies to address several of the limitations in the laboratory animal studies identified by the expert panel.

Figure 5. NTP Conclusions Regarding the Possibilities that Human Development Might be Adversely Affected by Consumption of Soy Infant Formula



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