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Center For The Evaluation Of Risks To Human Reproduction

DRAFT

**NTP-CERHR EXPERT PANEL REPORT on the
REPRODUCTIVE and DEVELOPMENTAL
TOXICITY of SOY FORMULA**

PREFACE

To be added after the Expert Panel meeting.

Reports can be obtained from the web site (<http://cerhr.niehs.nih.gov>) or from

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The findings and conclusions of this report are those of the expert panel and should not be construed to represent the views of the National Toxicology Program.

Abbreviations

ANCOVA	analysis of covariance
ANOVA	analysis of variance
AUC	area under the time-concentration curve
BMD ₁₀	benchmark dose, 10% effect level
BMD _{1 SD}	benchmark dose, 1 control standard deviation
BMDL	benchmark dose 95 th percentile lower confidence limit
bw	body weight
C _{max}	maximum plasma concentration
CASA	computer-assisted sperm analysis
CERHR	Center for the Evaluation of Risks to Human Reproduction
CI	confidence interval
CYP	cytochrome P450
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ER	estrogen receptor(s)
FDA	Food and Drug Administration
FSH	follicle-stimulating hormone
g	gram(s)
GC	gas chromatography
GD	gestation day(s)
GnRH	gonadotropin-releasing hormone
HDL	high-density lipoprotein
HPLC	high performance liquid chromatography
ip	intraperitoneal(ly)
IQ	intelligence quotient(s)
IU	international unit
iv	intravenous(ly)
kcal	kilocalorie(s)
kD	kilodalton(s)
kg	kilogram(s)
kJ	kilojoule(s)
LC	liquid chromatography
LDL	low-density lipoprotein
LH	luteinizing hormone
M	molar
mEq	milliequivalent(s)
mg	milligram(s)
mol	mole(s)
mRNA	messenger ribonucleic acid
MS	mass spectrometry
ng	nanogram(s)
NICHD	National Institute of Child Health and Human Development
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
nM	nanomolar
NTP	National Toxicology Program
OR	odds ratio
oz	ounce(s)

PAS	periodic acid-Schiff
PCR	polymerase chain reaction
pg	picogram(s)
pM	picomolar
PND	postnatal day(s)
PCNA	proliferating cell nuclear antigen
RIA	radioimmunoassay
RM3, RM1	Rat and Mouse No. 3, Rat and Mouse No. 1
RNA	ribonucleic acid
RR	risk ratio
RT	reverse transcription
sc	subcutaneous(ly)
SD	standard deviation
SDN-POA	sexually dimorphic nucleus of the pre-optic area
SEM	standard error of the mean
T _{max}	time to maximum plasma concentration
UDPGT	uridine diphosphate-glucuronosyltransferase
USDA	US Department of Agriculture
WISC-R	Wechsler Intelligence Scale for Children—Revised
μg	microgram(s)
μL	microliter(s)
μM	micromolar

TABLE OF CONTENTS

PREFACE	ii
Abbreviations	iv
TABLE OF CONTENTS	vi
List of Tables	vii
List of Figures	viii
1.0 CHEMISTRY, USE, AND HUMAN EXPOSURE	1
1.1 Chemistry	1
1.2 Use and Human Exposure	2
1.2.1 Production Information	2
1.2.2 Use	4
1.2.3 Occurrence and Infant Exposure	4
1.3 Utility of Data	9
1.4 Summary of Human Exposure Data	10
2.0 General Toxicology and Biological Effects	12
2.1 Toxicokinetics and Metabolism	12
2.1.1 Human	12
2.1.1.1 Absorption	12
2.1.1.2 Distribution	15
2.1.1.3 Metabolism	19
2.1.1.4 Excretion	23
2.1.2 Experimental animal	24
2.2 General toxicology	27
2.2.1 Thyroid	27
2.2.2 Cardiovascular	28
2.2.3 Menopausal symptoms	28
2.2.4 Estrogenicity	29
2.2.4.1 Human	29
2.2.4.2 Experimental animal	31
2.3 Genetic toxicity	36
2.4 Cancer	36
2.5 Potentially Sensitive Subpopulations	37
2.6 Summary of General Toxicology and Biological Effects	39
2.6.1 Toxicokinetics and Metabolism	39
2.6.1.1 Humans	40
2.6.1.2. Experimental animals	42
2.6.2 General Toxicology	42
2.6.3 Genetic Toxicity	43
2.6.4 Carcinogenicity	43
2.6.5 Potentially Sensitive Sub-populations	43
3.0 DEVELOPMENTAL TOXICITY DATA	45
3.1 Human data	45
3.1.1 Pregnancy exposure	45
3.1.2 Exposure of infants	46
3.1.2.1 Growth and Nutrition	46
3.1.2.2 Gastrointestinal effects	63
3.1.2.3 Allergy and immunology	67
3.1.2.4 Thyroid function	82
3.1.2.5 Reproductive endpoints	83
3.1.2.6 Cholesterol	86
3.1.2.7 Diabetes mellitus	87

3.1.2.8 Cognitive function.....	88
3.1.3 Exposure during adolescence.....	88
3.2 Experimental animal data.....	91
3.2.1 Development of the reproductive system	91
3.2.2 Mammary Development/Carcinogenesis.....	111
3.2.3 Brain Structure/Behavior	115
3.2.4 Other endpoints.....	123
3.2.4.1 Rat	123
3.2.4.2 Pig.....	126
3.3 Utility of Data	129
3.3.1 Human data.....	129
3.3.2 Experimental animal data	129
3.4 Summary of Developmental Toxicity Data	129
3.4.1 Human studies	129
3.4.2 Experimental animal studies.....	131
4.0 REPRODUCTIVE TOXICITY DATA.....	139
4.1 Human data	139
4.1.1 Female.....	139
4.1.2 Male	151
4.2 Experimental animal	154
4.2.1 Female.....	154
4.2.2 Male	160
4.3 Utility of Data	165
4.3.1 Human data.....	165
4.3.2 Experimental animal data	165
4.4 Summary of Reproductive Toxicity Data	165
4.4.1 Human data.....	165
4.4.2 Experimental animal data	166
5.0 Summaries, Conclusions, and Critical Data Needs	172
5.1 Summary and Conclusions of Reproductive and Developmental Hazards.....	172
5.2 Summary of Human Exposure	172
5.3 Overall Conclusions.....	172
5.4 Critical Data Needs	172
6.0 References	173

List of Tables

Table 1. Primary Ingredients in Powdered Enfamil and Isomil Brand Soy Formulas	1
Table 2. US Soy Formula Manufacturers and Brand Names	4
Table 3. USDA-IOWA State University Survey of Soy Infant Formulas.....	5
Table 4. Estimated Isoflavone Intake by Infants Consuming US Soy Formulas	6
Table 5. Comparison of Isoflavone Levels in Infant Soy Formulas from Different Countries.....	8
Table 6. Estimated Intake of Isoflavones in Infants Fed Soy Formula	8
Table 7. Blood Levels of Total Isoflavones by Population	13
Table 8. Blood Levels of Isoflavones After Ingestion of Soy Foods or Supplements	14
Table 9. Levels of Phytoestrogens in Maternal Plasma, Cord Plasma, and Amniotic Fluid.....	18
Table 10. Correlations Between Isoflavone Levels in Maternal Plasma, Cord Blood, and Amniotic Fluid	18
Table 11. Mean Recoveries of Genistein and Daidzein in Humans	23
Table 12. Isoflavone Levels in Plasma of Rat Dams and Offspring	25
Table 13. Isoflavone Serum Isoflavone Levels in Rodents of Offspring Fed a Soy-Based Diet	25
Table 14. Phytoestrogen Levels in Brains of Male Rats Fed a Phytoestrogen-Containing Diet.....	26

Table 15. Experimental Animal Toxicokinetic Data for Genistein and Metabolites	27
Table 16 . Summary of Studies Examining Estrogenicity Endpoints in Humans	29
Table 17. Uterotropic Responses in Rats Fed Infant Formulas or Diethylstilbestrol	31
Table 18. Soy Estrogenicity in Laboratory Animals	33
Table 19. Development of UDPGT Activity in Humans	39
Table 20. Plasma Biochemical Values at 3 Months of Age in Children Given Soy Formula or Evaporated Cow-Milk Formula	47
Table 21. Laboratory Values at 6 Months of Age	54
Table 22. Formula Composition.....	56
Table 23. Mean IgG Antibody Levels to β -Lactoglobulin	70
Table 24. Composition of Reconstituted Formulas	76
Table 25. Factors Associated with Thelarche Prior to 2 Years of Age on Multivariate Analysis.....	83
Table 26. Additional Findings in Subjects from the Strom et al. Study.....	85
Table 27. Serum Lipid Profiles and Fractional Cholesterol Synthesis Rates	87
Table 28. Association Between Dietary Soy During Adolescence and Adult Breast Cancer	89
Table 29. Statistically Significant Relationships Between Soy Intake and Breast Cancer.....	90
Table 30. The Effects of Diet and Soy Formula Given to Rats on PND 21–50	93
Table 31. The Five Diets Used by Odum et al.	95
Table 32. Effects of Diet on Pregnancy and Lactation in Rats.....	95
Table 33 Effects of Diet on Rat Developmental Landmarks and Reproductive Organ Weights	95
Table 34. Effects of Diet on Relative Uterine Weight	96
Table 35. Adverse Effects in Rats Treated with Ethinyl Estradiol and Given Soy-containing or Soy-Free Feed	99
Table 36. Effects of Neonatal Exposures to Soy-Free Diet or Genistein on the Reproductive System of Male Rats	100
Table 37. Dietary Effects on Steroidogenic Enzyme Activities in Rats	103
Table 38. Summary of Significant Effects in Wild-Type and Aromatase-Knockout Mice Fed Diets With and Without Soy	105
Table 39. Effect of Diet on Dimethylbenzanthracene-Induced Mammary Tumors in Female Rats	112
Table 40. Studies in Humans Exposed as Infants to Soy Formula.....	133
Table 41. Developmental Effects of Soy Products in Experimental Animals.....	137
Table 42. Statistically Significant Hormonal Effects in Women Fed Textured Vegetable Protein Containing 45 mg/Day Conjugated Isoflavone	140
Table 43. Effects of Soy Milk Consumption on Menstrual Cycle Length and Hormones.....	141
Table 44. Female Reproduction Study of Soy Extract Added to the Diet.....	156
Table 45. Effects on Adult Male Rats of Feeding Soy-Based Diet.....	162
Table 46. Summary of Reproductive Studies in Women	168
Table 47. Summary of Reproductive Studies in Men	171

List of Figures

Figure 1. Chemical Structures of Isoflavones Found in Soy Formula	3
Figure 2. Metabolism of Genistin.....	20
Figure 3. Metabolism of Daidzin.....	20
Figure 4. Growth Parameters in Children Assigned to Soy Formula or Cow-Milk Formula for 3 Months	49
Figure 5. Infant Blood Urea Nitrogen as a Function of the Protein Content of Food Source	50

1.0 CHEMISTRY, USE, AND HUMAN EXPOSURE

1.1 Chemistry

Soy formula refers to infant food made using soy protein isolate and other components. The terms “soy” and “soybean” are commonly used for the leguminous Asian plant *Glycine max*. Soybean is also used to designate the edible seed of this plant. In this report, the term “soy” is used as an adjective to denote products derived from the edible seed (e.g., soy milk, soy formula, soy meal) and “soybean” is used to refer to the edible seed itself. In the US, the nutrient composition of soy and other infant formulas is regulated by the Food and Drug Administration (FDA) (1). Table 1 lists the primary ingredients in some common brands of powdered soy infant formulas. Those ingredients include corn syrup, soy protein isolate, vegetable oils, and sugar (2). In addition, the formulas are fortified with nutrients such as iron, calcium, phosphorous, magnesium, zinc, manganese, copper, iodine, selenium sodium, potassium, chloride, choline, inositol and vitamins A, C, D, E, K, and B (B1, B2, B6, B12, niacin, folic acid, pantothenic acid, biotin). Before feeding, 8.7–9.3 g powdered formula is added to 2 fluid ounces of water (2). Soy formulas are also available as concentrated liquids and as ready-to-feed formulations (3).

Table 1. Primary Ingredients in Powdered Enfamil and Isomil Brand Soy Formulas

Ingredient	Percentage [weight assumed]
Corn syrup	40.8–55
Soy protein isolate	14.6–18
Vegetable oils	21–28.6
Sugar ^a	10.2–11

^aListed as sucrose in some formulations but not specified in others. From (2).

Contaminants of soy protein include phytates (1.5%), which bind minerals, and protease inhibitors, which have antitrypsin, antichymotrypsin, and antielastin properties (4). As discussed further in Section 1.2.1, formulas are fortified with minerals to compensate for phytate binding and heated to inactivate protease inhibitors. Aluminum is found in soy formulas at concentrations of 600–1300 ng/mL, levels that exceed aluminum concentrations in human milk, 4–65 ng/mL (4). Mineral salts added to soy formulas are the source of aluminum.

Soy products contain phytoestrogens of the isoflavone class (5-7). Isoflavones can be conjugated to glucose or other carbohydrate moieties. Carbohydrate conjugates are generically called glycosides, and glucose conjugates are called glucosides. Small amounts of isoflavones are present in soy products in their unconjugated (aglycone) forms. The 3 main aglycones found in soy formula are genistein, daidzein, and to a smaller extent, glycitein. The genistein content of soy formula has been the subject of commentary because of concern about exposure of infants to estrogenic compounds; however, soy formula and other soy-based foods contain many components of which genistein is only one. In this report, attention will be focused on the effects of feeding soy formulas and other soy-based products to humans or experimental animals. A separate CERHR Expert Panel Report on Genistein will focus on the effects of administering genistein itself, isolated from other plant components. When studies have been performed using administration of isoflavone mixtures, the Expert Panel will judge whether the isoflavone mixture is expected to be informative on possible effects of feeding soy products or the mixture is more appropriately discussed as estimating possible effects of genistein. The Expert Panel recognizes that it is not entirely satisfactory to discuss isoflavone mixtures as either representing soy foods or purified genistein.

1.0 Chemistry, Use, and Human Exposure

Chen and Walter (8) reported that only 3.2–5.8% of total isoflavone in soy formula consists of unconjugated genistein and daidzein, and that amounts can vary by batch. The majority (>65%) of isoflavones detected in soy formula are conjugated to sugar molecules to form glycosides (7). Glucose groups on glycoside compounds can be esterified with acetyl or malonyl groups to form acetyl- or malonylglycosides (6). Isoflavones detected in soy infant formula by Setchell et al. (7) and/or the UK Ministry of Agriculture, Fisheries, and Food (5) are listed in Figure 1, along with their structures.

Conjugation with glucose groups increases water solubility of isoflavones, which are low molecular-weight hydrophobic compounds (6). Glucoside compounds can be deconjugated to form the biologically active aglycone compound by gut microflora (5), under acidic conditions (6), or by metabolic enzymes (7). Therefore, exposure to a particular isoflavone (e.g., genistein) is theoretically the sum of the aglycone and respective glycoside compound concentrations converted on the basis of molecular weight. However, Chen and Rogan (8) report that isoflavones are glucuronidated and circulated primarily in conjugated form. Estimated infant exposures to particular isoflavones through soy formula are discussed below in Section 1.2.3.

Setchell et al. (7) stated that isoflavone levels in soybeans can vary as a result of geographic location, climate, and growing conditions. They noted that high temperatures during processing of formula could affect the profile of conjugated isoflavones but are not expected to affect total isoflavone levels. For example, malonyl groups are especially heat labile and decompose to form their respective acetylglycoside compounds.

1.2 Use and Human Exposure

1.2.1 Production Information

In the manufacture of soy infant formula, the hull of the soybean is removed and the pulp is processed into oil and flake (4). Soy protein isolate is extracted from the flake using a slightly alkaline solution and is precipitated at the isoelectric point of 4.5. The resulting isolate has a purity of $\geq 90\%$ soy protein. The soy protein isolate is fortified with L-methionine, L-carnitine, and taurine. L-methionine improves the biological quality of the protein. Carnitine is needed for oxidation of long-chain fatty acids. Taurine is an antioxidant that is a major conjugate of bile acids in infants. Both carnitine and taurine are added at concentrations found in human milk. Vegetable oils such as soy, palm, sunflower, olein, safflower, and coconut are added to provide fats. Corn starch, tapioca starch, and sucrose are used as carbohydrate sources. Phytates, which bind minerals, are present in soy proteins at 1.5%. Therefore, total phosphorus and calcium are added at concentrations that are 20% higher than in cow-milk formulas, and the formulas are supplemented with iron and zinc. Heat applied during the processing of soy protein removes 80–90% of protease inhibitor activity.

Manufacturers of soy formula and some brand names of soy formula sold in the US are listed in Table 2.

1.0 Chemistry, Use, and Human Exposure

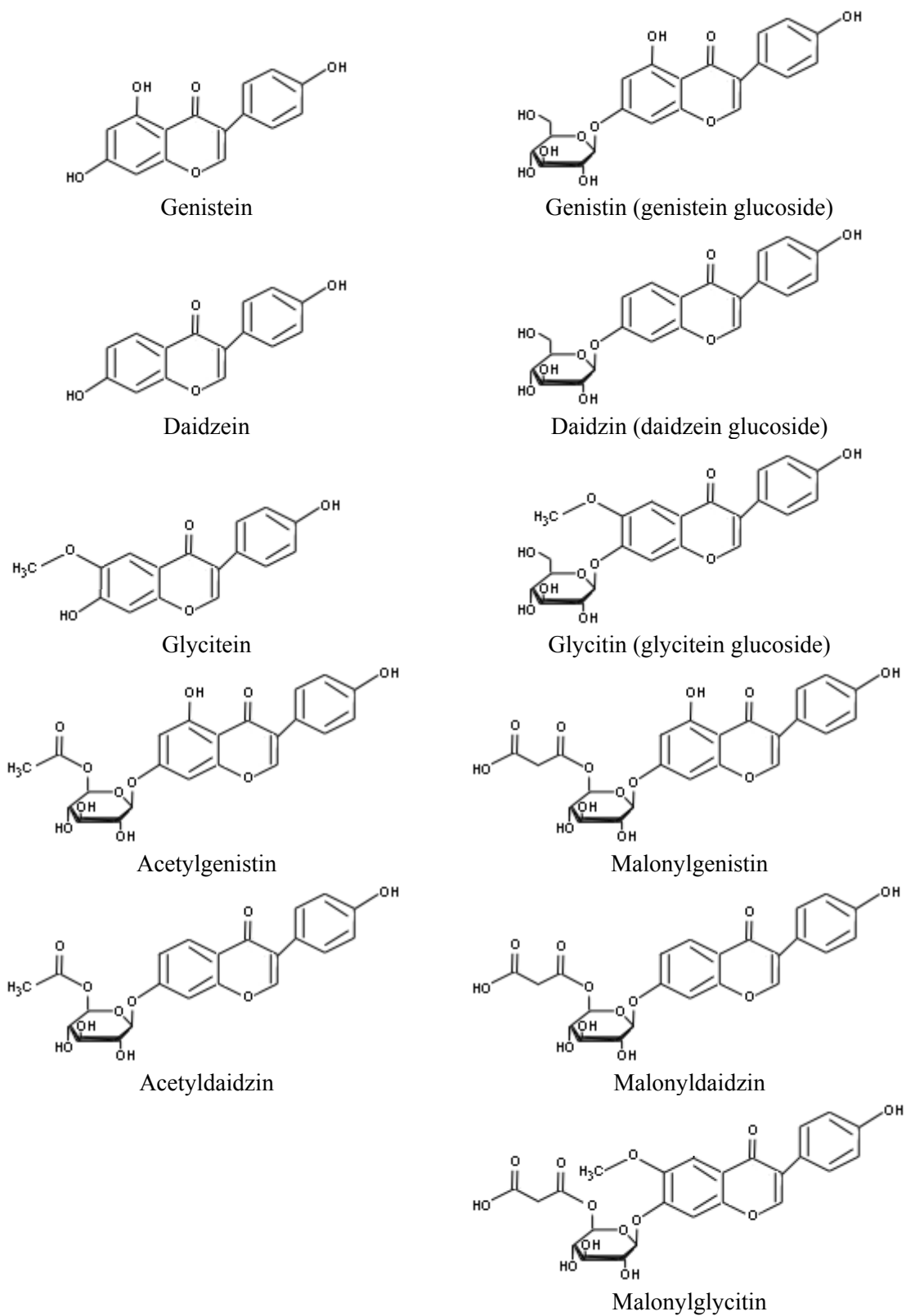


Figure 1. Chemical Structures of Isoflavones Found in Soy Formula

Table 2. US Soy Formula Manufacturers and Brand Names

Manufacturer	Brand names
Mead Johnson	Prosobee Gerber Soy
Ross	Isomil
Carnation	Alsoy
Wyeth-Ayerst	Nursoy
Wyeth (sold by PBM)	Various store brands (e.g. CVS, K-mart, Kroger, Safeway, Target)

Brand names are registered to their owners.
From (3, 9, 10)

1.2.2 Use

Soy formulas are generally used to feed infants who are allergic to dairy products or are intolerant of lactose, galactose, or cow-milk protein (11, 12). Infants are often changed from cow-milk to soy formula when they have symptoms such as colic, crying, diarrhea, and vomiting (13). Some parents feed their infants soy formula to maintain a vegetarian lifestyle or because of perceived health benefits of soy food consumption (14).

In 1996, Essex (12) reported that soy formulas represented 7% of infant formula sales in the UK, 13% in New Zealand, and 10–20% in the US. A 1998 infant-feeding survey conducted by Ross Products Division indicated that 18% of infants are fed soy formula during the first year of life (15). Based on the 2000 US Census estimate of 4 million American infants younger than 1 year of age, Strom et al. (15) estimated that 750,000 US infants per year are fed soy formula. According to marketing data and hospital discharge records, it has been estimated that 25% of newborns in the US are fed soy formula (14). A study conducted at Yale University examined formula changes in 189 breast-fed infants and 184 formula-fed infants and reported that a total of 87 infants [23%] received soy formula sometime during the first 4 months of life (13).

1.2.3 Occurrence and Infant Exposure

Levels of isoflavones in infant soy formula were reported in a survey of soy foods conducted by the US Department of Agriculture (USDA) and Iowa State University (3). The survey included results published by Murphy et al. (16), Nguyenle et al. (17), and Setchell et al. (18). Unpublished data and analyses conducted at Iowa State University were also included in the survey. Results were presented for the most common isoflavones, genistein, daidzein, and glycitein, although some studies did not include glycitein values. Glucoside values were converted to free-form (aglycone) values. Total isoflavones were calculated if values were available for daidzein and genistein, but it was noted that reported total isoflavone values may not equal values obtained by adding individual isoflavones. The survey included results for formula powders, liquid concentrates, and reconstituted or ready-to-eat formulas. Results of the infant soy formula survey are reported in Table 3. Reconstituted or ready-to-feed soy formulas contained genistein 1.58–2.26 mg/100 g formula [15.8–22.6 µg/g formula], daidzein 0.75–1.91 mg/100 g formula [7.5–19.1 µg/g formula], glycitein 0.28–0.35 mg/100 g formula [2.8–3.5 µg/g formula], and total isoflavones 2.63–4.17 mg/100 g formula [26.3–41.7 µg/g formula]. As expected, levels of isoflavone were higher in soy formula powders and liquid concentrates. Percentages of individual isoflavones were genistein 36.8–70.1%, daidzein 18.2–45.8%, and glycitein 7.9–13.0%.

Table 3. USDA-IOWA State University Survey of Soy Infant Formulas

Formula Description	Isoflavone	Mean (range), mg/100 g formula	Isoflavone % ^a	Confidence code ^b
ENFAMIL NEXT STEP, powder, not reconstituted	Daidzein	7.23 (7.15–7.30)	28.9	b
	Genistein	14.75 (14.50–15.00)	59.0	b
	Glycitein	3.00 (2.95–3.05)	12.0	b
	Total isoflavone	25.00 (24.90–25.10)		b
MEAD JOHNSON, GERBER, powder, not reconstituted	Daidzein	8.08 (6.50–9.65)	32.2	b
	Genistein	13.90 (12.80–15.00)	55.4	b
	Glycitein	3.12 (2.93–3.30)	12.5	b
	Total isoflavone	25.09 (22.23–27.95)		b
MEAD JOHNSON, PROSOBEE with iron, liquid concentrate, not reconstituted	Daidzein	1.10	18.2	c
	Genistein	2.22	36.8	c
	Total isoflavone	6.03 (3.32–8.75)		c
MEAD JOHNSON, PROSOBEE with iron, powder, not reconstituted	Daidzein	7.05 (6.90–7.20)	28.3	b
	Genistein	14.94 (14.45–15.43)	59.9	b
	Glycitein	2.95 (2.83–3.07)	11.8	b
	Total isoflavone	24.94 (24.18–25.70)		b
MEAD JOHNSON, PROSOBEE with iron, ready-to-feed	Daidzein	1.71	44.0	c
	Genistein	2.18	56.0	c
	Total isoflavone	3.89		c
ROSS, ISOMIL, with iron, powder, not reconstituted	Daidzein	6.03 (6.03–6.03)	28.7	b
	Genistein	12.23 (11.43–13.03)	58.3	b
	Glycitein	2.73 (2.70–2.77)	13.0	b
	Total isoflavone	20.99 (20.16–21.83)		b
ROSS, ISOMIL, with iron, powder, reconstituted as fed	Daidzein	0.78	28.8	none given
	Genistein	1.58	58.3	none given
	Glycitein	0.35	12.9	none given
	Total isoflavone	2.71		none given
ROSS, ISOMIL, with iron, ready-to-feed	Daidzein	1.91	45.8	c
	Genistein	2.26	54.2	c
	Total isoflavone	4.17		c
WYETH-AYERST, NURSOY, with iron, liquid concentrate, not reconstituted	Daidzein	1.02 (0.79–1.25)	25.4	b
	Genistein	2.82 (2.19–3.45)	70.1	b
	Glycitein	0.35	8.70	c
	Total isoflavone	4.02 (2.98–5.05)		b
WYETH-AYERST, NURSOY, with iron, powder, not reconstituted	Daidzein	5.70	21.9	c
	Genistein	13.55	52.1	c
	Glycitein	2.05	7.9	c
	Total isoflavone	26.00 (21.30–30.70)		b

1.0 Chemistry, Use, and Human Exposure

Formula Description	Isoflavone	Mean (range), mg/100 g formula	Isoflavone % ^a	Confidence code ^b
WYETH-AYERST,	Daidzein	0.75	28.5	c
NURSOY, with iron,	Genistein	1.60	60.8	c
ready-to-feed	Glycitein	0.28	10.6	c
	Total isoflavone	2.63		c

^aCalculated from mean values by CERHR.

^bAccording to the report, “Each mean is assigned a Confidence Code (CC) of a, b, or c. The Confidence code is an indicator of relative quality of the data and the reliability of a given mean value. A confidence Code of “a” indicates considerable reliability, due either to a few exemplary studies or to a large number of studies of varying quality.”

[The Expert Panel assumes that “a” means the highest confidence and “c” means the lowest confidence.]

Brand names are registered to their owners.

From (3).

In studies supported by Wyeth Laboratories, Protein Technologies International, and the National Institutes of Health (NIH), Setchell et al. (18) measured isoflavone levels in infant soy formula and in the blood of infants (n=7) consuming soy formula. Results of the study, as well as additional details about methodology, were published in a later report by Setchell et al. (7). Following extraction with methanol, isoflavone levels in 5 US infant formulas were measured by high performance liquid chromatography (HPLC). Genistein- and daidzein-related conjugates, mainly glycosidic conjugates, were the most abundant isoflavones identified. Mean percentages of isoflavones and their respective conjugates were reported at 67.1% genistein, 28.7% daidzein, and ~5% glycitein in soy infant formula. Levels of individual isoflavones, based on conversion to aglycone concentrations, are included in Table 3, which summarizes the USDA survey of soy formulas. Total average isoflavone concentrations were reported at 32–47 mg/L in prepared formulas. Table 4 lists estimated intakes of isoflavones and the assumptions used to estimate exposure. Depending on infant age, isoflavone intake was estimated at 6–12 mg/kg bw/day. A comparison of estimated isoflavone intake from soy formulas available in different countries is included in Table 5, which also includes estimates of individual isoflavone levels based on mean levels detected in formula.

Table 4. Estimated Isoflavone Intake by Infants Consuming US Soy Formulas

Infant age	Formula intake (mL/day)	Isoflavone intake (mg/day) ^a	Body weight (kg)	Dose (mg/kg bw/day)
1 week	500–550	22.5–24.8	2.5–3.8	5.7–7.3
1 month	700–800	31.5–36.0	2.9–3.8	6.0–11.9
2 months	800–830	36.0–37.0	3.6–5.9	6.1–10.0
4 months	800–1000	41.0–45.0	4.8–7.5	6.0–9.3

^aBased on a soy formula concentration of 45 µg/L. **[The unit of µg appears to be an error; units are listed as mg/L earlier in the report.]**

From Setchell et al.(7).

Franke et al. (19) measured isoflavone levels in 4 US brands of soy formula using methanol extraction and HPLC with diode-array ultraviolet detection. Isoflavone conjugation patterns were similar to those found in soy foods and included malonates (32–43%), glucosides (37–52%), acetates (6–7%), and aglycones (9–13%). Total isoflavones were measured at 155.1–281.4 µg/g. Percentages of each type of isoflavone included 55.3–57.7% genistein, 34–36.1% daidzein, and 7.4–8.5% glycitein. Based on an intake of 1 L formula, a body weight of 4.5 kg, and instructions for preparing formula, the authors estimated infant isoflavone exposure at ~7 mg/kg bw/day. The

1.0 Chemistry, Use, and Human Exposure

authors stated that isoflavone exposure in infants fed soy formula is 4–6 times higher than in adults eating a soy-rich diet (~30 g/day).

Irvine et al. (20) used an HPLC technique to measure isoflavone levels in infant soy formulas and foods such as cereals and pureed meats or vegetables purchased in New Zealand. In both soy formula and infant foods, the majority of genistein and daidzein were present as their respective glucosides. In soy formula, total concentrations of isoflavones (mean \pm SEM) were 87 ± 3 $\mu\text{g/g}$ genistein and 49 ± 2 $\mu\text{g/g}$ daidzein. **[If it is assumed that genistein and daidzein are the only isoflavones in the formulas, the percentages of total isoflavone represented by each compound are 64% genistein and 36% daidzein.]** Estimated infant exposures resulting from soy formula intake are summarized in Table 6. In 3 different infant cereals **[composition not indicated]** and 2 different infant dinners **[composition not indicated]**, genistein levels were measured at 3–287 mg/kg food and daidzein levels at 2–276 mg/kg food. The study authors noted that a single serving of 1 of the cereals could result in isoflavone exposures ranging from 0.01 to 0.8 mg/kg bw/day in a 4-month-old infant, thus increasing isoflavone intake by more than 25%.

Murphy et al. (16) analyzed 6 brands of soy formula sold in the US and reported that total isoflavone levels were similar across brands and were comprised of 59% genistein, 29% daidzein, and 12% glycitein. Levels of isoflavones are reported in the USDA survey (Table 3), and estimates of infant exposure are reported in Table 6.

Hoey et al. (21) used a liquid chromatography-mass spectrometry (LC-MS) method to measure isoflavone levels in 3 soy-based infant formulas from the UK. The isoflavone content of soy formulas consisted of ~58–67% genistein, 27–29% daidzein, and 6–16% glycitein. The study authors estimated that 4–6-month-old infants consumed between 17.5 and 33.0 mg/day isoflavones or 1.7–4.4 mg/kg bw/day. Infants fed the cow-milk formula with the highest isoflavone content (2.1 mg/kg formula) were estimated to consume 0.16–0.27 mg/day isoflavones, or 0.02–0.03 mg/kg bw/day.

The UK Ministry of Agriculture, Fisheries, and Food (5, 22) conducted a survey of isoflavone levels in 6 brands of infant soy formulas. The formulas were analyzed for 14 different isoflavones and 1 coumestan compound using methanol extraction followed by HPLC and LC-MS. Isoflavone levels were normalized to aglycone concentrations. Isoflavones were detected in all soy formulas at concentrations of 18–41 mg/L made-up formula. The majority of isoflavones were present as glycosides, but smaller amounts of acetyl and malonyl forms and aglycones were also present. The most abundant isoflavones, genistein-, daidzein-, and glycitein-related compounds, represented an average of 58, 36, and 6% of formulations on a molar basis, respectively. The isoflavones, glycitein, 6'-O-acetylglycitin, formononetin, and biochanin A, and the coumestan coumestrol were not detected. Eight batches of 1 soy formula, SMA Wysoy, purchased at different times and locations were analyzed to determine variation between batches. Isoflavone concentrations were 18–33 mg/L formula as made up. The UK Ministry of Agriculture, Fisheries, and Food concluded that isoflavone levels did not vary enough to significantly impact exposure. Statistical analyses by analysis of variance (ANOVA) and *F*-test did not find a significant difference at the 5% level in isoflavone levels between batches of the same brand and between different brands.

The UK Ministry of Agriculture, Fisheries, and Food summarized and compared levels of isoflavones measured in soy formula from other countries, and those values are outlined in Table 5. The Ministry of Agriculture, Fisheries, and Food concluded that isoflavone levels in soy formula from the UK were similar to concentrations reported in other countries; differences most

likely resulted from batch variations in soy isolate isoflavone levels, slight variations in formulas, and minor differences in analytic methodology.

The UK Ministry of Agriculture, Fisheries, and Food estimated isoflavone intake in 1–2-month-old and 4–6-month-old infants based on survey results, average body weight and intake data. Isoflavone intake was estimated at 5 mg/kg bw/day in 1–2-month-old infants and 4.5 mg/kg bw/day in 4–6-month-old infants. A comparison of the UK values with values obtained from other countries is included in Table 6.

Table 5. Comparison of Isoflavone Levels in Infant Soy Formulas from Different Countries

Country	Brands analyzed, n	Total isoflavones, mg/L formula as fed	Individual isoflavones, percent of total			Reference
			Genistein	Daidzein	Glycitein	
US	5	32–47	67.1	28.7	~5	Setchell et al. (18)
US	6	25–30	59	29	12	Murphy et al. (16)
US	4	155.1–281.4	55.3–57.7	34–36.1	7.4–8.5	Franke et al. (19)
Australia	4	18–21 ^a		not reported		Knight et al. (23)
UK	6	18–41	58	36	6	UK Ministry of Agriculture, Fisheries, and Food (5)

^aGlycitein was not measured

Table 6. Estimated Intake of Isoflavones in Infants Fed Soy Formula

Country, infant age (reference)	Intake (mg/kg bw/day) based on formula ingestion			
	Total isoflavone	Genistein	Daidzein	Glycitein
US, 4 months (18)	4.5–8.0; (6–12 ^a)	3.0–5.4 (4.0–8.0)	1.3–2.3 (1.7–3.4)	0.23–0.4 (0.3–0.6)
US, age not stated (16)	5–12	3.0–7.1	1.5–3.5	0.60–1.4
New Zealand, <1 month to 4 months, (20, 24)	2.9–3.8	1.9–2.4 ^b	1.0–1.4 ^b	Not known ^b
UK, 1–6 months (5)	4.5–5.0	2.6–2.9	1.6–1.8	0.27–0.30
US, 4.5 kg (19)	~1.6	~0.9	~0.5	~0.1
UK, 4–6 months old (21)	1.7–4.4	0.99–2.9	0.46–1.3	0.10–0.70

^aValues reported in a more recent publication by Setchell et al. (7).

^bPercentages of isoflavones are based upon levels of genistein and daidzein reported. It is not known if the formulas also contained glycitein.

Setchell et al. (7, 18) used a gas chromatography (GC)-MS method to compare isoflavone levels in soy formula and human milk and to measure isoflavone levels in human milk following soy ingestion. In comparison to soy formula, levels of isoflavones (measured by GC/MS) were substantially lower in cow-milk formula (below detection limit, which was not reported) and in milk collected from 9 healthy, omnivorous, lactating women (mean \pm SD = 5.6 \pm 4.4 μ g/L) (7). Equol was detected in 7 of 9 human-milk samples. A >10-fold increase in milk isoflavone levels was observed following ingestion of 30 mg isoflavones in the form of 10 g toasted soy nuts.

[Figure 2 in the study report suggests that milk daidzein levels increased >40-fold and genistein levels increased >10-fold 1 day after the ingestion of soy nuts.] The study authors noted that isoflavones occur predominantly as glucuronide conjugates in human milk. The UK

1.0 Chemistry, Use, and Human Exposure

Ministry of Agriculture, Fisheries, and Food (22) analyzed 3 cow-milk formulas for isoflavones using HPLC and LC-MS methods, and reported that isoflavone levels were below the detection limit of 0.25–0.5 mg/L. Irvine et al. (20) used an HPLC method to measure isoflavone levels in dairy-based formula and in human milk. Levels of genistein and daidzein were below the detection limit (0.1 µg/g) in human milk samples from 11 mothers and in the dairy-based formulas. Hoey et al. (21) used an LC-MS method to measure isoflavone levels in cow milk-based infant formulas from the UK. Isoflavones were not detected in the majority of the cow-milk infant formulas (0.5 mg/kg detection limit). King et al. (25) conducted HPLC analyses on Australian cow milk and reported mean isoflavone levels of <5 ng/mL daidzein, 4–29 ng/mL genistein, and 45–293 ng/mL equol.

After hydrolyzing samples to convert conjugated isoflavones to aglycones, Setchell et al. (7, 18) used a GC-MS method to measure total plasma isoflavone levels in seven 4-month-old male infants fed Isomil soy formula. Mean ± SD levels of genistein and daidzein were reported at 683 ± 442.6 and 295.3 ± 59.9 µg/L, respectively, and total isoflavones were reported at 552–1775 µg/L (mean 980 µg/L). **[Plasma glycitein levels were not measured.]** The study authors noted that they did not attempt to measure the extent of isoflavone conjugation in infant plasma. These total plasma isoflavone levels were 50–100-fold higher in infants fed soy formula compared to 4-month-old male infants fed human milk (mean ± SD 4.7 ± 1.3 µg/L, n = 7) and cow-milk formula (mean ± SD 9.3 ± 1.2 µg/L, n = 7). Plasma isoflavone levels in infants fed soy formula were also higher compared to adults (50–200 µg/L) and Japanese adults (40–240 µg/L) ingesting similar levels of isoflavones from soy-based foods.

The CERHR Expert Panel Report on Genistein provides estimates of adult exposures to isoflavones and allows for a comparison of isoflavone exposures in infants fed soy formula. Most estimates of adult exposure were based on dietary surveys, while infants exposures were based on isoflavone levels measured in soy formula and assumed body weight and formula intake. Based on limited information provided in the Genistein report, mean or median genistein exposures are estimated at ≤1 mg/day (0.01 mg/kg bw/day assuming a 70 kg bw) in US adults with no specified dietary preferences, 10–15 mg/day (0.1–0.2 mg/kg bw/day) in semivegetarian or vegetarians in the US, and 5–50 mg/day (0.1–1 mg/kg bw/day) in Asian adults. Based on mean mg/kg bw/day intake estimates, infants fed soy formula can be exposed to isoflavone levels that are 2–3 orders of magnitude higher than US adults with low isoflavone exposure, 1–2 orders of magnitude higher than vegetarians in Western countries, and within the same order of magnitude to 1 order of magnitude higher than Asians. Comparisons of isoflavone intake in infants and adults based on food or formula intake were fairly consistent with comparisons based on blood isoflavone levels presented in Section 2.

Differences in soy food exposure patterns throughout life were noted for Americans compared to Asians (14). In the US, typical diets are low in soy food intake, and the fetus is thus exposed to low levels of genistein. Significant exposure to genistein occurs in the approximately 25% of infants who are fed soy formula. After those infants are weaned, soy intake and genistein exposure drops and typically remain low over the lifetime. In Asian cultures consuming soy products, the fetus is exposed to genistein as a result of maternal soy food intake. At birth, most infants are either breast fed or fed cow-milk formula, so exposure to genistein is very low during infancy. Upon weaning, the infants begin receiving soy foods and exposure to soy products and genistein remain high over their lifetime.

1.3 Utility of Data

The human exposure data consist of a database reporting isoflavone levels in soy formulas in the US and other countries. Exposures of infants to isoflavones through consumption of soy formulas

have been estimated based on levels of isoflavones measured in formulas, formula intakes, and infant body weights. One study reported blood levels of isoflavones in infants fed soy formulas and compared the values to infants fed cow milk or human milk and to adults who ingested soy products. The available data provide a good foundation for estimating approximate exposure and dose within broad populations or within individuals when the soy formula and the infants' weight and age are known.

1.4 Summary of Human Exposure Data

Soy formula refers to infant food made using soy protein isolate and other components such as corn syrup, vegetable oils, and sugar (2). The soy protein isolate is fortified with L-methionine, L-carnitine, and taurine (4). The formulas are fortified with nutrients (2). Contaminants of soy protein include phytates (1.5%), which bind minerals, and protease inhibitors, which have antitrypsin, antichymotrypsin, and antielastin properties (4). Phosphorus, calcium, iron, and zinc are added to soy formula to compensate for phytate binding of minerals. Heat applied during the processing of soy protein removes 80–90% of protease inhibitor activity. Aluminum is present in soy formulas because of the addition of mineral salts.

Soy products contain phytoestrogens of the isoflavone class (5-7). The primary isoflavones detected in soybeans are derived from genistein, daidzein, and to a smaller extent, glycitein. The majority (>65%) of isoflavones in soy formula are conjugated to sugar molecules to form glycosides (7). Glucose groups in glycoside compounds can be esterified with acetyl or malonyl groups to form acetyl- or malonyl glycosides (6). Small amounts of genistein and daidzein (3.2–5.8%) are present in soy products in their unconjugated (aglycone) forms (8). Isoflavones detected in soy infant formula by Setchell et al. (7) and/or the UK Ministry of Agriculture, Fisheries, and Food (5) are listed in Figure 1 with their structures. Because glycosidic compounds can be deconjugated in the body to form the biologically active aglycone compound, exposure to a particular isoflavone (e.g., genistein) is theoretically the sum of the aglycone and respective glycoside compound concentrations converted on the basis of molecular weight (5-7). Isoflavone levels in soybeans are reported to vary as a result of geographic location, climate, and growing conditions (7).

It is estimated that 10–20% of infants in the US are fed soy formula (12, 15). Soy formulas are generally used to feed infants who are allergic to dairy products or are intolerant of lactose, galactose, or cow-milk protein (11, 12). Infants are sometimes given soy formula when they have symptoms such as colic, crying, diarrhea, or vomiting (13) or to maintain a vegetarian or perceived healthy lifestyle (14).

A number of studies measured isoflavone levels in infant formulas, determined percentages of individual isoflavones, and/or estimated infant exposures. In a USDA survey of infant formulas, isoflavone levels reported in reconstituted formulas or ready-to-feed formulas were reported at 15.8–22.6 µg genistein/g formula, 7.5–19.1 µg daidzein/g formula, 2.8–3.5 µg glycitein/g formula, and 26.3–41.7 µg total isoflavone/g formula (3). On a mg isoflavone/L ready-to-feed formula basis, isoflavone levels were reported at 25–281 mg/L in US soy formulas (7, 16, 19) and at 18–41 mg/L in formulas obtained from the UK and Australia (5, 22, 23). Percentages of individual isoflavones representing total isoflavone levels in US soy formulas were estimated at 36.8–70.1% genistein, 18.2–45.8% daidzein, and 5–13.0% glycitein (3, 7, 16, 18, 19). Percentages of individual isoflavones in formulas from the UK were reported at 58–67% genistein, 27–36% daidzein, and 6–16% glycitein (5, 21, 22).

Isoflavone exposure through soy formula intake has been estimated in the US and other countries based on isoflavone levels measured in soy formulas and assumptions of formula intakes and

1.0 Chemistry, Use, and Human Exposure

infant body weights. In the US, isoflavone intake by infants was estimated at 5–12 mg/kg bw/day (7, 16, 18, 19). In the UK and New Zealand, isoflavone intake by infants was estimated at 1.7–5 mg/kg bw/day (5, 20-22). A study conducted in New Zealand revealed that consuming cereals could further increase isoflavone exposure of infants by more than 25%.

Mean \pm SD plasma levels of isoflavones in infants fed soy formula were reported at 683 ± 442.6 $\mu\text{g/L}$ for genistein, 295.3 ± 59.9 $\mu\text{g/L}$ for daidzein, and 552–1775 $\mu\text{g/L}$ (mean 980 $\mu\text{g/L}$) for total isoflavones (7, 18). Mean plasma isoflavone levels were ~2 orders of magnitude higher than in infants fed cow-milk formula or human milk. Mean plasma levels of infants fed soy formulas were ~5–20 times higher than Japanese adults or adults ingesting similar levels of isoflavones from soy-based foods, ~50 times higher than vegetarian adults in Western populations, and ~500 times higher than omnivorous adults in Western populations (7, 18) (Table 7). Dietary exposure estimates comparing isoflavone intake in infants ingesting soy formula versus adults are fairly consistent to comparisons based on plasma isoflavone levels (see CERHR Expert Panel Report on Genistein).

2.0 General Toxicology and Biological Effects

2.1 Toxicokinetics and Metabolism

This section focuses on toxicokinetics and metabolism of 2 of the isoflavones, genistein and daidzein, found in soy formula and other soy foods. There are few studies available on toxicokinetics in infants. Therefore, a large part of this section is based on studies conducted in adults. Studies that focus on toxicokinetics following exposure to pure genistein are discussed in the CERHR Expert Panel Report on Genistein.

2.1.1 Human

2.1.1.1 Absorption

Genistein and daidzein exist mainly in their glycosidic forms in unfermented soy foods. Due to their high water solubility and molecular weight, glycosidic isoflavones are not readily absorbed across the gastrointestinal tract, as demonstrated in a study of adults by Setchell et al. (reviewed in (6)). For absorption to occur, isoflavones must first be hydrolyzed to their aglycones, which have some hydrophobicity and lower molecular weights. Absorption occurs primarily in the small and large intestine. Table 7 reports blood levels in infants and adults resulting from typical dietary exposures, and Table 8 reports blood levels in studies where humans were fed soy-based foods or supplements.

Infants are able to absorb isoflavones, and infants fed soy formula were demonstrated to have plasma isoflavone blood levels exceeding those of Japanese adults several-fold (Table 7) (7). Higher concentrations in infants are postulated to result from lower body weight, frequent feedings, and reduced metabolism.

Studies examining the bioavailability [**appearance of aglycone in plasma**] of isoflavones ingested as glycoside versus aglycone have reported conflicting findings (6). While some studies reported that bioavailability is increased when isoflavones are ingested as aglycones compared to glycosidic forms, other studies found no differences in bioavailability. One study reported that genistein levels peaked a mean of 6.6 hours after ingestion of the aglycone compared to 9 hours when ingested in glycosidic form (26). Bioavailability, as determined by area under the time-concentration curve (AUC), was reported to be greater for the glycosides than the aglycones. In a second study, Xu et al. (27) observed that bioavailability of isoflavones did not vary when they were administered to women as foods containing a high percentage of aglycones (e.g., tempeh) compared to a high percentage of glycosides (e.g., soybeans) but noted that a study by Hutchins et al. (28) found increased bioavailability for the aglycones. **[The Expert Panel notes that bioavailability in pharmacology and toxicology refers to the ratio of AUC after oral ingestion to the AUC after iv injection. The term bioavailability is often used in different ways by different authors.]**

A study by Xu et al. (27) found that increases in dietary fat, protein, and cholesterol had no effect on bioavailability of isoflavones administered 3 times daily to 8 women through powdered soy milk. Nevertheless, it has been postulated that bioavailability may be affected by food matrix, inter-individual variations, and intestinal microflora. Indeed, some studies reported that dietary fiber may lower isoflavone absorption (reviewed in (6, 29)).

Blood levels of genistein and daidzein (see Table 8) did not suggest saturated absorption in 12 women administered isoflavones at 0.7, 1.3, or 2.0 mg/kg bw through soy milk powder (44% genistein and 56% daidzein) (30). In a second study where groups of 8 women were given 0.4 or

2.0 General Toxicology and Biological Effects

0.8 mg/kg bw of ¹³C-labeled daidzein, the mean ± SEM AUCs at the higher doses were less than double the AUCs of the lower groups (5150 ± 1160 vs. 8700 ± 1610 nmol·h/L for daidzein and 6330 ± 1260 vs. 9770 ± 1320 nmol·h/L for genistein), suggesting decreased fractional absorption with increasing dose (31). [The Expert Panel notes that varying results obtained in studies by Xu et al. (30) and Setchell et al. (31) could reflect somewhat different fractional absorption for genistein from soy products compared to pure genistein.]

Absorption half-lives for both daidzin and genistin, presumably as glucuronides and sulfates of daidzein and genistein, were reported at ~1–3 hours following intake of foods containing ≤210 mg of each isoflavone or providing doses of ≤2 mg/kg bw/day of each isoflavone ((32, 33); reviewed in (34)). Genistein and daidzein equivalents were reported to peak at ~6–8 hours following ingestion of soy foods ((35); reviewed in (6, 34)).

Table 7. Blood Levels of Total Isoflavones by Population

Population and exposure condition	Plasma or serum aglycone equivalent levels, nM [µg/L] (mean ± SD)			Reference
	Genistein	Daidzein	Equol	
Seven 4-month old infants fed soy formula	2530 ± 1640 [684 ± 443]	1160 ± 230 [295 ± 58]	Not detected	Setchell et al. (7, 18)
Infants fed cow-milk formulas	11.6 ± 2.5 [3.1 ± 0.68]	8.1 ± 1.1 [2.1 ± 0.28]	16.9 ± 2.0 [4.1 ± 0.48]	Setchell et al. (7, 18)
Infants fed human milk	10.2 ± 2.7 [2.8 ± 0.76]	5.86 ± 0.51 [1.5 ± 0.13]	Not reported	Setchell et al. (1997) as cited in (8)
Men consuming traditional Japanese diet	90–1204 [24–325]	60–924 [15–235]	0.54–24.6 [0.13 ± 6.0]	Adlercreutz et al. (1994) as cited in (36)
Omnivorous Japanese men	276.0 [75]	107.0 [27]	5.5 [1.3]	Adlercreutz et al. (37)
Omnivorous Japanese men	206.1 [56]	72.5 [18]	Not reported	Arai et al. (2000) as cited in (34)
Japanese men	493.3 ± 604.4 [133 ± 163]	280.7 ± 375.5 [71 ± 95]	Not reported	Pumford et al. (35)
Japanese women	501.9 ± 717.6 [136 ± 194]	246.6 ± 369.4 [63 ± 94]	Not reported	Pumford et al. (35)
Japanese women	307.5 ± 325.4 [83 ± 88]	111.7 ± 187.8 [28 ± 48]	Not reported	Arai et al. (38)
Omnivorous Hong Kong men	Not reported	31.3 [8.0]	3.8 [0.92]	Morton et al. (1997) as cited in (34)
Vegetarian Finnish women	44.8 [12]	50 [13]	1.5 [0.36]	Adlercreutz et al. (39)
Vegetarian Finnish women	17.1 [4.6]	18.5 [4.7]	0.7 [0.17]	Adlercreutz et al. (1994) as cited in (34)
Lactovegetarian Finnish women	29.7 [8.0]	41.5 [11]	1.0 [0.059]	Adlercreutz et al. (1994) as cited in (34)
Omnivorous Finnish women	7.7 [2.0]	6.4 [1.6]	1.6 [0.39]	Adlercreutz et al. (39)
Omnivorous Finnish women	4.9 [1.3]	4.2 [1.1]	0.8 [0.19]	Adlercreutz et al. (1994) as cited in (34, 36)

2.0 General Toxicology and Biological Effects

Population and exposure condition	Plasma or serum aglycone equivalent levels, nM [$\mu\text{g/L}$] (mean \pm SD)			Reference
	Genistein	Daidzein	Equol	
Finnish men	6.3 [1.7]	6.2 [1.6]	0.8 [0.19]	Adlercreutz et al. (37)
Omnivorous Finnish men	0.5 [0.14]	0.6 [0.15]	0.1 [0.024]	Adlercreutz et al. (1993) as cited in (34)
Omnivorous Spanish men	Not reported	1.3 [0.33]	0.4 [0.10]	Morton et al. (1997) as cited in (34)
Omnivorous Canadian men	8.2 [2.2]	3.4 [0.86]	Not reported	Gooderham et al. (2000) as cited in (34)
Omnivorous British men	Not reported	8.2 [2.1]	0.6 [0.15]	Morton et al. (1997) as cited in (34)
British men	34.1 \pm 27.2 [9.2 \pm 7.4]	18.2 \pm 20.4 [4.6 \pm 5.2]	Not reported	Pumford et al. (35)
British women	30.1 \pm 31.2 [8.1 \pm 8.4]	13.5 \pm 11.6 [3.4 \pm 2.9]	Not reported	Pumford et al. (35)

Table 8. Blood Levels of Isoflavones After Ingestion of Soy Foods or Supplements

Population and exposure condition	Plasma or serum levels, nM [$\mu\text{g/L}$], aglycone equivalents, mean \pm SD		Reference
	Genistein	Daidzein	
Women ingesting 0.7 mg/kg bw isoflavones (44% genistein and 56% daidzein) through soy milk powder	740 \pm 440 [200 \pm 119]	790 \pm 40 [201 \pm 10]	Xu et al. (30)
Women ingesting 1.3 mg/kg bw isoflavones (44% genistein and 56% daidzein) through soy milk powder	1070 \pm 630 [289 \pm 170]	1220 \pm 670 [310 \pm 170]	Xu et al. (30)
Women ingesting 2.0 mg/kg bw isoflavones (44% genistein and 56% daidzein) through soy milk powder	2150 \pm 1330 [581 \pm 359]	2240 \pm 1180 [570 \pm 300]	Xu et al. (30)
Women consuming 4.5 $\mu\text{mol/kg}$ bw isoflavones through soy milk (48.9% genistein, 43.3% daidzein, 7.8% glycitein)	1700 \pm 1010 [459 \pm 273]	1040 \pm 610 [264 \pm 155]	Zhang et al. (40)
Women consuming 4.5 $\mu\text{mol/kg}$ bw isoflavones through soy germ (12.6% genistein, 48.5% daidzein, 38.9% glycitein) ^a	510 \pm 190 [138 \pm 51]	1630 \pm 1030 [414 \pm 262]	Zhang et al. (40)
Men consuming 4.5 $\mu\text{mol/kg}$ bw isoflavones through soy milk (48.9% genistein, 43.3% daidzein, 7.8% glycitein) ^a	1780 \pm 830 [481 \pm 224]	1290 \pm 500 [328 \pm 83]	Zhang et al. (40)
Men consuming 4.5 $\mu\text{mol/kg}$ bw	470 \pm 290	1160 \pm 440	Zhang et al. (40)

2.0 General Toxicology and Biological Effects

Population and exposure condition	Plasma or serum levels, nM [$\mu\text{g/L}$], aglycone equivalents, mean \pm SD		Reference
	Genistein	Daidzein	
isoflavones through soy germ (12.6% genistein, 48.5% daidzein, 38.9% glycitein) ^a	[127 \pm 78]	[295 \pm 11]	
Males ingesting cereal bar containing 8 g defatted soy grit (~20 mg isoflavones)	468 [126]	392 [100]	Pumford et al. (35)
Males ingesting cake containing 10.95 mg genistein and 8.54 mg daidzein for 3 days; day 3 values listed.	445 [120]	297 [75.5]	Pumford et al. (35)
Males ingesting isoflavones 16 mg/kg bw	7700 [2081] (total) 70 (free) [19]	Not reported	Busby et al. (2002) as cited in (6)
Males consuming soy protein-isolate beverage (60 g/day) for 28 days	907 \pm 245 [245 \pm 66]	498 \pm 102 nM [127 \pm 26]	Gooderham et al. (1996) as cited in (29)
Male ingesting soy supplement at dose of 35.6 mg/day daidzein and 5.6 mg/day genistein for 7 days	138 \pm 13 [37.3 \pm 3.5]	671 \pm 46 [171 \pm 12]	Doerge et al. (41)
Female ingesting soy supplement at dose of 35.6 mg/day daidzein and 5.6 mg/day genistein for 7 days	383 \pm 16 [104 \pm 4.3]	558 \pm 14 [142 \pm 3.6]	Doerge et al. (41)
Females ingesting 5 mg genistein or 5 mg daidzein	1220 \pm 470 ^b [330 \pm 127]	1550 \pm 240 ^b [394 \pm 61]	Setchell et al. (26)
Females ingesting 5 mg genistein or 5 mg daidzein	1260 \pm 270 ^b [341 \pm 73]	760 \pm 120 ^b [193 \pm 49]	Setchell et al. (26)

Equol was not reported in these studies. To convert nM genistein to genistein equivalents in $\mu\text{g/L}$, multiply by 0.27. To convert nM daidzein to daidzein equivalents in $\mu\text{g/L}$, multiply by 0.25.

^aPlasma glycitein values were reported at 200 \pm 80 nM [57 \pm 23 $\mu\text{g/L}$] in women consuming soy milk, 730 \pm 220 nM [208 \pm 63 $\mu\text{g/L}$] in women consuming soy germ, 220 \pm 80 nM [63 \pm 23 $\mu\text{g/L}$] in men consuming soy milk, and 850 \pm 250 nM [242 \pm 71 $\mu\text{g/L}$] in men consuming soy germ.

^bVariance not specified.

2.1.1.2 Distribution

One study reported volume of distribution for genistein and daidzein following intake of 50 mg of the compounds as aglycones and glycosides (26). Following intake of each compound in its glycosidic form, the mean volume of distribution for the bioavailable fraction was reported at ~161 L [2.7 L/kg bw assuming a 60 kg bw] for genistein and ~236 L [3.9 L/kg bw] for daidzein. Ingestion of the glycosidic compounds resulted in volumes of distribution for the bioavailable fraction of ~112 L [1.9 L/kg bw] for genistein and 77 L [1.3 L/kg bw] for daidzein. Similar mean volumes of distribution were reported for total genistein and daidzein in male and female volunteers who consumed 1–16 mg/kg bw formulations containing 70–100% unconjugated isoflavones; the volumes of distribution were ~1–6 L/kg bw for genistein and ~1–3

2.0 General Toxicology and Biological Effects

L/kg bw for daidzein (42, 43). In the same studies, volumes of distribution were higher for free isoflavones, ~16–877 L/kg for genistein and 15–245 L/kg bw/day daidzein. The study authors noted that the higher volumes of distribution for the free versus total isoflavones suggest that free genistein and daidzein are more likely to enter or be sequestered within tissues. **[There are problems in the reporting of volumes of distribution for the aglycones in these studies. The Expert Panel finds ranges of 16–877 and 15–245 L/kg bw/day to be implausible in this variability. In addition, volumes of distribution in the range of <10 L/kg bw in some reports are not consistent with volumes of distribution in the hundreds of L/kg bw in other reports. It is possible that the lower values were obtained from a mixture of aglycones and glucuronides with glucuronides predominating. The higher number may not take into consideration the bias toward the conjugated compounds in equilibrium.]**

Adlercreutz et al. (44) used a GC-MS method to measure maternal plasma, cord plasma, and amniotic fluid phytoestrogen levels in 7 healthy omnivorous Japanese women (20–30 years old) who had just given birth. Results for isoflavones and 2 metabolites are listed in Table 9, with total levels of isoflavone levels reported for maternal blood and unconjugated and conjugated levels reported for cord plasma and amniotic fluid. Conjugated and unconjugated values were not reported for maternal plasma because the authors stated that patterns in plasma are well established. Isoflavone and metabolite levels were variable between subjects, and 3 subjects had very low levels of equol. Levels of isoflavones and metabolites in cord blood and amniotic fluid were similar to maternal cord blood levels. Pearson correlations are shown in Table 10. Levels of *O*-demethylangolensin and equol in maternal plasma correlated significantly with levels in cord plasma and amniotic fluid. Significant correlations between cord plasma and amniotic fluid were found for all the isoflavones and metabolites. Lignans were also detected in maternal and fetal compartments but at much lower levels. The authors concluded that phytoestrogens cross the placenta. Levels in fetuses were similar to maternal levels, and conjugation patterns were reportedly similar to those observed in Japanese individuals.

Foster et al. (45) measured phytoestrogens in 57 human amniotic fluid samples collected between 15 and 23 weeks of gestation. Samples were collected in Los Angeles **[ethnic composition and dietary factors not discussed]**. Measurements were made by GC/MS after glucuronidase treatment to hydrolyze the conjugates. Genistein equivalent was measurable in 42 of the samples with a mean \pm SD concentration of 1.08 ± 0.91 ng/mL **[4.0 \pm 3.4 nM]** (range 0.4–4.86 ng/mL **[1.5–17.9 nM]**). In a different paper (46), these authors reported genistein concentrations in 59 amniotic fluid samples obtained from 53 pregnant women at 15–23 weeks of gestation (4 sets of twins and 1 woman who was sampled 3 times). There were 42 women with measurable amniotic fluid genistein concentrations. The mean \pm SD genistein equivalent concentration was 1.69 ± 1.48 ng/mL **[6.25 \pm 5.48 nM]** (maximum 6.54 ng/mL **[24.2 nM]**). **[In a table, the mean \pm SD is reported as 1.37 ± 1.00 ng/mL (5.07 \pm 3.7 nM) with a median of 0.99 ng/mL (3.7 nM). It is not known whether there are any samples represented in both papers.]**

Franke et al. (19, 47) measured isoflavone and metabolite levels in the milk and urine of 2 women who consumed 5, 10, and 20 g roasted soybeans at 0, 24, and 72 hours of the study, respectively. Plasma levels were also examined in 1 study (19). In the first study (47), isoflavone levels were measured in soybeans and found to be (in aglycone equivalents): daidzein 830 mg/kg soybean, genistein 913 mg/kg soybean, and glycitein 174 mg/kg soybean. Authors estimated the 3 daidzein doses at 0.08, 0.15, and 0.30 mg/kg bw and the 3 genistein doses at 0.08, 0.17, and 0.33 mg/kg bw. In the second study (19), the soybeans reportedly contained daidzein 0.85 mg/kg soybean and genistein 1.1 mg/kg soybean, and the authors stated that consumption of 20 g soybeans would result in intake of 37 mg isoflavones. **[Based on the authors' value for isoflavone intake, it appears that the units of isoflavones in soybeans should have been mg/g soybean instead of**

2.0 General Toxicology and Biological Effects

mg/kg soybean, consistent with data reported for mature soybeans in the USDA (3) survey. Assuming that the correct unit for the isoflavones is mg/g soybean, 20 g soybeans would contain ~17 mg daidzein and 22 mg genistein.] Milk samples were collected each time the infant was nursed, and blood samples were drawn in 1 study following an overnight fast. Isoflavones and metabolites were hydrolyzed enzymatically, extracted from samples using methanol or ethyl acetate, and analyzed by HPLC.

In both studies, isoflavone levels in milk were below the detection limit (1–3 pM) prior to hydrolysis, suggesting that isoflavones occur in milk as glucuronide and sulfate conjugates. Soybean intake resulted in a rapid and dose-dependent increase in genistein and daidzein derivatives in milk. Concentrations peaked 10–14 hours following consumption of soybeans and returned to baseline levels 2–4 days later. A biphasic pattern of isoflavone detection was observed in milk after consumption of 20 g soybeans. Milk samples did not contain glycitein or the metabolites equol and *O*-demethylangolensin at detectable levels. Milk contained higher concentrations of genistein than daidzein conjugates. Levels of genistein conjugates were also higher in plasma [**data were not shown**]. In urine, the ratio of isoflavones to metabolites suggested preferential excretion of metabolites [**data were not shown**]. In contrast to results for milk and plasma, urinary conjugates of daidzein were higher than conjugates of genistein. Glycitein and the isoflavone metabolites equol and *O*-demethylangolensin were also detected in urine.

In the Franke et al.(47) study, intake of 5, 10, and 20 g soybeans by a single subject resulted in maximum milk total genistein levels of 32, 46, and 71 nM and total daidzein levels of 16, 34, and 62 nM, respectively. The respective maximum urinary excretion rates for total genistein were 60, 300, and 300 nmol/hour and for total daidzein, 150, 500, and 600 nmol/hour. Milk and urine levels were compared to those of a Chinese woman who ate her usual diet, which included 1 serving of tofu soup a day. The woman's milk contained total genistein at 30–50 nM and total daidzein at 80–110 nM; urinary excretion rates ranged from 8 to 33 nmol/hour for total genistein and from 80 to 150 nmol/hour for total daidzein. In the Franke et al. (19) study, mean total isoflavone concentrations after consumption of 20 g soybeans were 0.2 µM in milk and 2 µM in plasma; urinary excretion rate for isoflavone was 3.0 µmol/hour. [**The data for “total isoflavones” were presented in the study abstract.**]

2.0 General Toxicology and Biological Effects

Table 9. Levels of Phytoestrogens in Maternal Plasma, Cord Plasma, and Amniotic Fluid

Analyte	Mean (range), nM						
	Maternal plasma (total)	Cord plasma			Amniotic fluid		
		Unconjugated and sulfates	Glucuronides and sulfoglucuronides	Total	Unconjugated and sulfates	Glucuronides and sulfoglucuronides	Total
Genistein	83.9 (9.16–303)	15.7 (3.51–37.3)	150 (35.6–387)	165 (39.8–417)	10.2 (2.93–24.4)	53.8 (3.86–198)	64 (11.4–212)
Daidzein	45.5 (2.04–243)	7.94 (1.09–15.1)	50.8 (8.84–122)	58.8 (10.2–137)	13.5 (2.11–29.4)	54.3 (5.03–143)	67.8 (15.6–156)
<i>O</i> -Demethylangolensin	31.2 (1.25–194)	11.2 (1.02–48.9)	12.9 (0–60.8)	24.1 (1.02–110)	3.09 (0–6.0)	20.5 (0.25–92.5)	23.6 (2.86–98.5)
Equol	71.1 (0.63–401)	11.5 (0–48.0)	39.4 (0–219)	50.9 (0–267)	3.80 (0–12.1)	64.2 (0.13–385)	68.0 (0.13–397)
Total isoflavones	232 (19.2–744)	46.4 (10.1–96.6)	253 (47.4–735)	299 (57.8–831)	30.6 (6.77–65.2)	193 (26.8–740)	223 (51.5–779)

To convert from nM to µg/L unconjugated equivalents, multiple by: genistein 0.27, daidzein 0.25, *O*-demethylangolensin 0.26, and equol 0.25.
From Adlercreutz et al. (44).

Table 10. Correlations Between Isoflavone Levels in Maternal Plasma, Cord Blood, and Amniotic Fluid

Isoflavone	Pearson correlation	
	Maternal plasma versus cord plasma	Maternal plasma versus amniotic fluid
Genistein	0.338	0.530
Daidzein	0.439	0.418
<i>O</i> -Demethylangolensin	0.967*	0.970*
Equol	0.997*	0.997*

* $P < 0.001$

From Adlercreutz et al. (44).

2.0 General Toxicology and Biological Effects

A mean \pm SD isoflavone level of 5.6 ± 4.4 $\mu\text{g/L}$ was reported in milk samples from 9 healthy lactating women (7). Equol was detected in 7 of 9 milk samples. A ≤ 10 -fold increase in milk isoflavone levels was observed in a woman who ate 30 mg isoflavones in the form of 10 g toasted soy nuts. **[Figure 2 in the study report suggests that milk total daidzein levels increased >15-fold and total genistein levels increased <10-fold 1 day after the ingestion of the soy nuts.]** The study authors noted that isoflavones occur predominantly as glucuronide conjugates in human milk. A second study reported that levels of total genistein and total daidzein were below the detection limit (0.1 $\mu\text{g/g}$) in milk samples from 11 mothers in New Zealand (20).

Isoflavone levels were measured in breast tissue from 4 women eating bread rolls supplemented with isoflavones 45 mg for 14 days prior to undergoing breast biopsy (35). Results were compared to those from 4 women who did not receive soy supplementation prior to biopsy. In women receiving soy supplementation, breast tissue contained genistein 0.655 (range 0.571–0.723) nmol/g wet tissue and daidzein 0.145 (range 0.083–0.128) nmol/g wet tissue. Breast tissue from women not undergoing supplementation contained genistein 0.120 (range 0.103–0.142) nmol/g wet tissue and daidzein 0.021 (range 0.017–0.028) nmol/g wet tissue.

Studies in humans reported that daidzein concentrations in prostate fluid were ~ 2 –4 times higher than plasma levels and equol concentrations in prostate fluid were 44 times higher than in plasma (reviewed by (34)).

2.1.1.3 Metabolism

The complete metabolic fates of genistein and daidzein are not known, but some information is available (Figure 2 and Figure 3). Most of the information was obtained from reviews describing metabolism of isoflavones following soy intake. However, some information obtained in studies with daidzein aglycone is also briefly discussed.

Metabolism of isoflavone glycosides begins with hydrolysis of the compounds to their respective aglycones, a step that must occur before the compounds can enter the systemic circulation. According to the UK Committee on Toxicity (6), there is disagreement about whether hydrolysis occurs in the stomach. Intestinal bacteria such as *Lactobacilli*, *Bifidobacteria*, and *Bacteroides* contain β -glycosidase activity that may facilitate the hydrolysis of isoflavone glycosides, but the specific types of bacteria involved have not been identified. Bacterial flora is scarce in the small intestine but abundant in the large intestine. One study suggested that enzymes in saliva can hydrolyze glycosidic isoflavones. β -Glucosidases in foods can also contribute to hydrolysis of glycosidic compounds (reviewed by Kurzer and Xu (36)), but their contribution to overall hydrolysis of the glycuronides is minor.

Before entering the systemic circulation, the majority of genistein and daidzein are further metabolized to their conjugates, as noted in Figure 2 and Figure 3 (6, 36, 48). It is thought that additional metabolites can be generated both by intestinal and liver enzymes and by intestinal microflora. The metabolic profile has been reported to vary among individuals. Some individuals produce little or no equol or *O*-demethylangolensin.

2.0 General Toxicology and Biological Effects

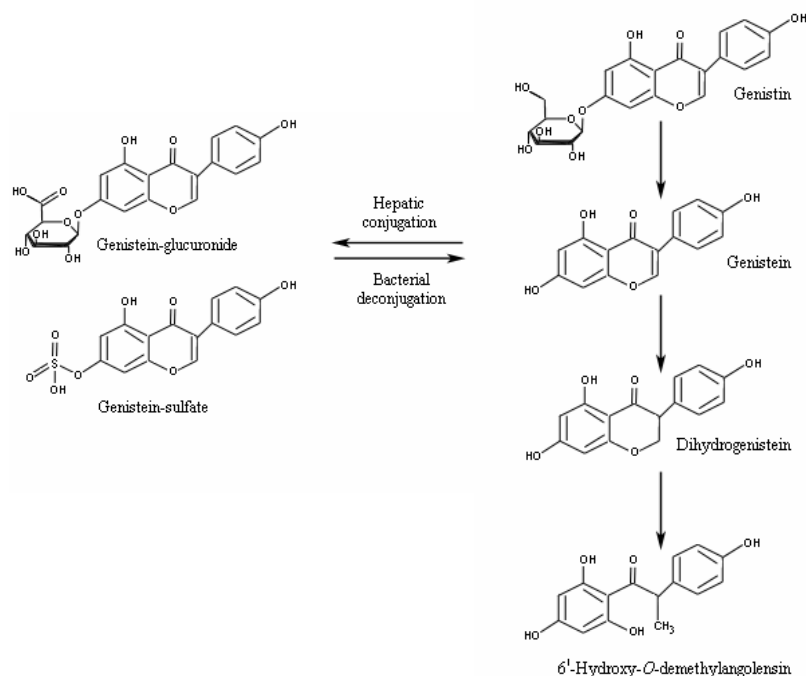


Figure 2. Metabolism of Genistin
Adapted from UK Committee on Toxicity (6).

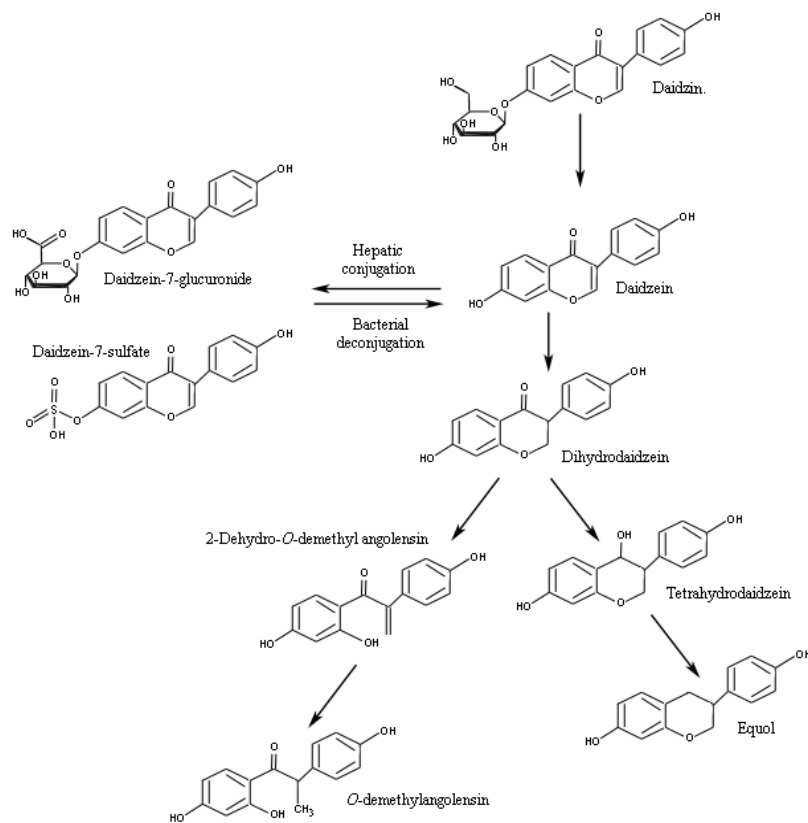


Figure 3. Metabolism of Daidzin
Adapted from UK Committee on Toxicity (6) and Joannou et al. (48).

2.0 General Toxicology and Biological Effects

Genistein and daidzein are conjugated with glucuronic acid by uridine diphosphate-glucuronosyltransferase (UDPGT) or to a much lesser extent, with sulfate by sulfotransferases. In a study described in greater detail in the CERHR Expert Panel Report on Genistein, the study authors concluded that the intestine plays a major role in the glucuronidation of genistein (41). In studies conducted with daidzein, the ability to glucuronidate daidzein was the same for microsomes from kidney and liver, while microsomes from colon had no measurable activity (41). The 1A10 isoform of UDPGT, which is present in the gut but not in liver, lacked activity toward daidzein, while the 1A1 and 1A9 isoforms, found in gut and liver, had the greatest activity. Thus, study authors concluded that the majority of daidzein glucuronidation occurs in liver (41).

The glucuronide and sulfate conjugates enter the systemic circulation, and it has been reported that the majority of isoflavones in the circulation are present in conjugated form. In individuals who ingested a soy supplement consisting of glycosidic isoflavones, glucuronides represented 69–98% of circulating genistein and 40–62% of circulating daidzein (41). In the same study, sulfate conjugates represented 4% of circulating daidzein compounds in 1 male but were not detected in a female subject. Genistein sulfates were not detected in the male or female. A second study reported mean steady-state circulating unconjugated isoflavones at 2.7% for daidzein and 1.6% for genistein when the compounds were taken as aglycones or in glycosidic form (26). The conjugated isoflavones undergo enterohepatic circulation, and on return to the intestine, they are deconjugated by bacteria with β -glucuronidase or arylsulfatase activity. The metabolites may be reabsorbed or further metabolized by gut microflora.

Limited ability of infants to produce equol was demonstrated by Setchell et al. (7, 18), who detected equol in plasma of 4 of 7 infants fed soy milk formulas but with a peak area 1–2 orders of magnitude lower than in infants fed cow-milk formula. Equol was detected in cow-milk samples from Australia (25). The finding was consistent with previous studies that found no equol in urine of infants fed soy formula and others that reported age-related increases in β -glucosidase activity (reviewed in (7)).

The ability of infants to absorb and metabolize isoflavones was demonstrated by Hoey et al. (21). The study examined 60 infants and children, aged 4 months to 6 years, who were assigned to either a soy-formula or control group (N = 30 in each) depending on whether they had consumed soy or cow-milk formula during infancy. Subjects were also divided into 4 groups according to age (4–6 months, 7–12 months, 1–3 years, and 3–7 years). Genistein, daidzein, and glycitein were present in the urine of all soy-fed infants in the 4–6-month age group, while *O*-demethylangolensin and equol were detected in 75 and 25% of soy-fed infants, respectively. In contrast, isoflavonoids were very low or not detected in the 4–6-month control group. In subjects aged 7 months to 7 years who were given a soy challenge, *O*-demethylangolensin was present in the urine of 75% of soy subjects and 50% of control subjects, while equol was present in the urine of 19% of soy subjects and 5% of control subjects. These percentages were similar between groups in the 3–7-year age group. Analysis of fecal samples showed that the total bacterial count was significantly higher in cow milk-fed infants than in the soy-fed infants. More specifically, the bacterial counts for *Bifidobacteria*, *Bacteroides*, and *Clostridia* were significantly higher in the cow milk-fed group, while the combined count for *Lactobacillus* + *Enterococcus* was not significantly different between groups. There were no significant differences between the soy-formula and control groups in fecal enzyme activities, pH, or short-chain fatty acid concentrations. The researchers concluded from the 4–6-month age group data that the isoflavones genistin, daidzin, and glycitin were well absorbed after hydrolysis in the gut because significant concentrations of their glucuronides were found in urine samples. They also deduced that the ability to hydrolyze glycosides to aglycones developed by the age of 4–6 months because

2.0 General Toxicology and Biological Effects

such hydrolysis is required for absorption. Although a higher percentage of soy-fed infants than cow milk-fed infants of the younger age groups were able to convert daidzein to equol, the percentages were similar among the older children. Thus, the authors concluded that isoflavone exposure early in life has no lasting effect on isoflavone metabolism. They also noted the influence of formula type on the composition of the microflora present in the gut of infants.

Setchell et al. (26) reported that no adult females administered daidzein produced equol but that 2 of 4 women given daidzin produced equol; the study authors speculated that daidzin may have traveled further down the intestinal tract, where it was metabolized by bacteria. Half-lives for equol formation were reported at 2–4 hours in adults eating soy foods (32, 33). A review by Price and Fenwick (49) reported that the majority of equol was excreted as glucuronide, with smaller amounts excreted as the sulfate conjugate in some humans.

Setchell (50) reported that recent studies conducting detailed qualitative analysis of human urine identified numerous diphenolic metabolites generated in intermediates steps of genistein and daidzein biotransformation. At the time the Setchell review was published, the intermediate metabolites had not yet been identified by MS.

Mean recoveries of genistein and daidzein within 24–48 hours are well below 100%, as outlined in Table 11. Incomplete recoveries suggest the formation of additional metabolites such as *p*-ethylphenol or 2,4,6-trihydroxybenzoic acid (reviewed in (51)). In some reviews such as the one conducted by Whitten and Patisaul (34), recoveries were considered to represent bioavailability. Because recovery of daidzein and its metabolites usually exceeds that of genistein and its metabolites, it has been concluded that daidzein is more bioavailable than genistein; however, this conclusion is not supported by blood data that sometimes indicate higher levels of total genistein than total daidzein (Table 7 and Table 8). Two studies by Setchell et al. (26, 31) reported greater bioavailability of genistein and derivatives than of daidzein and its metabolites based on blood levels measured over time; the authors noted that some studies reporting greater bioavailability of daidzein measured only 2 time points in the elimination period and could have reached erroneous conclusions. Setchell et al. (31) also noted that urinary daidzein concentrations are consistently higher than genistein glucuronide concentrations when the 2 compounds are administered at equimolar concentrations, which is expected because of the greater plasma clearance rate of daidzein. In two trials of a study where 0.4 mg/kg bw of the ¹³C-labeled compounds were administered as aglycones to 2 different groups of 8 women, the mean ± SEM plasma clearance rates were reported at 26.73 ± 4.29 L/h for daidzein and 20.17 ± 3.50 L/h for genistein.

There is some evidence that cytochrome P450 (CYP) may be involved in the metabolism of isoflavones. Unidentified metabolites considered to be hydrolysis products have been detected following in vitro incubation of genistein with human recombinant CYP1A1, 1A2, 1B1, 2E1, or 3A4 isoforms (6).

The role of gut microflora in the metabolism of isoflavones was clearly established (6). Experiments conducted with cultured human fecal bacteria demonstrated the formation of equol from textured vegetable protein and the conversion of daidzein to dihydrodaidzein, benzopyran-4,7-diol, 3-(4-hydroxyphenol), and equol. Information on genistein metabolites is provided in the CERHR Expert Panel Report on Genistein.

In a review, Munro et al. (52) reported that variations in metabolic pathways of isoflavones can occur as a result of differences in microflora, intestinal transit time, pH, or redox potential, factors that can be affected by diet, drugs, intestinal disease, surgery, and immunity.

Table 11. Mean Recoveries of Genistein and Daidzein in Humans

Population and exposure condition ^a	Percent intake excreted within 24–48 hours ^b			Reference
	Genistein	Daidzein	Total isoflavones	
Four infants fed soy formulas containing 81–92 mg/kg genistein and 44–55 mg/kg daidzein	13 ± 3 (SEM)	38 ± 4	Not reported	Irvine et al. (24)
Women consuming soy milk powder (56.0% genistein and 44.0% daidzein) with 0.9 mg/kg bw isoflavone/meal 3 times daily with various background diets	18–20	26–27	25–27	Xu et al. (27)
Women consuming soybeans, texturized vegetable protein, tofu, or tempeh (54–60% genistein and 40–46% daidzein)	9–16	38–51	21–34	Xu et al. (27)
Women consuming 0.7, 1.3, and 2.0 mg isoflavones through soy milk (44% genistein and 56% daidzein)	5–11	19–23	15–19	Xu et al. (30)
Women drinking soy milk providing doses of ~85 mg/day genistein and 69 mg/day daidzein	11	35	22	Lu et al. (53)
Women drinking soy milk containing 80–200 mg of each isoflavone	24	66	Not reported	Lu and Anderson (32)
Men drinking soy milk containing 80–200 mg of each isoflavone	15	47	Not reported	Lu and Anderson (32)
Men drinking soy milk containing ~100 mg each of genistein and daidzein	15	47	Not reported	Lu et al. (33)
Men consuming baked soybean powder	18	36	Not reported	Watanabe et al. as cited in (6)
Women and men consuming 4.5 µmol isoflavones through soy milk (48.9% genistein, 43.3% daidzein, 7.8% glycitein) ^c	27.6	48.6	Not reported	Zhang et al. (40)
Women and men consuming 4.5 µmol isoflavones through soy germ (12.6% genistein, 48.5% daidzein, 38.9% glycitein)	29.7	43.8	Not reported	Zhang et al. (40)
Women consuming soy protein 60 g/day (25.08 mg/day daidzein and 19.85 mg/day genistein) during 1 menstrual cycle	[0.4–12]	[0.8–16]	1.8–12.9	Cassidy et al. (54)
Men and women consuming 1–16 mg/kg bw formulations containing 70–100% unconjugated isoflavones	8–18	26–53	34–74	Bloedon et al. (43) and Busby et al. (42)

^aIsoflavone content assumed to be expressed in aglycone equivalents.

^bExcretion percentages were reported for urine or urine and feces; however, the inconsistency is not expected to have a significant impact on values because percent fecal excretion is minor (≤4%).

^cPercent excretion for glycitein was 55.3 in subjects fed soy milk and 54.5 in subjects fed soy germ.

2.1.1.4 Excretion

Reviews that primarily addressed isoflavone exposure through soy product intake reported that most ingested genistein is excreted in urine, with very little excreted in feces (reviewed in (6, 29)). In studies in which volunteers ate soy-based foods, isoflavone excretion was reported at ~30% in urine and 1–4% in feces ((27). An additional study in which volunteers drank soy milk reported peak urinary excretion at 8–10 hours, and 95% of excretion occurring within 24 hours; total urinary excretion consisted of 1% aglycones and 99% glucuronidated metabolites (32). A second study reported that following ingestion of isoflavone mixtures containing ≥70% aglycones, <0.3% of the dose was excreted as free genistein or daidzein (43).

2.0 General Toxicology and Biological Effects

Elimination half-lives were reported at ~4–6 hours for genistein glucuronide, 3–4 hours for daidzein glucuronide, and 5–10 hours for equol following intake of ≤ 100 mg of each isoflavone through soy milk (33). Studies in which male and female volunteers consumed formulations containing 70–100% unconjugated isoflavones at 1–16 mg/kg bw reported longer mean half-lives for total isoflavones (~6–13 hours for genistein and 4–16 hours for daidzein) compared to free isoflavones (~2–7 hours for genistein and daidzein) (42, 43). **[A value of 21.2 hours for free daidzein appeared to be an outlier.]**

Numerous studies demonstrated that urinary excretion of isoflavones increased with increasing soy intake (6). However, 1 study demonstrated that percent excretion of total genistein and daidzein was not affected at isoflavone levels of 0.7, 1.3, or 2.0 mg/kg bw/day administered through soy milk powder (30). Mean in vitro fecal degradation half-lives for 14 volunteers were reported at ~8.9 hours for genistein and ~15.7 hours for daidzein (40).

It has been reported that urinary levels of daidzein and genistein derivatives were slightly lower in infants compared to adults fed equivalent amounts of isoflavones. This difference could indicate slower renal clearance in early life or slower glucuronidation and as a consequence, decreased renal secretion (reviewed in (7)).

Irvine et al. (24) measured urinary excretion of genistein and daidzein in 4 infants fed soy formula. The soy formulas contained genistein equivalents at 81–92 mg/kg formula and daidzein equivalents at 44–55 mg/kg formula as determined by HPLC. Based on measured isoflavone levels, recommendations by formula manufacturers, and infant weights, the authors estimated that the infants received isoflavones 2.9–3.8 mg/kg bw/day from 2 to 16 weeks of age. Once per week until 8 weeks of age and once every 2 weeks up to 16 weeks of age, 3–5 used diapers not containing feces were collected over a 24-hour period. Urine was diluted with water, squeezed from diapers, and pooled over 24 hours. Samples were hydrolyzed, methanol-extracted, and analyzed by HPLC. Recovery of isoflavones from diapers was verified. Data were presented as mean \pm SEM and analyzed by ANOVA. Average concentrations of isoflavones in urine were daidzein 2.9 ± 0.3 mg/L and genistein 1.5 ± 0.2 mg/L. Mean percentages of daily isoflavone intake detected in urine and normalized for creatinine levels were daidzein equivalents $38 \pm 4\%$ and genistein equivalents $13 \pm 3\%$. Percent excretion was not affected by age. Isoflavone excretion rates varied little between infants and ranged from 0.37 ± 0.03 to 0.58 ± 0.06 mg/kg bw/day for daidzein and from 0.15 ± 0.03 to 0.32 ± 0.04 mg/kg bw/day for genistein. Age did not affect excretion rate. Urine from 25 infants fed dairy-based formula was also analyzed, and no isoflavones were detected **[detection limit not specified]**.

2.1.2 Experimental animal

Placental and lactational transfer of dietary isoflavones in rats was examined in a developmental toxicity study that is discussed in detail in Section 3.2 (55). Throughout pregnancy and lactation, 10 Sprague Dawley rats received a commercially available high-phytoestrogen diet. The diet contained phytoestrogens 603 $\mu\text{g/g}$ feed consisting mainly of daidzin, genistin, and glycitin. The diet was soy-based, according to Lephart et al. (56). At gestation day (GD) 20.5 **[day of plug not specified]** and on postnatal day (PND) 3.5 **[day of birth not specified]**, blood was collected and pooled from dams and offspring ($n = 3\text{--}5$ litters at each collection period) for measurement of total plasma genistein, daidzein, and equol levels by GC-MS. Blood levels of phytoestrogens in adult male rats fed the same diets were also examined. As noted from the study results listed in Table 12, gestational and lactational transfer of isoflavones was demonstrated. GD 20.5 dams were noted to have lower phytoestrogen plasma levels than male rats. The study authors proposed that changes in phytoestrogen metabolism or increased circulatory volume in late pregnancy were possible reasons for the lower plasma phytoestrogen levels in GD 20.5 dams.

Table 12. Isoflavone Levels in Plasma of Rat Dams and Offspring

	Plasma aglycone equivalent levels ($\mu\text{g/L}$) [nM]		
	Genistein	Daidzein	Equol
Fetus, GD 20.5	106.39 [394]	67.53 [266]	51.58 [213]
Dam, GD 20.5	232.31 [860]	193.80 [762]	578.12 [2387]
Pup, PND 3.5	234.85 [869]	341.13 [1342]	161.85 [668]
Dam, PND 3.5	441.22 [1633]	339.46 [1335]	906.22 [3742]
Adult male	420.95 [1558]	390.27 [1535]	932.37 [3850]

From Weber et al. (55).

Brown and Setchell (57) measured serum levels of isoflavones in adult mice and rats and in fetuses and pups of rats fed various rodent diets. In adult FVB mice fed 1 of 3 soy-based diets (Purina 5008 or 5010 or NIH-07), serum levels of total genistein and daidzein [**$\leq 100 \text{ ng/mL}$ each; $\leq 370 \text{ nM}$ genistein, $\leq 393 \text{ nM}$ daidzein**] were about an order of magnitude lower than serum total equol levels [**$\sim 1300\text{--}2300 \text{ ng/mL}$**]. Similar results were observed in Sprague Dawley rats fed a soy-based diet (Purina 5001), with serum levels of total equol [**$\sim 2100 \text{ ng/mL}$**] greatly exceeding those of total genistein and daidzein [**$\sim 100 \text{ ng/mL}$ each; $\leq 370 \text{ nM}$ genistein, $\leq 393 \text{ nM}$ daidzein**]. Urine levels of genistein and daidzein glucuronide were about 25% of equol glucuronide levels in mice and 50% of equol glucuronide levels in rats. Results of serum analysis in rat pups of dams fed soy-based diets are presented in Table 13. According to the study authors, serum isoflavones in newborn pups prior to nursing represent maternal-fetal transfer during gestation. The authors stated that pups do not start eating feed until 15–16 days of age, and therefore serum levels of isoflavones in pups before that time point were also of maternal origin. Equol levels were very high at birth and rapidly declined during the postnatal period. Stomach contents of newborn rats, presumed to be swallowed amniotic fluid, also contained high levels of isoflavones consisting of 44% genistein derivatives, 37% equol derivatives, and 19% daidzein derivatives.

Table 13. Isoflavone Serum Isoflavone Levels in Rodents of Offspring Fed a Soy-Based Diet

Pup age	Aglycone equivalent levels, ng/mL^a [nM]		
	Genistein	Daidzein	Equol
Newborn ^b	100 [370]	50 [197]	550 [2271]
6-day-old	90 [333]	50 [197]	80 [330]
12-day-old	110 [407]	25 [98]	70 [289]
16-day-old	10 [37]	5 [20]	60 [248]

^aValues estimated from a graph by CERHR.

^bMeasured in pups prior to nursing.

From Brown and Setchell (57).

Lund et al. (58) also reported serum total equol levels [**$\sim 3000\text{--}4000 \text{ ng/mL}$; $12,389\text{--}16,519 \text{ nM}$**] that were about an order of magnitude higher than levels of total genistein [**$\sim 300\text{--}500 \text{ ng/mL}$; $1110\text{--}1850 \text{ nM}$**] or daidzein [**$\sim 300\text{--}400 \text{ ng/mL}$; $\sim 1180\text{--}1573 \text{ nM}$**] in adult rats fed soy-based diets containing phytoestrogens at 600 $\mu\text{g/g}$ diet.

In rats fed a soy-based diet containing ~ 30 ppm each of genistin and daidzin, serum total genistein levels were measured at $\sim 620 \text{ nM}$ in females and 350 nM in males (59). Serum total daidzein concentrations were measured at 250 nM in females and at 200 nM in males.

2.0 General Toxicology and Biological Effects

Isoflavones were detected in brains of adult male rats fed a soy-based diet containing 600 ppm phytoestrogens (60, 61). As noted in Table 14, total isoflavones were greatest in frontal cortex > cerebellum > amygdala > hippocampus. In hippocampus, isoflavone levels were similar to those of rats fed a phytoestrogen-free diet. In the frontal cortex and amygdala, the concentration of equol was > genistein > daidzein. In the cerebellum, genistein > daidzein > equol. The study authors stated that cerebellum and frontal cortex contained an abundance of estrogen receptor (ER) β . Levels of phytoestrogens in the medial basal hypothalamic and preoptic areas were reported at 4.4 ng/g [0.017 nmol/g] daidzein, 3.5 ng/g [0.013 nmol/g] genistein, and 126 ng/g [0.52 nmol/g] equol (61). Levels of genistein and equol were significantly higher than in rats fed a phytoestrogen-free diet.

Table 14. Phytoestrogen Levels in Brains of Male Rats Fed a Phytoestrogen-Containing Diet

Brain region	Isoflavone levels in $\mu\text{g}/\text{kg}$ [nmol/g] ^a			
	Daidzein	Genistein	Equol	Total
Frontal cortex	271.0 \pm 29.8 [1.07 \pm 0.12]	295.5 \pm 35.4 [1.09 \pm 0.13]	705.3 \pm 63.4 [2.91 \pm 0.26]	1272 \pm 129 [5.07 \pm 0.51]
Hippocampus	3.4 \pm 0.4 [0.013 \pm 0.0016]	3.1 \pm 0.3 [0.011 \pm 0.0011]	28.5 \pm 3.4 [0.12 \pm 0.014]	35.0 \pm 4.1 [0.14 \pm 0.017]
Amygdala	5.5 \pm 0.6 [0.022 \pm 0.0024]	9.9 \pm 1.1 [0.037 \pm 0.0041]	57.5 \pm 5.2 [0.24 \pm 0.021]	72.9 \pm 6.9 [0.30 \pm 0.028]
Cerebellum	58.8 \pm 29.8 [0.23 \pm 0.12]	126.8 \pm 29.8 [0.47 \pm 0.12]	33.4 \pm 29.8 [0.14 \pm 0.12]	219.0 \pm 29.8 ^b [0.84 \pm 0.36]

^aData presented as mean \pm SEM

^b[SEMs reported by study authors; there appears to be an error because all were the same.]

From (60).

Whitten and Patisaul (34) reviewed animal toxicokinetic data on genistein and daidzein derivatives (Table 15). In a review, Setchell (50) reported that high levels of equol equivalents have been detected in portal venous blood and bile of rats, thus indicating that isoflavones undergo enterohepatic circulation. Because equol was primarily found as a conjugate of glucuronic acid in venous portal blood, it was suggested that conjugation within the intestinal wall may occur during absorption. Intestinal conjugation of genistein was confirmed in studies using everted rat intestinal preparations (62); the study is described in detail in the CERHR Expert Panel Report on Genistein.

2.0 General Toxicology and Biological Effects

Table 15. Experimental Animal Toxicokinetic Data for Genistein and Metabolites

Source/route	Species	Dose, mg/kg bw/day	C _{max} ^a , nM [µg/L]	T _{max} , hours	Plasma half-life, hours	Recovery, %
Genistein/oral	Rat	~8	600–900 [162–243]	Not reported	Not reported	Not reported
Genistein/oral	Rat	20	11,000 [2970]	2	8.8	20
Genistein/oral	Rat	45	2200 [594]	2	Not reported	Not reported
Genistein/oral	Rat	~40	6000–8000 [1620–2160]	Not reported	3–4	Not reported
Soy-derived genistein/oral	Rat	20	4900 [1323]	2	8.8	18
Soy-derived genistein/oral	Rat	20	9500 [2565]	2	Not reported	15
Genistein/oral	Mouse	45	2600 (free) [702]	0.3	4.8	20
Genistein/oral	Mouse	54–180	4100 (free) [1107]	0.05	4.7	21
Genistein/oral	Mouse	50	1500 (free) [405]	0.5	8	11
Genistein/iv	Mouse	52	237,000 [63,990]	N/A	Not reported	Not reported
Genistein/oral	Rhesus macaque	7	55 (free+sulfate) [15]	Not reported	Not reported	Not reported
Soy-derived daidzein/oral	Rat	21	5000 [1350]	2	Not reported	19
Daidzein/oral	Rhesus macaque	3	21 (free+sulfate) [6]	Not reported	Not reported	Not reported

C_{max} = maximum plasma concentration; T_{max} = time to C_{max}, iv = intravenous.

^aConcentrations expressed in aglycone equivalents.

From Whitten and Patisaul (34)

2.2 General toxicology

2.2.1 Thyroid

Concerns about soy-induced thyroid toxicity arose in the 1930s when goiters were observed in rats fed soybeans (reviewed in (6, 63)). In the 1950s and 1960s, cases of altered thyroid function, mostly goiter, were reported in infants fed soy formula. The problem was eliminated by adding more iodine to the formulas and replacing soy flour with soy protein isolate. Studies addressing possible thyroid toxicity resulting from soybean or isoflavone intake in developing humans or animals are discussed more fully in Section 3, while the remainder of this section focuses on effects in adults.

A study in Japanese men fed 30 g soybeans/day for 1 or 3 months reported signs of thyroid toxicity including increased thyroid-stimulating hormone levels, decreased thyroxine, and diffuse goiter (reviewed in (6, 63)). Symptoms of constipation, fatigue, and lethargy were observed in about half the subjects treated for 3 months. Recovery was observed following cessation of the soybean diet. Based on levels of isoflavones in Japanese soybeans, Fitzpatrick (63) estimated intakes of 23 mg/day genistein and 10 mg/day daidzein. Other studies in humans ingesting up to 132 mg/day isoflavones, through soy consumption in most cases, reported small and variable

2.0 General Toxicology and Biological Effects

effects on thyroid hormone levels that the study authors did not consider clinically significant (reviewed in (6)). Both Fitzpatrick (63) and the UK Committee on Toxicity (6) expressed concerns that individuals with hypothyroidism may be more susceptible to thyroid effects following soy intake or that soy may interfere with medications used to treat the condition. Though limited, epidemiologic studies suggest no association between phytoestrogen exposure through soy consumption and thyroid cancer risk in humans.

Evidence of thyroid toxicity (e.g., increases in thyroid weight and thyroid-releasing hormone level, decreased thyroxine level, and histological changes) was observed in iodine-deficient rats fed soybeans, but there was no effect when rats were fed soy providing genistein concentrations of 60 mg/kg diet or isoflavones at ≤ 2000 mg/kg diet (reviewed in (6)). One study of iodine-deficient rats fed soybeans reported an increase in thyroid carcinoma, but no evidence of carcinogenicity was observed in a second study examining effects of genistein intake (≤ 250 mg/kg diet) in rodents (reviewed in (6)).

2.2.2 Cardiovascular

The FDA (64) and the UK Committee on Toxicity (6) reviewed numerous human studies examining the effects of soy foods on cholesterol levels. Based on well conducted human studies, both the FDA and UK Committee on Toxicity concluded that consumption of soy foods as part of a diet low in saturated fat and cholesterol can lower total blood cholesterol and low density lipoprotein levels. As noted in the Expert Panel Report on Genistein, there is no conclusive evidence that the hypocholesteremic properties of soy products are due to isoflavones. The UK Committee on Toxicity examined the effects of soy product intake on other risk factors associated with cardiovascular disease (e.g., high blood pressure, thrombosis, and atherosclerosis), and concluded that there are insufficient data to reach a conclusion. A study using a transgenic mouse model of hypertrophic cardiomyopathy (65) showed that feeding a soybean-based diet resulted in more dilated ventricles and poorer cardiac function than did feeding a casein based diet. This effect was seen only in males.

2.2.3 Menopausal symptoms

Some perimenopausal and menopausal women experience hot flashes and vaginal dryness; these symptoms can vary by culture (36). One study noted that fewer menopausal Japanese compared to Canadian women experienced hot flashes. It was postulated that weak estrogenic effects associated with a phytoestrogen-rich diet could be the cause of reduced menopausal symptoms in Japanese women. Therefore, the effects of soy diets and isoflavone supplements on hot flashes were investigated. Of the 12 studies reviewed by the UK Committee on Toxicity (6), half reported that soy diets or isoflavone supplementation reduced the frequency of hot flashes, while the other half reported no effect on hot flashes.

The effects of soy-product or genistein intake on vaginal cytology in postmenopausal women are reported in the discussion of *in vivo* estrogenicity effects.

Animal studies reviewed by the UK Committee on Toxicity (6) consistently demonstrated that soy products prevented bone loss in ovariectomized rodents. Epidemiologic studies reviewed by the UK Committee on Toxicity (6) reported higher bone mass in populations consuming more soy products. A small number (~6) of short-term intervention studies in humans consistently demonstrated small but statistically significant soy-associated increases in bone mineral density in the lumbar spine. Other studies examining effects at other sites produced equivocal findings, with some reporting beneficial effects on bone mineral density and others reporting no effect. The UK Committee on Toxicity stated that long-term studies are needed before conclusions can be made about the effectiveness of phytoestrogens in improving bone health.

2.0 General Toxicology and Biological Effects

2.2.4 Estrogenicity

2.2.4.1 Human

Estrogenic effects were examined in women receiving soy diets or supplements, summarized in Table 16. Three of the studies did not demonstrate changes in vaginal cytology related to soy product intake. In the more detailed of these studies (66, 67), women were exposed to up to 1 mg/kg bw/day genistein for 30–93 days. A fourth study that provided very limited detail and included alternating exposure to non-soy-based phytoestrogens reported increased vaginal cell maturation after women received soy flour supplements for 2 weeks (68). A fifth study with a longer exposure period (5 years) demonstrated estrogenic effects on the endometrium (69). However, a limitation of the Unfer et al. (69) study was noted in a letter to the editor by Foth and Nawroth (70). They noted that at baseline and at the 30-month evaluation period 25% of endometrium samples were inaccessible in the treated and placebo groups. **[The Expert Panel notes that 20–25% of endometrium samples were also inaccessible at the 5-year evaluation period.]** It does not appear that women with inaccessible endometrium samples at baseline were excluded for evaluation at future time points. Therefore, it is not known if endometrial hyperplasia was present at baseline in women with inaccessible endometrium samples. It was also noted that no information was provided about endometrial thickness or bleeding patterns. A sixth study reported increased proliferation of breast lobular epithelium and progesterone receptor expression in women who ate bread rolls containing 60 g soy supplement as textured vegetable protein (71).

Table 16 . Summary of Studies Examining Estrogenicity Endpoints in Humans

Study description ^a	Endpoint	Effect	Reference
For 93 days, postmenopausal women (n = 17–18) received soy protein powders providing total isoflavone intakes of 0.11 ± 0.01 (control), 1.00 ± 0.01 (low), or 2.00 ± 0.02 (high) mg/kg bw/day. [Based on reported percentages of isoflavones, the diets resulted in intakes of about 0.06, 0.58, and 1.2 mg/kg bw/day genistein; 0.04, 0.33, and 0.66 mg/kg bw/day daidzein; and 0.01, 0.09, and 0.18 mg/kg bw/day glycitein.]	Vaginal cytology and endometrial biopsy Plasma levels of 17β-estradiol, estrone, dehydroepiandrosteron e-sulfate, sex hormone-binding globulin, insulin, thyroid-binding globulin	No significant change Slight decreases compared to baseline values, but the study authors stated effects were modest and unlikely to be of physiologic significance	Duncan et al. (66)
Postmenopausal women received a soy diet (n=66) with soy products representing one-third of caloric intake or their usual diet (control group, n=25) for 4 weeks. Foods were analyzed by HPLC, and it was determined that the soy diets provided 165 mg/day isoflavones [40.3 mg/day genistein and 124.8 mg/day daidzein. Based on 58 kg bw: 0.69 mg/kg bw/day genistein and 2.2 mg/kg bw/day daidzein] .	Vaginal superficial cells Serum FSH, LH, 17β-estradiol, or sex hormone-binding globulin levels	No significant change No significant change	Baird et al. (67)

2.0 General Toxicology and Biological Effects

Study description ^a	Endpoint	Effect	Reference
Postmenopausal women were randomized to receive 46 g/day of either soy (n = 23) or wheat (n = 24) flour for 12 weeks.	Vaginal maturation index	No significant change	Murkies et al. (72)
In a study with Latin-square design, postmenopausal women (n = 23) were given 45 g soy flour, 10 g red clover sprouts, or 25 g linseed daily, during separate 2-week periods. Vaginal smears were taken after each supplementation period, and cumulative effects of the 3 foods were compared at 6 weeks and at 2 and 8 weeks following supplementation.	Vaginal cytology	Significant changes in vaginal cytology after 6 weeks of supplementation; changes persisted for 2 weeks.	Wilcox et al. (68)
	Vaginal maturation index FSH/LH levels	Increased after soy supplementation Concentrations remained higher than premenopausal values.	
Healthy menopausal women were randomized to receive for 5 years placebo (n=197) or 3 extract tablets/day (n=179), each containing 150 mg total isoflavones consisting of genistein 40–45%, daidzein 40–45%, and glycitein 10–20%.	Endometrial proliferation or hyperplasia	Baseline: Proliferative endometria in 2 women of the soy extract group and 3 women of the placebo group; hyperplasia was an exclusion criterion. At 30 months: No cases of proliferation or hyperplasia. At 5 years: No proliferation or hyperplasia in placebo group; 5 biopsies (3.2%) were proliferative and 6 biopsies (3.8%) were hyperplastic in the soy-extract group.	Unfer et al. (69)
Prior to scheduled biopsy, women with benign or malignant breast conditions but normal menstrual cycles were assigned to a control group (n = 29) or a soy-supplement group (n = 19). Women in the soy-supplement group ate bread rolls containing 60 g soy supplement as textured vegetable protein (45 mg isoflavones) for 14 days. Normal breast tissue was collected near lesions.	Proliferation of breast lobular epithelium and progesterone receptor expression.	Both endpoints increased when controlled for stage of menstrual cycle and age.	McMichael-Phillips (71)

FSH = follicle-stimulating hormone, LH = luteinizing hormone

^aIsoflavone content assumed to be expressed in aglycone equivalents.

2.0 General Toxicology and Biological Effects

2.2.4.2 Experimental animal

Ashby et al. (73) examined uterotrophic effects of infant formulas. From PND 21/22 through 24/25, Alpk rats were given access to infant formula in drinking water bottles and RM1, a standard diet consisting of 6% soy protein. Three soy-based and 1 cow-milk formula were prepared as recommended by manufacturers. Rats fed the RM1 diet and not given infant formula served as negative controls, and rats given diethylstilbestrol or 17 β -estradiol served as positive controls. Data were analyzed by analysis of covariance (ANCOVA). **[It is not clear how many animals were examined in each group, but if numbers above the bars in a graph indicated the numbers of animals, then each group contained 5–29 animals.]** Results are summarized in Table 17. Both cow-milk and soy-based formulas prepared at full strength increased uterine weight, with greater responses generally noted with recommended concentrations of soy compared to cow-milk formula. Testing of 3 different concentrations of 1 of the soy formulas (Infasoy®) showed dose-related responses. It was noted that rats consumed Infasoy at a level 3 times the recommended intake for an infant on a mg/kg bw basis. The cow-milk formula, SMA Gold, and the 33%-strength Infasoy formula were consumed at levels similar to those recommended for infants on a mg/kg bw basis. Faslodex, an ER α and ER β antagonist, inhibited the uterotrophic effects of soy and cow-milk formulas and of diethylstilbestrol **[data not shown for cow-milk formula]**. The aromatase inhibitor anastrozole attenuated the uterotrophic response of Infasoy but not of 17 β -estradiol **[data not shown]**. The gonadotropin-releasing hormone (GnRH) antagonist antarelix inhibited the uterotrophic effect of cow-milk and soy formulas but not of diethylstilbestrol **[data not shown for cow-milk formula]**. Neither the Infasoy nor cow-milk formula induced an uterotrophic response in ovariectomized adult rats **[data not shown]**. Dietary effects on puberty were also examined and are discussed in Section 3. The study authors concluded that both cow-milk and soy formulas induced estrogenic effects in rodents independent of formula phytoestrogen content. The authors further stated that infants fed recommended quantities of formulas would consume similar quantities of formula (on a mg/kg bw basis) as rats given the 33% soy formula or cow-milk formula, resulting in exposures at the threshold of estrogenic activity in rats.

Table 17. Uterotrophic Responses in Rats Fed Infant Formulas or Diethylstilbestrol

Treatment	Strength, % of recommended dilution for feeding infants	Uterine wet weight, % of negative control ^a
Cow-milk formula		
SMA gold	100	132% ^b
Soy formula		
Wysoy	100	140% ^c
Farley's	100	125% ^c
Infasoy	33	116%
	100	148–179% ^c
	200	167% ^c
Diethylstilbestrol, 10 μ g/L		156–196% ^c

^aEstimated from graphs by CERHR.

^b $P < 0.05$; ^c $P < 0.01$ compared to control.

From Ashby et al. (73).

Results of estrogenicity testing in laboratory animals fed soy-based diets are summarized in Table 18. No evidence of estrogenicity was observed in studies in which rats or monkeys were fed soy-based diets (74-77); however, estrogenic effects occurred when soy isoflavones were added to a soy-free diet (78). In the monkey studies (76, 77), the genistein dose in the soybean extract was stated to be equivalent to that of a woman receiving genistein 99.7 mg /day [**~ 2 mg/kg bw/day assuming a 50 kg bw**]. A study in immature female mice found a soy-based diet to increase

2.0 General Toxicology and Biological Effects

relative uterine weight and to antagonize the weight increase associated with feeding diethylstilbestrol, demonstrating weak estrogen agonist and antagonist properties (79). In a study in which rats were fed soy protein with and without the addition of an estrogen, there were no additive effects between soy diets and estrogen (75). Soy diets that were not alcohol extracted to remove isoflavones antagonized the effects of estrogens on increased lactoferrin staining and epithelial luminal cell height. In some studies, increases in rodent uterine weight were greater in rats fed soy-free versus soy-containing diets (73, 74, 80, 81).

2.0 General Toxicology and Biological Effects

Table 18. Soy Estrogenicity in Laboratory Animals

Animal	Design	Endpoint(s)	Results	Reference
Alpk rat, 21 days old	Rats were fed RM1 (standard) diet or AIN-76A (phytoestrogen-free diet) for 3 days. Some rats fed AIN-76A diet were also administered the anti-estrogen Faslodex.	Uterine weight	Higher in rats fed AIN-76A diet compared to RM1 diet, an effect that was eliminated with Faslodex treatment. The authors had no explanation for the results.	Ashby et al. (74); abstract; Ashby et al. (73)
Sprague Dawley rat, > 40 days old, ovariectomized	Rats were fed a soy diet containing 117.8 mg isoflavone/1800 calories or an alcohol-extracted soy diet with 11.6 mg isoflavone/1800 calories for 2 months. Rats were fed casein diets or soy diets containing low or high isoflavone levels (as described above) + conjugated equine estrogen at 0.313 or 0.625 mg/1800 calories.	Vaginal cytology, uterine weight, endometrial cell proliferation, lactoferrin expression, luminal epithelial cell height, or apoptosis Vaginal cytology, uterine weight, endothelial cell proliferation, and apoptosis. Lactoferrin staining Luminal epithelial cell height	No significant effects Estrogen induced changes in all parameters examined; soy isoflavones did not further affect these parameters. High isoflavones attenuated staining induced by estrogen. High isoflavones attenuated increase induced by estrogen	Tansey et al. (75)
Female Sprague Dawley rat, 24 days old	Multigeneration design using casein-based diet or alcohol-washed, isoflavone-poor soy protein diet. A commercial soy extract was added to the isoflavone-poor diet, providing isoflavone levels of 31.7–1046.6 mg/kg feed. Juvenile F ₂ females were evaluated on PND 4. Some females were given sc ethinyl estradiol or bisphenol A from PND 21.	Uterine weight, peroxidase, and epithelial height	All 3 estrogenic endpoints were increased by the highest isoflavone diet (1046.6 mg/kg feed). There was no interaction with ethinyl estradiol or bisphenol A except additivity between ethinyl estradiol and isoflavones at the highest dietary level.	Wade et al. (78)
Female F344 rat, 3 months old	For 14 weeks, rats (10–14/group) were fed either a casein-based diet, a diet containing 100 g/kg isolated soy	Uterine wet weight and histopathology	No significant effects.	Nakai et al. (82)

2.0 General Toxicology and Biological Effects

Animal	Design	Endpoint(s)	Results	Reference
Male adult NMRI mouse exposed to diethylstilbestrol as neonate and castrated in adulthood.	protein (2.14 mg aglycones/g isoflavone), a diet containing 200 mg/kg isolated soy protein, a casein-based diet containing 17.2 g/kg isoflavones (11.37 mg aglycones/g isoflavones), or a casein-based diet containing 34.4 g/kg isoflavones. Mice were fed soy-free diets or diets containing 7% roasted soy meal, for up to 10–20 days following castration; 17β-estradiol was given to some mice in each dietary group.	Prostatic metaplastic transformation and expression of <i>c-fos</i> oncogene (endpoints of estrogenic action)	Soy diets did not affect either endpoint and did not alter estrogenic effects in mice exposed to 17β-estradiol.	Mäkelä et al. (83)
Female Han-NMRI mouse, 16 days old	Mice weaned at 16 days old to soy-free diet or diet containing 7% roasted soy meal. diethylstilbestrol was added to some diets (6 µg/kg [kg feed assumed]).	Relative uterine weight after 7 days on diet	Relative uterine weight increased 10–15% by soy diet. Soy diet decreased the diethylstilbestrol-associated increase in relative uterine weight.	Mäkelä et al. (79)
Female CD-1 mouse, 15 days old	Mice were weaned at 15 days of age and fed 1 of the following diets for 3, 5, or 7 days: Rodent Chow #5002 (no information on dietary components); Rodent Chow 5001 (reported to have high isoflavone levels and assumed to be soy based); Mouse Chow #5015 (reported to have high isoflavone levels and assumed to be soy based); NIH-07 (12% soybean meal); NIH-31 (5% soybean meal); or AIN-76A (casein based).	Uterine:body weight ratios	Compared to the 5002 diet, uterine weight:body weight ratios were higher with the 5015 diet, NIH 31 diet, and the AIN-76A diet on days 3, 5, and 7; no significant increases in uterine weight were noted for the 5001 or the NIH-07 diets compared to the 5002 diet.	Thigpen et al. 1987 (80) and Thigpen et al. 1999 (81)
Adult cynomolgus (<i>Macaca</i>)	Monkeys (n=12) were fed a soy protein isolate diet providing a dose of 26.6 mg free genistein/monkey/day (the	Vaginal maturation and karyopyknotic indices	Not significantly affected by soy diet	Cline et al. (76)

2.0 General Toxicology and Biological Effects

Animal	Design	Endpoint(s)	Results	Reference
<i>fascicularis</i>) monkey, ovariectomized	equivalent of a women receiving 99.7 mg genistein/day [~2 mg/kg bw/day assuming a 58 kg bw] ^b). A control group (n=13) was given isoflavone-extracted soy diet and a positive control group the extracted soy diet supplemented with estrogen (n = 15). Animals were fed the diets for 6 months.			
Adult cynomolgus (<i>Macaca fascicularis</i>) monkey, ovariectomized	For 36 months, monkeys (n=57–62/group) were fed soy protein isolate that was alcohol treated to remove isoflavones (negative control), untreated soy protein isolate (~91 mg genistein, 31 mg daidzein, and 7 mg glycitein), or alcohol-extracted soy protein isolate containing conjugated equine estrogens (positive control).	Breast and uterine proliferation, sex steroid receptor expression, and serum estrogen level	In the soy protein isolate group, there was no increase in breast or uterine proliferation or steroid receptor expression; mammary gland thickness and serum estrone and 17β-estradiol levels were significantly reduced.	Wood et al. (77).

^aSince statistical significance was not clearly indicated, only obvious effects are listed.

^bAssumptions used in dose estimates obtained from (84).

2.0 General Toxicology and Biological Effects

2.3 Genetic toxicity

No soy formula genotoxicity studies were identified. Genotoxicity of genistein is discussed in the CERHR Expert Panel Report on Genistein.

2.4 Cancer

Studies examining cancer in humans or rodents exposed to soy during prenatal or postnatal development are discussed in Section 3. This section is limited to studies examining soy intake in adulthood and is based on reviews. Possible mechanisms by which genistein could modulate breast cancer effects were examined and are discussed in the CERHR Expert Panel Report on Genistein. Other components of soy that were suggested as having possible chemopreventive properties include protease inhibitors, phytic acid, and β -sitosterol (reviewed in (85)).

It has been observed that breast cancer rates are lower in Asian than Western populations (86). Breast cancer rates are higher in Asian women who immigrate to the US prior to age 35 and in Asian women born in the US than in women born in Asian countries who do not immigrate to the US. Higher soy product intake in Asian women has been investigated as a possible reason for lower breast cancer rates in Asian populations. Several epidemiologic studies have been conducted to determine if soy product intake in adulthood is related to breast cancer incidence, and these studies were addressed in reviews (6, 85). The reviews reported conflicting results for epidemiologic studies, with some studies demonstrating protective effects of soy product intake and others demonstrating no effect. A meta-analysis conducted by Bouker and Hilakivi-Clarke (86) indicated that soy products may reduce breast cancer risk in premenopausal women but may have no effect in postmenopausal women. The UK Committee on Toxicity (6) noted that most rodent studies examining links between soy product intake and chemically induced mammary cancer reported protective effects of soy product intake, but 1 study demonstrated no effect. The final conclusion in each of the reviews was that there was no convincing evidence that soy products or isoflavones have beneficial or adverse effects on development of breast cancer (6, 85). Bouker and Hilakivi-Clarke noted that evaluations of associations between soy product intake and breast cancer risks need to consider confounding factors such as timing and duration of exposure, interactions between different components of soybeans, and other environmental or lifestyle factors that may contribute to lower breast cancer rates in Asian women (86).

It was observed that Asian men have lower rates of invasive prostate cancer and mortality, although rates of latent, small, and non-infiltrative prostate cancer are similar in Asian and Western men (6). Studies examining the association between soy intake and prostate cancer in adults were reviewed by the UK Committee on Toxicity (6) and Adlercreutz (85). Studies of fermented soy intake by Asian men most often showed no associations, while studies of non-fermented soy consumption by Western men most often showed protective effects. Most of the rodent studies involved tumor implants or chemically induced cancers (reviewed in (6)). In the majority of experimental animal studies, tumor growth was inhibited in animals fed soy (reviewed in (6, 85)). The UK Committee on Toxicity concluded that evidence of protective effects against prostate cancer is greater in experimental animals than in humans and noted that interpretation of human studies is complicated by confounding factors such as differing lifestyle and diet. In contrast Adlercreutz concluded that there was fairly strong evidence that a diet high in soy and isoflavones protects against prostate cancer.

According to the UK Committee on Toxicity (6), human studies generally have indicated an increased risk of colorectal cancer with consumption of fermented soy products and a decreased risk of colorectal cancer with consumption of non-fermented soy products. Studies in experimental animals have been reviewed (6, 85)). Adlercreutz (85) concluded that soy products did not seem to offer protection against colon cancer.

2.0 General Toxicology and Biological Effects

Genistein was postulated to have chemopreventive properties, and a discussion is included in the CERHR Expert Panel Report on Genistein. Other compounds with possible chemopreventive properties were identified in soy, and they include Bowman-Birk protease inhibitors (compounds that affect cell transformation), inositol phosphates, phytosterols, and saponins (reviewed in (53)). Ingestion of soy in the diet alters the urinary excretion pattern of estrone and 17 β -estradiol metabolites, shifting the ratio of 2- and 16-hydroxylated forms toward the more favorable 2-hydroxyestrone and -estradiol (87, 88).

It has been postulated that soy protein isolate can reduce carcinogenicity of procarcinogens such as dimethylbenzanthracene by inhibiting CYP enzymes involved in bioactivation ((89) reviewed in (14)). Rats fed diets containing soy protein isolates compared to casein had reduced levels of CYP1A1 protein in liver and CYP1A1, CYP1A2, and CYP1B1 proteins in mammary gland. Similar reductions in CYP protein expression were observed in rats fed whey-based diets. Feeding of soy protein isolate to rats exposed to dimethylbenzanthracene lowered levels of dimethylbenzanthracene-deoxyribonucleic acid (DNA) adducts in tissues (reviewed in (14)). Thus, it has been suggested that down-regulation of CYP1A1 and CYP1B1 is a possible mechanism of cancer protection in dimethylbenzanthracene-treated rats. Soy protein diets treated to remove isoflavones also provided protection against dimethylbenzanthracene-induced cancer in rats, suggesting that the protective effects of soy are not due to isoflavones.

Reactive oxygen species can damage DNA, cellular proteins, and lipids and may be involved in carcinogenesis (6). Soy isoflavones have antioxidant properties. Studies in humans ingesting soy products with ≥ 12 mg genistein or ≥ 56 mg isoflavones demonstrated reductions in oxidized low-density lipoprotein, lipoprotein peroxidation, or oxidative DNA base damage (reviewed in (6)).

2.5 Potentially Sensitive Subpopulations

It has been reported that bacterial β -glucosidase is lower in infants compared to adults and increases with age (reviewed in (7)).

Production of equol was found to vary greatly among individuals, and it is estimated that only 30–40% of the human population is capable of producing equol, an estrogenic metabolite of daidzein (6, 31, 36, 87, 90, 91). A study by Lu et al. (33) reported that only 1 of 6 men produced equol following ingestion of soy milk. Higher levels of parent compound and precursor metabolites were detected in urine of low-equol metabolizers. By contrast, a study by Kelly et al. (92) found equol to increase markedly in the urine of 11 of 12 women given soy flour, and a study by Cassidy et al. (54) reported that equol was detected in 5 of 6 women fed soy protein, with high concentrations detected in 2 women. Excretion of equol and its precursor daidzein appeared to be inversely related but were not statistically evaluated. Setchell et al. (93) reviewed the role of metabolism of daidzein or formononetin (a clover isoflavone) to equol in experimental animal and human intestine and argued that much of the estrogenic activity of dietary soy products was due to the equol. They indicated, for example, that the menstrual cycle study of Cassidy et al. (54), reviewed in Section 3.1, showed the greatest effect of soy foods on menstrual cycle lengthening in the 2 women whose urinary equol excretion was high. The variability in human equol excretion after soy product intake has been attributed to variations in intestinal microflora, although the relevant microorganisms have not been identified (91). As discussed above, Setchell et al. (7, 18) reported that infants fed soy formula excreted little, if any, equol, presumably due to immaturity of their bowel flora. The rat, by contrast, was characterized as an “equol-producing machine” because of its large cecum and abundant microflora (93). Thus, susceptibility to the estrogenic effects of dietary soy products may vary from one individual to the

2.0 General Toxicology and Biological Effects

next and may be low in infants fed soy formula. **[These data suggest that the rat pup may not be a good model for the human infant.]**

Some studies suggested that diet can influence equol production because increased levels of production were noted with a high-carbohydrate and low-fat diet. Variations in excretion of *O*-demethylangolensin, a metabolite of daidzein, and 6'-hydroxy-*O*-demethylangolensin, a metabolite of genistein, have also been reported.

A study by Lu and Anderson (32) reported sex-specific differences in metabolism of isoflavones in soy milk. On study days 3, 16, 17, 30, and 31, six men and 6 women living in a metabolic unit ingested 1.065 L soy milk following an overnight fast. With each dose, subjects received 80–210 mg of each isoflavone (80% in glycosidic form). **[Isoflavone levels were also reported as 34 ± 15 (SD) total daidzein and 38 ± 15 mg total genistein.]** Urine was collected prior to and for up to 48 hours following each exposure. Recovery of the ingested dose was reported at 24% for genistein, 66% for daidzein, and 28% for equol in women and 15% for genistein, 47% for daidzein, and 15% for equol in men. Authors noted the higher excretion of daidzein in women compared to men. Over time, there was a progressive decrease in percent urinary recovery and shortened half-life in women but not men. Increased metabolism or cyclic variations in hormones were discussed as possible reasons for changes in urinary excretion in women. Elimination half-lives in women (~4.4 hours for daidzein and 6.7 hours for genistein) were longer than elimination half-lives in men (~2.9 hours for daidzein and 3.8 hours for genistein). One of 6 men and 1 of 6 women were capable of producing equol (16–28% of daidzein intake) at the start of the study. Two weeks into the study, 3 additional women, but no additional men, developed the ability to produce equol.

A review by Setchell (50) reported that some studies demonstrated no sex-related differences in biotransformation and excretion of soy isoflavones.

As noted in the toxicokinetics section (Section 2.1), most genistein and daidzein is present in the circulation as glucuronide conjugates. Studies in humans suggest that infants may have decreased ability to glucuronidate isoflavones because UDPGT activity is low in the fetus and neonate but gradually increases to adult levels in the time period of months (reviewed in (94)). Coughtrie et al. (95) examined the ontogeny of UDPGT in humans. Activity was measured in postmortem liver microsome samples obtained from premature or full-term infants and adults. Results of this analysis are listed in Table 19. Activities for isoenzymes catalyzing glucuronidation of bilirubin, testosterone, and 1-naphthol were very low at birth in premature and full-term infants. Activities increased with age for the isoenzymes catalyzing glucuronidation of bilirubin (~80% of adult levels by 8–15 weeks of age) and 1-naphthol (~30% of adult levels at 8–15 weeks of age). During the first 55 weeks of life, no consistent increase in activity was noted for the isoenzyme catalyzing glucuronidation of testosterone. Using an immunoblot technique with antibodies developed toward liver testosterone/4-nitrophenol and kidney naphthol/bilirubin, 1 immunoreactive protein was observed in microsomes of 18- and 27-week-old fetuses, 3 immunoreactive proteins were observed in microsomes of term infants, and most isoenzymes present in adults were observed within 3 months of age at levels ~25% those of adults.

Table 19. Development of UDPGT Activity in Humans

Age	UDPGT activity towards substrate (nmol/min/mg protein) ^a		
	Bilirubin	Testosterone	1-Napthol
30 weeks gestation	0.05	0	0.56
30 weeks gestation with 10 weeks survival	0.4, 1	0.14, 0.85	3.0, 1.8
Full-term infants surviving 1–10 days (n = 7)	0.07 ± 0.04	0.10 ± 0.06	0.75 ± 0.68
Full-term infants surviving 8–15 weeks (n = 6)	0.64 ± 0.32	0.12 ± 0.05	2.4 ± 1.1
Full-term infants surviving 22–55 weeks (n = 5)	0.99 ± 1.1	0.09 ± 0.06	3.6 ± 2.1
Adult males (n = 3)	0.76±0.43	0.46 ± 0.61	7.2 ± 2.2

^aData presented as individual values or mean ± SD.

From Coughtrie et al. (95).

Despite the possibility of lowered UDPGT activity in infants, a letter to the editor providing few details but a reference for the analytical method used reported no detectable levels of unconjugated isoflavones in plasma from 4 infants (2.5–5.5 months old) exclusively fed soy formula for at least 2 weeks (96); blood samples had been measured before and after hydrolysis with β -glucuronidase and sulfatase, but the percentages of each conjugate were not specified.

[The Panel was not able to verify this information due to lack of experimental details and data. This reference is presented for completeness and will not be considered further.]

Coughtrie et al. (95) also measured activity and expression of UDPGT in hepatic microsomes of WAG rats from GD 17 to PND 75. Consistent results were obtained using methods to measure enzyme activity and protein levels via immunoreactive probes. Activity of the isoenzyme catalyzing the glucuronidation of testosterone was barely detectable in fetuses, increased to ~20% of adult levels at birth, and continued to increase until reaching adult levels between 26 and 30 days of age (with the exception of a decrease on PND 40). Activity of the isoenzyme catalyzing glucuronidation of bilirubin was barely detectable in fetuses, increased at birth to reach 75% of adult levels on PND 2–16 (with the exception of a decrease on PND 5), and attained or exceeded adult levels by PND 20 (with the exception of a decrease on PND 40). The isoenzyme catalyzing glucuronidation of 2-aminophenol had ~30–60% of adult activity in fetuses, reached or exceeded adult activity on PND 2–5, had ~30% of adult activity on PND 10–20, and reached or exceeded adult activity by PND 26.

A study in rats examined the effects of intestinal microflora on interindividual variations in isoflavone metabolism (reviewed in (6)). Feeding of soy protein to germ-free rats lacking intestinal microflora resulted in urinary excretion of daidzein and genistein but not equol or *O*-demethylangolensin. When the rats were colonized with bacterial flora from a human able to convert daidzein to genistein, the rats excreted equol but not *O*-demethylangolensin. No equol was excreted by the rats when they were colonized by bacteria from a human unable to produce equol.

2.6 Summary of General Toxicology and Biological Effects

2.6.1 Toxicokinetics and Metabolism

This section focuses on toxicokinetics and metabolism of 2 isoflavones, genistein and daidzein, found in soy formula and other soy foods. Detailed toxicokinetic data on other soy isoflavones were not located. Studies that focus on toxicokinetics following exposure to pure genistein are discussed in the CERHR Expert Panel Report on Genistein, although some highly relevant information for genistein is presented in this report.

2.0 General Toxicology and Biological Effects

2.6.1.1 Humans

Genistein and daidzein exist mainly in their glycosidic forms in unfermented soy foods. Before isoflavone glycosides can be absorbed into the systemic circulation, they must first be hydrolyzed to their aglycones, which have greater hydrophobicity and lower molecular weights (reviewed in (6)). The majority of isoflavones are glucuronidated prior to entering the systemic circulation (41). Absorption half lives for both daidzein and genistein were reported at ~1–3 hours following intake of foods containing ≤ 210 mg of each isoflavone or providing doses of ≤ 2 mg/kg bw/day of each isoflavone ((32, 33); reviewed in (34)). Genistein and daidzein were reported to peak at ~6–8 hours following ingestion of soy foods ((35); reviewed in (6, 34)).

Genistein and daidzein were detected in blood of individuals who consume diets containing soy products (Table 7) and volunteers consuming soy-based foods or supplements in scientific studies (Table 8). Infants are able to absorb isoflavones, and infants fed soy formula were demonstrated to have plasma isoflavone blood levels exceeding those of Japanese adults (Table 7) (7). The highest genistein/daidzein blood levels were reported for infants fed soy formula (~2530/1160 nM [**683/295 $\mu\text{g/L}$ aglycone equivalent**]), and those values exceeded blood levels reported for Asian populations (~90–1200/60–900 nM [**24–324/15–229 $\mu\text{g/L}$ aglycone equivalent**]). Genistein/daidzein blood levels in infants fed human milk or cow-milk formula were reported at ~10–12/6–8 nM [**2.7–3.2/1.5–2.0 $\mu\text{g/L}$ aglycone equivalent**]. In Finland and Canada, genistein/daidzein blood concentrations were reported at 0.5–8/0.6–6 nM [**0.14–2.16/0.15–1.5 $\mu\text{g/L}$ aglycone equivalent**] in omnivores and 17–45/19–50 nM [**4.6–12/4.8–13 $\mu\text{g/L}$ aglycone equivalent**] in vegetarians. Higher concentrations in infants are postulated to result from lower body weight, frequent feedings, and reduced metabolism.

In a study by Hoey et al. (21), the presence of genistein, daidzein, and glycitein in urine samples of 4–6 month-old infants fed soy formula led study authors to conclude that isoflavones were well absorbed by infants. It was also deduced that the 4–6 month-old infants had the ability to hydrolyze glycosides to aglycones because such hydrolysis is required for absorption of isoflavones.

Blood levels of genistein and daidzein did not suggest saturated absorption in 12 women administered isoflavones (Table 8) (30), but a study in which 8 women were dosed with ^{13}C -labeled genistein demonstrated that the AUC at the higher dose was less than double the AUC at the lower dose, suggesting a decrease in fractional absorption with increasing doses (31). [**The Expert Panel notes that varying results obtained in studies by Xu et al. (30) and Setchell et al. (31) could reflect different fractional absorption for genistein from soy products versus pure genistein.**]

Two studies detected genistein in human amniotic fluid at up to 212 nM [**57 $\mu\text{g/L}$ aglycone equivalent**] (44, 45). One of the studies demonstrated that 84% of genistein in amniotic fluid and 91% in cord blood was present as a glucuronide conjugate (44). Studies by Franke et al. (19, 47) reported distribution of genistein and daidzein to human milk. Isoflavone concentrations peaked 10–14 hours following consumption of soybeans and returned to baseline levels 2–4 days later. Isoflavone levels in milk were below the detection limit (1–3 pM) prior to hydrolysis, suggesting that isoflavones occur in milk as glucuronide and sulfate conjugates. In the Franke et al. (47) study, intake of 5, 10, and 20 g soybeans by a single subject resulted in maximum milk genistein levels of 32, 46, and 71 nM [**8.6, 12, and 19 $\mu\text{g/L}$**] and daidzein levels of 16, 34, and 62 nM [**4.1, 8.6, and 16 $\mu\text{g/L}$**]. Milk isoflavone levels in a Chinese woman who ate 1 serving of tofu soup a day were reported at 30–50 nM genistein [**8.1–14 $\mu\text{g/L}$**] and 80–110 nM daidzein [**20–28 $\mu\text{g/L}$**]. A third study reported a mean \pm SD isoflavone level of 5.6 ± 4.4 $\mu\text{g/L}$ in milk samples from 9 healthy lactating women (7). Equol was detected in 7 of 9 milk samples.

2.0 General Toxicology and Biological Effects

Metabolism of genistein is outlined in Figure 2, and metabolism of daidzein is outlined in Figure 3. Prior to absorption, most genistein and daidzein are conjugated with glucuronic acid by UDPGT in the gut wall; a smaller amount is conjugated to sulfate by sulfotransferases (6, 36, 48). Conjugation of isoflavones can occur in liver and intestine. The glucuronide and sulfate conjugates can enter the systemic circulation, and the majority of isoflavone compounds in the circulation are present in conjugated form.

Conjugated isoflavones undergo enterohepatic circulation, and on return to the intestine, they can be deconjugated by bacteria with β -glucuronidase or arylsulfatase activity. The metabolites may be reabsorbed or further metabolized by gut microflora. Isoflavones can undergo further biotransformation that ultimately leads to the formation of 6'-hydroxy-*O*-demethylangolensin from genistein and *O*-demethylangolensin from daidzein (6, 36, 48). The metabolic profile varies among individuals, with some individuals producing little or no *O*-demethylangolensin or equol, an intermediate metabolite of daidzein that is biologically active.

Limited ability of infants to produce equol was demonstrated by Setchell et al. (7, 18), who detected equol in plasma of 4 of 7 infants fed soy formulas but with a peak area 1–2 orders of magnitude lower than in infants fed cow-milk formula. Equol was detected in cow-milk samples from Australia (25). In a study of children who had consumed soy or cow-milk formulas, Hoey et al. (21) reported that genistein, daidzein, and glycitein were present in the urine of all soy-fed infants in the 4–6-month age group, while *O*-demethylangolensin and equol were detected in 75 and 25% of soy-fed infants, respectively. In subjects aged 7 months to 7 years who were given a soy challenge, *O*-demethylangolensin was present in the urine of 75% of soy-fed subjects and 50% of control subjects, while equol was present in the urine of 19% of soy-fed subjects and 5% of control subjects. Although a higher percentage of soy-fed infants than cow milk-fed infants of the younger age groups were able to convert daidzein to equol, the percentages were similar among the older children. Thus, the authors concluded that isoflavone exposure early in life has no lasting effect on isoflavone metabolism.

Elimination half-lives were reported at ~4–9 hours for genistein and 3–9 hours for daidzein following intake of ≤ 100 mg or ≤ 2 mg/kg bw/day of each isoflavone in aglycone or glycosidic form ((26, 33) and reviewed in (6, 34)). In volunteers who consumed formulations containing 70–100% unconjugated isoflavones at 1–16 mg/kg bw, mean half-lives were reportedly longer for total isoflavones (~6–13 hours for genistein and 4–16 hours for daidzein) compared to free isoflavones (~2–7 hours for genistein and daidzein) (42, 43). **[A value of 21.2 hours for free daidzein appeared to be an outlier.]** In subjects ingesting soy milk, urinary excretion peaked at 8–10 hours and 95% of excretion occurred within 24 hours; total urinary excretion consisted of 1% aglycones and 99% glucuronidated metabolites (32). One study demonstrated that percent excretion of genistein and daidzein was not affected at isoflavone levels of 0.7, 1.3, or 2.0 mg/kg bw/day administered through soy milk powder (30).

It has been reported that urinary levels of daidzein and genistein are slightly lower in infants than in adults fed equivalent amounts of isoflavones. This difference could possibly indicate slower renal clearance in early life (reviewed in (7)). In a study measuring urinary excretion of genistein and daidzein in 4 infants fed soy formula, average concentrations of isoflavones in urine were daidzein 2.9 ± 0.3 mg/L and genistein 1.5 ± 0.2 mg/L (24). Mean percentages of daily isoflavone intake detected in urine and normalized for creatinine levels were daidzein $38 \pm 4\%$ and genistein $13 \pm 3\%$. Isoflavone excretion rates varied little between infants and ranged from 0.37 ± 0.03 to 0.58 ± 0.06 mg/kg bw/day for daidzein and from 0.15 ± 0.03 to 0.32 ± 0.04 mg/kg bw/day for genistein. Percent isoflavone excretion and excretion rate were not affected by age.

2.0 General Toxicology and Biological Effects

2.6.1.2. *Experimental animals*

As noted by the detection of isoflavones in blood of experimental animals fed soy-containing feed, isoflavones are absorbed following oral exposure (55, 57-59).

Studies in rats demonstrated the distribution of isoflavones and metabolites to fetuses during pregnancy or pups during lactation following ingestion of soy-containing feed by the dam (55, 57). In one study, GD 20.5 fetuses and PND 3.5 pups contained about half the genistein concentration and about one-tenth the equol concentration measured in dams; daidzein concentrations were about one-third lower in GD 20.5 fetuses and about the same in PND 3.5 pups compared to dams (55). A second study reported that in pups born to dams fed a soy-containing diet, genistein levels remained steady between birth and PND 12, while daidzein levels were reduced by half during the same time period (57). On PND 16, the time when pups likely began eating feed, there was a 5- to 10-fold drop in genistein and daidzein concentrations compared to earlier time periods. In contrast, equol levels in pups decreased steadily following birth.

Studies in adult rats and mice fed soy-containing feed reported that blood equol levels were 2–10 times higher than blood genistein and daidzein levels (55, 57, 58). In most cases, equol levels were lower than or within the same order of magnitude as genistein and daidzein levels in fetuses or pups of rat dams fed soy-based diets (55, 57).

The half-life for genistein following ingestion of soy products by rats was reported at 8.8 hours (Table 15). No data were found for half-life of daidzein following ingestion through soy foods. Urinary levels of genistein and daidzein were reported to be ~25% of equol levels in mice and ~50% of equol levels in rats.

2.6.2 *General Toxicology*

The FDA (64) and the UK Committee on Toxicity (6) reviewed numerous human studies examining the effects of soy foods on cholesterol levels. Based on well conducted human studies, both the FDA and UK Committee on Toxicity concluded that consumption of soy as part of a diet low in saturated fat and cholesterol can lower total blood cholesterol and LDL levels.

In women receiving soy diets or supplements, there were no estrogenic effects on vaginal cytology in 3 studies (66, 67, 72). A fourth study that provided very limited details and alternating exposure to non-soy based phytoestrogens reported increased vaginal cell maturation after women received soy flour supplements for 2 weeks (68). A fifth study with a longer exposure period (5 years) demonstrated estrogenic effects on the endometrium (69), but the study was limited by the inability to obtain endometrial samples in 20–25% of subjects during each of 3 examination periods (70). A sixth study reported increased proliferation of breast lobular epithelium and progesterone receptor expression in women who ate bread rolls containing 60 g soy as textured vegetable protein (71).

A study examining uterotrophic effects of infant formulas in immature rats reported estrogenicity effects for both soy and cow-milk formulas (73). Both cow-milk and soy formulas increased uterine weight independent of phytoestrogen content, with greater responses generally noted with soy compared to cow-milk formula when both were used at the concentration recommended for infant feeding. No evidence of estrogenicity was observed in studies in which rats or monkeys were fed soy-based diets (74-77); however, estrogenic effects occurred when soy isoflavones were added to a soy-free diet (78). A study in immature female mice found a soy-based diet to increase relative uterine weight and to antagonize the weight increase associated with feeding

2.0 General Toxicology and Biological Effects

diethylstilbestrol, thus demonstrating weak estrogen agonist and antagonist properties (79). In a study in which rats were fed soy protein with and without the addition of conjugated equine estrogens, there were no additive effects between soy diets and equine estrogens (75). Soy diets that were not alcohol-extracted to remove isoflavones antagonized the effects of estrogens on increased lactoferrin staining and epithelial luminal cell height. In some studies, increases in rodent uterine weight were greater in rats fed soy-free versus soy-containing diets (73, 74, 80, 81).

2.6.3 Genetic Toxicity

No genotoxicity studies were identified for soy formula or other soy foods.

2.6.4 Carcinogenicity

Because Asian populations have lower rates of some types of cancer, a possible role of soy products in protecting against carcinogenicity has been examined in reviews. Components of soybeans that were suggested as having possible chemopreventive properties included genistein, protease inhibitors, phytic acid, and β -sitosterol (reviewed in (85)).

The following conclusions regarding soy intake and cancer were made by the UK Committee on Toxicity (6):

- There was no convincing evidence that soy products have beneficial or adverse effects on development of breast cancer,
- Evidence of protective effects against prostate cancer is greater in experimental animal than in humans, and
- Soy products do not seem to offer protection against colon cancer.

Possible mechanism suggested for protection against cancer have included changes in ratios of estrogen compounds (87, 88), inhibited bioactivation of procarcinogens (89), and antioxidant activity (reviewed in (6)).

2.6.5 Potentially Sensitive Sub-populations

As noted in the toxicokinetics section, most genistein is present in the circulation as glucuronide conjugates. Human infants may have decreased ability to glucuronidate isoflavones because UDPGT activity is low in the fetus and neonate but gradually increases to adult levels in the first months to years of life (reviewed in (94)).

Sex-specific differences in metabolism of isoflavones in soy milk were reported (32). Compared to men, women excreted higher percentages of daidzein, experienced a progressive decrease in percent urinary recovery and shortened half-life of isoflavones, and had longer elimination half-lives (~4.4 hours for daidzein and 6.7 hours for genistein in women versus ~2.9 hours for daidzein and 3.8 hours for genistein in men). One of 6 men and 1 of 6 women were capable of producing equol (16–28% of daidzein intake) at the start of the study. Two weeks into the study, 3 additional women, but no additional men, developed the ability to produce equol.

Production of equol was found to vary greatly among individuals, and it is estimated that only 30–40% of the human population is capable of producing the daidzein metabolite equol, an estrogenic metabolite of daidzein (6, 31, 36, 87, 90, 91). Setchell et al. (93) reviewed the role of metabolism of daidzein or formononetin (a clover isoflavone) to equol in experimental animal and human intestine and argued that much of the estrogenic activity of dietary soy products was due to the equol. They indicated, for example, that the menstrual cycle study of Cassidy et al. (54), reviewed in Section 4.1, showed the greatest effect of a soy diet on menstrual cycle

2.0 General Toxicology and Biological Effects

lengthening in the 2 women whose urinary equol excretion was high. The variability in human equol excretion after soy product intake has been attributed to variations in intestinal microflora, although the relevant microorganisms have not been identified (91). As discussed above, Setchell et al. (7, 18) reported that infants fed soy formula excreted little, if any, equol, presumably due to immaturity of their bowel flora. The rat, by contrast, was characterized as an “equol-producing machine” because of its large cecum and abundant microflora (93). Thus, susceptibility to the estrogenic effects of dietary soy products may vary from one individual to the next, may be low in infants fed soy formula, and may not be accurately modeled by rats.

3.0 DEVELOPMENTAL TOXICITY DATA

3.1 Human data

3.1.1 Pregnancy exposure

North et al. (97), supported by the UK Medical Research Council, the UK Department of Health, the UK Department of the Environment, the UK Ministry of Agriculture, Fisheries, and Food, the BBC, Nutricia, Nestlé, “and other companies,” performed a case–control study of hypospadias as part of the Avon Longitudinal Study of Pregnancy and Childhood. This population-based project involved women with estimated delivery dates in 1991 and 1992. About 85% of the eligible population enrolled. Women completed questionnaires during pregnancy at 8, 18, and 32 weeks and at various times after birth. The 18- and 32-week pregnancy questionnaires included items identifying illness and drug exposures, and the 32-week questionnaire included dietary items. Cases of hypospadias ($n = 51$) were identified based on questionnaire responses in the first 3 years after birth, as well as “birth notifications,” neonatologist records, and postmortem records. There were 7877 boys without hypospadias in the Avon database. Between approximately 5700 and 6600 of the pregnancies giving rise to these boys without hypospadias were used for various comparisons. Initial comparisons were made using the chi-squared test followed by unadjusted logistic regression and then multivariate logistic regression analysis.

The univariate analyses showed a significant association between hypospadias and the use of iron supplementation during the first 18 weeks of pregnancy (odds ratio [OR] 1.87, 95% confidence interval [CI] 1.02–3.46), vegetarianism during pregnancy (OR 3.53, 95% CI 1.56–7.98), and influenza during the first trimester (OR 3.08, 95% CI 1.46–6.48). Specific questions about use of soy milk or imitation meat did not show significant associations with hypospadias in the offspring, but the number of exposed cases was small (2 case children whose mothers used soy milk and 5 case children whose mothers used imitation meat). The use of “pulses” (peas, beans, lentils, chickpeas) 4 or more times/week was associated with hypospadias (OR 7.56, 95% CI 2.25–25.42), based on 3 affected children. In the multivariate model considering iron supplementation and vegetarianism separately, the association of hypospadias with maternal vegetarianism persisted (OR 3.88, 95% CI 1.69–8.92); the association between hypospadias and iron supplementation was no longer statistically significant (OR 1.81, 95% CI 0.92–3.55). Influenza exposure remained significantly associated with hypospadias (OR 3.24, 95% CI 1.52–6.88). In a multivariate model combining iron supplementation and diet, using omnivorous diet without iron supplementation as the reference group, the association between vegetarianism and hypospadias was statistically significant (OR 4.99, 95% CI 2.10–11.88). The authors concluded that the association between maternal vegetarianism and hypospadias may be due to estrogenic constituents of soy and other plant products. As an alternative explanation, they proposed greater exposure of vegetarian women to pesticides used on food plants.

Strengths/Weaknesses: This population-based, relatively large study had a high participation rate. The prospective study design with information on potential confounders, the multiple sources of information for complete ascertainment of outcome, and the multivariate analysis were strengths. Dietary information was collected prospectively but only by interview and only at 32 weeks’ gestation. The rarity of the defect created sample size problems, and the information used for classifying outcome varied in consistency and reliability. There were no biological measures of exposure and the prevalence of exposure was low in this sample. Possible confounding by pesticide exposure is an additional weakness.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful with limitations.

3.0 Developmental Toxicity Data

3.1.2 Exposure of infants

3.1.2.1 Growth and Nutrition

Reports on the ability of soy formula to support normal growth and to provide adequate nutrition are presented. Case series are presented first followed by controlled studies. Although several of these reports involved premature infants, soy formula is not currently recommended for premature infants.

Kulkarni et al. (98) (funding not indicated) reported 22 cases of rickets in very low birth-weight (< 1500 g) infants fed a soy-isolate formula. The soy formula contained 700 mg/L calcium, 500 mg/L phosphorus, and 400 IU/L vitamin D. When feeding was tolerated, the formula was supplemented by a multivitamin; additional calcium lactate and/or vitamin D were also provided depending on neonatologist preference. The mean daily intake of each of these supplements was measured during infant hospitalization periods. The lowest mean serum phosphorus concentration occurred at 7–10 weeks, which preceded the active phase of rickets by about 2 weeks. The highest serum levels of alkaline phosphatase occurred at 11–14 weeks, 2 weeks prior to the healing phase. The lack of a control group prevented the authors from drawing conclusions from the data, and no statistical analyses were performed. However, the authors suggested further studies investigating calcium, phosphorus, and vitamin D in very low birth-weight infants with rickets, in particular those fed soy formula. This report led to a controlled prospective study (99), which is discussed below.

Strengths/Weaknesses: This case series involved a special population of very low birth-weight infants.

Utility (Adequacy) for CERHR Evaluation Process: Although this report may be useful for hypothesis-generation, it is not useful in the CERHR evaluation process.

Fomon (100), supported by Mead Johnson and Ross Laboratories, evaluated growth and nitrogen retention in 4 infants aged 4–6.5 months receiving a soy formula with 6.8% caloric protein. The author reported that weight gain was normal in these infants, and nitrogen retention was at least as great as that estimated in 3 of the infants during a previous month during which they consumed human milk.

Strengths/Weaknesses: This paper involved a small sample (n = 4) with no comparison group.

Utility (Adequacy) for CERHR Evaluation Process: This paper is not useful in the CERHR evaluation process.

Sellars et al. (101), supported by Borden, Inc., reported length and weight measurements for 1583 Caucasian infants during the first year of life. Mothers were encouraged to breast feed as long as possible. Infants not being breast-fed, or being weaned from breast feeding, were assigned to cow-milk or soy formula based on family history of allergy [**although the assignment strategy was not discussed**]. The comparison groups for evaluation of height and weight included 401 breast-fed infants, 239 infants on soy formula, and 839 infants on cow-milk formula. Infants were evaluated monthly until the age of 6 months and bi-monthly thereafter. Height and weight were plotted on growth curves, and statistical comparisons were made by an unspecified method. There were no differences in growth between the 3 feeding groups. The authors concluded that any of the 3 feeding methods would support normal growth.

3.0 Developmental Toxicity Data

Strengths/Weaknesses: The large sample, repeated measurements of weight and lengths, and 1-year follow up are strengths. The study was limited to 1 racial group, and there may have been selection bias in mothers who did not breast feed or who discontinued breast feeding, as well as in assignment to soy formula group on the basis of family history of allergy. Head circumference was not evaluated. Growth curves should have differed between breast-fed and formula-fed infants at least before introduction of solid foods; so, it is unclear how statistical comparisons (whatever they were) could have shown no differences. There were apparently no adjustments for potential confounders and baseline differences between groups. The introduction of solid food by pediatricians at 1 month is another weakness.

Utility (Adequacy) for CERHR Evaluation Process: This paper is of minimal utility in the evaluation process.

Kay et al. (102), supported by the Borden Company, followed normal, healthy infants fed soy formula (Mull-Soy®) or evaporated cow-milk formula (Silver Cow®) to evaluate differences in weight, length, hemoglobin, hematocrit, and plasma biochemical values. [An additional 32 infants on unspecified feeding methods were evaluated at birth or at 3 months of age and are not considered here.] At 24 hours of age, 14 infants were started on soybean formula and 14 were started on evaporated cow-milk formula and followed for 3 months. Efforts were made to match patients for sex, race, and birth weight. Infant weight and length were measured at clinical visits every 2 weeks. Hemoglobin, hematocrit, and plasma biochemical values were measured from blood drawn shortly after birth and at 3 months of age. Plasma protein electrophoresis was performed to quantify albumin, α 1-globulin, α 2-globulin, β -globulin, and γ -globulin.

Growth increases in the soybean formula and evaporated milk formula were similar and paralleled normal growth curves (based on Iowa Growth Charts). There was no statistical feeding group difference in hemoglobin, hematocrit, or plasma biochemistry values except as shown in Table 20. The study authors concluded that nutritive value and infant growth measurements were similar in infants fed soy formula and evaporated cow-milk formula from birth to 3 months of age.

Table 20. Plasma Biochemical Values at 3 Months of Age in Children Given Soy Formula or Evaporated Cow-Milk Formula

Laboratory test	Group (n)	Mean \pm SD	P-value by <i>t</i> test
Cholesterol, mg. %	Soy (14)	126 \pm 34	0.01
	Cow milk (14)	163 \pm 32	
Total plasma protein, g %	Soy (14)	6.7 \pm 0.8	0.03
	Cow milk (14)	6.0 \pm 0.5	
α 2-Globulin	Soy (7)	8 \pm 4	0.01
	Cow milk (8)	14 \pm 5	
γ -Globulin, %	Soy (7)	9 \pm 3	0.001
	Cow milk (8)	15 \pm 4	

From Kay et al. (102)

Strengths/Weaknesses: Some effort was made to match groups at the outset on important potential confounders. The use of blood measures at two time points is a strength. Although there was a control group, diet was unrestricted and there was no comparison to breast-fed infants. It was unclear if infants were exclusively formula-fed for the duration of study and how they were selected for feeding groups. The small sample size provided limited power for the conclusion of no differences in growth curves, and there was no measurement of head circumference. Only about half of the already small sample size were available for the full set of biochemical measures

3.0 Developmental Toxicity Data

at 3 months of age. Intake was not reported, and it is like that the evaporated milk group would have had down-regulation of intake. Without knowing whether feedings were isocaloric, the groups cannot be meaningfully compared. The differences in total protein α 2-globulin levels are small and not clinically significant.

Utility (Adequacy) for CERHR Evaluation Process: This paper is useful in the evaluation process.

Cherry et al. (103), supported by Gerber Products Co., tested the acceptance, tolerability, and nutritional value of a new (in 1968) soy formula provided to healthy newborn infants. When discharged from the hospital, mothers were given a 3-month supply of the soy formula or a marketed cow-milk formula (Modilac®). Formulas were packaged in identical containers. The soy formula contained isolated soy protein (15.1%), soy oil (37.5%), and carbohydrate (47.4% as sucrose and corn syrup solids). **[The Expert Panel notes that this soy formula contains more fat and less carbohydrate than soy formulas in current use. As noted in Section 1.1, current soy formulas include soy protein isolate (14.6–18%), vegetable oils (21–28.6%), and carbohydrate as corn syrup (40.8–55%) and sugar (10.2–11%).]** Formulas were approximately isocaloric. Mothers recorded the amount of formula consumed at each feeding, stool characteristics (color, consistency, and frequency), vomiting, acceptability of the feeding, and satiety after the feeding. Clinic visits included standard well-baby physical examinations, immunizations, measurements (weight, length, and head circumference), and blood work. Other procedures included muscle reflex patterns (twice in 6 months), x-rays of the tibia (at 6–8 weeks and again at 4–6 months), and more extensive blood work (at 2, 4, and 6 months). A reference population for growth measurements consisted of 200 children of similar socioeconomic and racial background chosen from the Collaborative Child Development Project at Charity Hospital in New Orleans. Fifty-eight of the 73 infants initially enrolled completed 6 months of follow-up. Proportions of infants with symptoms were compared by chi-squared testing, and growth parameters were compared using *t*-tests. Multiple regression was used to evaluate the relationship between nutritional components of the formulas and weight gain and ANCOVA was used to evaluate growth data and food intake.

Growth data are illustrated in Figure 4. Soy-fed infants, especially girls, lagged behind cow milk-fed infants with significant differences primarily between 2 and 4 months of age. The reference population growth parameters (not shown) were generally between those of the cow-milk and soy groups. Formula intake level averaged 2 oz/day more in the cow-milk group than in the soy group, resulting in a 10% difference over the initial 56 days. The cow-milk group gained significantly more weight per oz of formula than the soy group. Cow milk-fed infants had greater intakes of methionine **[which today is added as a supplement to soy formulas]**, calcium, and folic acid, and soy-fed infants had greater intakes of iodine, thiamine, vitamin C, and vitamin B₁₂. Maternal observations showed more vomiting in the milk-fed group (21% slight, 5% frank) compared to the soy-fed group (13% slight, 1% frank, *P* = 0.001). Eighteen percent of the infants in the milk-fed group were categorized as hungry after feeding compared to 7% in the soy-fed group (*P* = 0.001). Loose stools were more frequently reported in the soy-fed group (27%) than the milk-fed group (19%, *P* = 0.001). There were no instances of anemia, and hematologic parameters did not differ consistently by feeding group.

The study authors concluded that the slower growth in soy-fed girls might be of concern.

Strengths/Weaknesses: Strengths including the blinding of participants to feeding-group assignment, measurements of head circumference and blood parameters, use of x-rays, an additional outside reference population, and assessment of differences in growth by infant sex. It

3.0 Developmental Toxicity Data

is a strength that there was specific recording of the amounts of formula consumed at each feeding, allowing for recognition that the cow-milk group consumed more formula; however, there is no way to know if the accuracy of recording in the two groups differed. There could have been selection bias in mothers who elected not to breast feed and therefore could participate. The drop-out rate was high, with 15 of 73 subjects not completing the 6 months' evaluation. The sample was very small, providing limited power, especially for stratified analysis on infant sex. There was no comparison to exclusively breast-fed infants, and the introduction of solids was not specified. A repeated-measures type of analysis would have been appropriate for growth over time.

Utility (Adequacy) for CERHR Evaluation Process: This paper is marginally useful for the evaluation process, limited by concerns about accuracy of intake recording and differences in modern soy formula from the product used at the time of the study.

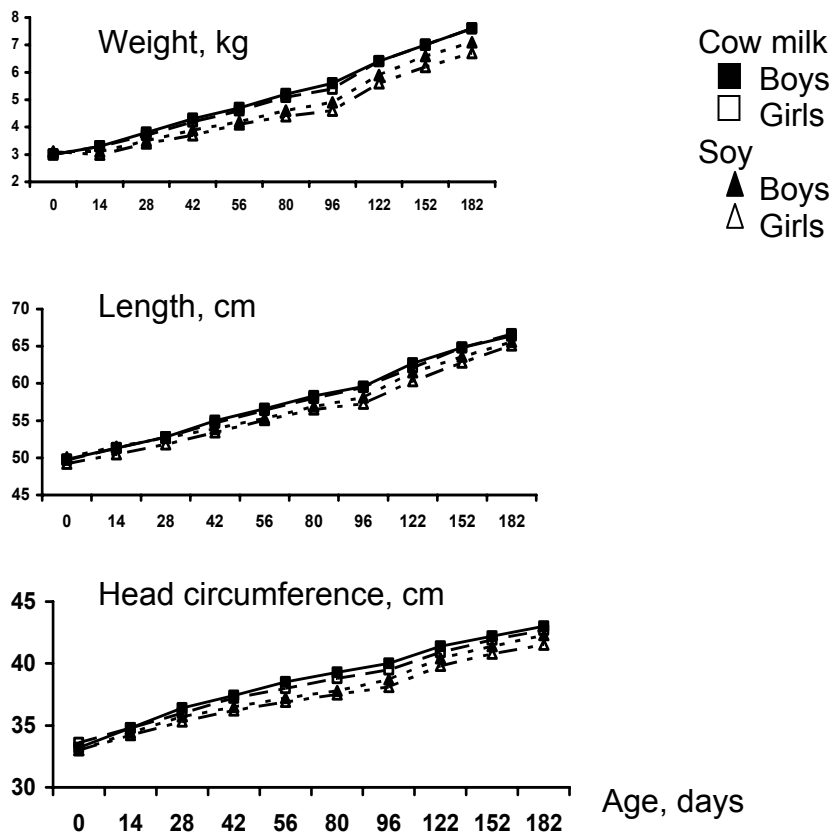


Figure 4. Growth Parameters in Children Assigned to Soy Formula or Cow-Milk Formula for 3 Months

n = 14–20 per data point, error bars omitted for clarity. From data presented by Cherry et al. (103).

3.0 Developmental Toxicity Data

Dean (104), supported by Ross Laboratories, evaluated growth and development over the first 6 months of life in children fed a commercial soy formula or a commercial cow-milk formula, or who were breast-fed. Babies whose mothers chose to breast feed were enrolled into the breast-fed group. There were 26 such babies at the beginning of the study, but only 6 remained on breast feeding by 6 months of age. Among babies whose mothers chose to bottle-feed, 26 were assigned to soy formula (of whom 24 completed the 6-month trial) and 29 were assigned to cow-milk formula. There were 35 children in the cow-milk group at the end of the study because some breast-fed children who “failed” breast feeding within the first month of life were reassigned to the cow-milk formula group. **[The method of assignment to soy or cow-milk formula was not discussed.]** All babies were at least 2800 g at birth and were normal on examination by the investigator. Solid foods were added at 6–12 weeks of age. Weight, length, and head circumference were measured monthly. The Denver Developmental Screening test was administered **[how often and by whom is not indicated]**. Blood was obtained at different visits in different children for chemistry and hematology evaluation.

The only abnormality noted in the children on soy formula was perianal redness that responded to zinc and cod-liver oil cream. The 24 children in this group who finished the study followed their centile growth curves during the study period. There was more disparity in growth in the cow-milk formula group, with 5 of 39 children having weight percentiles that exceeded their length percentiles. **[The number of children (39) in the study Results section is different from the number (35) in the study Methods section and from the number (40) in 1 of the study data tables.]** All infants were said to be “neurologically normal or advanced” except for 1 child in each of the 3 feeding groups. The laboratory values were normal in all children, but the blood urea nitrogen was highest in the cow-milk formula group, intermediate in the soy formula group, and lowest in the breast-fed group, proportional to the nitrogen content of the formulas/milk (Figure 5). The author concluded that soy formula was an adequate nutrition source.

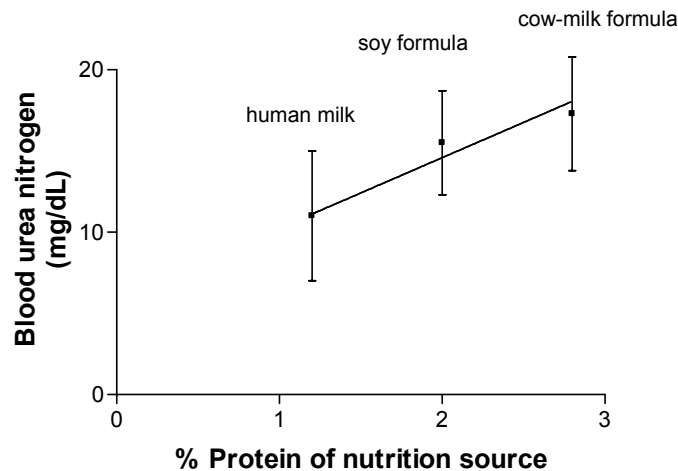


Figure 5. Infant Blood Urea Nitrogen as a Function of the Protein Content of Food Source Human milk protein 1.2%, soy formula protein 2.0%, and cow-milk formula protein 2.8%. Data expressed as mean \pm SD, from Dean (104).

Strengths/Weaknesses: Strengths include the use of 2 formula-fed groups and a breast-fed comparison group, repeated measurements of growth including head circumference, and developmental assessments. In spite of these strengths, the sample size is very small, and dropouts could have influenced the results. It seems inappropriate for infants who “failed” breast feeding to have been included in the cow-milk group. There could have been selection bias in mothers’ assignment to feeding groups. The introduction of solid foods was not taken into

3.0 Developmental Toxicity Data

account, and there was no report of how many calories were given to the groups. A repeated-measures analysis would have been useful.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility.

Jung and Carr (105), support not indicated, studied the nutritional and growth differences between 20 infants on soy formula (Isomil®) and 20 infants on cow-milk formula (Similac® with Iron). Healthy infants were assigned by gestational age, weight, race, and sex to receive 1 of the formulas for 16 weeks. Cereal was introduced at 4 weeks, and vegetables were introduced at 8 weeks. Infants were assessed for formula tolerance, weight, length, and head circumference. The researchers reported that general acceptance of both formulas was good, and no infants needed formula changes. All growth measurements were normal, and there were no significant differences in body weight, length, or head circumference between the groups. Hemoglobin, hematocrit, protein, albumin, calcium, phosphorous, glucose, urea nitrogen, and alkaline phosphorous were also within the normal range and did not significantly differ between groups. The authors conclude that the soy formula used in this study imposed no significant nutritional differences compared to cow-milk formula.

Strengths/Weaknesses: There was some attention to prospective matching of infants on important confounders. The introduction of specified solids at specified times in both groups is a strength, as is the use of blood measures and measurement of head circumference. The sample size was small, however, with limited power to detect differences between groups. There was inadequate adjustment for potential confounders, and there was no breast-fed comparison group.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility.

Köhler et al. (106), support not indicated, reported the growth and food intake of 59 infants aged 6–25 months on human milk, cow-milk formula, or soy formula. Participants were selected by 6 weeks of age from 2 well-baby clinics, 1 of which provided infants with cow milk-based formula (Similac®; n = 20), and the other of which provided infants on soy formula (Isomil®; n = 13). The remaining children (n = 26) were fully breast-fed. Mothers recorded 48-hour intake at 6, 14, 22, and 26 weeks. Cow-milk and soy formula consumption was measured by administering a standard formula volume and weighing the amount remaining after feeding. Breast-fed infants were weighed by their mothers before and after feedings. There were no significant differences in consumption between formula groups, but breast-fed infants consumed significantly less per 24 hours than the formula-fed infants. There were no statistically significant differences in energy intake between infants on soy and cow-milk formulas. The difference in intake between breast-fed and all artificially fed infants was statistically significant at 6 and 14 weeks [**statistical methods not indicated**].

Growth was measured by weight, length, and skin-fold thickness and followed for 52 weeks. Infants on soy formula gained less weight in the first 6 weeks than breast-fed infants and those on cow-milk formula. The authors suggested that this difference might be explained by infants in the soy group being significantly heavier by an average of 200 g at birth. After 6 weeks of age, there were no significant differences in mean weight between the 3 groups. No significant differences were found between the 3 groups in length or sum of skin-fold thicknesses.

Radiographs of the left hand were taken at 3 and 6 months of age to measure bone mineralization and maturation. The internal (d) and external (D) diameters of the narrowest part of the diaphysis of the 2nd metacarpal bone were used to “represent” the cortical cross-sectional area as $D^2 - d^2$. [**The Expert Panel notes that the cross-sectional areas would be $\pi(D^2 - d^2)/4$.**] This

3.0 Developmental Toxicity Data

measurement, which the authors cite as representative of bone ash content per unit length, was used to indicate bone mineralization. Ossification centers were measured to characterize skeletal maturation. At 3 months of age, the soy formula group showed slower mineralization and bone maturation than both the breast-fed group and the cow-milk group; however, by 6 months these differences in skeletal development were no longer significant.

The authors concluded that although they believed birth weight differences could explain differences in weight gain during the first 6 weeks of life, they could not exclude the possibility that nutrients were less well absorbed from soy formula than from human or cow milk.

Strengths/Weaknesses: It is a strength that this study compared three feeding groups with recording of infant intake and measures of bone mineralization. Multivariate analysis should have been used to adjust for differences such as birth weight between groups. There could have been selection bias among mothers who elect to breast feed or not. Assignment to formula feeding groups was by clinic location and not within clinics, and there could have been differences between women associated with a site that were not taken into account. There was 1-year growth follow-up, but no head circumference measures were taken, and there was no mention of adjusting for supplementary foods, including milk in the breast-fed group. The sample size was small with inadequate adjustment for other potential confounders.

Utility (Adequacy) for CERHR Evaluation Process: This study is of marginal utility.

Steichen and Tsang (107), supported by the National Institute of Child Health and Human Development (NICHD), the Children's Hospital Research Foundation, the Veteran's Administration of Cincinnati, the Jewish Hospital of Cincinnati, Ross Laboratories, and Gerber Laboratories, performed a prospective, randomized, double-blind, longitudinal study over 1 year to determine differences in growth and skeletal mineralization between infants fed soy formula and those fed cow-milk formula. Subjects were healthy term infants appropriate for gestational age and born to healthy, middle-class white mothers. Infants were given a soy formula (Isomil® with Iron, n = 18) or a cow-milk formula (Similac® 20 with Iron, n = 17). Infants were fed exclusively with the assigned formula from birth until 6 months of age, when baby food was provided by investigators and introduced to the diet. At 6 weeks and 3, 6, 9, and 12 months, measurements were made of weight, length, head circumference, and bone mineral content by dual photon absorptiometry of the midshaft and wrist of the left radius and ulna. Student *t*-test and ANOVA with Duncan multiple range test were used in the comparisons.

There were no significant differences at the start of the study in weight, length, or head circumference. Energy intake during the study was the same in both groups, and the groups did not significantly differ in weight, length, head circumference, or the rates at which these measurements increased over the study period. All infants were within the normal growth range based on the National Center for Health Statistics growth curves. The ratio of bone mineral content to bone width was significantly lower in the soy formula group at 3, 6, 9, and 12 months of age. Both groups showed a decrease in bone mineral content per bone width until 26 weeks of age and then a subsequent increase until 52 weeks. In the infants on cow-milk formula, bone mineral content rebounded to initial levels, but in the soy formula-fed infants, bone mineral content remained significantly lower than initial values.

The authors noted that the lower bone mineral content of the soy formula-fed infants may not have been clinically significant and was similar to that of breast-fed infants measured at 3, 6, and 12 months of age in a previous study at the same center. They further suggested that cow-milk formula may cause bone to be hypermineralized, rather than soy formula causing below-normal

3.0 Developmental Toxicity Data

bone mineralization. Although the authors did not analyze whether there was benefit or harm due to increased bone mineral content, they noted that in rats, bone hypermineralization early in life was found to decrease long-term mineral retention, and they questioned a relationship to osteoporosis. The authors also speculated that the lower bone mineral content in soy-fed infants could have been due to decreased availability of calcium and protein.

Strengths/Weaknesses: The prospective, randomized, double-blind design is a strength. There was 1-year follow-up with multiple measures, including head circumference and bone mineral content. Use of a homogeneous racial and socioeconomic group is a strength, although it limits generalizability of the study. Solid food was introduced at the same time and food was provided by investigators, and there were similar proportions of subjects in the formula groups receiving solid food. Weaknesses include the lack of a concurrent breast-fed comparison group and the small sample size. There could have been selection bias in women who elected to formula feed, and there was no adjustment for potential confounders.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility, except for the bone mineral density portion of the study, which may be of particular utility, despite very small sample size.

Hillman et al. (108, 109), supported by Mead Johnson Co., studied 31 term infants receiving either human milk ($n = 9$), the cow-milk formula Enfamil® ($n = 11$), or the soy formula ProSobee® ($n = 11$). The decision to breast- or formula-feed was made by each infant's mother. Within the formula group, babies were placed on soy formula by their private pediatricians for "colic or spitting up." Cereal was allowed at 4 months of age, and other solid foods at 6 months. Breast-fed infants were given vitamin D 400 IU/day as a supplement (both formulas also included this level of vitamin D). The infants were examined at entry into the study (age <3 weeks) and then at 2, 4, 6, 9, and 12 months. Samples were taken for measurement of serum vitamin D, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, parathyroid hormone, alkaline phosphatase, calcium, phosphorus, magnesium, and albumin. One urine sample was also taken randomly from each subject for measurement of calcium, phosphorus, magnesium, and creatine. Weight, body length, head circumference, bone width, and bone density (single-photon absorptiometry, mid-humerus) were measured at each visit. Trends in data over time were analyzed by linear regression, and mean slopes were analyzed using the 2-tailed Dunnett *t*-test. ANOVA was performed for 6-month values, and differences were identified by post hoc Duncan test.

There were no significant differences by feeding type in the rate of increase of bone width, bone mineral content, or bone mineral content as a function of body length. Serum and urine measurements are shown in Table 21. The differences noted in vitamin and mineral levels were considered to reflect homeostatic adjustments to differing mineral levels in the food sources. The authors concluded that infant bone mineralization was not affected by food type when adequate vitamin D and mineral intake were ensured.

3.0 Developmental Toxicity Data

Table 21. Laboratory Values at 6 Months of Age

Analyte	Human milk (n = 8)	Enfamil (n = 11)	ProSobee (n = 11)
Serum measurement			
Vitamin D (ng/mL)	2.2 ± 0.9	2.0 ± 0.8	2.3 ± 1.2
25-Hydroxyvitamin D (ng/mL)	25.6 ± 3.3	23.8 ± 3.1	24.0 ± 5.3
1,25-Dihydroxyvitamin D (pg/mL)	29.3 ± 11.3	45.4 ± 15.9 ^a	47.7 ± 10.2 ^a
Calcium (mg/dL)	10.0 ± 0.6	9.9 ± 0.6 ^b	10.5 ± 0.5 ^{a,b}
Phosphorus (mg/dL)	5.5 ± 0.3	6.0 ± 0.7 ^a	6.6 ± 0.9 ^a
Magnesium (mEq/L)	1.81 ± 0.13	1.79 ± 0.13 ^b	1.95 ± 0.15 ^{a,b}
Alkaline phosphatase (IU/L)	78 ± 22	59 ± 8	70 ± 23
Parathyroid hormone (μIEq/mL ^c)	3.2 ± 2.5	2.2 ± 0.4	3.1 ± 0.8
Albumin (g/dL)	4.7 ± 0.4	4.5 ± 0.3	4.5 ± 0.6
Urine measurement			
Calcium (mg/mg creatinine)	0.37 ± 0.31	0.25 ± 0.14	0.29 ± 0.28
Phosphorus (mg/mg creatinine)	0.71 ± 0.44	1.4 ± 0.3 ^a	1.5 ± 0.66 ^a
Magnesium (mEq/mg creatinine)	0.018 ± 0.008	0.018 ± 0.005 ^b	0.029 ± 0.017 ^{ab}

Values are mean ± SD.

^aDifferent from human milk: $P < 0.05$.

^bDifference between ProSobee® and Enfamil®, $P < 0.05$.

^cUnits as in the original.

From Hillman et al. (109).

Strengths/Weaknesses: The use of 3 feeding groups is a strength, as are the multiple measurements of growth, including measurements of head circumference and bone density and the blood and urine measures. The specification of times when solid foods were allowed is a strength, but it is a weakness that solid food was started early and there was no documentation of the dietary intakes of the 3 groups. Other weaknesses include the small sample size and the lack of adjustment for potential confounders. In addition, the soy group was selected for a specific indication, and these children could have been on cow-milk formula before enrollment (up to 3 weeks). Using “colic” as an indication for recommending soy infant formula introduces a potential bias in group assignment.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility, except for the bone mineral density portion of the study, which may be of particular utility, despite very small sample size.

Lasekan et al. (110), supported by Ross Products Division, Abbott Laboratories, performed a randomized, masked, parallel 1-year clinical feeding study to determine the effects of soy protein-based formulas and supplemental free nucleotides on the growth of infants. Healthy term infants were recruited from 9 private pediatric practices. Infants were given a soy formula (Isomil®, n = 73), the soy formula with 72 mg/L supplemental free monomeric nucleotides (n = 73), or mixed feeding (n = 67) consisting of 2 months exclusive breast feeding followed by a cow-milk formula (Similac® with Iron supplementation), without nucleotide fortification. Assignment was randomized by sex in the 2 soy groups. Infants were enrolled in the mixed-feeding group if their mothers chose to breast feed. Children were allowed to eat solid foods after 4 months of age. Formula tolerance and growth measurements (weight, length, and head circumference) were measured at enrollment and at 0.5, 1, 2, 4, 6, 7, and 12 months of age. Growth data were analyzed by 2-way ANOVA with feeding and sex as interaction terms.

3.0 Developmental Toxicity Data

At birth, infants on soy formula were heavier and longer than infants on nucleotide-supplemented soy formula (mean \pm SEM weight: soy 3616 \pm 66 compared to soy + nucleotides 3254 \pm 78 g; length: soy 52 \pm 0.4 compared to soy + nucleotides 50 \pm 0.4 cm). There was a significantly larger weight gain between 2 weeks and 1 month of age in the mixed-feeding group (mean \pm SEM: 42.6 \pm 1.8 g/day) compared to infants fed unsupplemented soy formula (34.1 \pm 1.3 g/day) or soy formula with supplemental nucleotides (36.7 \pm 1.3 g/day). Between 2 and 4 months of age however, infants fed unsupplemented (24.2 \pm 0.7 g/day) and supplemented soy formula (24.2 \pm 0.8 g/day) gained significantly more weight than mixed-feeding infants (20.3 \pm 0.3 g/day). After 4 months of age, there were no significant weight gain differences between the groups. Children in the unsupplemented soy group were significantly longer than mixed feeding infants at 7 and 12 months of age. Mean head circumference for infants on soy formula with supplemental nucleotides was larger than the mixed-feeding group at 6 months of age. Over the course of the entire study, length and head circumference measurements did not significantly differ between groups. All growth measurements were consistent with National Center for Health Statistics normal values.

Mean hemoglobin concentration, plasma albumin, and blood urea nitrogen were within the normal ranges in all groups. There was a significantly higher level of blood urea nitrogen in both soy groups than in the mixed-feeding group ($P < 0.0001$, mean \pm SEM not reported). The authors noted that this finding was expected due to the typically higher levels of protein in soy formula than in human milk.

The study authors concluded that over the first year of life, growth of healthy term infants fed soy formula or soy formula with supplemental nucleotides was similar to infants on a mixed-feeding regimen of human milk followed by cow milk-based formula. They also noted that there were no significant effects of supplemental nucleotides on growth.

Strengths/Weaknesses: Strengths include the use of 3 defined feeding groups, the relatively large sample size, random assignment by sex to soy-feeding groups, the specified time for introduction of solid foods, and the multiple measures of growth including head circumference. The mixed-feeding was group defined by length of exclusive breast feeding followed by cow-milk formula. It is a weakness that solid food was started early. There may have been selection bias in mothers who elect to breast feed. Random assignment by sex to the two formula groups did not result in equality between the groups; with 73 children in each group, there was still a mean difference of almost 400 g in birth weight between the groups, suggesting that there was some other fundamental difference between the groups at baseline. There was inadequate adjustment for potential confounders, and a repeated-measures analysis would have been preferable.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility.

Callenbach et al. (111), support not indicated, reviewed the records of 65 surviving infants who were born weighing less than 1500 g and for whom radiologic and biochemical data were available. There were 37 infants with radiologic evidence of rickets and 28 infants without such evidence. Soy-isolate formulas had been given to 32 of the children with rickets and 26 of the infants without rickets [$P = 0.69$, Fisher exact test]. The authors observed that these infants were very ill, and that long periods of parenteral feeding had been required during the initial weeks of life.

Strengths/Weaknesses: Strengths include the focus on rickets and the evaluation of a homogenous special population of very low birth-weight infants with radiologic findings.

3.0 Developmental Toxicity Data

Weaknesses include the retrospective nature of the record review, the lack of adjustment for confounders, and the small amount of variability in exposure: it appears that 58 of 65 infants in the sample had been given soy formula.

Utility (Adequacy) for CERHR Evaluation Process: This paper is not useful in the evaluation process.

Kulkarni et al. (99), supported by Ross Laboratories, performed a clinical trial in 46 very low birth-weight (≤ 1500 g) infants given 1 of 3 formulas to ascertain the relationship between formula type and rickets. Infants were randomly assigned to soy formula (Isomil®), cow-milk formula (Similac® with Iron), or a hypercaloric cow milk-based formula designed for low birth-weight infants (Similac® 24 LBW), and followed until 3–4 months of age. Formula compositions are given in Table 22.

Table 22. Formula Composition

Component	Soy	Cow milk	Hypercaloric cow milk
Energy, kcal/L	680	680	810
Protein, g/L	20.0	15.5	22.0
Fat, g/L	36.0	36.1	44.9
Calcium, mg/L	700	510	730
Phosphorous, mg/L	500	390	560
Vitamin D, IU/L	400	400	480

From Kulkarni et al. (99)

Formula was introduced by continuous nasogastric drip until a weight of 1600 g was reached and infants could tolerate bolus feeding. Roentgenograms of 1 knee and 1 wrist were taken in all infants when they were between 8 and 17 weeks old. Infants who did not reach a daily alimentary intake of 80 kcal/kg by 5 weeks old were not included in the study. Initial numbers of infants in the soy formula, cow milk-based formula, and hypercaloric cow milk-based formula groups were 15, 18, and 13, respectively; however, these numbers dropped to 9, 8, and 8.

Nutrient intake was analyzed by repeated measures ANOVA and the Scheffé method of multiple comparisons. There were no significant formula-group differences in intake of calories, protein, phosphorus, or vitamin D. Calcium intake was significantly higher in the soy-fed infants than those fed cow milk-based formula. Blood chemistry data collected during hospitalization were analyzed using the Bonferroni multiple comparison test. Data collected after discharge were averaged for each infant, and the values were analyzed using ANOVA followed by the Duncan multiple range test.

No significant differences were found prior to 3 weeks of age. Infants on soy formula had significantly lower serum levels of phosphorus at weeks 3, 5, and 8–14 and higher serum levels of alkaline phosphatase at week 5 and weeks 8–14 than infants in 1 or both of the cow-milk formula groups. Active rickets was diagnosed by x-ray based on the concurrence of 2 radiologists. Of the 15 infants in the soy formula group, 9 developed rickets. In contrast, only 1 case of rickets was diagnosed in the 2 cow-milk groups combined. The study authors speculated that the higher incidence of rickets in very low birth-weight infants fed soy formula may have been due to decreased absorption or solubility and bioavailability of minerals and discouraged the use of soy isolate formula for this population.

Strengths/Weaknesses: Strengths include the study of a homogeneous special population of very low birth-weight infants, random assignment to 1 of 3 formula groups, similarity of caloric,

3.0 Developmental Toxicity Data

protein, phosphorus, and vitamin D intake between groups, follow-up to 3–4 months, specification of the mode of delivering formula, specification of exclusionary criteria, use of appropriate repeated-measures method of analysis, and the use of 2 radiologists to confirm the diagnosis of rickets. Weaknesses include the small sample size and high drop-out rate.

Utility (Adequacy) for CERHR Evaluation Process: This study is relevant to the risk for rickets in very low birth-weight infants, but the drop-outs and excluded infants could be important limitations on its utility.

Naude et al. (112), support not indicated, conducted a 35-day trial in 40 preterm infants to compare the effects of feeding a cow-milk formula compared to a soy formula. Infants who weighed between 1500 and 1800 g at birth, appeared healthy, were appropriate for gestational age, and whose mothers had decided not to breast feed were randomly assigned to either a cow-milk or a soy formula feeding group. The children received formula amounts up to 180 mL/kg bw/day. All infants were given supplemental vitamins. Weights were taken on study days 1 and 2 and then on alternate days for the duration of the study. Head circumference and body length were measured on day 1 and then weekly. Blood samples were taken once between days 7 and 10 and again between days 32 and 35. Samples were analyzed for serum concentration of albumin, calcium, phosphorus, alkaline phosphatase, and sodium, and for blood urea nitrogen and hemoglobin. Student *t* test was used for statistical analysis.

The researchers report that it became immediately evident that the infants in the soy formula group were not progressing well. Of the first 10 infants allocated to this group, 7 were withdrawn because of weight loss or poor weight gain, and 1 died with necrotizing enterocolitis. Only 2 infants completed the 35-day trial. Only 2 of the first 10 infants in the cow-milk group were withdrawn, 1 due to jaundice and the other due to vomiting and a diaphragmatic hernia. The remaining 8 were said to progress “satisfactorily.” Due to the low success rate, the researchers changed the feeding regimen for the 20 subsequent subjects, increasing the daily intake from 180 to 200 mL/kg bw/day. The remaining infants allocated to the soy-formula group progressed more satisfactorily with the increased intake, and only 2 were withdrawn due to jaundice. On the higher-volume feeds, infants on soy formula gained an average of 305 g less weight and acquired 1.6 cm less height than infants on cow-milk formula. **[The data table shows a height difference of 16 cm, which the Expert Panel assumes should be 16 mm.]** Serum albumin and phosphatase levels were lower in soy formula-fed infants. The study authors recommended that caution be exercised in the feeding of soy formulas to preterm babies.

Strengths/Weaknesses: Strengths include the random assignment to soy or cow-milk formula, the multiple growth measurements including head circumference, the use of blood measures, and the use of a homogeneous special population sample of preterm, low birth-weight infants. Weaknesses include the small sample size and inability to adjust for potential confounders. In addition, the change in strategy mid-stream made it unclear whether reduced growth in subsequently enrolled soy formula-fed infants was due to a still-inadequate supply of formula.

Utility (Adequacy) for CERHR Evaluation Process: This study is somewhat useful in the evaluation process.

Shenai et al. (113), supported by Ross Laboratories, prospectively studied nutritional retention over 28 days in 19 very low birth-weight (<1530 g) infants on either soy formula (similar to Isomil®; n = 10) or cow milk-based formula (Similac® with Iron; n = 9). Infants with a gestational age of 31 weeks or less were recruited from the neonatal intensive care center of the University of Oregon Health Science Center. The very low birth-weight infants were of normal

3.0 Developmental Toxicity Data

size for gestational age and did not have metabolic or congenital anomalies. Within 7 days of birth, infants were fed at 3-hour intervals with the assigned formula in addition to a multivitamin and vitamin E 25 IU/day. When the infant had achieved full oral intake of the formula for 8 days, a 96-hour metabolic balance study was performed. Infants were weighed daily and occipitofrontal circumference and crown-heel length were measured weekly. Stool and urine samples were frozen until analysis for calcium, phosphorus, and nitrogen; stools were also analyzed for fat. Blood samples were taken at approximately 4, 14, 21, and 28 days postnatally. The 2 groups were studied successively [**not randomized**]; however, stool and urine analyses were concurrent. Statistical analysis was performed by *t*-test and ANCOVA.

There were no formula-group differences in fat intake or absorption. The soy formula-group had a significantly higher intake of nitrogen and calcium and lower intake of phosphorus than the cow-milk group. Because there were no significant differences in calcium excretion, absorption, or retention rates, the authors proposed that the absence of lactose in soy formula did not interfere with calcium retention or homeostasis. Though nitrogen intake was higher in the soy formula-than cow-milk formula-group, there was significantly lower nitrogen retention in the soy-formula group. Soy-fed infants had significantly lower phosphorus intake, absorption, and urinary excretion and a higher fecal excretion than cow-milk formula-fed infants. However, there was no significant difference in overall phosphorus retention between groups.

There were no significant differences between groups at any time in serum calcium or alkaline phosphate concentrations. Mean serum phosphorus levels were lower in soy-fed than cow milk-fed infants on PND 14, 21, and 28. No significant differences were found in mean serum total protein and albumin values at any time, and measurements were within the normal range for very low birth-weight infants. Mean blood urea nitrogen values were significantly higher in the soy-formula group on PND 14, 21, and 28 compared to the cow-milk group. Weight gain, crown-heel length, and occipitofrontal circumference did not significantly differ between the 2 feeding groups.

The authors stated that the study period was too short for these measurements to be valuable in determining growth effects. The authors also noted that the study may not have shown effects of sustained soy-formula feeding due to the small sample size, short duration, and lack of long-term follow-up. They specifically questioned whether long-term use of soy formula in very low birth-weight infants would have led to phosphorous-deficiency rickets.

Strengths/Weaknesses: The use of a homogeneous, special population sample of very low birth-weight infants and the repeated measurements for growth including head circumference and blood samples are strengths; however, the follow-up period of less than 1 month was too short, and the small sample size gave very limited power to detect differences.

Utility (Adequacy) for CERHR Evaluation Process: This study is not useful in the evaluation process.

Hall et al. (114), supported by Mead-Johnson Laboratories, conducted a randomized, controlled study to compare a calcium- and phosphorus-supplemented soy formula with a whey-predominant premature formula with regard to nutrition and growth in very low birth-weight infants. Forty infants from 3 to 8 weeks of age who had birth weights lower than 1500 g and were not breast feeding were recruited for the study. Formula assignment was made at the time of feeding initiation and all infants were given supplemental vitamins. Seventeen infants fed soy formula and 15 infants fed whey formula completed the study. Infants were put on cow-milk formula close to the time of hospital discharge. Body weight measurements were taken daily, and

3.0 Developmental Toxicity Data

measurements of length and head circumference were taken weekly. Data were analyzed using Student *t* test.

There were no significant differences in calorie or protein intake or in formula tolerance between groups. At 8 weeks of age, the mean body weight of infants receiving whey formula was significantly higher than that of infants receiving soy formula. There was a significantly higher mean weight gain per day in the whey formula group during the last 2 weeks of the study, in addition to greater caloric efficiency at 7 weeks in this group. Similarly, mean weight gain over the duration of the study was significantly greater in the whey formula group. Mean serum protein and albumin concentrations were within the normal range for both groups, although values were significantly higher in the whey group at the end of the study. Mean serum calcium and phosphorus were significantly higher at 8 weeks in the whey formula group, although the values for both groups were within normal ranges. Concentrations of alkaline phosphatase, parathyroid hormone, and 25-hydroxyvitamin D were within normal ranges and did not differ between groups. No cases of rickets were identified in either group by wrist radiography.

The study authors concluded that premature whey formula was preferable to supplemented soy formula for very low birth-weight infants.

Strengths/Weaknesses: The prospective design, use of a homogeneous, special population sample of very low birth-weight infants, and the repeated measurements for growth including head circumference are strengths. Weaknesses include the small sample size and the initiation of subjects into the study at 3 weeks of age or older without adjustment for baseline differences.

Utility (Adequacy) for CERHR Evaluation Process: This study is not useful in the evaluation process.

Churella et al. (115), support not indicated, conducted a controlled, randomized, blinded, parallel clinical trial of 2 soy formulas in 64 healthy term infants. Infants were randomly assigned to Isomil® (protein 2.91 g/100 kcal) or a lower-protein (2.45 g/100 kcal) soy formula. Parents agreed to exclusively feed infants the assigned formula from approximately 2 to 112 days of age. Infants were evaluated at 8, 28, 56, and 112 days of age. Weight, length, and head circumference were measured at each evaluation. Blood samples were taken at 56 and 112 days and analyzed for plasma urea nitrogen, total protein, and albumin. Data were analyzed using ANOVA. There were no differences between the groups in weight, length, or head circumference or in changes in these parameters. Mean energy intakes calculated from 3-day formula intake records did not differ. Mean protein intake was lower in the group fed the lower protein formula. Despite the significant differences in protein intake, there were no significant differences between the 2 groups in total plasma protein concentrations, plasma albumin concentrations, or median plasma transthyretin concentrations. Plasma urea nitrogen concentration was lower in the low-protein formula group. The authors noted that the growth of the infants in their study was similar to that of infants in other studies who were fed human milk or cow-milk formula. They concluded that because transthyretin, a sensitive indicator of protein status, was present at similar concentrations in both groups of infants, the content and quality of the protein in both formulas was adequate for growing infants.

Strengths/Weaknesses: Strengths include the controlled randomized design, the exclusive feeding for duration of trial, the multiple measures of growth including head circumference, use of blood measures, follow-up to about 3 months of age, and, relative to other studies, the moderate sample size (32 in each group). The power to show a group difference was limited by

3.0 Developmental Toxicity Data

sample size, however, and the comparison was only between soy formulas without a formal comparison to other kinds of formulas or to breast feeding.

Utility (Adequacy) for CERHR Evaluation Process: This study is not useful in the evaluation process.

Chan et al. (116), supported by Ross Laboratories, randomized 10 male and 10 female term newborns per group to receive 1 of 2 soy formulas that differed only in carbohydrate source (100% glucose polymers compared to 50% glucose polymers + 50% sucrose). A comparison group of 5 male and 5 female newborns who were exclusively breast-fed was used for comparisons up to 4 months of age, and a historical control group was used for comparisons at 6 and 12 months. Breast-fed infants were given supplemental vitamins and fluoride. Weight, length, and head circumference were determined at 2 weeks, 2 months, and 4 months of age, and serum was obtained for total calcium, phosphorus, magnesium, 25-hydroxycholecalciferol (a D-vitamin precursor), and alkaline phosphatase. Plasma and erythrocyte copper and zinc were measured, and bone mineral content (bone mineral density/bone width) of the distal radius was measured **[technique not specified, but probably single-photon absorptiometry, which was in widespread use during the time period]**. There were no differences among groups in any of the measures of growth or serum chemistries. Plasma but not erythrocyte zinc was higher in breast-fed than formula-fed infants at 4 months (mean \pm SD 74 ± 11 $\mu\text{g/dL}$ in the breast-fed group compared to 63 ± 15 and 59 ± 6 $\mu\text{g/dL}$ in the formula-fed groups). Bone mineral content was higher in the breast-fed group than in the formula-fed groups at 2 and 4 months. **[Mean bone mineral content was estimated from a graph: 2 months 0.1 g/cm^2 in the breast-fed group compared to 0.08 and 0.06 g/cm^2 in the formula-fed groups, 4 months 0.11 g/cm^2 in the breast-fed group compared to 0.08 and 0.06 g/cm^2 in the formula-fed groups. SEM appears to be about 0.02 for all data estimates.]** At 6 and 12 months, bone mineral content was the same in the formula-fed infants as in the historical control group of breast-fed infants. The authors posited that phytic acid (inositol hexaphosphoric acid) in soy formulas may interfere with calcium and zinc absorption, but that adaptation occurs and mineral absorption returns to normal over time. **[No comment was made concerning the effects of supplemental fluoride on measured bone mineral density.]**

Strengths/Weaknesses: Strengths include random assignment by infant sex, use of 2 soy formulas and a breast-fed comparison group, multiple measures of growth including head circumference, and use of blood measures and bone mineral content. Weaknesses include the small sample size (10 in each group) and the lack of adjustment for confounders. The separate historical control group used for breast-fed comparisons at 6 and 12 months was not optimal, and there could have been selection bias for breast-fed infants.

Utility (Adequacy) for CERHR Evaluation Process: This study is of marginal utility in the evaluation process.

Venkataraman (117), supported by Ross Laboratories, evaluated growth and bone mineral content in 56 healthy term infants exclusively fed human milk or 1 of 2 study formulas until 4 months of age, when baby foods were added to their diets. Infants receiving human milk were also given vitamin D 400 IU daily. Seventeen infants received human milk, while the remaining infants were randomly assigned to cow-milk formula (Similac®, $n = 19$) or soy formula (Isomil®, $n = 20$) using a block for equal sex distribution. The soy formula included modifications from previous soy formulas in calcium source and suspension characteristics. The feeding groups did not differ at the beginning of the study in weight, length, or head circumference. Infants were evaluated at 8, 16, and 24–26 weeks of age for length, weight, head

3.0 Developmental Toxicity Data

circumference, bone mineral content (single photon absorptiometry of the distal radius), and serum concentrations of calcium, magnesium, phosphorus, alkaline phosphatase, and parathyroid hormone. Data were analyzed using chi-squared for discrete variables and ANOVA or non-parametric methods for continuous variables. The Tukey Student range test was used to evaluate differences between pairs.

No significant differences were seen between the 3 groups in growth measurements or in serum concentrations of calcium, magnesium, alkaline phosphatase, or parathyroid hormone at any time point. Bone mineral content and bone width were significantly greater in the soy group than in the human-milk group at 16 weeks, and the difference in bone mineral content remained significant at 24–26 weeks. Although serum phosphorus levels were lower in the human-milk group and in the cow-milk formula group at 8 weeks, differences did not remain significant at the 16- or 24–26-week evaluation times.

The authors suggested that decreased bone mineral content seen in previous studies in infants receiving soy formula could be attributed to decreased delivery of calcium salts to the infants due to sedimentation. They also suggested that the increased bone width and bone mineral content in soy-fed infants compared to breast-fed infants could be a result of lower nutrient density of human milk. The researchers concluded that the decreased bone mineralization associated with soy-formula feeding in infants could be prevented through improved suspension characteristics of the minerals used and also noted the importance of these characteristics in the interpretation of studies involving bone mineral status.

Strengths/Weaknesses: Strengths include the use of 3 feeding groups including a breast-fed group, the random assignment of formula-fed infants, with procedures for assuring approximate equal distribution by infant sex, the multiple measurements of growth including head circumference measured and bone mineral content, and the restriction of solid foods during most of the study period. There were, however, apparently no adjustments for confounders. Selection bias may have occurred for breast-fed infants, and the sample was small with 17–20 in each group. There was a difference in fluid consumption between the groups.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process.

Mimouni et al. (118), supported by the NIH, Children’s Hospital Research Foundation, and Ross Laboratories, studied 72 healthy term infants receiving human milk (n = 10), cow-milk formula (Similac®, n = 20), or 1 of 2 soy formulas (Prosobee®, n = 21 or Isomil® n = 21). Breast-fed infants were recruited from infants whose mothers had decided to breast feed and were given vitamin D 400 IU. Supplementation of feeds with cow-milk formula was permitted. Infants whose mothers had decided not to breast feed were randomly assigned to a formula group. Solid food was withheld until 3 months for breast-fed infants and until 4 months for formula-fed infants. The infants were examined at entry into the study (2–7 days of age) and then at 8, 16, 26, and 52 weeks for measurement of weight, length, head circumference, bone mineral content (single-photon absorptiometry, distal radius and ulna), and serum calcium, phosphorus, magnesium, alkaline phosphatase, and 1,25-dihydroxyvitamin D. Data were analyzed using chi-squared for discrete variables and ANOVA or nonparametric methods for continuous variables. The Tukey Studentized range test was used to identify pair-wise differences.

Weight and head circumference did not differ by feeding group, but length gain was significantly lower in the breast-fed group than in the 3 formula-fed groups from 1 to 26 weeks. Bone mineral content and bone width were similar between groups throughout the study. There were no

3.0 Developmental Toxicity Data

significant feeding group effects on levels of serum total or ionized calcium, magnesium, or alkaline phosphatase. Serum phosphorus was significantly lower in the breast-fed group than in the combined formula groups at 8 weeks and lower than in the cow-milk formula group at 26 weeks. Serum levels of 1,25-hydroxyvitamin D were higher in the Prosobee® group than in the other groups at 8, 16, and 26 weeks.

The authors suggested that elevated concentrations of 1,25-dihydroxyvitamin D in Prosobee®-fed infants could indicate inadequate mineral intake or high mineral need. They indicated, however, that the increase in 1,25-dihydroxyvitamin D was apparently sufficient to increase calcium absorption and resulted in adequate bone mineral content. The authors concluded that the concentration and suspension characteristics of minerals in available soy-based formulas permitted adequate growth and bone mineralization.

Strengths/Weaknesses: Strengths include the use of 4 feeding groups, including 2 types of soy formula, and random assignment to formula-fed groups. The 1-year follow-up and multiple measurements of growth including head circumference and bone mineral content are additional strengths. There could have been selection bias for breast-fed infants, however, and supplementation with cow-milk formula was allowed for breast-fed infants. Solid foods were restricted for differing lengths of time in breast-fed and formula-fed infants and were introduced early. The sample size was relatively small.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process.

Stettler et al. (119), supported by the NIH and the International Formula Council, evaluated the relationship between rate of weight gain in early life and overweight in adults. Subjects included adults (20–32 years old, median age 26 years) who had participated in infant feeding studies between 1965 and 1978 in Iowa. The feeding studies had been restricted to infants who were not breast-fed at all. In these studies, assignment was made to whichever cow-milk or soy formula was being studied in the research unit at the time of the child's birth. Infants were enrolled at 8 days of age and were followed to 112 days of age. The adults who were subsequently recruited for follow-up had eaten their assigned formula for the duration of the study period. Additional foods were permitted after 28 days of age. From the 952 children in the original infant studies, 653 adults were located, met inclusion criteria, and agreed to participate. The subjects were contacted by telephone. Information was obtained from the subjects on current height and weight and on whether the parents of the subjects were overweight. The adult subjects were considered overweight if their body-mass index was ≥ 25 kg/m² and obese if their body-mass index was ≥ 30 kg/m². **[Because there were not enough obese subjects for planned analyses, the analyses involved overweight subjects, who represented 32% of the cohort.]** Infant weight had been obtained at 7 intervals through 112 days of age and was expressed corrected for age as a z-score. The period between birth and 8 days of age was identified as the most sensitive for predicting adult overweight, and subsequent analyses focused on this time period. The relationship between adult overweight and weight gain during this period was evaluated by multiple logistic regression with respect to potential confounders.

The unadjusted analysis showed absolute weight gain during the first week of life and from birth to 112 days to be significantly associated with adult overweight. Use of soy formula was not significantly associated with adult overweight in this analysis. With adjustment for birth weight, overweight parents, age in adulthood at the time of the survey, subject income, and first-week infant weight gain, there was a significant association between use of soy formula and adult overweight (OR 1.47, 95% CI 1.01–2.13, $P = 0.046$). When infant weight gain from birth to 112

3.0 Developmental Toxicity Data

days of age was included in the model, statistical significance was lost (OR 1.37, 95% CI 0.95–1.98, $P = 0.1$). The authors called the association between soy formula and overweight an incidental finding and suggested that it be treated with caution due to the inconsistency in results between the analytic models and due to the borderline level of statistical significance. They concluded that “soy-based formulas should be further investigated as a possible risk factor for overweight.”

Strengths/Weaknesses: Strengths are that infants were exclusively formula-fed for at least 28 days and were quasi-assigned to soy or cow milk by site rather than by mother or pediatrician preference. The multivariate analyses addressed a variety of other factors in infancy that could have been important predictors of adult overweight. If soy formula really is associated with adult overweight relative to cow-milk formula, adjusting for the higher infant weight gain up to 112 days of age (the intervening variable) in the multivariate model would have removed some of the effect of soy formula. Weaknesses are the relatively small proportion of subjects available for follow-up, the lack of information on other potential confounders that could have been important in the 26 years to follow-up but weren't measured, the lack of specific information on solid food that was given between 28 and 112 days, and the lack of information on the reasons for mothers selecting exclusive formula feeding. Infants usually lose weight in the first week and regain their birth weight by 2 weeks of age, so weight gain during the first week is not a usual observation in clinical practice.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

3.1.2.2 *Gastrointestinal effects*

Reports on gastrointestinal effects of soy formula are presented. Case series are followed by controlled studies. Some reports include what may be gastrointestinal manifestations of allergic disease, and these studies might just as reasonably have been discussed in Section 3.1.2.3.

Ament and Rubin (120), supported by the NIH, the Public Health Service, the Children's Orthopedic Hospital and Medical Center, and the University of Washington, presented a case of a 6-week-old infant with an immediate response to soy feeding consisting of fever, leukocytosis, cyanosis, vomiting, blood-tinged mucoid diarrhea, dehydration, and metabolic acidosis, all of which disappeared after 3 days of iv feeding. Subsequent challenges with soy protein were associated with violent systemic symptoms and jejunal abnormalities on endoscopy consisting of loss of villi and surface ulcerations. There was no reaction to gluten, soy lecithin, lactose, or sucrose. A moderate reaction to cow milk disappeared by 10 months of age, whereas the soy reaction continued to occur. The authors suggested that this response was unlikely to be allergic. They noted a similarity of the intestinal lesion to that of celiac disease and wondered if the underlying mechanism of injury might similarly be based on a genetic abnormality.

Strengths/Weaknesses: This paper represents a single case report of what may be a unique reaction.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Iyngkaran et al. (121), funding not indicated, investigated the effects of soy protein on the small bowel mucosa of 18 infants who suffered from acute gastroenteritis. Infants were hospitalized for correction of fluid and electrolyte status, following which they received a protein hydrolysate formula (such as Pregestimil®, Nutramigen®, Alfare®, or prehydrolysed human milk) for 6–8

3.0 Developmental Toxicity Data

weeks. Infants were readmitted again for soy-protein challenge (Prosobee®) and evaluated by biopsy for histologic appearance and enzyme levels in the jejunal mucosa before and 20–24 hours after the challenge. Ten (55.5%) of the 18 infants developed soy-protein enteropathy. The infants were divided into 3 groups based on their histologic and clinical results. Group 1 (n = 3) demonstrated histologic and clinical reactions between the 3rd and 9th hour of soy-protein challenge. Histologic reactions included depletion of the mucosal enzymes lactase, sucrase, maltase, and alkaline phosphatase and depressed blood xylose levels; clinical reactions were diarrhea, weight loss, vomiting, lethargy, fever, rhinitis, and skin rash. In Group 2 (n = 7), infants experienced only histologic reactions to the initial challenge. As a group, they had less depression of mucosal enzyme activity and blood xylose absorption compared to Group 1, but individual reactions varied considerably. Group 3 (8 infants) did not have any histologic, enzymatic, or clinical reactions to the soy-protein challenge. Significant differences in mucosal enzymes and xylose absorption were not found on comparison of Groups 1 and 2 to Group 3.

Strengths/Weaknesses: The clinical evaluation after soy challenge and the inclusion of biopsies are strengths, but this sample is highly select group of children with acute gastrointestinal episodes apparently due to a variety of causes. It was not possible to determine exactly which of the 18 infants had been previously exposed to soy prior to the acute episode and soy challenge. There was no comparison group.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Halpin et al. (122), funding not indicated, reported 4 infants, aged 2–4 months, with suspected intolerance to soy protein. Infants were admitted to the hospital, and proctosigmoidoscopy with rectal biopsy was performed within 24 hours. Gross and microscopic evidence of acute colitis was present in all 4 infants. After 1 month, all infants had recovered and sucrose and lactose tolerance tests were normal. Proctosigmoidoscopy with biopsy was repeated to confirm mucosal normalcy. Soy-protein challenge was given by feeding increasing amounts of soy formula (Isomil®) over 8 hours. If the feeds were tolerated, the soy formula was continued every 4 hours for up to 5 days. Stools were weighed and tested for occult blood and for the presence of reducing sugars. If the stool results were abnormal, a proctosigmoidoscopy with rectal biopsy was performed. Soy-protein challenge produced symptoms in the infants within 3 hours to 5 days. Proctosigmoidoscopy revealed friable mucosa, loss of vascular pattern, acute colitis (polymorphonuclear leukocytes present in the lamina propria or in the walls of the rectal glands), and an increase in mitotic cells in the crypt. Other findings included frank mucosal hemorrhage (2 infants), crypt abscess (1 infant), and depleted rectal-gland mucus (2 infants). Abnormal stool tests (low pH and increased reducing sugars) occurred in 2 infants, but these infants had no symptoms and their biopsies were normal. The authors suggested that the normal biopsies could be a result of rapid recovery, mild infection, or patchy mucosal damage. When biopsies from symptomatic children were evaluated blindly against biopsies from children with other colitides, the authors were unable to distinguish infectious colitis or mild ulcerative colitis from soy-induced colitis. They were able to distinguish severe ulcerative colitis and granulomatous colitis, nodular lymphoid hyperplasia, and normal bowel from the specimens taken in this study. The authors concluded that soy-induced intestinal mucosal damage is under-reported, and that soy protein may have the same potential for mucosal injury as does cow-milk protein.

Strengths/Weaknesses: In this case series, all 4 infants were known to have been fed soy and were suspected to be intolerant. The withdrawal and soy challenge under controlled conditions is a strength, but there was no comparison group. This population may represent a uniquely susceptible subgroup.

3.0 Developmental Toxicity Data

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Powell (123), support not indicated, reported 9 infants, aged 4–27 days at onset of symptoms, with protracted diarrhea before and after milk and soy-formula challenges. A control group consisted of 11 infants (age 2–9 months) who were hospitalized for chronic diarrhea not caused by cow-milk or soy formula. **[Not all of these control infants were tested in the same manner as the formula-intolerant infants, and results for the control infants are not clearly presented.]** Infants in both groups were managed with iv therapy if needed and with a casein hydrolysate formula (Nutramigen®). Once normal stools were observed and appropriate weight was gained, the children were challenged with cow-milk formula and, at least 1 week later, with soy formula. Stools were tested 12 hours prior to the challenge and 48 hours after the challenge. Complete blood counts, serum β 1C globulin, and serum protein electrophoresis were determined before and after the challenge. A positive response to the challenge was defined as liquid stools or stools that contained blood or leukocytes within 24 hours of the challenge. In the group with formula intolerance, 8 of 9 infants had positive responses to the cow-milk challenge and 6 of 9 had positive responses to the soy challenge. Serum protein electrophoresis remained unchanged in all but 1 infant. There was a statistically significant increase in serum β 1C globulin levels after the positive responses 90 minutes after the challenge but not thereafter. Leukocytosis occurred 6–10 hours after the positive challenges. No changes in eosinophils (<6% of white blood cell count), hemoglobin, or hematocrit values were observed.

The authors concluded that the severity of responses may have been unique to this age group. They also concluded that soy formula can be just as damaging as cow-milk formula if fed during this stage in the infant's life or after a reaction to cow milk.

Strengths/Weaknesses: This paper included infants with known cow-milk or soy gastrointestinal sensitivity. A comparison group with similar symptoms that were known not to be due to formula was used, and challenge with both cow-milk and soy formula occurred in each child. Weaknesses include the apparent non-random order of challenge (cow milk and then soy) and it was not clear if the control group received the same challenges and what the results were.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Lothe et al. (124), funding not indicated (but formula provided by Mead Johnson), carried out a double-blind crossover study to determine the effect of cow milk on infantile colic. Sixty-five cow-milk formula-fed infants with symptoms of infantile colic were identified at the Children's Hospital in Malmö, Sweden and enrolled. Of these, 60 infants between the ages of 2 weeks and 3 months completed the study. Identical-appearing cans of formula were provided to the parents for use over a 2-week period. The cans contained 1 week of cow-milk formula (Enfamil®) or 1 week of soy formula (ProSobee®). **[Randomization of order was not discussed.]** Five infants also received some human milk, and their mothers were instructed not to drink cow milk during the study period. Parents completed a daily questionnaire noting symptoms of colic (including paroxysmal abdominal pain, sustained severe crying, distended abdomen due to gas, and frequent wish to suck). Parents were also asked to note "whether the infant seemed to thrive" on the test formula. Infants who appeared to have improved with soy the first week and then worsened the second week on cow milk were put back on soy formula at the end of the study period. Infants who received soy formula the second week and appeared to improve were given a cow-milk formula challenge 1, 3, and 9 months later to exclude spontaneous recovery. Infants whose

3.0 Developmental Toxicity Data

symptoms did not improve with either the soy or cow-milk formulas were then given a hydrolyzed casein formula (Nutramigen®) containing neither cow milk nor soy. Infants who improved on the casein hydrolysate formula were also rechallenged with cow-milk formula **[results not discussed here]**.

Seventeen infants (29%) had what appeared to be a spontaneous recovery and tolerated the cow-milk formula. In 11 infants (18%) who did not tolerate cow-milk formula, colic disappeared within 48 hours of being put on soy formula. On rechallenge with cow milk 1 month later, 8 of the 11 infants had recurrent symptoms. Of the remaining 32 infants (53%) whose symptoms did not diminish on either cow-milk or soy formula, all were symptom-free within 48 hours of being placed on casein hydrolysate formula, and 14 of the 32 infants had recurrent symptoms when rechallenged with cow milk.

The authors concluded that cow milk is a significant factor in the development of infantile colic in formula-fed infants. They hypothesized an allergic cause and suggested dietary treatment with alternative formulas, such as those based on soy or casein hydrolysate.

Strengths/Weaknesses: All infants were said to have severe colic and to have been fed with cow milk-based formula. Infants were treated with both cow-milk and soy formula successively, with the mother blinded to formula content. To rule out spontaneous recovery, infants who recovered were challenged again later with cow milk formula. It was unclear, however, how formula was assigned and if it made a difference which formula was given first. Later challenges to rule out spontaneous recovery were only performed with cow milk even if the infant had not improved on soy formula. In addition, there was no inclusion of a group of infants without colic.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Poley et al. (125), supported by NIH, reported observations from scanning electron microscopy and light microscopy of small intestine biopsies from 2 infants with soy protein-induced villous atrophy. The first biopsy was performed on admission, and a follow-up biopsy was performed 6 weeks later. Mucosa obtained from a 7-month-old infant who had been on a sucrose-restricted diet with no diarrhea for 4 weeks preceding biopsy was used as a control. The soy-sensitive patients showed flattening of the villi, indistinct cell borders, lack of glycocalyx, and exposed microvilli. The degree of villous atrophy was similar to that seen in gluten-sensitive enteropathy. The authors estimated that 50–75% reconstruction of villous height had occurred at the 6-week follow-up biopsies. Tissue disaccharidase activity **[measured as the production of glucose after incubation of disaccharide with a tissue homogenate]** was depressed during soy feeding but showed recovery after 6 weeks. The authors described the recovery after cessation of soy exposure as “remarkable.”

Strengths/Weaknesses: The detailed evaluation of the clinical and histological picture of the disease and course of recovery is a strength, as is the inclusion of a control patient. It is a weakness that only 2 infants were included.

Utility (Adequacy) for CERHR Evaluation Process: This report is useful and shows that some infants are sensitive to soy protein.

Burks et al. (126), supported by NIH and Ross Laboratories, studied 43 infants under the age of 18 months to evaluate whether formula protein source was associated with enterocolitis. All subjects were suspected to have milk- and/or soy-protein enterocolitis due to vomiting or other

3.0 Developmental Toxicity Data

gastrointestinal symptoms. Patients with suspected IgE-mediated adverse food reactions were excluded. Formula challenges were performed in the hospital when the children were symptomatic. The challenge groups were:

Group 1 (n=23): cow-milk formula, powdered soy formula, liquid soy formula,
Group 2 (n=12): cow-milk formula, liquid soy formula, powdered soy formula, and
Group 3 (n=8): cow-milk formula, then soy formula challenges in randomized order.

[The grouping was not part of the original study design; study investigators modified the order of formula administration after results from the first group failed to support their hypothesis that the last formula would be most likely to produce a reaction. Thus, testing of the 3 groups was successive, and only the last group had a randomization component.]

The subjects fasted for 4–6 hours prior to challenge, and there were 48 hours between challenges. Patients were observed and stools were collected during the 24 hours following challenge. Reactions were considered positive if 2 or more of the following criteria were met: 1.) vomiting or diarrhea developed; 2.) absolute neutrophil count increased 6–8 hours after the challenge by at least 3500/mm³ compared to blood drawn immediately before challenge; 3.) blood was identified in stool; 4.) leukocytes were present in Wright-stained fecal mucus; and 5.) Charcot-Leyden crystals or eosinophilic debris were noted in stool after Hansel staining. Laboratory personnel were blinded to formula type. Statistical analysis methods were not given in detail. **[Subjects appear to have been combined without regard to group.]**

Twenty-one infants (48.8%) had a negative challenge to milk and soy formulas, 12 (27.9%) reacted only to soy, 4 (9.3%) reacted only to cow milk, and 6 (14.0%) reacted to both cow milk and soy. **[Statistical comparisons were not reported; chi squared test by CERHR gives $P = 0.1$ for any soy reaction compared to any cow-milk reaction.]** Patients who reacted to only 1 type of soy formula tended to react to whichever formula was administered first. Because the order of administration rather than the type of protein isolate was associated with enterocolitis, the authors concluded that processing soy into a powdered form did not render it less likely to cause a reaction. The authors interpreted the ordering effect as evidence that milk- and soy-protein enterocolitis may be caused by an unknown local immune mechanism with depletion of gastrointestinal immune mediators by the first challenge, regardless of protein source.

Strengths/Weaknesses: Strengths include the evaluation of all infants with enterocolitis thought to be due to formula intolerance and sequential administration of three types of protein. The non-random order of the challenge sequence and the lack of accounting for formula type at baseline are weaknesses.

Utility (Adequacy) for CERHR Evaluation Process: This report is somewhat useful in the evaluation process.

3.1.2.3 Allergy and immunology

Mortimer (127), funding not indicated, reported a case study of soybean anaphylaxis in a male child with asthma and eczema. The anaphylactic reaction occurred at 20 months of age. The child had previously been exposed to soy formula, and the family lived near a soy-processing facility. At age 3 years, the child was slowly re-introduced to soy milk to test the hypothesis that allergenicity would decrease with avoidance of antigen. No reaction was seen. The author concluded that soybean allergenicity should be re-examined to determine the potential for soy to approach cow milk as an allergen in light of increasing exposure in the general population.

3.0 Developmental Toxicity Data

Strengths/Weaknesses: This case report is described in detail, but it is still just a case report.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Whittington (128), support not indicated, presented case reports on 4 infants with soy-protein intolerance. The 4 infants developed diarrhea during the first month of life while on cow-milk formula. Switching to soy-based formula resulted in clinical deterioration. Responses to soy-challenge tests included diarrhea, vomiting, hypotension, lethargy, and fever. Switching to a diet free of soy or cow-milk protein was followed by recovery and weight gain.

Strengths/Weaknesses: This case series involves severe clinical courses in infants sensitive to soy formula. It is a weakness that all infants first demonstrated sensitivity to cow milk formula before responding poorly to soy formula.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Perkkio et al. (129), supported by the Finnish Foundation for Pediatric Research and the Paolo Foundation, conducted a morphometric and immunohistochemical study of jejunal biopsy specimens from 5 infants with soy allergy, all of whom also had cow-milk allergy. The biopsies were taken when soy was the only food source for the infants, and they were compared to biopsies taken before soy feeding and after soy had been eliminated from the diet. Biopsies were either processed for routine histology or examined by direct immunofluorescence or by the direct immunoperoxidase technique. In 4 infants, serial blood samples were taken for measurement of immunoglobulins, complement fractions, and soy antibodies. During soy feeding, biopsies of the 5 study patients showed partial or total villous atrophy associated with crypt hyperplasia. Inflammation of the lamina propria and the epithelium was seen and was similar both to that seen in intestinal cow-milk allergy with malabsorption and to that seen in celiac disease. There were also increased numbers of plasma cells and IgA- and IgM-containing cells in the lamina propria, as well as increased numbers of intraepithelial lymphocytes. Soy antibodies appeared and IgA also increased in the serum at the time of the reaction to soy. When soy feeding was stopped, the morphology of the jejunum improved and cell numbers were reduced to normal. The authors concluded that intestinal soy allergy was similar to intestinal cow-milk allergy and was readily reversible.

Strengths/Weaknesses: This report includes careful clinical and histological descriptions of infants with gastrointestinal sensitivity to soy formula, including comparison of biopsies before and after soy feeding and demonstration of reversal of changes. It is a weakness that all infants had known previous cow-milk formula sensitivity as well.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Halpern et al. (130), supported by Borden, Inc., followed 1753 Caucasian infants (893 male) who were fed either human milk, soy formula, or cow-milk formula from birth to 6 months of age to observe the influence of diet, family history, and sex on the development of childhood allergy. Assignment to the 3 dietary groups was not randomized, but was as “unprejudiced as possible if the mother did not insist on a particular regimen.” Breast feeding occurred in 352 infants, soy formula was given to 317 infants, and cow-milk formula was given to 1081 infants. The soy formula was Mull-Soy®, and the cow-milk formula was a proprietary liquid formula (Bremil®),

3.0 Developmental Toxicity Data

Similac®, Enfamil®, or SMA®). The mothers of the breast-fed infants were advised to limit their own cow-milk intake to 1 pint per day. The infants were followed for varying periods of time up to age 7 years. Diagnosis of food allergy was made solely on clinical grounds, with at least 3 episodes of characteristic symptoms being required to verify each diagnosis. Of the 1753 children, 401 were diagnosed as either definitely or possibly allergic. Children with a family history of allergy were significantly more likely to develop allergy, but diet had no significant effect on the development of childhood allergy. The authors noted that only 0.5% of infants fed soy milk developed allergy in the first 6 months of life, while 1.8 % of infants fed cow milk became allergic to it ($P < 0.05$, chi-squared). The authors concluded that this finding agreed with the conclusions of other investigators that “soy milk is a less potent sensitizer.”

Strengths/Weaknesses: Strengths include a large sample size from 9 different pediatric offices with 3 feeding groups and prospective ascertainment and follow-up for as long as 7 years. The authors evaluated a broad spectrum of allergic endpoints and included family history. There was, however, a lack of randomization to feeding group; women who had a preference could choose method of feeding and more women with immediate family history of allergy chose soy formula. Pediatrician preference also played a role. Many of the groups were mixed-feeding groups and mothers could have changed methods of feeding during the study period. Infants were followed for varying lengths of time, and there was substantial attrition at each follow-up point. No time variable was included in the analyses, and the denominator for the incidence of allergy was the entire sample regardless of the length of follow-up. Not all children were evaluated for allergies by a specialist, only those referred by the pediatrician. Multivariate analysis should have been used to control for multiple potential confounders simultaneously.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Kjellman and Johansson (131), supported by Förenade Liv, Semper Fund for Nutritional Research, Linköping University, and the Swedish Medical Research Council, performed a randomized clinical trial of soy formula ($n = 23$) or cow-milk formula ($n = 25$) in children with biparental atopic disease. Mothers were encouraged to breast feed, and infants were placed on a randomly assigned formula from weaning until age 9 months. At age 9 months, soy-fed infants were slowly introduced to cow milk. Children were followed to age 4 years. They were evaluated periodically, and atopic disease was rated as obvious, probable, possible, or none based on the number of episodes of atopic dermatitis, bronchial asthma, allergic rhinitis, allergic urticaria, or gastrointestinal allergy. Cord blood and venous blood drawn at 3, 9, 12, 18, and 36 months of age was analyzed for IgE and IgG antibodies to cow-milk and soybean protein and for levels of IgE, IgG, IgA, IgM, hemoglobin, cholesterol, and albumin. Statistical methods were not discussed.

Obvious and/or probable atopic disease occurred in two-thirds of children during the 4-year follow up period. There was no statistically significant difference in development of obvious or probable atopic disease by sex or formula type. Four children in each formula group were found to have IgE antibodies to cow milk. IgG antibodies to β -lactoglobulin, a protein, were present in the cord blood of all but 3 infants. The cow-milk formula group had significantly higher levels of IgG antibody to β -lactoglobulin from age 3 to 18 months, but the difference was no longer significant at 3 years (Table 23). **[The Expert Panel cannot evaluate these data because the graph in the original paper did not indicate variance, and the statistical methods were not given.]**

Table 23. Mean IgG Antibody Levels to β -Lactoglobulin

Age (months)	Formula		P
	Soy	Cow milk	
Newborn	26	38	Not significant
3	28	120	< 0.01
9	24	355	< 0.001
12	85	270	< 0.05
18	74	285	< 0.05
36	83	237	Not significant

Data given as mean % of a reference serum, estimated from a graph. The reference serum was not defined, variances were not shown, and the statistical methods were not given.

From Kjellman and Johansson (131).

IgE antibodies to soybean protein did not differ significantly between groups. Serum IgG antibodies to soybean protein were significantly higher at 9 and 12 months of age in the soybean formula group: mean \pm SD 65.7 ± 164.1 (9 months) and $63.3 \pm 104.5\%$ (12 months) of reference serum compared to the cow-milk formula group: 9.5 ± 2.0 (9 months) and $24.5 \pm 52.5\%$ (12 months) of reference serum. IgE levels did not differ significantly between healthy and atopic children, and there were no significant differences at birth or at 3, 18, or 36 months.

Overall, there were very few side effects of soy-formula intake, and all children in the soy group tolerated the formula, except for 1 child with a malabsorption syndrome who was put on a soy-free diet until age 3 years. The authors concluded that delaying introduction of cow milk by feeding infants soy formula until 9 months of age did not diminish the incidence of cow-milk intolerance.

Strengths/Weaknesses: The children in this study had a strong family history of allergy. Strengths include the random assignment to formula-feeding group and specified time that children were fed in that manner. There was long-term follow-up with repeated blood measures of immune status. The small sample size is a weakness. There was no breast-fed comparison group, and all infants were breast-fed for varying lengths of time before being randomized to formula group. Mothers could mix breast feeding and formula feeding, making effects of 1 food source impossible to determine. Statistical methods were not shown.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Gruskay (132), support not indicated, conducted a prospective evaluation of 908 children, 328 of whom had a family history of major allergy, and a control group of 580 children with no such family history, with the objective of determining if diet during the early months of life was related to the development of atopy later in childhood. The study population consisted of consecutive, healthy full-term newborns under the care of the author or participating pediatricians. In the families with a history of allergy, breast feeding was chosen by the mothers of 48 subjects. The remaining infants were assigned non-randomly to soy formula (every fourth child, $n = 79$) or cow-milk formula ($n = 201$) [**formula composition not given**]. Children were evaluated every month from 1 to 6 months of age, then every 2 or 3 months until age 2, and every year for up to 17 years thereafter. Children without a family history of allergy all received cow-milk formula if bottle-feeding was elected by the mother. Comparisons were made in the proportion of children at different ages with allergy. Statistical procedures were not discussed.

3.0 Developmental Toxicity Data

After 15 years, 50% of infants with a family history of allergy had developed allergic disease, while only 15% of control infants had developed allergy [**level of significance not given**]. In infants with a positive family history, allergy developed over 15 years with the same frequency in those fed cow-milk formula as in those fed soy formula (53%). Allergy was present significantly less often in children with a family history of allergy if they had been breast-fed, but differences in allergy rates between bottle- and breast-fed infants did not become statistically significant until after 3 years of age. The author concluded that breast feeding may play a role in decreasing the incidence of allergic disease. He also concluded that soy formula has no apparent advantage over cow-milk formula in the prevention of allergy.

Strengths/Weaknesses: Strengths include the large sample size with both positive and negative family history groups. There was some attempt to randomly assign mothers in the positive family history group to soy or cow-milk formula groups. The long-term follow-up is a strength, although the statistical methods did not include time in follow-up. The authors appeared to use appropriate denominators at each follow-up time point. Weaknesses include the possible selection bias inasmuch as mothers could elect to breast feed. Mixed feeding was allowed, and there was no adjustment for other foods when introduced. In the end, it appears authors only compared soy-fed to breast-fed infants in the positive family history group. Soy was not formally compared to cow-milk formula, although rates of allergy incidence were similar overall in both soy and cow-milk formula groups. There was no adjustment for potential confounders.

Utility (Adequacy) for CERHR Evaluation Process: This study's utility is limited to a comparison of soy formula feeding and breast feeding in infants with a positive family history.

Moore et al. (133), supported by the UK Medical Research Council, conducted a study of the breast-fed infants of atopic parents. The study objective was to determine the effectiveness of breast feeding in the prevention of atopy. Women in an experimental group (n = 250) were asked to breast feed their babies for the first 3 months, avoid giving them solids, and to use a soy formula if any extra food was necessary. Women in the control group (n = 275) used a cow-milk formula. The infants were seen daily until their discharge from the hospital and then at 3, 6, and 12 months. The mothers kept feeding diaries during the first 3 months. At 3 months, detailed feeding histories were obtained from the mothers, and the infants were clinically examined. The proportion of infants having eczema at 3 months of age was related to the time they were introduced to cow milk, with more than twice as many of those who were given cow milk in the first 4 weeks developing eczema as those introduced to cow milk during weeks 5–8 [**level of significance not indicated**]. Significant differences were found in the prevalence of eczema at 3 and 6 months between infants who had received cow milk in weeks 1–4 and those who had not. There were no significant differences between groups at 12 months. Multivariate analysis identified breast feeding as a protective factor. There was no advantage of using soy as opposed to cow-milk formula as an artificial food source.

Strengths/Weaknesses: Strengths include use of a homogeneous group with positive history of eczema or asthma. Women who agreed to participate could be randomly assigned to breast feeding for at least 3 months or to standard advice. The multivariate analysis and the initially large sample size are additional strengths. The study was, however, primarily focused on comparing breast feeding to cow-milk formula. It appears that few women actually fed their infants soy formula, and almost none used this method exclusively. Consequently, the sample was not adequate for a comparison of soy-fed infants to cow milk- or breast-fed infants.

3.0 Developmental Toxicity Data

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Miskelly et al. (134), supported by Wyeth Laboratories and the Welsh Scheme for the Development of Health and Social Research, compared the effects of feeding cow-milk or soy formula on the development of allergic symptoms in 487 infants at risk of allergic disease because of family history. Women randomized to the intervention group were advised not to give their infants cow milk and to restrict their own cow-milk intakes to a half-pint per day. They were supplied with a soy formula to supplement or replace breast feeding. Mothers in the control group were not given instructions on any diet restrictions, and most infants in this group received cow milk. Babies were examined for allergic symptoms at 3, 6, and 12 months by a physician who was unaware of the babies' formula types. Skin tests for milk, cod, egg, wheat flour, and soy antigens were performed at 6 and 12 months. Feeding diaries were kept by the mothers. The proportions of infants with symptoms were evaluated using a chi-squared test with Yates correction.

Mothers in the intervention group were less likely to breast feed than those in the control group. The authors suggested that the decreased breast feeding in the intervention group was due the free supply of soy formula given to women in this group. The only significant symptomatic difference in the first year of life between the 2 groups was in the incidence of oral thrush (51% soy formula compared to 40% breast feeding, $P < 0.05$). When the effects of breast feeding were evaluated, infants who were never breast-fed had a significantly higher incidence of wheezing and of diarrhea than infants who were breast-fed. There was also a numerically higher incidence of nasal discharge (not statistically significant). Skin testing showed that the only food antigen associated with eczema was egg, with positive tests occurring in 15% of babies with a history of eczema compared to 2% of babies without eczema ($P < 0.001$). The authors concluded that breast feeding was highly advantageous and that their findings gave no support to the hypothesis that withholding cow milk reduces the risk of allergic disease.

Strengths/Weaknesses: Strengths include the large sample size and the homogeneous group of infants at risk of allergy due to family history. The intervention was a combination of breast- and soy formula-fed infants, which is an important weakness. There does not appear to have been a separate analysis for soy formula-fed infants, so lack of differences between groups could have been due to failure to compare subgroups of the intervention group to the control group.

Utility (Adequacy) for CERHR Evaluation Process: This report is of marginal utility in the evaluation process.

Chandra et al. (135), supported by the National Health Research Development Programme of Health and Welfare Canada, and the Mead Johnson Canada division of Bristol Myers, examined the effects of maternal diet during breast feeding and the effects of 3 different types of formula on the development of atopic eczema in infants with family histories of atopic disease. A mother was recruited if either she or the baby's father had a family history of atopy. Mothers planning to breast feed exclusively were randomly assigned to an unrestricted-diet group or an experimental group asked to exclude dairy products, eggs, fish, peanuts, and soybeans from their diet and to take a 1 g calcium supplement daily for 6 months or the duration of lactation. Compliance with the requested restrictions was assessed by questioning, analysis of food diaries, and testing for β -lactoglobulin and ovalbumin in random samples of human milk. Mothers who did not plan to breast feed were randomly given either cow-milk formula (Enfalac®, $n = 40$), soy formula (Prosobee®, $n = 41$), or casein-hydrolysate formula (Nutramigen®, $n = 43$), which they were told to feed the infant for at least 6 months. The mothers and examiners were unaware of the formula

3.0 Developmental Toxicity Data

type. Infants were examined by a physician at 2, 4, 6, 12, and 18 months, and an eczema score was constructed based on affected parts of the body, type of eruption, and severity. Student *t* test was used to compare square-root transformed eczema scores, and proportions were compared using the chi-squared test with Yates correction. The 3 formula groups were compared using the Tukey W test for transformed scores and chi-squared test for proportions.

Among breast-fed infants, maternal dietary restriction was associated with a lower mean eczema score and a lower proportion of affected infants [**not further discussed for the purposes of this report**]. A comparison of the 3 formula-fed groups shows a lower proportion of affected infants and lower eczema scores in the group given the casein hydrolysate than in the other 2 groups. Only 9 infants in the casein-hydrolysate group developed eczema, while 36 infants in the soy group and 38 infants in the cow-milk group developed eczema. There was no difference in the incidence of eczema between the soy and cow-milk groups. The authors recommended that formula-fed infants with family history of atopy be given a casein hydrolysate formula.

Strengths/Weaknesses: Strengths include use of a homogenous group with positive family history of atopic disease, random assignment to 1 of 3 formula groups, 1 of which was restricted to soy formula for at least 6 months, randomization after maternal selection of breast or bottle, blinding of mothers and observers to formula type, and separate analysis of the formula group due to self-selection. Weaknesses include the relatively small sample size of soy formula-fed infants (*n* = 41) and the lack of mention of introduction of other foods. There were no multivariate analyses, and although baseline characteristics were said not to differ, no statistical comparisons were made.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful and shows no apparent benefit of soy formula over cow-milk formula in a high risk population.

Chandra et al. (136, 137), supported by the National Health Research Development Program of Health and Welfare Canada, Carnation Nutritional Products, and the Nutrition Research Education Foundation, conducted a prospective randomized controlled study to examine the effects of exclusive breast feeding and the feeding of 1 of 3 different types of formula on the incidence of atopic disease in predisposed infants. Mothers were recruited if either they or the baby's father had a family history of atopy. Mothers planning to breast feed (*n* = 60) were instructed to breast feed exclusively but were not given any other instructions regarding their own diets. Mothers who did not plan to breast feed were randomly given either cow-milk formula (Similac®, *n* = 24), soy formula (Isomil®, *n* = 25), or whey-hydrolysate formula (Good Start H.A.®, *n* = 68), which they were told to feed their infants exclusively for 6 months. The mothers and examiners were unaware of formula assignment. After 6 months, families were given usual advice for the feeding of infants with family history of allergy. Infants were examined at 6, 12, and 18 months of age for clinical manifestations of possible allergy. Skin tests were conducted on symptomatic infants using commercial antigens, and IgE antibodies to milk and soy were measured using commercial kits.

Infants in the whey-hydrolysate group had significantly lower incidence of atopic symptoms than those in the cow-milk and soy-formula groups. No significant differences were apparent between the cow-milk and soy-formula groups or between the whey-hydrolysate and breast-fed groups. Serum IgE antibodies to cow-milk or soy proteins were described as more common in infants fed the respective protein source [**statistical comparisons not reported**]. Skin prick tests in symptomatic infants did not identify significant differences between cow milk- and soy-fed infants. The authors concluded that there was a lower incidence of atopy in predisposed infants

3.0 Developmental Toxicity Data

who were breast-fed or fed hydrolysate compared to predisposed infants fed cow-milk or soy formula.

Strengths/Weaknesses: Strengths include use of a homogeneous group with positive family history and randomization to formula groups after the maternal decision not to breast feed. The use of 3 formula groups with exclusive feeding for 6 months and the blinding of mothers and observers to formula type are additional strengths. The sample sizes were moderate with only 25 subjects in the soy-formula group. Breast feeding mothers were self-selected. There was no mention of solid foods, and although baseline characteristics were said to not differ, there were no multivariate analyses.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful and shows no apparent benefit of soy formula over cow-milk formula in a high-risk population.

Chandra et al. (138, 139) presented 5-year results from the above study. The prevalence of eczema by 60 months was 6/60 [10%] in the breast-fed group, 10/68 [15%] in the whey-hydrolysate group, 19/68 [28%] in the soy-formula group, and 20/67 [30%] in the cow-milk formula group. The prevalence of asthma by 60 months was 4/60 [7%] in the breast-fed group, 7/68 [10%] in the whey-hydrolysate group, 14/68 [21%] in the soy formula group, and 16/67 [24%] in the cow-milk formula group. Skin prick testing and double-blind placebo-controlled food challenges showed a lower incidence of food allergy in the whey-hydrolysate and breast-fed groups. The authors concluded that breast feeding is preferred in children with a high risk of atopy, and that among formulas, the whey-hydrolysate formula was preferred to cow-milk or soy formula. [The Expert Panel notes that the whey-hydrolysate formula was marketed by Carnation, a sponsor of this study.]

Strengths/Weaknesses: This 5-year follow-up has the same strengths and weaknesses as the earlier reports (136, 137).

Utility (Adequacy) for CERHR Evaluation Process: This study is useful and shows no apparent benefit of soy formula over cow-milk formula in a high-risk population.

Odze et al. (140), funding not indicated, reported biopsy findings from 20 infants with allergic proctocolitis. The authors evaluated a possible association between numbers of eosinophils in colonic mucosal biopsy specimens and infant food source. The diagnosis of allergic proctocolitis was based on the presence of rectal bleeding in infants younger than 1 year who responded to a change of diet and in whom no other cause of rectal bleeding was found. Of the 20 infants, 7 had been fed human milk, 5 cow-milk formula, 5 soy formula, and 3 a combination. Cow milk-fed infants presented at a mean age of 21 days, soy-fed infants at 36 days, breast-fed infants at 85 days, and combination infants at 144 days. These differences were not significant. The signs and symptoms of proctocolitis were similar in the different feeding groups. No significant relationship was found between the number of eosinophils in the mucosa and infant diet.

Strengths/Weaknesses: A strength of this case series is the use of biopsies with quantification of eosinophils.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the evaluation process.

Lack et al. (141), supported by the Medical Research Council, the Wellcome Trust, and several UK government agencies, used data from the Avon Longitudinal Study of Parents and Children

3.0 Developmental Toxicity Data

to determine what factors, including soy intake, affected development of peanut allergy. From a large prospective cohort study of 13,971 preschool-aged children, 49 children with peanut allergy were identified and compared to controls using data retrospectively collected by masked telephone interview. One control group of 70 atopic children was randomly selected from those who were reported to have eczema or whose mothers had a history of eczema. A second control group consisted of 140 randomly selected children who did not have peanut allergy. The telephone survey of case and control parents was designed to collect additional information on maternal peanut consumption during pregnancy and lactation, family history of peanut allergy, and use of peanut oil-containing skin lotions and creams. Of the 49 children identified, 36 underwent skin testing and 29 tested positive for peanut allergy. There was a statistically significant association between soy product consumption and both peanut allergy and positive peanut challenge. Soy-milk or soy-formula consumption in the first 2 years occurred in 8.3% of the children in the total cohort, compared to 24.5% with peanut allergy and 34.8% with positive peanut challenge. In addition, 9 of the 10 children for whom consumption history was available were exposed to soy prior to peanut reaction. Step-wise logistic regression analysis, controlling for significant risk factors (rash over joints and in skin creases and oozing, crusted rash), confirmed that early soy consumption was an independent risk factor for peanut allergy (OR 2.61, 95% CI 1.31–5.20) and positive peanut challenge (OR 3.15, 95% CI 1.27–7.80).

The possibility that peanut allergy was associated with underlying cow-milk allergy rather than with soy product intake was examined, but cow-milk allergy was determined not to be a confounder. Only 4 of the 289 children in the cohort who were allergic to milk also had peanut allergy. The authors speculated that although no child in the study reacted to both peanuts and soy products, an immunologic co-reactivity between peanuts and soybeans could have caused cross-sensitization without clinical soy product allergy. Alternatively, the authors noted that an unidentified confounding factor could have been responsible for the observed relationship.

Strengths/Weaknesses: This nested case control study within a large prospective cohort study included 2 comparison groups, 1 “diseased” (atopic children) and 1 normal. It appears that soy-product feeding data were collected prospectively with respect to outcome. There were appropriate multivariate analyses. The reasons for soy product consumption in the first place could have confounded the association between soybean and peanut allergy. This possibility was likely addressed by atopic control group, but the reported comparisons seem to have been conducted with both control groups combined.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Zoppi et al. (142), funding not indicated, evaluated full-term infants given soy formula for the first 4 months of life. Three different protein levels were used (2.5, 4.0, and 5.5 g/kg/day [**kg bw or kg formula was not specified; assignment method was unclear**]). There were 39 infants in the study, 3 of whom were hospitalized for illness and were not included in the results. Diet was adjusted every 2 weeks based on infant body weight. Blood samples were taken early in the fifth study month for determination of hemoglobin, red blood cell count, protein electrophoresis, immunoglobulins, urea nitrogen, creatinine, glucose, electrolytes, acid-base status, and amino acids. Data were compared using Student *t*-test.

Gains in weight and length over the 4-month observation period did not differ significantly between groups. Hematologic parameters were within the normal range and did not differ between groups. Concentrations of urea nitrogen reflected the different nitrogen intakes. Serum protein concentration was described as slightly higher in the high-protein group, although not

3.0 Developmental Toxicity Data

significantly different from the other 2 groups. Serum albumin and α - and β -globulins were similar between groups. γ -Globulins increased with increasing protein intake. An increase in immunoglobulin concentration with increasing protein level was also described; however, due to the large SDs, only differences in IgG were significant. The authors noted that the serum levels of γ -globulin and immunoglobulins in the infants on the high-protein diet were similar to those reported in a different study in infants fed less than half this amount of cow-milk protein. The authors also reported that morbidity was higher in the low-protein group than in the other 2 groups, with the low-, mid-, and high-protein groups having 3.18, 2.13, and 2.00 infections per infant, respectively.

Strengths/Weaknesses: It is a strength that blood measures after 4 months of feeding were associated with the level of soy protein. Infections were not well described, and no significance testing was used. The sample size was small.

Utility (Adequacy) for CERHR Evaluation Process: This study is somewhat useful in the evaluation process.

Zoppi et al. (143), supported by “CNR and MPI grants,” compared immunocompetence in infants fed formulas containing either cow-milk protein or soy protein. Infants were assigned to 1 of 4 feeding groups, as shown in Table 24 [method of assignment unclear; previous study cited]. Two different protein concentrations were obtained for each formula by diluting with water and adding dextralmaltose to maintain caloric content. The infants received their assigned formulas from birth to 4.5 months and were examined every 2 weeks for weight, length, and illness (chiefly infection). After 4.5 months, blood samples were evaluated for hemoglobin, hematocrit, white blood cell count, protein electrophoresis, urea nitrogen, creatinine, glucose, electrolytes, iron, transferrin, cholesterol, and triglycerides. Immunoglobulin, complement, and amino acid determinations were performed. Tests of immune function were performed on isolated monocytes and included rosette formation to sheep erythrocytes, estimation of surface immunoglobulins, and mitogen response to phytohemagglutinin, pokeweed mitogen, concanavalin A, and *Staphylococcus* protein A. Data were analyzed using the 2-sample *t* test or the Behrens-Fisher test and multivariate linear discriminant analysis.

Table 24. Composition of Reconstituted Formulas

Protein (n)	Protein concentration,		Protein:calorie ratio, g protein/100 calories
	(% kcal from protein)	Calories, kJ/100 mL	
Cow-milk formula			
2.0 g/kg/day (7)	1.6 (8.6)	74.2 (310.4)	2.2
4.0 g/kg/day (7)	3.0 (15.9)	75.4 (315.5)	4.0
Soy formula			
2.0 g/kg/day (13)	1.6 (8.6)	74.2 (310.4)	2.2
5.0 g/kg/day (14)	3.5 (21.0)	70.5 (295.0)	5.3

[Per kg assumed to be per kg bw].

From Zoppi et al. (143).

Head circumference and mean gains in weight and length did not differ between the groups. There were more episodes of infection in infants fed the low-protein soy formula (3.4 episodes per infant) than in infants in the other groups (1.8–2.6 episodes per infant [significance levels not given]). Hematologic parameters fell within normal ranges for all groups; although in comparison to other groups, the authors noted a significantly lower hematocrit in the low-protein soy group, a higher white blood cell count in the high-protein soy group, and higher iron concentration (with

3.0 Developmental Toxicity Data

lower transferrin values) in infants fed either soy formula. Serum cholesterol and triglycerides were lower in the 2 soy groups. **[Levels of significance were not given for these comparisons.]**

Total serum protein, albumin, and β -globulin, and γ -globulin levels were described as higher in the cow-milk than soy groups, although the differences were not significant. Serum gamma-globulins, IgA, IgG, and IgM were significantly lower in the low-protein soy group than in the other groups. Complement C3 levels were lower in soy-fed infants than in those receiving cow milk. Complement C1 and C3 levels were lower in the 2 low-protein groups compared to the 2 high-protein groups. There were no statistically significant differences between groups in monocyte function, although the authors reported a “tendency” for T cells from infants in high-protein groups to show a greater response to mitogens. Discriminant analysis incorporating γ -globulins, transferrin, complement (C4 and C1), activated rosette formation, and mitogenic response showed clear separation of groups by protein amount and source (cow milk or soy). Using only T-cell function parameters, a separation between groups was evident with a comparison of low-protein soy and high-protein cow-milk groups and, to a lesser extent, between low- and high-protein cow-milk groups. The study authors concluded that soy protein was of lower nutritional value than cow-milk protein, and that low-protein formulas were suboptimal with respect to immune function.

Strengths/Weaknesses: The strengths and weaknesses of this study are similar to those of the previous study (143).

Utility (Adequacy) for CERHR Evaluation Process: This study is somewhat useful in the evaluation process.

Zoppi et al. (144), funding not indicated, studied the relationship between formula type and antibody response to vaccination in 62 healthy infants born at term. The study included 27 infants whose mothers decided to breast feed. Other study infants were randomly assigned to a high-protein cow-milk formula (4.4 g/kg /day; n = 7), a low-protein cow-milk formula (1.6 g/kg/day; n = 9), an adapted cow-milk formula with a casein/albumin ratio of 40:60 (protein 1.8 g/kg/day; n = 10), or a soy formula (protein 4.6 g/kg/day; n= 9). Infants received their assigned formula exclusively until 5 months of age when they were all switched to the same diet (with ~3.5 g/kg/day of protein) and followed to 1 year of age. **[The Expert Panel assumes the per kg designations are per kg bw.]**

Weight and length were measured every 2 weeks. Infants were vaccinated against poliovirus, diphtheria, tetanus, and pertussis at 2 and 4 months of age. Blood was sampled at 5 and 8 months of age and analyzed for antibodies, immunoglobulins, hemoglobin, hematocrit, white blood cell count, protein electrophoresis, urea nitrogen, creatinine, glucose, electrolytes, iron, transferrin, cholesterol, and triglycerides. Data were evaluated using ANOVA **[post hoc tests not designated except as orthogonal]**.

All groups had normal hematologic variables, blood urea nitrogen, amino acid levels, serum protein, immunoglobulin, zinc, transferrin, and mean gains in weight, height, and head circumference. Groups differed in antibody response to vaccination. Breast-fed infants and infants fed the high-protein cow-milk formula had protective antibody levels at age 5 and 8 months. Antibody levels in infants fed the adapted formula were significantly higher at 5 months but lower at 8 months than those of breast-fed infants. In infants fed the low-protein cow-milk or soy formulas, antibody levels were significantly lower than in breast-fed infants. Infants fed soy formula had significantly lower levels of antibodies to tetanus, diphtheria, and pertussis than did the breast-fed group or the high-protein cow-milk group. However, infants fed soy, low-protein

3.0 Developmental Toxicity Data

cow-milk, and adapted cow-milk formulas had significantly higher levels of antibodies to poliovirus type 2 than did breast-fed infants. The mean number of episodes of infection per infant was highest in the soy-fed group (2.9), compared to breast-fed (0.5), high-protein cow milk (1.6), low-protein cow milk (2.7), and adjusted formula (2.4) [**statistical significance not reported**]. The authors concluded that soy-fed infants had an impairment of antibody response to common viral and bacterial vaccinations, with the exception of poliovirus type 2. They suggested that recommended protein intakes should be re-assessed to take into account both the quality and quantity of dietary protein. They recommended an animal protein intake of 3 g/kg bw/day and concluded that vegetable protein should not be given to infants during the first months of life.

Strengths/Weaknesses: Strengths included a concrete measure of immune function in infants, vaccination response, and the use of 4 feeding groups with random assignment after breast feeding-preference selection. Weaknesses included the small numbers, although significant differences were still identified on some measures, the possible selection bias for breast-fed mothers, and the lack of demonstrated clinical significance of the observed differences in immune response.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

May et al. (145), supported by the Public Health Service and the National Dairy Council, measured serum antibodies to 5 major milk proteins and a soy-protein isolate in 67 infants fed 1 of 2 cow-milk preparations, a soy product (EdiPro A), or a mixture of 73% cow milk/27% soy isolate from birth to 112 days of age. The infants were then switched to another product. Serum samples were obtained at 28, 112, 140, 168, and 196 days of age. Samples were also obtained from 7 infants who were breast-fed from birth to 112 days. Additional data were obtained from 60 older children up to 16 years of age from a separate food-sensitivity study in which subjects received unrestricted diets including cow milk. The serum antigen binding capacity (total antibody content) was determined using radiolabeled antigens. Data were analyzed using the Student *t*-test. After 112 days, binding of soy protein in the serum of soy-fed infants was significantly less than binding of milk proteins by infants fed only cow milk. Feeding a soy product from birth resulted in a comparable or greater antibody response to subsequent cow-milk introduction than was seen in infants fed cow milk from birth. In light of this finding and a decreased antibody response to heat-treated milk, the authors suggest that heat-treated cow-milk formula rather than soy formula may be a preferred substitute for human milk.

Strengths/Weaknesses: There were 4 feeding groups with defined exposures, and there was some information about solid food intake. It is a weakness that the data were not originally collected for this study but rather were from different sites with different initial protocols. The group sizes were small.

Utility (Adequacy) for CERHR Evaluation Process: This study is somewhat useful for the evaluation process.

McDonald et al. (146), funding not indicated, conducted a study to evaluate immunologic mechanisms involved in food protein-induced enterocolitis. Eighteen infants (14 male) presented with vomiting and diarrhea that continued after switching to soy formula and had stools containing mucus, blood, and leukocytes. Symptoms disappeared within 2–3 days of removing whole protein from the diet. Class-specific antibodies to ovalbumin, soy, and cow milk were measured before food challenges to obtain baseline levels. Infants were then challenged with sequential oral 0.6 g protein/kg bw doses of egg white, soy formula, and skim milk. Forty-eight

3.0 Developmental Toxicity Data

hours or 5 days were allowed for re-attainment of baseline values following either a negative or positive challenge, respectively.

Infants with positive challenges to soy formula and egg had significantly higher IgG anti-soybean antibody concentrations than those with negative responses. Similarly, children with positive challenges to soy formula and egg also had higher IgA food-antibody titers. IgM levels did not differ between positive and negative challenge responders. However, in 12 infants who were followed after a single soy formula challenge, IgM anti-soybean antibody was found to increase with a negative response and decrease with a positive response. The authors stated that these findings suggested altered immunologic responses to ingested antigens in infants having food protein-induced enterocolitis.

Strengths/Weaknesses: The report included a well defined group with confirmed disease and looked at a range of antibodies. It is a strength that baseline, challenge, and post-challenge values were included. Weaknesses include the small sample size, the unknown or poorly described feeding histories, and the broad subject age range.

Utility (Adequacy) for CERHR Evaluation Process: This study is somewhat useful for the evaluation process.

Jenkins et al. (147), funding not indicated, studied 46 children with colitis diagnosed within a 4-year period. They reported that the age distribution of the children showed peaks at <2 years and 6–13 years, with all 38 children in the older group suffering from either ulcerative colitis or Crohn’s disease. The researchers compared the remaining 8 children with selected controls from the older group. Of these 8 subjects, 6 presented before 4 months and were switched from a cow-milk to a soy-milk diet. The remaining 2 subjects were receiving a mixed diet and were switched to an egg-free cow-milk diet. For each of these 8 patients and selected controls, dietary history, family history of allergy, blood count, serum immunoglobulins, IgE, and IgE antibodies to whole cow milk, β -lactoglobulin, and ovalbumin were studied. Colonoscopies were also performed in 7 of the patients and in the controls. Biopsies were taken before and after the implementation of the exclusion diets and examined for eosinophils and IgE-containing cells.

Symptoms disappeared in all 8 patients when switched to soy formula. Symptoms recurred within 2–6 weeks of the switch in 3 of the 8 patients but resolved after a chicken-based formula was substituted. Two patients who were given cow milk again 9–12 months after diagnosis showed symptoms again within 24 hours. Another patient who was given beef broth at 1 year also showed symptoms. All symptoms resolved after return to the exclusion diet. The researchers found that positive family history was significantly more common in the 8 patients with food-allergy colitis than in the selected controls. Blood eosinophil counts, serum IgE concentrations, and antibodies were also higher in the study group. Colonoscopies of the study group were distinctly different from those of controls, with an increase in eosinophils and IgE-containing cells. The authors concluded that colitis arising during infancy was most likely a result of food allergy and was best treated with an exclusion diet.

Strengths/Weaknesses: It is a strength that biopsies were taken before and after exclusion diets, but a weakness that infants were compared with much older “selected” controls.

Utility (Adequacy) for CERHR Evaluation Process: This study is not useful for the evaluation process.

3.0 Developmental Toxicity Data

Fort et al. 1990 (148), funding not indicated, conducted a retrospective study of 59 children with autoimmune thyroid disease, their 76 healthy siblings, and 54 healthy, unrelated control children to determine whether feeding soy formula or breast feeding was associated with the development of thyroid disorders. The children with autoimmune thyroid disease included 52 with Hashimoto's thyroiditis and 7 with Graves' disease. A nutritionist evaluated the history of infant feeding early in life, including the type of formula given and the age at which solid food was introduced. Proportions were evaluated using a chi-squared test. No significant differences were seen in the prevalence or duration of breast feeding or in the age at which solid foods were introduced. Among children with thyroid disease, there were no significant differences in the initial serum values of thyroxine or thyroid-stimulating hormone or in anti-thyroid antibody titers between breast-fed and bottle-fed infants [**data not shown**]. Of children with thyroid disease, 31% had received soy formula as infants compared to 12% of healthy siblings ($P < 0.01$) and 13% of healthy unrelated controls ($P < 0.02$). The authors concluded that they were unable to document an advantage of breast feeding with regard to the subsequent development of autoimmune thyroid disease. The greater prevalence of soy-feeding among children with thyroid disease compared to children without thyroid disease was proposed as being due to a possible decrease in cow-milk tolerance among children predisposed to developing thyroid disease or to possible adverse effects of soy on the developing thyroid.

Strengths/Weaknesses: The use of sibling controls plus a healthy unrelated control group is a strength. Weaknesses include the retrospective data collection and the small sample. As the authors suggested, the reasons for selecting soy formula may have confounded the association between this product and subsequent thyroid disease. This study cannot make that distinction.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Ostrom et al. (149), supported by the Ross Products Division, Abbott Laboratories, conducted a feeding trial to evaluate the immune status and morbidity of infants fed soy protein-isolate formulas with and without added nucleotides. Infants of mothers who decided not to breast feed before enrollment were randomly assigned to receive soy formula ($n = 92$) or soy formula with monomeric nucleotides added at 74 mg/L ($n = 94$) exclusively, from enrollment to 4 months. Both of these formulas, based on Isomil®, contained nucleotides 300 mg/L prior to supplementation. A third non-randomized group of 81 infants received human milk exclusively for up to at least 2 months and then received cow-milk formula supplements (Similac® with Iron; total nucleotides, 7 mg/L). Recommended immunizations were administered at 2, 4, and 6 months according to the 1994 immunization schedule of the American Academy of Pediatrics. Immune status of the infants was assessed by comparing morbidity and antibody responses to specific immunizations. Blood samples were taken at 6, 7, and 12 months of age. Antigen-specific immune responses, immune cell populations, and serum IgG and IgA were measured. ANOVA and ANCOVA models with the Tukey-Kramer method were used to compare continuous data from the 3 groups, controlling for site. Proportions of children with diarrhea, otitis media, and antibiotic usage were analyzed using chi-squared test.

There were no significant differences between the 3 groups at 6, 7, or 12 months of age in total serum IgG or IgA concentrations or in serum concentrations of antibodies against poliovirus (IgA), diphtheria (IgG), or tetanus (IgG). Although there were no significant differences in response to *Hemophilus influenza* b between infants fed soy formula with or without added nucleotides, infants fed nucleotide-supplemented soy formula had significantly higher geometric mean levels of antibody to *Hemophilus influenza* b at 7 and 12 months than did infants fed human milk. Although diarrhea reported by parents did not differ by feeding groups, physician-reported

3.0 Developmental Toxicity Data

diarrhea was significantly less frequent in the human milk-fed group compared to both soy-formula groups. There were no feeding-group differences for otitis media or antibiotic usage.

The authors concluded that infants fed soy-based formulas have normal immunoglobulin levels, normal responses to vaccine antigens, similar morbidity associated with otitis media, and thus normal immune development compared with breast-fed infants. They also concluded that nucleotide supplementation of soy formula provided no benefit other than a marginal benefit in response to *Hemophilus influenzae* b immunization.

Strengths/Weaknesses: Strengths include random assignment of infants whose mothers did not choose breast feeding, randomization within site, blinding of assignment, and the block design accounting for infant sex. The sample size was relatively large and an appropriate repeated-measures analysis was used for vaccine response data. Weaknesses include the varying lengths of time for which breast-fed infants were fed exclusively by that method and the lack of baseline immune function data prior to vaccination. There could have been selection bias in women who elected to breast feed.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Cordle et al. (150), supported by the Ross Products Division, Abbott Laboratories, reported additional results from the trial reported by Ostrom et al. (149). There were no feeding-group differences in total white blood cell count or in numbers or percentages of the 5 major leukocyte populations; however, at 6 months, the breast-fed group had significantly higher numbers and percentages of eosinophils than the group given nucleotide-supplemented soy formula. The breast-fed group also had a higher percentage of lymphocytes at 12 months than the group given nucleotide-supplemented soy formula. No feeding-group differences were found in numbers or percentages of B, pre-B, or mature B lymphocytes. No significant differences were found in the numbers or percentages of total T, helper T, or cytotoxic/suppressor T cells between the breast-fed group and the group given unsupplemented-soy formula. At 7 months, the group given supplemented-soy formula had a higher percentage of total T lymphocytes, higher numbers and percentages of helper T lymphocytes, and higher percentages of non-natural killer-like T cells. Despite the differences, all values were reportedly within normal ranges.

The researchers also found no feeding-group differences in expression of the human leukocyte antigen or in the maturation state of helper T lymphocytes, with the exception that the supplemented-soy group had higher percentages of naïve helper T cells than the breast-fed group at 6 and 7 months and a higher number of memory/effector helper T cells than the breast-fed group at 7 months. The study authors reported that the concomitant increase in CD45RA and CD45RO cells suggested a balanced, antigen-driven conversion of the helper T-cell population. No differences were seen in numbers or percentages of natural killer lymphocytes or natural killer T lymphocytes between the 3 feeding groups, with the exception of the supplemented soy group having lower numbers and percentages of natural killer cells compared with the breast-fed group and the unsupplemented soy group having lower percentages of natural killer T cells than the breast-fed group at 12 months.

The authors concluded that there were no consistent significant differences in immune status, maturation, or level of immunocompetence between infants fed a soy-based formula and those fed human or cow milk. They also reported that their study failed to demonstrate a strong effect of nucleotide supplementation of soy formula on immune status of infants.

3.0 Developmental Toxicity Data

Strengths/Weaknesses: Strengths and weaknesses are similar to those for Ostrom et al. (149).

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

3.1.2.4 Thyroid function

Shepard et al. (151), supported by the Platex Park Research Institution, reported 3 children with goiter related to the feeding of soy formula. The infants were clinically euthyroid but had low-normal levels of protein-bound iodine and high ^{131}I uptakes. When soy formula was discontinued, ^{131}I uptake returned to normal. In 2 of the cases, the goiter decreased in size or disappeared with the discontinuation of soy formula. Reduction in goiter size occurred in the third case when iodine was added to the diet and the brand of soy formula was changed. The authors concluded that lack of iodine was the cause of the soy formula-associated goiter.

Strengths/Weaknesses: These cases involve a special population of infants with congenital hypothyroidism. The case series format is a weakness.

Utility (Adequacy) for CERHR Evaluation Process: This report is useful for the evaluation process.

Chorazy et al. (152), support not indicated, presented a case report of a child with congenital hypothyroidism who was placed on soy formula at birth due to parental concern about a family history of milk allergy. The child's thyroid-stimulating hormone could not be suppressed into the normal range with oral thyroxine supplementation until cow-milk formula was substituted for soy formula. Stool frequency decreased from 5–7/day on soy to 2–3/day on cow milk. The authors believed this report and a similar report from 1965 provided evidence that soy interferes with thyroxine absorption from the intestine through fecal wastage. They noted that although iodine supplementation of formulas and a reduction in the fiber content of soy formulas decreased the risk of thyroxine malabsorption, formula intolerance might have led to an increase in stool frequency and a risk of fecal wasting of thyroxine in their patient.

Strengths/Weaknesses: This case involves a special population of infants with congenital hypothyroidism. The case report format is a weakness.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Jabbar et al. (153), funding not indicated, reported 3 infants with congenital hypothyroidism who experienced apparent malabsorption of thyroxine while on soy formula. Two of the infants were found on laboratory monitoring to have elevated thyroxine levels after they had been switched from soy to cow-milk formula. The thyroxine levels normalized with the thyroid replacement dose was decreased. The third infant did not suppress thyroid-stimulating hormone levels on thyroxine replacement doses as high as 19 $\mu\text{g}/\text{kg}$ bw/day. Switching from soy formula to cow-milk formula resulted in normalization of thyroid-stimulating hormone with an administered thyroxine dose of 14 $\mu\text{g}/\text{kg}$ bw/day. The authors concluded that soy formula could result in decreased absorption of administered thyroxine and decreased enterohepatic reabsorption of thyroxine with consequent fecal wastage.

Strengths/Weaknesses: These cases involve a special population of infants with congenital hypothyroidism. The case series format is a weakness.

3.0 Developmental Toxicity Data

Utility (Adequacy) for CERHR Evaluation Process: This report is useful for the evaluation process.

3.1.2.5 Reproductive endpoints

Freni-Titulaer et al. (154), funding not indicated, conducted a case-control study to evaluate factors associated with a 3-fold increase in the number of reported patients with premature thelarche between 1978 and 1981 in Puerto Rico. Potential cases included girls between the ages of 6 months and 8 years with palpable breast tissue at least 1.5 cm in diameter at the time of diagnosis of premature thelarche in the absence of other evidence of premature sexual development. Of the 552 potentially eligible subjects diagnosed between 1978 and 1982 with premature thelarche, 397 parents returned consent forms, and 130 representative case subjects were systematically selected for the study. An age-matched control subject was selected for each case subject, including where possible, a friend of the case. Parents were interviewed to determine family history and possible exposures. Data were analyzed by the computation of OR with 95% CI using a matched-pairs analysis followed by step-wise logistic regression on all the variables. Multivariate analysis was used to control for confounding factors.

Consumption of soy formula was found to be associated with premature thelarche in the univariate analysis (OR 2.2, 95% CI 1.0–5.2, $P = 0.05$ based on 22 exposed cases and 10 exposed controls). A maternal history of ovarian cysts was also significantly associated with premature thelarche on univariate analysis (OR 3.8, 95% CI 1.5–11.5, $P = 0.002$). Multivariate analysis showed no significant associations overall. When the analysis was restricted to girls with onset of thelarche before age 2 years, consumption of soy formula remained significantly associated in multivariate analysis (Table 25). The study authors noted that in more than 50% of the subjects, there was no exposure to any of the risk factors for which statistical associations were found. They suggested that premature thelarche resulted from a variety of environmental and familial factors.

Table 25. Factors Associated with Thelarche Prior to 2 Years of Age on Multivariate Analysis

Factor	OR (95% CI)	<i>P</i> value (two-sided)
Soy formula	2.7 (1.1–6.8)	0.029
Maternal ovarian cysts	6.8 (1.4–33.0)	0.017
Chicken consumption	4.9 (1.1–21.9)	0.035
Corn consumption	0.2 (0.0–0.9)	0.039

From Freni-Titulaer (154).

Strengths/Weaknesses: The case-control study design is a strength. Other strengths are the use of age-matched controls, the appropriate matched and multivariate analyses, and the reasonable sample size. Weaknesses include the broad age range of the girls with varying lengths of delay in collecting retrospective history and the use of friend controls in some cases but not in others. There could have been recall bias; for example, factors associated with thelarche prior to age 2 (in multivariate analyses) should have controlled for the age at which the maternal interview was conducted. Other potential confounders were not addressed, and the reasons why soy formula was used initially could have been a confounding association.

Utility (Adequacy) for CERHR Evaluation Process: This report is useful for the evaluation process.

Strom et al. (15), supported by Ross Products, Nestlé, and Mead Johnson, compared endocrine and reproductive outcomes in adults who had been fed soy or cow-milk formula during infancy.

3.0 Developmental Toxicity Data

Subjects were identified from a group of 952 individuals (248 fed soy formula and 563 fed cow-milk formula) who participated in controlled non-randomized infant feeding studies conducted at the University of Iowa between 1965 and 1978. In the feeding study, infants were enrolled before 9 days of age and fed either a cow-milk or soy formula through 16 weeks of age. The current study was conducted from March through August of 1999. Subjects included 248 individuals (120 male, 128 female) fed soy formula and 563 individuals (295 males and 268 females) fed cow-milk formula. Most subjects were Caucasian and were between 20 and 34 years of age. Information was obtained by structured and standardized telephone interviews with study participants. Men and women were asked about adult heights and weights, sexual maturation, and education level. Women were also questioned about menstrual cycles (cycle length, menstrual duration, flow, and pain) and reproductive outcomes (number of pregnancy, deliveries, and complications). Men were also asked about pregnancy outcomes in partners. Additional questions included congenital malformations in offspring, hormonal disorders, and homosexual orientation in both sexes, and testicular cancer in men. These outcomes were considered secondary and were expected to occur too infrequently to provide definitive data. Ordinal variables were analyzed by chi-squared or Fisher exact tests, and unadjusted and adjusted relative risks were calculated. Continuous variables were analyzed by *t*-test or Wilcoxon rank sum test followed by linear regression. Confounding factors considered in the analysis included birth weight, age, usual body mass, parental weight and height, hormone disorders, smoking, alcohol intake, soy-food consumption, vegetarian diet, herbal supplement use, recreational drug use, physical activity, sexually transmitted diseases, and use of hormonal birth-control methods.

No significant differences were noted in adult height and weight, body mass index, or sexual maturation in men and women. Duration of menstrual bleeding was slightly longer in women from the soy-formula group (adjusted mean difference = 0.37 days, 95% CI = 0.06–0.68), but bleeding was not heavier. Borderline significance was obtained for the number of subjects experiencing discomfort during menstruation in the soy-formula group (unadjusted risk ratio (RR) = 1.77, 95% CI = 1.04–3.00 for extreme versus no or mild discomfort; adjusted RR was not calculable). No significant differences were seen for pregnancy outcomes in women. **[No reproductive parameters were reported for male subjects with the exception of sexual maturation. Although men were questioned about pregnancy outcomes in partners, the results were not reported.]** No significant effects were observed for secondary outcomes (i.e., cancer, reproductive organ disorders, hormonal disorders, sexual orientation, or defects in offspring **[data were not shown]**).

The study authors concluded that “Exposure to soy formula does not appear to lead to different general health or reproductive outcomes than exposure to cow-milk formula. Although the few positive findings should be explored in future studies, our findings are reassuring about the safety of infant soy formula.”

In a letter to the editor, Goldman et al. (155) discussed several perceived limitations of the Strom et al. (15) study:

- Failure to mention in study abstract a higher use of asthma and allergy medications in subjects fed soy-based formula ($P=0.08$ in males and $P=0.047$ in females),
- Disregarding the slightly increased duration of menstrual cycle and greater menstrual discomfort findings in soy formula-fed women, when those findings could indicate endometriosis or uterine fibroids, theoretical outcomes of early-life estrogen exposure,
- Inadequate assessment of female fertility by not considering time to pregnancy and use of fertility technologies and only asking subjects about subjective outcomes,

3.0 Developmental Toxicity Data

- Lack of reporting of male fertility outcomes or semen quality,
- Lack of assessment of cancer risk, and
- Lack of consideration of exposure to estrogenic compounds in various soy-based formulas.

In response to this letter, Strom et al. provided incidences for thyroid disease (a possible autoimmune disease), endometriosis, uterine fibroids, low sperm count, and cancer in subjects fed soy versus cow-milk formula (Table 26). They noted the low number of subjects affected.

Table 26. Additional Findings in Subjects from the Strom et al. Study

Outcome	Females, n (%)			Males, n (%)		
	Soy (n=128)	Cow milk (n=268)	P	Soy (n=120)	Cow milk (n=295)	P
Thyroid disease ^a	5 (3.9)	6 (2.2)	0.34	1 (0.8)	0 (0)	0.29
Endometriosis	0 (0)	6 (2.2)	0.18	Not applicable		
Uterine fibroids	0 (0)	2 (0.8)	>0.99	Not applicable		
Low sperm count	N/A	N/A	N/A	1 (0.8)	1 (0.3)	0.50
Cancer ^b	4 (3.1)	3 (1.1)	0.22	0 (0)	1 (0.3)	>0.99

^aMainly hypothyroidism.

^bCervical in females, testicular in males.

From the Strom et al. response to Goldman et al. (155).

Strengths/Weaknesses: Strengths of the study include the large sample, the prospective collection of feeding information, and the appropriate statistical analyses. Feeding assignment was, however, not randomized, soy-formula exposure was short-term (0–16 weeks of age), and information on subsequent feeding other than current vegetarian diet was not collected. Sample sizes for some analyses (cancer, reproductive organ disorders, hormonal disorders, libido dysfunction, sexual orientation, and birth defects in the offspring) were too small to be definitive. It is possible that there was a bias in subjects who were able to be located compared to those not located 30 years later, and there may have been inaccurate recall for some measures collected at follow-up. The measures of infertility were weak, and the clinical relevance of the subjective measure of menstrual bleeding is questionable.

Utility (Adequacy) for CERHR Evaluation Process: This report is useful for the evaluation process.

Giampietro et al. (156), funding not indicated, conducted a retrospective study to determine the hormonal and metabolic effects of long-term feeding of soy formula in children. The study population consisted of 48 children age 7–96 months who had been fed soy formula exclusively for at least 6 months. Of these children, 30 were given soy formula for documented cow-milk allergy and 18 for family history of allergy. The 48 children were divided into 3 age groups, 7–24 months (n = 20), 25–72 months (n = 20), and 73–96 months (n = 8). An additional 18 healthy children aged 12–96 months who had not received soy formula during the first months of life were enrolled as a control group. The children were evaluated for height, weight, presence of breast budding, testicular size, presence of pubic/axillary hair, signs of precocious puberty in girls, and the appearance of gynecomastia in boys. Also studied were radiologic and biochemical markers of increased bone metabolism, including bone age, urinary deoxyypyridoline, calcium, creatinine, and phosphate and serum levels of bone alkaline phosphatase, osteocalcin, 17β-estradiol, and parathyroid hormone. Univariate analyses were performed on the data, using the

3.0 Developmental Toxicity Data

Fisher exact test and chi-squared test for discrete variables and the Mann-Whitney test for continuous nonparametric variables.

Height and weight were in the normal range for all children, and there were no differences between soy-fed and control groups. No signs of precocious puberty in girls or of gynecomastia in boys were found. All 17β -estradiol concentrations were below the method detection limit of 20 pg/mL. No significant differences were seen in serum or urinary measurements, except that soy-fed infants had significantly lower urinary calcium and significant higher urinary phosphate in children 7–24 months old compared to control children.

The authors concluded that long-term feeding of soy formula in infants did not produce estrogen-like hormonal effects. They suggested that their findings confirm the theory that phytoestrogens have a low affinity for ERs and therefore produce only weak biologic effects. The authors suggested that the isolated differences in urinary calcium and phosphate should be confirmed in larger studies before conclusions are drawn.

Strengths/Weaknesses: It is a strength that exclusive feeding of soy formula was apparently at least 6 months in duration. Use of a comparison group of unexposed infants in the first months of life and multiple measures of possible hormonal effects are other strengths. Weaknesses include the retrospective enrollment, in some cases many years after infancy, and the lack of clarity on how feeding patterns in early life were validated. The feeding practices in the comparison group were not well described. There were no multivariate analyses or controls for potential confounders. Sample sizes in each age group were small, raising the question of the power to identify group differences.

Utility (Adequacy) for CERHR Evaluation Process: This report is of limited utility for the evaluation process.

3.1.2.6 Cholesterol

Cruz et al. (157), supported by NIH, the Perinatal Research Institute, USDA, and Ross Laboratories, conducted a prospective, partially randomized, controlled study to evaluate the effects of cholesterol and phytoestrogen intake on cholesterol-synthesis rates in infants. Thirty-nine normal term Caucasian male infants were recruited from area hospitals. Of the 33 infants who completed the study, 12 received human milk (cholesterol 2.59–3.88 mM) for the first 4 months of life, with occasional supplemental cow-milk formula (Similac® with Iron; cholesterol 0.28–0.85 mM). Twenty of the 26 remaining infants were randomized to receive either cow-milk formula or soy formula (Isomil® with Iron; cholesterol 0 mM). The remaining 6 infants were non-randomly assigned to receive a modified-soy formula with the same cholesterol concentration (0.28 mM) as the cow-milk formula. Infants received their assigned formulas beginning at age 3–7 days and continuing until age 4 months, with no additional nutrition received other than multivitamins. At 4 months, cholesterol-synthesis rates and urinary isoflavone excretion were determined, with measurements taken over a 4-day period. Serum cholesterol and lipid profiles were determined using validated enzymatic techniques. Serum LDL cholesterol concentrations were calculated from serum total cholesterol. Cholesterol fractional synthesis rate [**the percent cholesterol synthesized per day**] was determined from the rate of incorporation of deuterium into red blood cell-membrane cholesterol. Urinary isoflavones were determined by GC-MS. Data were analyzed using 1-way ANOVA with post hoc Tukey-Kramer test. Correlations between cholesterol intake and cholesterol fractional synthesis rates were determined using a nonparametric test for ordered alternatives.

Data for serum lipid profiles and fractional synthesis-rate determinations are shown in

3.0 Developmental Toxicity Data

Table 27. The highest values for serum total and LDL cholesterol were found in breast-fed infants. These infants had the lowest fraction of synthesized cholesterol. There was a significant negative association between cholesterol intake and the fractional cholesterol-synthesis rate ($P < 0.0001$). Isoflavone excretion was associated with soy-formula consumption. Multiple regression analysis controlling for group effect found total urinary isoflavone excretion to be negatively associated with fractional cholesterol-synthesis rate but not with serum cholesterol concentration.

The study authors concluded that infants responded to different dietary cholesterol intakes through altered cholesterol-synthesis rates and that infant diet can affect serum lipid profiles, cholesterol-synthesis rates, and urinary isoflavone excretion.

Table 27. Serum Lipid Profiles and Fractional Cholesterol Synthesis Rates

Food	Serum concentration (mM)				Fractional cholesterol synthesis rate (%/day)
	Cholesterol			Triglycerides	
	Total	HDL	LDL		
Human milk	4.47 ± 0.20 ^a	1.32 ± 0.09	2.54 ± 0.21 ^a	1.30 ± 0.20	2.97 ± 0.49 ^b
Formula					
Cow milk	3.34 ± 0.24	1.22 ± 0.11	1.45 ± 0.25	1.44 ± 0.25	9.43 ± 1.12
Soy	3.11 ± 0.26	1.43 ± 0.12	0.99 ± 0.27	1.38 ± 0.27	12.02 ± 1.36
Modified soy	3.40 ± 0.28	1.26 ± 0.13	1.49 ± 0.29	1.39 ± 0.29	10.55 ± 1.05

Mean ± SEM. HDL = high-density lipoprotein; LDL = low-density lipoprotein.

^aHuman milk value statistically different from all formula values. [**Authors presented different P values, all 0.03 or lower.**]

^bAll values in column statistically different from one another, except cow-milk formula compared to modified-soy formula for which $P = 0.1$.

From Cruz et al. (157).

Strengths/Weaknesses: After selection for breast feeding, random assignment to primary soy or cow-milk formula groups and documentation of no additional nutrition to 4 months are strengths. The inclusion of only males is a weakness. Selection bias was possible for breast feeding mothers. The modified soy-formula group was not included in randomization, and the multivariate analysis results were not described very clearly.

Utility (Adequacy) for CERHR Evaluation Process: This report is useful for the evaluation process.

3.1.2.7 Diabetes mellitus

Fort et al. (158), funding not indicated, examined feeding histories of 95 diabetic children and controls to study the effect of breast feeding on the development of insulin-dependent diabetes mellitus. The feeding histories of the diabetic children were compared to those of a control group consisting of 194 non-diabetic siblings and an additional 95 non-diabetic friends living in the same geographic area. Data were analyzed by a sign test and chi-squared test. Eleven of the 95 diabetic children were excluded from statistical analysis because they changed addresses or could not be matched. There were no significant differences in the incidence or duration of breast feeding among the groups, or in the introduction of solid foods. Almost twice as many diabetic children had been fed soy formulas compared to controls. [**Data on soy formulas were not shown. The authors noted that most children on formula received cow-milk formula and that the group differences in soy-formula intake were not statistically significant.**] The authors postulated that diabetic infants might have a higher incidence of gastrointestinal alterations giving rise to a switch to soy formula.

3.0 Developmental Toxicity Data

Strengths/Weaknesses: The case control design is a strength as is the use of sibling controls and unrelated controls matched by geographic area. Weaknesses include the retrospective collection of infant feeding information an average of 14 years later. The soy-formula exposure was not specified and could have been with breast feeding or not. Specific numbers were not presented for soy formula; an association was identified but there were no statistically significant differences, making conclusions difficult.

Utility (Adequacy) for CERHR Evaluation Process: This report is somewhat useful for the evaluation process

3.1.2.8 Cognitive function

Malloy and Berendez (159), funding not indicated, studied the relationship between breast feeding during infancy and cognitive performance in 9- and 10-year-old children. Cognitive testing and health results were obtained for children in a 1978–1979 birth cohort. All 518 infants in the study had received soy formula. Of these infants, 342 had been breast-fed and 176 had never been breast-fed. Dietary information was obtained through questionnaires completed by the parents. The Wechsler Intelligence Scale for Children—Revised (WISC-R) verbal, performance, and full-scale intelligence quotients (IQ) were administered to children in their homes. Data were analyzed using chi-squared and Student *t*-test. A linear regression model was used to adjust least-square means for potential confounding variables. Differences were considered significant at $P \leq 0.05$.

The researchers initially found higher WISC-R verbal and full-scale mean scores among the breast-fed children. After adjustment for maternal education, paternal education, and annual family income, the differences were no longer significant. The authors concluded that in this relatively socio-economically advantaged population, additional advantages of breast feeding might be more difficult to demonstrate.

Strengths/Weaknesses: The use of a birth cohort, standardized testing in school-age children within a narrow age range, and the large sample size are strengths. Weaknesses include the retrospective collection of infant feeding information only by questionnaire 9–10 years later and the comparison only between children who were breast-fed for any length of time and children who were exclusively soy formula-fed. If any real differences existed, they could have been diluted by soy exposure in the breast-fed group.

Utility (Adequacy) for CERHR Evaluation Process: This study is somewhat useful in the evaluation process.

3.1.3 Exposure during adolescence

Shu et al. (160), supported by the National Cancer Institute, used a case-control design to evaluate the association between breast cancer in adulthood and soy-food intake during adolescence. Women between the ages of 25 and 64 years with newly diagnosed breast cancer were recruited. The women, who were permanent residents of Shanghai, were selected from a cancer registry. Controls were selected from a registry of residents of Shanghai and were matched to cases on age (within 5-year intervals). Of the women identified, 91.1% of cases ($n = 1459$) and 90.3% of controls ($n = 1556$) agreed to participate. The height and weight of each participant was recorded, and participants were interviewed regarding demographic factors, menstrual history, reproductive history, hormone use, past illnesses, physical activity, tobacco and ethanol use, weight, and family history of cancer. A food frequency questionnaire was used to determine adult and adolescent (age 13–15 years) dietary intake. In a subgroup of 569 cases and 659 controls who

3.0 Developmental Toxicity Data

were less than 45 years old at interview, attempts were made to contact the participants' mothers for assessment of their daughters' adolescent dietary intake as well as pregnancy and lactational exposures. Interviews were completed for 79.6% of case mothers and 81.8% of control mothers. Soy intake was estimated by combining the soy-protein content of specific foods and was divided into quintiles. Unconditional logistic regression was used to obtain OR and 95% CI with control for potential confounders (age, education, family history of breast cancer, history of fibroadenoma, body mass index, waist:hip ratio, and exercise level.) **[The data table also indicates adjustments for rice and wheat intake, pregnancy experience, age at menarche, and age at menopause.]**

Results are summarized in Table 28. There was a significant inverse association between adolescent soy intake and risk of breast cancer. This significant inverse association was found when total soy intake was considered and when individual categories of soy food (tofu, soy milk, other soy food) were considered. There was an inverse association between rice/wheat and milk intake and risk of breast cancer. Controlling for soy intake did not eliminate the rice/wheat or milk associations. Intake of fresh legumes and other categories of foods was not associated with breast cancer risk. The inverse association between adolescent soy intake and breast cancer persisted when adjusted for adult soy intake, suggesting to the authors that adolescent soy intake was not just a proxy for lifetime or adult soy intake. The authors recognized the potential inaccuracy of diet recall, but they believed that any recall errors would be non-differential because the hypothesized protective effect of soy against breast cancer was not known in China during the study period.

Table 28. Association Between Dietary Soy During Adolescence and Adult Breast Cancer

Soy intake quintile (soy protein cut-off, g/day)	Adjusted OR (95% CI)	
	Subject recall	Mother recall
1 (<2.20)	Reference	Reference
2 (2.20–4.40)	0.75 (0.60–0.93)	0.70 (0.45–1.09)
3 (4.41–6.60)	0.69 (0.55–0.87)	0.59 (0.32–1.07)
4 (6.61–11.00)	0.69 (0.55–0.86)	0.60 (0.37–0.99)
5 (≥11.01)	0.51 (0.40–0.65)	0.35 (0.21–0.60)
Trend test <i>P</i> value	< 0.01	0.0002

OR adjusted for intake of rice and wheat products, education, family history of breast cancer, history of fibroadenomas, waist:hip ratio, age at menarche, physical activity, ever giving birth, age at first delivery, menopausal status, and age at menopause.

From Shu et al. (160).

Strengths/Weaknesses: The case-control design using age-matched controls is a strength. The use of an Asian population likely to have high exposures, the large sample, and the high participation rate are additional strengths. The dose-response relation shown in the recall of the women and the women's mothers reinforces the identification of the association. Weaknesses include the lumping of pre- and post-menopausal breast cancer cases, the potential for recall bias, and the possibility that the food frequency questionnaire may not have represented true intake. In addition, for the purposes of this evaluation, the evaluation of soy food rather than soy formula is a weakness.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful in the evaluation process.

Wu et al. (161), in a study supported by the California Breast Cancer Research Program, examined the relationship between soy intake in adolescence and breast cancer in adulthood.

3.0 Developmental Toxicity Data

Volunteers selected for the case-control study were of Asian-American descent, specifically Chinese, Japanese, or Filipino, and lived in Los Angeles county. From 1995, 501 cancer cases were selected from the Los Angeles County Cancer Surveillance program. The patients were 25–74 years old at the time of breast cancer diagnosis. Controls (n=594) were selected from neighborhoods where cancer patients lived at the time of diagnosis and were matched according to ethnicity and age. Volunteers were interviewed about demographic characteristics, migration history, menstrual and reproductive history, body size, physical activity, and diet history. Soy intake in adulthood was estimated by asking volunteers about the frequency and volume of various soy foods ingested (e.g., miso, tofu, vegetarian meats, soybeans, and natto). Crude estimates of soy product intake in adolescence (12–18 years of age) were based on reported frequency of tofu ingestion. Isoflavone intake in adulthood was estimated using data from the Hawaii Food Composition Database. ORs were calculated by conditional logistic regression analysis with matching for age and specific Asian ethnicity. ORs were adjusted for education, birthplace, age at menarche, parity, current body mass, menopausal status, and use of menopausal hormones. A second set of adjustments was made for intake of dark leafy vegetables during adolescence (considered an indication of a traditional Asian diet), smoking history, alcohol intake, physical activity, and family history of breast cancer. ORs were calculated for quartiles of soy intake during adolescence (<1 times/month, 1–3 times/month, 1–3 times/week, ≥4 times/week) and isoflavone intake during adulthood (≤1.79, >1.79–6.24, >6.24–12.68, or >12.68 mg/1000 kcal).

Soy product intake was highest in Chinese (26.8 mg/day), intermediate in Japanese (18.4 mg/day), and lowest in Filipino (9.3 mg/day) volunteers. Statistically significant ORs (i.e., 1 not included in 95% CI) are summarized in Table 29. There were some significant and inverse associations between soy intake in adolescence or adulthood and risk of breast cancer in adulthood. When exposures during both adolescence and adulthood were considered, only high exposures during both adolescence and adulthood suggested a significant protective effect against breast cancer. There was no significant effect of high-soy exposure in adolescence and low-soy exposure in adulthood (OR, 95% CI: 0.77, 0.51–1.10). There was also no significant association for low-soy exposure in childhood and high-soy exposure in adulthood and decreased risk of breast cancer (OR, 95% CI: 0.93, 0.58–1.48). The study authors concluded that the study demonstrated an association between high intake of soy in childhood and reduced breast cancer risk in Asian Americans; the study authors also noted that breast cancer risk may be reduced further by soy intake in adulthood.

Table 29. Statistically Significant Relationships Between Soy Intake and Breast Cancer

Exposure Group	Subjects, n		Adjusted OR (95% CI) ^a	
	Cases	Controls	a	b
Tofu intake of 1–3 times/week in adolescence	189	270	0.56 (0.38–0.82)	0.62 (0.42–0.92)
Tofu intake of ≥4 times/week in adolescence	55	85	0.51 (0.31–0.84)	0.65 (0.38–1.10)
Intake of >12.68 mg isoflavone/1000 kcal in adulthood	104	166	0.51 (0.33–0.78)	0.61 (0.39–0.97)
High tofu intake in adolescence (≥1 time/week intake) and high isoflavone intake (>6.24 mg isoflavone/1000 kcal in adulthood)	164	261	0.53 (0.36–0.78)	0.65 (0.43–0.97)

^aAdjusted for birthplace, education, age at menarche, parity, current body-mass index, menopausal status, hormone use.

^bFurther adjusted for dark leafy green intake in adolescence, smoking, alcohol ingestion, physical activity, and family history of breast cancer.

From Wu et al. (161).

3.0 Developmental Toxicity Data

Strengths/Weaknesses: This well constructed case-control study had a large sample size with appropriate matching of controls using matched analytical methods. Multivariate analyses controlled for important potential confounders. Weaknesses include the very wide age range of breast cancer cases and controls, so time-to-recall of adolescent tofu intake apparently ranged from about 6 years to about 55 years. There is also potential for recall bias in a case-control study design with normal controls. Measure of adolescent intake was based solely on recall of tofu intake, which could have misrepresented total intake.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

3.2 Experimental animal data

3.2.1 Development of the reproductive system

The effects of developmental soy exposure on reproductive endpoints were examined in numerous rat studies, 1 marmoset study, 1 rhesus monkey study, and 1 mouse study. Studies in rats are presented first, in order of female effects followed by male effects. Within in each sex, studies with exposures commencing during the prenatal period are presented prior to studies with exposures beginning in the postnatal period. Studies in mice and monkeys are presented at the end of this section.

Hughes et al. (162), supported by EPA, examined the offspring of dams treated with soy milk during the lactation period. A similar study was conducted in rats dosed with genistein and is discussed in the CERHR Expert Panel Report on Genistein. This study was conducted in Long Evans hooded rats that were fed a phytoestrogen-free AIN-93G diet in which the soy oil was replaced with corn oil. Dams were randomly assigned to groups given 2%-fat rice milk (controls, n = 2) or 2%-fat soy milk (n = 3) instead of water from PND 1 (day of delivery) to PND 21. The study authors estimated that intake of isoflavones through soy formula was 3.5–8.5 mg/day or 10–30 mg/kg bw/day. Pups had some direct exposure to the soy or rice milk once they were old enough to drink from the dam's bottle. Milks were replaced with water from PND 21 to the end of the study. Litter size was evaluated at birth, and pups were sexed, weighed, and evaluated for anogenital distance. Body weights and anogenital distance were again measured at weaning. Pups were monitored for onset of puberty. Estrous cycles were monitored for 3 weeks in females. On PND 60, offspring were killed and male reproductive organs were weighed. A total of 14 male and 22 female pups exposed to soy milk and 8 male and 18 female pups exposed to rice milk were evaluated. Uteri from 6 offspring/group were fixed in 4% paraformaldehyde for histomorphometry examination and immunohistochemical analyses for proliferating cell nuclear antigen (PCNA), ER α , and progesterone receptor. Statistical analyses included ANOVA and Kruskal-Wallis test. The individual pups rather than the litter were considered the statistical unit. The pup-based analysis was said to have been used because intrauterine position of pups, which was not considered, was said to have a greater impact on variances of outcomes than differences between dams.

Soy milk had no effect on anogenital distance, age of preputial separation, age of vaginal opening, or estrous cycle length. Body weight was significantly higher and anogenital distance adjusted for body weight was significantly lower in female offspring of the soy group at weaning. Soy milk had no effect on relative (to body weight) weights of testis, seminal vesicles, or prostate in adult offspring. Relative epididymis weight was significantly reduced [**by 14%**] in the soy group compared to the rice-milk control. [**Absolute organ weights were not reported.**] The only

3.0 Developmental Toxicity Data

significant effect noted in the uterus was a [12%] increase in progesterone receptor expression in glandular epithelial cells in females of the soy-milk group. Soy milk had no effect on uterine gland numbers, luminal epithelial cell height, uterine proliferation, ER α expression in luminal and glandular epithelial cells, or progesterone receptor expression in luminal epithelial cells. As discussed in the CERHR Expert Panel Report on Genistein, gestational and lactational exposure to genistein also increased expression of the progesterone receptor in uterine glandular epithelial cells. The study authors concluded that exposure of developing rats to isoflavones approximating human exposure levels induced an effect in an estrogen-responsive uterine marker long after cessation of exposure. Concerns were expressed because the progesterone receptor is involved in several reproductive processes.

Strengths/Weakness: Strengths of this study were use of a phytoestrogen-free chow, exposure during an appropriate period (lactation), exposure of rats to soy milk, and estimation of daily isoflavone intake. Weaknesses were the small numbers of litters used/group (2–3) and that the litter was not considered the experimental unit.

Utility (Adequacy) for CERHR Evaluative Process: This study is of limited utility due to the small numbers of animals used.

Ashby et al. (73) examined the effects of Infasoy®, an infant soy formula, and animal feeds on uterotrophic responses and initiation of puberty in rats. The uterotrophic effects are summarized in Section 2.2.4.2. Two separate experiments were conducted. In the first experiment, a control group of 34 rats from 5 litters was fed RM1 [Rat and Mouse No. 1, a soy-based diet] and a comparison group of 29 rats from 6 litters was fed AIN-76 (a phytoestrogen-free diet). In the second experiment, a control group of 49 rats from 9 litters was fed the RM1 diet and a comparison group of 61 rats from 10 litters was given Infasoy® in drinking water. In both experiments, rats were exposed to each diet or treatment on PND 21–55. Body weights and age of vaginal opening and first estrus were monitored. Data were analyzed by ANOVA and ANCOVA, with the litter as the unit of analysis. Results are summarized in Table 30. Vaginal opening and first estrus occurred at a younger age in rats fed the AIN-76A compared to the RM1 diet. Body weights of rats fed the AIN-76-A diet were higher on PND 35 but lower on day of vaginal opening. Age of vaginal opening and first estrus were accelerated in the rats given Infasoy® compared to control rats fed the RM1 diets. Body weights of rats given Infasoy® were higher than control rats on PND 46 but were lower than control rats on the day of vaginal opening and first estrus. As explained in greater detail in Section 2.2.4.2, uterotrophic responses were attenuated by the ER α and ER β antagonist Faslodex, the aromatase inhibitor anastrozole, and the GnRH antagonist antarelix. The study authors noted that sexual development in rodents can be accelerated by exogenous synthetic or dietary estrogens interacting with tissue ERs or through a centrally mediated increase in endogenous estrogens. The study authors concluded that dietary components other than contaminant synthetic estrogens or phytoestrogens can accelerate sexual development in rodents and may therefore be a factor in precocious human development.

Strengths/Weakness: Strengths of the study were use of soy milk, exposures only postnatally (PND 21–55), adequate sample size (n = 9–10), and use of the litter as the experimental unit. Weaknesses include the lack of information on phytoestrogen content of the chow, the failure to monitor liquid consumption, resulting in unknown exposure to soy, the use of a soy-based chow (RM1) with experimental exposure to Infasoy, resulting in 2 sources of soy exposure, the use of only 1 soy formula dose level, and the lack of clarity on how animals were chosen for analysis (presumably all females from all litters evaluated).

3.0 Developmental Toxicity Data

Utility (Adequacy) for CERHR Evaluative Process: This study is of low utility based on the single dose level of soy formula and the complication of exposure to soy in both the diet and the liquid.

Table 30. The Effects of Diet and Soy Formula Given to Rats on PND 21–50

Endpoint ^a	Experiment 1		Experiment 2	
	RM1 diet (control)	AIN-76A diet	RM1 diet (control)	RM1 diet + Infasoy®
Body weight (g)				
PND 21	40.8 ± 4.7	40.2 ± 3.7	37.1 ± 5.7	37.3 ± 5.6
PND 35	107.1 ± 9.1	120.5 ± 8.3 ^b	106.0 ± 12.0	107.6 ± 17.0
PND 46	Not determined	Not determined	156.2 ± 12.3	165.3 ± 14.4 ^b
At vaginal opening	108.6 ± 11.6	97.7 ± 9.0 ^b	102.4 ± 13.2	91.5 ± 10.8 ^b
At first estrus	Not determined	Not determined	115.2 ± 19.8	108.3 ± 18.7
Age (days)				
At vaginal opening	35.1 ± 1.9	31.5 ± 1.2 ^b	34.5 ± 2.0	32.4 ± 1.2 ^b
At first estrus	38.1 ± 2.8	34.6 ± 3.5 ^b	37.1 ± 3.9	35.3 ± 2.7 ^c

^aData expressed as mean ± variance [unspecified but listed as SD in other figures of this report].

^b $P < 0.01$; ^c $P < 0.05$ compared to control

From Ashby et al. (73).

Odum et al. (163), supported by the Alkyl Phenol Ethoxylate Research Council, the Japanese Chemical Industries Association, and the Food Standards Agency [a UK government agency], investigated the effects of different rodent diets on sexual development in the rat. Animals were Wistar-derived Alpk:APfSD rats. The diets included Rat and Mouse No. 3 (RM3), a standard diet in the UK for pregnancy and lactation, RM1, a standard diet for maintenance of rodents, Teklad Global 2016 (from Harlan Teklad), a natural diet made without soy or alfalfa, AIN-76A (from Harlan Teklad), a diet with sucrose, casein, and maize instead of soy and alfalfa, and Purina 5001 (from Purina Mills), a common US diet based on soy and alfalfa. Features of these diets are shown in Table 31. Diet combinations included a specified diet during pregnancy and lactation followed by the same or a different diet for weaned offspring. The pregnancy/weaning treatment diets were RM3/RM1, AIN-76A/AIN-76A, RM3/AIN-76A, Global 2016/Global 2016, and 5001/5001. Pregnant females were begun on their assigned diets on the day sperm were detected in the vaginal smear (GD 0). There were 12 females assigned to each of the 5 diet combinations. Genistein and daidzein content were measured in each diet by GC-MS. Females were maintained on the assigned diets through pregnancy and lactation. Offspring were maintained on their assigned diets until PND 68. From PND 70, offspring were all given the RM1 diet [the diet between PND 68 and 70 was not specified]. All females from 6 litters per treatment group were killed on PND 26, and sex organs were weighed. The remaining females were housed together by litter. Males were culled at weaning to 4 per litter (housed together). Developmental landmarks were monitored, including eye opening, testis descent, vaginal opening, and preputial separation. After vaginal opening, daily vaginal smears were taken until first estrus was identified. Daily vaginal smears were also taken on PND 52–69 to evaluate time spent in estrus. On PND 68, males from 6 litters per treatment group were killed and liver, kidney, and sex organs were weighed. On PND 68, the remaining females were culled to 4 per litter (housed together). The remaining males were killed on PND 128, and the remaining females were killed in estrus on PND 140–144 (after being on the RM1 diet since PND 70). Liver, kidneys, and sex organs were weighed. The RM3/RM1 diet combination was considered the control condition because this combination (for pregnancy/weaning) was used in most of the authors' previous studies. The 5001/5001 diet combination was also evaluated as a control diet, because it is used in many regulatory studies in the US. [Analysis using the 5001 diet as a control was not shown in the paper.] Statistical analysis was performed by ANOVA or ANCOVA (taking weaning weight as a

3.0 Developmental Toxicity Data

covariate) with post hoc Student *t*-test. The litter was the statistical unit. The proportion of animals recorded with a developmental landmark each day was evaluated by Fisher exact test.

Pregnancy outcome and offspring weight results are summarized in Table 32. **[Post-weaning food consumption and weight varied over the course of the experiment. The data graphs are difficult to interpret, but it appears that AIN-76A and Global 2016 may be suboptimal feeds for the post-weaning period in terms of body weight gain, feed consumption, or both. The authors note that the high-energy density of these diets may have influenced feed consumption. The authors imply that during the “recovery phase” after PND 70, when all animals were given RM1, feed consumption and weight tended to become similar among groups, although it did not reach parity in all groups.]** There were no effects of diet on eye opening. The effects of the diets on sex-related developmental landmarks are summarized in Table 33. Post-weaning exposure to AIN-76A appeared to consistently advance male and female pubertal landmarks compared to RM1, while the other 2 diets had less consistent effects. Effects on body weight and reproductive organ weight are summarized in Table 33. Absolute body weight and absolute organ weights were not evaluated for statistical differences; Table 33 shows the effects of diet only with regard to body weight corrected for weaning body weight and with regard to relative organ weights. The authors noted that AIN-76A was associated with body weight gain that persisted during the recovery period, i.e., during the weeks following PND 70 when all animals were on the RM1 diet.

In a second experiment, weanling female rats were obtained at 20–21 days of age. Animals were weaned on RM3 and then switched to RM1, AIN-76A, Global 2016, or 5001 (*n* = 24/group) for 4 days. Half of the animals in each group received, respectively, 0 or 300 µg/kg bw/day sc of the GnRH antagonist antarelix for 3 days starting on PND 20 or 21. Animals were killed 24 hours after the last dose of antarelix. Body and uterine weights were compared with ANCOVA, considering initial body weight (for final body weight) and final body weight (for uterine weight) as covariates. The individual female was the statistical unit. Results are summarized in Table 34 for final body weight (adjusted for initial body weight) and relative uterine weight. Absolute body and uterine weights were not analyzed statistically. Antrelax increased feed consumption by 24–40% **[data not shown]**.

The authors concluded that diet could affect sexual development, but they observed that the effects on sexual development were not consistent with presumed estrogenicity of diets like 5001 that are high in phytoestrogens. They noted, for example, that the greatest effect on sexual development appeared with post-weaning exposure to AIN-76A, a soy- and alfalfa-free diet with negligible phytoestrogen content. They further noted that in the uterotrophic assay, AIN-76A and 5001 produced an increase in relative uterine weight that was prevented with a GnRH antagonist, suggesting that the effects of diet involved alterations in hypothalamic function rather than simple estrogen exposure.

3.0 Developmental Toxicity Data

Table 31. The Five Diets Used by Odum et al.

Component	Diets				
	RM1	RM3	AIN-76A	Global 2016	5001
Protein content (%)	14.7	22.3	20.0	16.7	23.4
Energy (kJ/g)	10.9	11.5	15.7	13.3	12.7
Soy content (%)	6.0	13 ^a	0	0	>18 ^a
Alfalfa content (%)	0	0	0	0	3 ^a
Genistein (ppm) ^b	45	105	Negligible	Negligible	175
Daidzein (ppm) ^b	30	70	Negligible	Negligible	110

^aEstimated by study authors.

^bEstimated by CERHR from a graph in the paper.
From Odum et al. (163).

Table 32. Effects of Diet on Pregnancy and Lactation in Rats

Parameter	Diet combinations (pregnancy/weaning) compared to RM3/RM1			
	AIN-76A/AIN-76A	RM3/AIN-76A	Global 2016/Global 2016	5001/5001
Dam body weight				
Pregnancy	↔	↔	↔	↔
Lactation	↓3% ^a	↔	↓7% ^a	↔
Litter survival to weaning	“↑”	“↑”	↔	↔
Offspring weight ^b				
Birth	↓	↔	↔	↔
Weaning	↔	↔	↓	↓
Post-weaning	↑	↑	↔	↑

↑, ↓, ↔ Significant increase, decrease, no change compared with RM3/RM1 diet. “↑” indicates study author conclusion of an increase without statistical significance.

^aEstimated from a graph.

^bResults reported as suggested by the text of the paper. The data figures are not marked for significant differences.
From Odum et al. (163).

Table 33 Effects of Diet on Rat Developmental Landmarks and Reproductive Organ Weights

Parameter	Diet combinations (pregnancy/weaning) compared to RM3/RM1			
	AIN-76A/AIN-76A	RM3/AIN-76A	Global 2016/Global 2016	5001/5001
Testis descent				
Age	↑3%	↓3%	↔	↔
Body weight	↔	↔	↓8%	↔
Preputial separation				
Age	↓5%	↓7%	↔	↓4%
Body weight	↔	↔	↔	↔
Vaginal opening				
Age at onset	↓7%	↓10%	↔	↔
Body weight at completion	↔	↓12%	↓11%	↔
Age at first estrus	↔	↓11%	↔	↓8%
Body weight (adjusted for weaning weight) and relative organ weight)				
Females on PND 26				
Body weight	↑10%	↑11%	↓5%	↑14%
Uterus (blotted)	↑37%	↑83%	↑28%	↑77%
Uterus (dry)	↑30%	↑78%	↑24%	↑70%
Vagina	↑17%	↑42%	↑16%	↑27%
Cervix	↔	↑45%	↔	↔

3.0 Developmental Toxicity Data

Parameter	Diet combinations (pregnancy/weaning) compared to RM3/RM1			
	AIN-76A/AIN-76A	RM3/AIN-76A	Global 2016/Global 2016	5001/5001
Ovary	↔	↑11%	↔	↔
Males on PND 68				
Body weight	↑16%	↑9%	↔	↑13%
Liver	↑12%	↑10%	↔	↔
Kidney	↑7%	↑26%	↔	↑22%
Testis	↓10%	↔	↔	↔
Epididymis	↓9%	↔	↔	↔
Seminal vesicle	↔	↔	↔	↔
Prostate	↔	↔	↔	↔
Males on PND 128				
Body weight	↑10%	↑14%	↔	↔
Liver	↔	↔	↔	↔
Kidney	↔	↔	↔	↑9%
Testis	↓9%	↔	↔	↔
Epididymis	↔	↔	↔	↔
Seminal vesicle	↔	↔	↔	↔
Prostate	↔	↔	↔	↔
Females on PND 140–144				
Body weight	↑9%	↑13%	↔	↔
Liver	↔	↔	↔	↔
Kidney	↑22%	↑22%	↔	↔
Uterus (blotted)	↔	↔	↔	↔
Uterus (dry)	↓12%	↔	↔	↔
Vagina	↔	↔	↔	↔
Cervix	↔	↔	↔	↔
Ovary	↔	↔	↔	↔

↑, ↓, ↔ Significantly increased (older), decreased (younger), unchanged compared with RM3/RM1 diet.
From Odum et al. (163).

Table 34. Effects of Diet on Relative Uterine Weight

Endpoint	Diet and antarelix dose, µg/kg bw/day							
	RM1		AIN-76A		Global 2016		5001	
	0	300	0	300	0	300	0	300
Body weight ^a	Control	↔	↑12%	↔	↔	↔	↑14%	↔
Relative uterine weight	Control	↔	↑50%	↔	↔	↔	↑68%	↔

↑, ↔ Significant increase or no change compared to control.

^aAdjusted for initial body weight

From Odum et al. (163)1}.

Strengths/Weakness: Strengths of the study were analysis of diet for genistein and daidzein content and analysis of appropriate endpoints. Weaknesses were differences in dietary protein and metabolizable energy content. Exposure through chow is a strength in its relevance to human exposure and a weakness in permitting only imprecise estimates of dose levels.

Utility (Adequacy) for CERHR Evaluative Process: This study is of low-to-moderate utility based on the fairly large number of animals examined and the appropriateness of endpoints.

3.0 Developmental Toxicity Data

Lund et al. (58), supported by the National Science Foundation and Brigham Young University, conducted a series of experiments to examine reproductive and developmental effects in Long Evans rats given phytoestrogen-containing feed. The F₁ rats used in these studies were born to 5 F₀ dams/group that were fed throughout the gestation and lactation periods with a soy-based diet containing phytoestrogens 600 µg/g diet (phyto-600) or a phytoestrogen-free fish meal-based diet. The offspring were weaned on PND 30 to the same diets as their dams. Diets were similar in protein, fat, amino acid, mineral, and vitamin content. Assessment of estrous cycles in F₀ rats is discussed in Section 4.2. Data were analyzed by ANOVA followed by Bonferroni post hoc comparison.

In the first part of the experiment, female offspring (n = 16/group) were monitored for day of vaginal opening and estrous cyclicity from 60 to 73 days of age. Body weights were measured in male and female offspring at 50 days of age (n = 20/sex/group). In the phyto-600 compared to phytoestrogen-free group, day of vaginal opening was significantly delayed (mean ± SEM = 39.3 ± 0.5 days compared to 35.0±0.3 days in the control). Diet had no effect on length or regularity of estrous cycles [**data were not shown**]. Body weights at 50 days of age were significantly lower in males and females fed the phyto-600 compared to the phytoestrogen-free diet [**by ~10% in females and ~6% in males**].

In the second part of the study, 5–7 rats/sex/group given the phyto-600 diet continued to receive that diet, and 6 rats/sex/group were switched to the phytoestrogen-free diet at 80 days of age. Rats remained on each diet for 40 more days (until 120 days of age), at which time they were killed. Blood from 2 rats/group was pooled for measurement of serum phytoestrogen levels by GC-MS and measurement of 17β-estradiol and testosterone levels by radioimmunoassay (RIA). Body and prostate weights and volume of the sexually dimorphic nucleus of the pre-optic area (SDN-POA) were determined (n = 5–7/sex/group). Body weights of male and female rats switched to the phytoestrogen-free diet were significantly higher than body weights of rats that remained on the phyto-600 diet [**7% higher in males and 13% higher in females**]. Males switched to the phytoestrogen-free diet also had higher absolute [**~ 21%**] and relative [**~ 15%**] prostate weights. Diet had no effect on serum testosterone or 17β-estradiol level in males. Males that remained on the phyto-600 diet had a significantly higher SDN-POA volume than males switched to the phytoestrogen-free diet. An increase in SDN-POA volume in females switched to the phytoestrogen-free diet compared to females remaining on the phyto-600 diet approached statistical significance. Total brain weight did not differ between dietary groups.

The study authors concluded that, “. . . phytoestrogens have considerable effects on hormonally sensitive somatic, reproductive organ, and neuroendocrine parameters. . .”

Strengths/Weakness: Strengths of this study were detailed descriptions of diets, measurement of serum phytoestrogens, and examination of appropriate endpoints. Weaknesses were comparison of only 1 phytoestrogen-containing chow to phytoestrogen-free chow, and the small number of animals used (n = 5). Exposure through chow is a strength in its relevance to human exposure and a weakness in permitting only imprecise estimates of dose levels. In addition, the litter was not considered the experimental unit, and reproductive capability of animals was not determined.

Utility (Adequacy) for CERHR Evaluative Process: This study is of limited utility. The effect of switching the diet at 80 days of age is not relevant to developmental events.

Masutomi et al. (164), in a study supported in part by grants from the Japanese Ministry of Health, Labor, and Welfare, examined the effects of maternal diet in rats perinatally exposed to ethinyl estradiol. Pregnant Sprague Dawley rats (n = 15/group) were divided into groups fed

3.0 Developmental Toxicity Data

CRF-1, a regular rodent diet containing soy-derived proteins, or a soy-free diet. The CRF-1 diet contained genistin at 10.2 mg/100 g diet and daidzin at 8.7 mg/100 g diet. Soy-free diet was prepared according to the NIH-07 formulation, except that soy meal and oil were replaced with ground corn, wheat, and corn oil. **[It also appears that soy-free diets may have used fish or gluten meal as the source of protein.]** On GD 15 (day of vaginal plug = GD 0), 7 dams from each dietary group were given 0.5 ppm ethinyl estradiol through diet. Dosing was continued through PND 10 (day of delivery = PND 1). Pup anogenital distance was measured on PND 2. Five offspring/sex/group were necropsied on PND 21 for measurement of organ weight. Eight offspring/sex/group were weaned to the CRF-1 diet and evaluated for onset of puberty. Estrous cyclicity was monitored from 8 to 11 weeks of age. Offspring were killed on PND 77–79, with females killed during diestrus. Organs were weighed and examined histologically. The litter was considered the statistical unit in evaluations conducted during the lactation period. For offspring data collected after weaning, individual animals were considered the statistical unit. Statistical analyses included ANOVA, Fisher exact test, and Mann Whitney *U*-test.

Because the purpose of this Expert Panel evaluation is to evaluate the effects of soy formula, discussion of results will focus on effects in groups fed the soy-containing versus soy-free diets and dietary effects on ethinyl estradiol responses. Diets did not affect maternal body weight gain during gestation or lactation. Litter size was also unaffected by diet. On PND 2, male pups from the soy-free diet group weighed significantly less than males in the soy diet group. Offspring body weight gains on PND 10–12 in both sexes were significantly lower in the soy-free than the soy-diet group. Diet alone had no effect on absolute anogenital distance, but anogenital distance adjusted for body weight was slightly higher in females of the soy-free versus soy diet group. **[Diet was said to interact with ethinyl estradiol effects on anogenital distance in females, but the type of interaction was not clear.]** At the PND 21 necropsy, body weights of females in the soy-free groups were lower than in the soy diet group. Ovary weight relative to body weight was higher in the soy-free than the soy-diet group.

In offspring evaluated in adulthood, body weight gain on PND 21–42 was significantly reduced only in males from the ethinyl estradiol/soy-free diet group. Day of vaginal opening was significantly accelerated and body weight at vaginal opening was significantly lower in females from the ethinyl estradiol/soy-diet group. Body weight at preputial separation was significantly lower in the soy-free diet compared to the soy-diet group. Necropsies conducted in adult offspring revealed that the soy-free compared to the soy diet significantly increased relative pituitary weights in males, decreased absolute and relative pituitary weights in females, and increased absolute and relative adrenal weights in males. Ethinyl estradiol induced a significant reduction in absolute and relative ovary weight only in offspring of rats fed the soy diet. The most prominent effects in female offspring exposed to ethinyl estradiol were irregular estrous cycles and histopathologic changes in reproductive tissues as outlined in Table 35. The number of rats with estrous cycle irregularities was statistically altered only in the soy-diet group, but the numbers were increased in both dietary groups exposed to ethinyl estradiol in utero. Incidence and severity of lesions in the ovary, uterus, vagina, mammary gland, and pituitary were greater in offspring of rats exposed to ethinyl estradiol and fed soy compared to soy-free diets.

The study authors concluded that typical estrogenic responses to ethinyl estradiol were enhanced by soybean-derived factors.

3.0 Developmental Toxicity Data

Table 35. Adverse Effects in Rats Treated with Ethinyl Estradiol and Given Soy-containing or Soy-Free Feed

Parameter (n affected of 8/group)	Ethinyl estradiol:	Soy diet		Soy-free diet	
		0	0.5 ppm	0	0.5 ppm
Irregular estrous cycles	0	8 ^a	1	5	
Persistent diestrus	0	0	0	3	
Persistent estrous	0	6	0	1 ^b	
Increased ovarian follicles/decreased corpora lutea (±/ +/+/+/++)	0	8 (0/1/0/7)	0	3 (0/2/0/1) ^b	
Uterine epithelial hypertrophy (+/+/++)	0	8 (3/5) ^a	0	2 (0/2) ^b	
Endometrial squamous metaplasia (+/+/++)	0	4 (3/1)	0	1 (0/1)	
Vaginal mucosal hyperplasia	0	8 ^a	0	1 ^b	
Vaginal diestrus mucosal change	8	0	8	6 ^b	
Mammary lobular hyperplasia (+/+/+/++)	0	8 (1/6/1) ^a	1 (1/0/0)	4 (3/1/0) ^b	
Anterior pituitary hyperplasia	0	8 (0/2/6) ^a	0	3 (1/2/0) ^b	

± minimal, + slight, ++ moderate, and +++ severe.

^aStatistically significant compared to same diet without ethinyl estradiol.

^bStatistically significant compared to ethinyl estradiol group fed soy diet.

From Masutomi et al. (164).

Strengths/Weakness: Strengths of the study were use of soy-free chow with similar nutritional contents as soy-containing chow, oral exposure route, determination of feed consumption, standardization of litter size (PND 10), and killing of adult females at the same stage of the estrous cycle. Phytoestrogens were measured in chow, but no methods and few data were presented. Weaknesses were the use of only 7 animals/treatment group, and lack of determination of reproductive capability.

Utility (Adequacy) for CERHR Evaluative Process: The study is of limited utility based on the small number of animals used.

Mäkelä et al. (165), support not indicated, examined prostate effects in offspring of rats fed soy-containing or soy-free diets. From 1 week prior to mating through gestation and lactation, Sprague Dawley rats were fed a commercial rat diet containing 13% soy (control group, n = 2 dams) or a soy-free, casein-based diet (n = 3 dams). Pups were weaned at 21 days of age to the commercial (soy-containing) diet. Offspring were killed at 10 weeks of age, and prostates were removed and weighed in 13 or 14 animals/group. 5 α -Reductase production was measured by determining levels of metabolites following incubation of prostates in media for 30 minutes. Prostates were then fixed in Bouin fluid for sectioning and morphometric analysis. Data were analyzed by ANOVA followed by *t*-test with Bonferroni correction. Prostate weights were significantly higher [**by ~40%**] in offspring of rats fed the soy-free diet. Diet had no effect on prostate 5 α -reductase activity or volume density of epithelium, lumina, or intra-acinar stromal tissues.

Strengths/Weakness: Weaknesses of the study were the lack of comparison of chows, the small number of animals/group (n = 2–3), and evaluation of only males.

Utility (Adequacy) for CERHR Evaluative Process: The study is not useful because of the small numbers of animals used and the very small number of endpoints examined.

Atanassova et al. (166), supported by the European Center for the Ecotoxicology of Chemicals and AstraZeneca, examined the effects of neonatal exposure to weak and strong estrogens on pubertal spermatogenesis and long-term changes in the reproductive system of male rats. As part

3.0 Developmental Toxicity Data

of this study, adult female Wistar rats were fed standard diets (15.5% soy meal) or soy-free diets (soy substituted by fishmeal and cereal content increased from 64 to 78%) for 3 weeks prior to mating and through mating, pregnancy, and lactation. Male offspring of rats fed soy-free diets were maintained on soy-free diets from weaning until termination. An unspecified number of males born to mothers on the soy-free diets received sc injections of 4 mg/kg bw/day genistein [purity not specified] in phosphate-buffered saline vehicle on PND 2–18, and that part of the study is discussed in the CERHR Expert Panel Report on Genistein. Males from the soy-free control group were compared to males in the standard diet control group. [The total number of rats treated was not stated, but 7–14 rats/group were evaluated.] On PND 18 and 25, rats were killed and testes were fixed in Bouin fluid. Testicular cell numbers and seminiferous tubule lumen formation were determined by standard point counting of cell nuclei. Apoptosis was assessed by DNA fragmentation detected by in situ DNA 3'-end labeling. Plasma FSH and inhibin B were measured by RIA and enzyme-linked immunosorbent assay (ELISA), respectively. In addition, mating and fertility were examined in adult rats (80–90 days old) by placing them in a cage with an unexposed female for 7 days. Statistical significance was determined by ANOVA.

Results and statistical significance for endpoints characterizing pubertal spermatogenesis in 18- and 25-day-old rats are listed in Table 36. The study authors noted that the increase in spermatocyte nuclear volume per Sertoli cell nuclear volume in rats fed soy-free compared to standard diets on PND 18 suggested that dietary soy retarded an aspect of pubertal spermatogenesis. For parameters also assessed on PND 25, the only significant effect that remained was the increase in spermatocyte nuclear volume per Sertoli cell nuclear volume in soy-free compared to standard-diet controls. Testis weights in adult rats (90–100 days old) from the soy-free group were significantly (8%) higher compared to rats in the standard-diet group, and testis weights of rats in the genistein group were similar to those in the soy-free group. Animals in the soy-free control group were not mated.

In a larger study reported in this paper, body weight, testis weight, and plasma FSH levels were compared in 24 litters from soy-free groups and 29 litters from standard-diet groups. Male rats were evaluated at 90–95 days of age. Rats in the soy-free group had significantly higher body (5.7%) and testis weights (3.6%) and significantly reduced plasma FSH levels (11.1%).

The study authors concluded that “the presence or absence of soy or genistein in the diet has significant short-term (pubertal spermatogenesis) and long-term (body weight testis size, FSH levels, and possibly mating) effects on males.”

Table 36. Effects of Neonatal Exposures to Soy-Free Diet or Genistein on the Reproductive System of Male Rats

Effect	Soy-free control compared to standard-diet control	Genistein 4 mg/kg bw/day (soy-free diet) compared to soy-free control
Germ cell apoptotic index, PND 18	↔	↑
Germ cell apoptotic index, PND 25	↔	↔
Seminiferous tubule lumen formation, PND 18	↔	↓
Plasma inhibin B, PND 18	↔	↔
Sertoli cell nuclear volume/testis, PND 18	↑	↔
Plasma FSH, PND 18	↔	↓
Plasma FSH, PND 25	↔	↔
Spermatocyte/Sertoli cell nuclear volume, PND 18	↑	↓
Spermatocyte/Sertoli cell nuclear volume, PND 25	↑	↔

↑,↓ Statistically significant increase, decrease; ↔ no effect. From Atanassova et al. (166).

3.0 Developmental Toxicity Data

Strengths/Weakness: A strength of this study was the large numbers of litters examined (n = 24–29). Weaknesses included lack of comparison of dietary nutrients, examination of only males, and lack of determination of reproductive capability. Exposure through chow is a strength in its relevance to human exposure and a weakness in permitting only imprecise estimates of dose levels.

Utility (Adequacy) for the CERHR Evaluative Process: The endpoints examined in this study are of limited utility alone in determining developmental effects.

McVey et al. (167), of Health Canada, reported serum and testicular androgen levels in F₁ rats exposed to a soy supplement in a multigeneration study in Sprague Dawley rats. **[The full report of the multigeneration study is not yet available (G. Cooke, personal communication, December 2, 2004).]** Six diets were used in the study. The control diet was a soy-free casein-based diet (AIN93G) in which there were no detectable isoflavones by HPLC after β -glucuronidase digestion. A second diet was similar to the control diet but included a commercial soy-protein concentrate (Pro Fam 930) in place of casein. This diet was supplemented with a commercial soy-isoflavone concentrate (Novasoy®) at 4 different levels of supplementation. The total isoflavone levels in the soy-containing diets were 31.7, 36.1, 74.5, 235.8, and 1046.6 mg/kg feed [ppm]. Of the total isoflavones that were measured by HPLC after β -glucuronidase, 33–39% was daidzein, 8–10% was glycitein, and 52–59% was genistein. The authors noted that the range of isoflavone content in the diets represented a range of human consumption of soy isoflavones from the negligible amounts in North American diets to the high levels consumed by infants on soy formula. Diets were provided ad libitum from 50 days of age to male and female rats. After 70 days on the diets, animals were mated to produce an F₁ generation, which was weaned on PND 21 to the same diet assignment as the parents. F₁ males were killed on PND 28, 70, 120, 240, or 360 for measurement of testosterone and dihydrotestosterone in serum and in homogenized testes using a commercial ELISA kit. ANOVA with post hoc Tukey test was used for comparisons.

Based on feed consumption and body weight data, estimated F₁ isoflavone intakes in the 6 diet groups at PND 28 were 0, 0.9, 1.2, 2.0, 6.3, and 29.5 mg/kg bw/day. On PND 120, estimated F₁ isoflavone intakes were 0, 0.9, 1.0, 2.1, 7.3, and 28.7 mg/kg bw/day. There were no treatment-related alterations in mean body weight or feed consumption at any time during the study. Testis weights were increased by about 30% in the PND 28 males on the Novasoy®-containing diets (total isoflavone intakes ≥ 2.0 mg/kg bw/day, n = 5 or 6/treatment group). Serum testosterone peaked in all groups at the PND 70 measurement, consistent with puberty in rats, and remained elevated at PND 120 in the groups exposed to the diets with the 2 highest isoflavone levels. There was no difference between groups at any time point other than PND 120 (n = 12/group/time point except n = 6/group on PND 360). Serum dihydrotestosterone followed the same pattern, with PND 120 levels statistically higher in the 2 highest isoflavone groups than in the other groups. There was no significant alteration in the ratio between testosterone and dihydrotestosterone at any time point. Intra-testicular testosterone reached a peak on PND 120, at which time values in all soy-supplement groups appeared graphically higher than those in the control group but without evidence of dose-dependency and with a statistical difference from control only for the second-highest isoflavone-diet group. Intra-testicular dihydrotestosterone (ng/g testis) appeared to show a numerical decrease on PND 28 **[the only time point for which data were given]** as dietary isoflavone concentration increased, but no statistically significant changes were identified by the study authors. **[Trend test performed by CERHR was significant at P = 0.015.]** The authors concluded that developmental exposure to isoflavones could alter testicular weight and

3.0 Developmental Toxicity Data

androgen levels, although the mechanism for the apparent modulation of Leydig cell androgen production was not known.

Strengths/Weaknesses: Strengths of the study were determination of dietary isoflavone content, use of 5 different soy doses in chow, and monitoring of food consumption and soy intake. Weaknesses included examination of only males, lack of dose-response relationship for altered endpoints (e.g., plasma and/or testicular testosterone and dihydrotestosterone), lack of determination of reproductive capability, and lack of indication of the number of litters represented.

Utility (Adequacy) for CERHR Evaluation Process: This study is of low-to-moderate utility based on the limited usefulness of the endpoints.

McVey et al. (168), support not indicated, examined the effects of lifetime exposure to isoflavones on steroidogenic enzyme activities in rat testes. Throughout gestation and lactation, female Sprague Dawley rats [number not reported] were fed one of six diets containing casein or soy as the protein source. The casein diet served as the control. The soy diets were alcohol-extracted to remove naturally occurring isoflavones and then fortified with Novasoy, an isoflavone supplement, at the isoflavone concentrations listed in Table 37. Male offspring were weaned on PND 21 and fed the same diets as their mothers. Male offspring (5–9/group) were killed on PND 28, 70, 120, 240, or 360. Enzyme activities were determined for 3 β -hydroxysteroid dehydrogenase, CYP17, 17 β -hydroxysteroid dehydrogenase, and 5 α -reductase. Expression of messenger ribonucleic acid (mRNA) and protein was determined for 3 β -hydroxysteroid dehydrogenase, CYP17, and P450 SCC, the mitochondrial cholesterol side chain cleavage enzyme involved in the first step of androgen production. Data were analyzed by linear and non-linear regression, ANOVA, and Tukey test. Enzymes activities declined with age in all dietary groups. As noted in Table 37, the only effects on enzyme activities occurred on PND 28 and included increased 3 β -hydroxysteroid dehydrogenase activity in rats fed diets containing isoflavones 235.6 and 1046.6 mg/kg diet and increased CYP17 activity in rats fed diets containing isoflavones 235.6 mg/kg diet. There were no differences compared to controls at later time periods or in other enzyme activities. There were also no differences in mRNA or protein expression. According to the study authors, these results suggest that isoflavones at levels consistent with infant exposures alter testicular enzyme activities in rats during development.

Table 37. Dietary Effects on Steroidogenic Enzyme Activities in Rats

Protein Source ^a	Isoflavones (mg/kg feed [ppm])				Results
	Genistein	Daidzein	Glycitein	Total	
Casein	Not detected	Not detected	Not detected	Not detected	Control diet
Soy	18.6	10.5	2.6	31.7	↔
	21	12.3	2.8	36.1	↔
	39.3	27.6	7.6	74.5	↔
	124.4	90.9	20.5	235.6	↑3β-Hydroxysteroid dehydrogenase enzyme activity/testis on PND 28; ↑CYP17 enzyme activity/microsomal protein and /testis on PND 28
	544.8	412.3	89.5	1046.6	↑3β-Hydroxysteroid dehydrogenase enzyme activity/microsomal protein and /testis on PND 28

↔ No significant effect; ↑Statistically significant increase

^aThe soy-based diets were alcohol extracted to remove naturally-occurring isoflavones and then supplemented with varying amounts of isoflavones.

From McVey et al. (168).

Strengths/Weaknesses: Strengths of this study include the use of 5 different dose levels of soy, including doses with relevance to human exposure, and the measurement of isoflavone content of the diet. Weaknesses for this evaluation include the lifetime exposure to soy, the examination of only male offspring, the fairly small numbers of animals per group (n = 5–9), and the lack of consideration of litter effects. The endpoints evaluated (steroid metabolizing enzyme activities and mRNA and protein expression) are of little utility alone, but may provide mechanistic information useful in interpreting results from other studies.

Utility (Adequacy) for CERHR Evaluation Process: This study is of low utility based on inappropriate exposure time. It is also difficult to reconcile the results of this study with those reported earlier by this group using the same dietary conditions (167). In their previous study, the authors demonstrated that serum testosterone and dihydrotestosterone were slightly (but not significantly) decreased at PND 28 compared to casein-exposed controls and were increased at PND 120. However, in this study, enzyme activities were altered at PND 28 but not at later time points, including PND 120. Additionally, the enzyme activity changes reported in this study were not dose-related.

Robertson et al. (169), supported by the National Health and Medical Research Council of Australia, examined the effects of a soy-based diet on aromatase-knockout mice. The knockout mice, which are unable to synthesize endogenous estrogens, are initially fertile, but at around 18 weeks of age, spermiogenesis is disrupted as a result of a post-meiotic defect. Groups of wild-type and aromatase-knockout mice were fed a diet that contained 10% soy meal or a soy-free diet. Isoflavone levels were reported at 146 mg/g soy-based diet. The study authors stated that each type of diet was fed to mice beginning at birth. **[It is not clear if the diets were fed to offspring and parents. Most likely, the parental rats were exposed because the authors stated that all knockout mice were fed the soy-free diet for 2 years prior to the study, with the exception of 10 mating pairs fed the soy-containing diet.]** One set of wild-type and knockout mice (n=6–12/group) fed either the soy-free or soy-containing diet and exposed during the same time period

3.0 Developmental Toxicity Data

were killed at 14 weeks of age. A second set of wild-type and knockout mice fed the soy-free diet (n=11–12/group) were killed at 1 year of age, and data obtained from these mice were compared with data obtained from mice fed the soy-containing diet in a previous study (n=6–8/group). **[The Expert Panel notes that 1-year-old animals from the two different diet groups were not exposed concurrently.]** Parameters examined included terminal body weight, testis weight, testicular morphology, spermatogenic germ cells in the testis, and serum LH and FSH levels. Statistical analyses included ANOVA and least-squares significant difference test.

Terminal body weights were significantly higher in 14-week-old wild-type **[24% higher]** and knockout mice **[17%]** and in 1-year-old knockout **[25%]** mice fed the soy-free versus soy-containing diet. Testis weight was not affected by diet or genotype. Qualitative evaluations of testes revealed more pronounced disruption of spermatogenesis in 1-year-old mice fed the soy-free versus soy-containing diet **[data not shown]**. Quantitative testicular observations that were statistically significant are summarized in Table 38. Few and minimal effects were observed in 14-week-old mice. The study authors noted that at 1 year of age, knockout mice fed the soy-containing diet had increased spermatogenic cell numbers compared to knockout mice fed the soy-free diets. Compared to knockout mice fed soy-free diet, the knockout mice fed the soy-containing diet had significantly lower **[40%]** serum FSH levels, but there were no significant effects of dietary exposure on LH levels in knockout mice. The study authors concluded that low levels of dietary phytoestrogens exert biological effects on the testis that are independent of effects on the pituitary-gonadal axis.

3.0 Developmental Toxicity Data

Table 38. Summary of Significant Effects in Wild-Type and Aromatase-Knockout Mice Fed Diets With and Without Soy

Parameter	Genotype and dietary comparisons ^a			
	KO-soy ⁺ vs. KO-soy ⁻	KO- soy ⁺ vs. WT- soy ⁺	KO- soy ⁻ vs. WT- soy ⁻	WT-soy ⁺ vs. WT- soy ⁻
<i>14 weeks of age</i>				
Seminiferous tubule diameter	↑13%	↔	↔	↑8.3%
Seminiferous tubule length	↓12%	↔	↔	↓13%
Testicular spermatogonia numbers	↓29%	↓29%	↔	↔
Testicular round spermatid numbers	↑22%	↔	↔	↔
Sertoli cell numbers	↔	↑7.7%	↔	↓7.1%
<i>1 year of age</i>				
Seminiferous tubule lumen volume	↑550%	↔	↓80%	↔
Testicular epithelial volume	↑160%	↓32%	↓79%	↓21%
Testicular interstitial volume	↔	↔	↑109%	↑45%
Seminiferous tubule diameter	↑31%	↔	↓33%	↔
Seminiferous tubule length	↔	↔	↓50%	↓14%
Testicular spermatocyte numbers	↑317%	↔	↓81%	↔
Testicular round spermatid numbers	↑360%	↓54%	↓90%	↔
Testicular elongated spermatid numbers	↑400%	↓56%	↓92%	↔
Total germ cell numbers/Sertoli cell numbers	↑240%	↓55%	↓86%	↔

^aPercent changes were estimated from a graph by CERHR.

↑, ↓, ↔ Significantly increased, decreased; KO aromatase-knockout mice; WT wild-type mice; soy⁺ containing diet, soy⁻ soy-free diet.

From Robertson et al. (169).

3.0 Developmental Toxicity Data

Strengths/Weaknesses: Strengths are that chow was analyzed for isoflavone content and that the study used reasonably sized groups (n = 6–12). The lifetime exposure to soy and the use of only 1 dose level are weaknesses.

Utility (Adequacy) for CERHR Evaluation Process: This study is of low utility based on the inappropriate exposure period and use of only a single dose level of soy. However, this study did demonstrate that estrogen is required for normal spermatogenesis and that the phytoestrogens in soy can partially compensate for the inability to synthesize estrogen in aromatase-knockout mice.

Mäkelä et al. (79), supported by Turku University Foundation, Yrjö Jahnsson Foundation, and Emil Aaltonen Foundation, evaluated the anti-estrogenic effects of dietary soy in developing male Han-NMRI mice. Animals were given either a standard laboratory chow containing 7% roasted soybean meal or a commercial soy-free feed. Female mice were fed either the soy diet or the soy-free diet during pregnancy and lactation [**n not given**]. At weaning, half the males in each litter were continued on their dams' diet and half were switched to the opposite diet (n = 27–29/group). At 2 months of age, urethrostomatic blocks were harvested for dissection of seminal vesicles, coagulating gland, ventral prostate, and dorsolateral prostate. Relative weights of these organs and testes were recorded, and histopathology was evaluated on the reproductive organs of 5 animals/group. An additional experiment was performed using males exposed during pregnancy, lactation, and thereafter to 1 of the 2 diets (15–19/group) with harvesting and examination of reproductive organs at 12 months of age. Other experiments described in this paper used the same design (exposure during pregnancy, lactation, and thereafter), but also treated male pups with sc diethylstilbestrol on the first 3 days of life. Evaluations of the reproductive organs of these males were performed at 2, 9, or 12 months of age (n = 13–19/group). Statistical analysis used the Student-Newman-Keuls multiple-range test, Fisher exact test, or ANOVA with post hoc *t*-test. [**The number of animals in the data tables were lower than the number of animals identified in the Methods section for some experiments.**]

Coagulating gland and combined prostate lobe relative weights were increased in 2-month-old males fed a soy diet after weaning, following a soy-free diet given to the dam during pregnancy and lactation, when compared to animals exposed to soy during pregnancy and lactation and after weaning. The relative weight of the prostate lobes was also significantly higher than the comparable organ weights of males placed on a soy-free diet after weaning, regardless of which diet their dams had been fed. The increase in prostatic lobe relative weight in the group fed soy after being weaned from soy-free dams was 11–19%. There were no body weight changes or accessory gland histologic alterations associated with soy feeding status. When males were evaluated at 12 months after exposure to the same soy-free or soy-containing diets as their dams received during pregnancy and lactation, relative reproductive organ weights were increased by soy feeding. The magnitude of the increase was 40% for ventral prostate, 60% for coagulating gland, 63% for dorsal prostate, 35% for seminal vesicles, and 20% for testes. There were no body weight changes or histological differences associated with soy feeding status. In neonatally estrogenized males evaluated at 2 months of age, soy feeding of dams and offspring was associated with a 46% increase in relative weight of the coagulating gland and an 81% increase in relative weight of dorsolateral prostate, without a change in body weight. There was a soy-associated decrease in the proportion of animals with severe dysplasia when the accessory reproductive organs were histologically evaluated at 9 months of age (3/10 compared to 8/10). The authors also stated that the incidence of severe dysplasia was decreased by soy feeding at 12 months, although the difference between groups was not statistically significant (7/11 compared to 12/14 [**P = 0.76, Fisher exact test**]).

3.0 Developmental Toxicity Data

The authors concluded that there was an anti-estrogenic effect of feeding a soy diet during male development because of amelioration of diethylstilbestrol-associated prostatic growth inhibition and because of a delay in diethylstilbestrol-associated prostatic dysplasia. There was no conclusion concerning the differential effects of soy exposure of dams during pregnancy and lactation or soy exposure of offspring after weaning.

Strengths/Weaknesses: A strength was that the 2 diets were analyzed and found to be similar in protein, vitamin, and mineral content. In addition, urinary isoflavones and lignans were measured. Weaknesses were lack of indication of the number of animals/group and apparent lack of consideration of the litter as experimental unit. Exposure through chow is a strength in its relevance to humans and a weakness in permitting only imprecise estimates of dose levels.

Utility (Adequacy) for CERHR Evaluation Process: The study is of low utility based on lack of experimental details.

Sharpe et al. (170), support not indicated, fed male marmoset offspring a soy-based infant formula (Wysoy®) or a cow milk-based formula (SMA Gold™). The animals included 13 pairs of co-twins assigned to different formulas to permit paired statistical tests and 4 additional animals. At 4–5 days of age, the marmoset offspring were intermittently separated from their mothers by a wire mesh divider, permitting mothers and young to be within sight of one another. The periods of separation included the 8-hour work-day on weekdays and about 2 hours on weekends. During this time, the infants remained with their fathers and siblings in the family cage. Because marmoset young typically receive care (other than feeding) from their fathers and older siblings, these periods of separation did not appear to distress either the infants or the mothers. During the periods of separation, infants were hand-fed their assigned formulas using a soft tube connected to a syringe. The formulas were prepared according to the instructions for human infant feeding and were administered in quantities limited only by the marmoset infant's appetite. Animals were formula-fed 3 or 4 times on weekdays and once or twice on weekend days. Except for periods of separation, the marmoset infants were caged with their mothers and permitted to nurse. Based on the composition of the formulas and the amount consumed, the investigators estimated that the marmoset infant intake of isoflavones during the 5–6 weeks of the study was 1.6–3.5 mg/kg bw/day. On PND 18–20 and 35–45, infant blood was taken for plasma testosterone determination by ELISA. In 7 pairs of twins, the second blood sample was obtained by cardiac puncture after the animals were killed for evaluation. The earlier blood sample in these animals and both blood samples in the animals that remained alive were obtained from the femoral vein. Testes and pituitaries were removed from the animals killed on PND 35–45. Testes were weighed after fixation in Bouin fluid. Testes were sectioned and evaluated for Sertoli and germ cell number, Leydig cell number per testis using immunostaining for 3β-hydroxysteroid dehydrogenase, and intensity of staining for 17α-hydroxylase/C₁₇₋₂₀ lyase. Pituitaries were fixed in Bouin fluid, sectioned, and immunostained for the β-subunits of LH and FSH. Statistical comparisons were made using paired *t* tests.

There were no differences between treatment groups in body weight at the beginning or the end of the treatment period. Formula intake did not differ between the treatment groups. Plasma testosterone was similar between the treatment groups on PND 18–20, but was lower in the soy formula-fed group on PND 35–45 when analysis was restricted to the 13 pairs of twins (mean ± SD 1.3 ± 2.1 ng/mL compared to 2.8 ± 3.9 ng/mL in the cow-milk formula-fed group, *P* = 0.004 by paired *t*-test on log-transformed data). The proportion of 35–45-day-old animals with plasma testosterone < 0.5 ng/mL was 1/15 in the group given cow-milk formula and 12/15 in the group given soy formula (*P* < 0.001, Fisher exact test). Testis weights were comparable between the 2 groups, as were Sertoli and germ cell numbers/testis. Leydig cells/testis were increased 74% in

3.0 Developmental Toxicity Data

the soy formula-fed group at 35–45 days of age. Intensity of staining for 17α -hydroxylase/ C_{17-20} lyase in testis sections, and numbers of cells immunopositive for β -subunit LH or FSH in pituitary sections did not differ by treatment group. Because blood concentrations of LH and FSH cannot be reliably measured in the marmoset, the authors could not determine whether the decrease in plasma testosterone was due to an effect of soy constituents on the pituitary or on the Leydig cell, but they believed the decrease in plasma testosterone to be potentially important, particularly in light of the normal increase in testosterone that occurs in neonatal primates, including humans.

Strengths/Weaknesses: Strengths of this study included use of a non-human primate species, appropriate exposure period, exposure to soy milk, and adequate numbers of animals/group. A weakness is that because animals were allowed to nurse, total soy exposure was unknown. Specifically, maternal chow was not described and possible exposure occurring through mother's milk was unknown. In addition, only one soy dose was used.

Utility (Adequacy) for CERHR Evaluation Process: The study is of low-to-moderate utility based on appropriateness of exposure period and species used. The endpoints are of limited utility alone in determining reproductive effects, but may be helpful in interpreting results from other studies.

Tan et al. (171), funded by the UK Medical Research Council, extended the report of Sharpe et al. (170) by describing outcomes in the 7 marmoset twin pairs that were continued to 120–138 weeks of age. The treatment regimen was as described above. At the end of the formula-feeding period (35–45 days of age), these animals were maintained in their family groups until 80–140 weeks. Thereafter, each male was caged singly with an adult female and any resulting offspring until termination. Between 40 and 120 weeks of age, plasma was sampled every 10 weeks for measurement of testosterone by RIA. After death, testis, epididymis, prostate, seminal vesicles, thymus, spleen, and pituitary were dissected, fixed in Bouin fluid, and weighed. The right testis was sectioned, infiltrated with resin, and stained with Harris hematoxylin for enumeration of Sertoli cells. The remaining tissues were embedded in paraffin and sectioned for light microscopy. Immunostaining for 3β -hydroxysteroid dehydrogenase was used to quantify Leydig cell number, nuclear diameter, and volume. Cytoplasm staining for 3β -hydroxysteroid dehydrogenase was expressed as relative volume of the testis and multiplied by testis weight for an estimate of Leydig cell volume. Stereologic methods were used to estimate seminiferous epithelium and luminal volumes. Statistical analysis was performed using paired *t* tests.

There was no effect of formula type on body weight or terminal weights of thymus, spleen, prostate, seminal vesicles, or pituitary, and penile length was comparable between the feeding groups. Testis weight was 14% higher in the soy formula-fed than the cow-milk formula-fed group, and in 6 of the 7 twin pairs, testis weight was higher in the soy-fed twin than in its co-twin. A slightly lower testis weight in 1 of the cow-milk formula-fed twins compared to its co-twin was attributed to Sertoli-only tubules, considered most likely to be a result of blockage of the excurrent duct system. The timing of puberty onset, determined by an increase in plasma testosterone, was not altered by infant feeding method, and there were no significant group differences in plasma testosterone at any time point. Two of the soy-fed marmosets had plasma testosterone levels that were consistently at the lower end of the normal range. All but 1 of the marmosets produced young; the animal that did not produce young was 1 of the soy formula-fed animals with consistently low-normal testosterone plasma levels. No determination was made of the female partner's fertility status, precluding the conclusion that this male was infertile. There was no formal assessment of mating behavior.

3.0 Developmental Toxicity Data

Except for the 1 cow-milk formula-fed marmoset with Sertoli-only tubules, testicular histology was normal. Sertoli and Leydig cell numbers/testis were higher after soy-formula feeding in each of the 7 individual twin comparisons, and group means were significantly higher for both cell types in the soy formula-fed animals. Leydig cell numbers were increased to the greatest degree (55 and 214% compared to the control co-twin) in the 2 soy formula-fed animals with the consistently low-normal plasma testosterone levels. The volumes of the seminiferous epithelium and of the tubular lumina were described as higher in the soy formula-fed group; however, there was substantial variability among twin pairs, and the group means were not statistically different.

The authors attributed the higher testis weights in the soy formula-fed group to a presumed larger number of germ cells secondary to the large number of Sertoli cells. They concluded that these results were generally reassuring in showing normal reproductive outcome after soy formula feeding during infancy; however, they postulated that the soy-associated increase in testis weight and Leydig cell numbers may have represented a state of compensated Leydig cell failure. This possible Leydig cell failure was most evident in the 2 animals with low-normal plasma testosterone levels that had the largest increase in Leydig cell numbers compared to their co-twins. The authors stated that because their design did not permit 100% formula-feeding, soy-formula intake of their marmosets would have been only 40–87% of the intake of a human infant exclusively fed soy formula. The exclusive feeding of soy infant formula in humans, they suggested, might be more likely to produce adverse reproductive effects.

Strengths/Weaknesses: The use of a co-twin design to minimize the number of animals used and to control for litter effect and large inter-animal variability is a potential strength of the design; however, no data were presented to verify the assumption that male twins are more comparable for reproductive parameters than unrelated males of the same age or to demonstrate that the rate of maturation is the same in co-twins. The variance of untreated co-twins compared to other untreated marmosets of similar age and body weight was not presented. Maintaining family groups during treatment to minimize stress is a strength. Marmosets were exposed orally to soy formula during the neonatal period with feeding on demand, which is relevant to human infants. The authors estimated that isoflavone intake for soy-fed males was 40–87% of 4-month-old human infants maintained on an soy formula; thus, exposure levels were realistic. When determining Leydig cell numbers, the authors based the number of fields counted per animal on obtaining a percentage standard error value of <5%, which is a strength, as is reporting the detection level and intra- and inter-assay coefficients of variation for testosterone assays. When determining testosterone levels, sample from co-twins were processed at the same time. Offspring in both treatment groups reportedly consumed similar amounts of formula. The decrease in plasma/serum testosterone levels and increase in Leydig cell numbers with soy formula treatment agreed with results seen at 35–40 days of age in soy-fed marmosets in the previous Sharpe et al. study (170).

The study compared only one concentration of soy formula, so it is not possible to evaluate dose-response relationships. As is common in primate studies, the sample sizes were small, but the use of 7 co-twins disagrees with the original report (170) in which only 6 sets of co-twins were maintained to adulthood (plus 4 male non-twins). It would have been useful if the authors had presented some historical control data on untreated control laboratory values for marmosets. As the data are presented, it is not possible to determine how the testicular parameters in cow-milk formula-fed controls relate to typical marmoset values, and the variances for measured parameters are not identified. Marmosets were returned to their biological mother for 16 hours/day on weekdays and 22 hours/day on weekends; thus, breast feeding during this interval may have masked some effects of an entirely soy-based diet. There were no data on blood levels

3.0 Developmental Toxicity Data

of genistein or daidzein, so it is difficult to determine how marmoset kinetics might differ from those of human infants. The authors used previously reported values of aglycone/L formula to estimate marmoset exposures, which is a weakness. It is not clear why the authors fixed the reproductive organs prior to collecting organ weights. It is not possible to determine whether the authors controlled for diurnal variation in their measurements of hormones by collecting all samples at the same time each day. Fertility in co-habited females was not proven. Co-twin number 7 had a pronounced incidence of seminiferous tubules that contained only Sertoli cells. The authors hypothesized that this abnormality was due to partial blockage of the efferent ducts or rete and was unlikely to be related to soy treatment; however, they did not exclude this animal (or its co-twin) from other testicular measures. The authors reported that soy formula attenuated the neonatal rise in testosterone (170); however, marmosets appear to be more resilient to disturbances in neonatal testosterone levels than humans (172). The authors hypothesized that the soy formula-treated males may exhibit a degree of “compensated Leydig cell failure” based on increased Leydig cell numbers with normal testosterone levels. The authors were unable to measure marmoset serum LH levels, but hypothesized that elevations of gonadotropin levels (both LH and FSH) could provide an explanation. According to Li et al. (172), it is difficult to extrapolate marmoset data to humans because marmosets differ markedly in some areas of testicular development, including the lack of a regular LH/LH receptor system, the presence of a positive feedback loop for 17 β -estradiol in normal male marmosets, and a generalized steroid resistance due to high circulating levels of testosterone, estrogen, and other steroids.

Utility (Adequacy) for CERHR Evaluation Process: This report of limited utility in the evaluation process.

Anthony et al. (173), funding not indicated, presented a limited examination of hormonal levels and reproductive organ weights in rhesus monkeys (*Macaca mulatta*) fed soy protein diets as part of a study examining the effects of soy isoflavones on cardiovascular risk. Peripubertal male (1.3–2.1 years old) and female (1.3–4.1 years old) monkeys were fed diets containing casein and lactalbumin as the source of protein for 3 weeks. The monkeys were then stratified randomly according to age and cholesterol levels. For a 24-week period, 1 group of 7 females and 5 males was fed a soy-based diet containing 1.27 mg genistein and 0.42 mg daidzein per g soy protein. The diet resulted in isoflavone intake of 9.41 mg/kg bw. **[Assuming that genistein and daidzein were the only isoflavones present, intake of each respective isoflavone would be ~7 and 2 mg/kg bw.]** A second group of 7 females and 6 males was fed a diet in which the soy protein was alcohol-extracted to remove the majority of isoflavones. Levels of isoflavones in the isoflavone-reduced diet were 0.121 mg genistein and 0.052 mg daidzein per g soy protein. The reduced-isoflavone diet provided an isoflavone intake of 0.97 mg/kg bw **[genistein ~0.7 mg/kg bw and daidzein ~0.3 mg/kg bw]**. As part of the cross-over study design, each group of monkeys received 1 of the diets for 24 weeks and was then switched to the opposite diet for 24 weeks. Blood levels of 17 β -estradiol, testosterone, dehydroepiandrosterone sulfate, and thyroxine were measured by RIA, and sex hormone-binding globulin was measured by a saturation assay. It appears that results were pooled for animals of the same treatment groups regardless of exposure period. Following the second exposure period, monkeys were killed and prostate, testis, and uterus weights were obtained. Data were analyzed by ANCOVA and paired *t*-tests. The presence of dietary isoflavones in the soy diet had no effect on hormone levels or reproductive organ weights. The authors reported the intact isoflavone protein had favorable effects on plasma lipid and lipoprotein concentrations. **[As noted by study authors, all male monkeys were prepubertal at the start of the study and some reached sexual maturity during the study. Results could have been affected by imbalances in male sexual maturity.]**

3.0 Developmental Toxicity Data

Strengths/Weaknesses: A strength of this study was use of rhesus monkeys, which is presumably one of the most relevant species for humans. The cross-over design allowed for measurements from the same animal on both diets (soy-based and alcohol-extracted), which is valuable given the small sample sizes. Monkeys were randomized based on age and the ratio of total plasma cholesterol to HDL cholesterol, one of the primary confounders in examining the soy effects on plasma lipid and lipoprotein concentrations. Diets were kept frozen until needed, which may have improved test material stability. Levels of genistein and daidzein were determined in both the soy-based and alcohol-extracted soy protein. Proteins were also analyzed for the presence of β -sitosterol. Samples were analyzed in duplicate. Statistical analyses seemed appropriate with analyses for main effects of treatment and period as well as any interaction between those terms. Some weaknesses were also noted. As is often the case in studies using monkeys, this study contained small sample sizes (n = 14 females and 13 males at study start). Results were pooled for all animals receiving the same diet, regardless of differences in age or other factors. After the second 24-week exposure, animals were killed and reproductive organ weights were measured. It was assumed that the endpoints measured reflected the last diet to which the animals were exposed (i.e., that there were no long-term effects of soy-based diet because both groups had received this diet prior to necropsy). It was not possible to control for differences in the state of sexual maturity of the animals at the beginning versus the end of the study, making it unclear whether differences in hormone measurements or reproductive organ weights were masked. Data on these parameters and their variances were not provided. Furthermore, there was no evidence that the authors controlled for diurnal variation in their measurements of hormone levels. Despite attempts to control dietary components, the authors noted that “differences in the concentration of the protein in the products somewhat affected the percent of energy as protein, fat and carbohydrate,” among other factors that could influence study outcome.

Utility (Adequacy) for CERHR Evaluative Process: This report is not useful in the CERHR evaluation process.

3.2.2 Mammary Development/Carcinogenesis

Hakkak et al. (174), funded by the USDA, examined the effects of diet containing soy or whey protein on the development of dimethylbenzanthracene-induced mammary tumors in rats. Breeder Sprague Dawley rats were fed AIN-93G diets in which soybean oil was replaced by corn oil and the protein source was either casein, whey, or soy-protein isolate. The soy diet contained isoflavones 430 mg/kg diet, specifically, genistein 276 mg/kg diet and daidzein 132 mg/kg diet. The study authors estimated that a 333 g rat eating 25 g diet/day would be exposed to genistein at 20.4 mg/kg bw/day. Rats from the same dietary groups were bred to produce F₁ offspring, and upon maturity, the F₁ offspring from the same dietary groups were bred to rats from different litters to produce F₂ offspring. **[The number of rats bred was not stated for any generation, and it is not known how many litters were represented in F₁ rats selected for breeding.]** At weaning, the F₁ and F₂ offspring were fed the same diets as their mothers and continued to receive the diets throughout their lifetimes. **[Based on a body weight figure presented in the study, it was estimated that a weanling (21-day-old) rat weighs 50 g. The EPA (84) assumption for food intake in a weanling female Sprague Dawley rat is 0.150 kg/kg bw/day. Therefore genistein intake in weanling rats was estimated at ~830 mg/kg bw/day.]** At 50 days of age, ≥ 19 F₁ rats/group **[inconsistent values reported for number of whey-fed rats in various tables]** and ≥ 31 F₂ rats/group were gavaged with dimethylbenzanthracene 80 mg/kg bw and the development of palpable mammary tumors was assessed. Rats were killed when all rats from the casein diet had at least 1 palpable mammary tumor. Tumors were examined, fixed in buffered formalin, and sectioned. Statistical analyses included ANOVA, Kaplan-Meier analysis, Wilcoxon test, Fisher exact test, and Kruskal-Wallis test.

3.0 Developmental Toxicity Data

Beginning at 8 weeks of age, body weight gain in both generations of casein-fed rats was slightly but significantly higher than body weight gain in the soy- or whey-fed rats. Vaginal opening was significantly accelerated by 1 day in the soy-fed compared to casein- or whey-fed rats (PND 37 versus PND 38). No differences were noted for relative organ weight, estrous cycle, successful breeding, sex ratio, or litter size [data not shown]. Results for mammary carcinogenesis are outlined in Table 39. Latency, in terms of the number of post-treatment days for 50% of rats to develop at least 1 tumor, was significantly increased in both generations of the soy- and whey-fed rats compared to casein-fed rats. Time course for tumor development was accelerated in the casein-fed rats, as noted by a significantly lower percentage of soy- and whey-fed rats with tumors at the time when 100% of casein-fed rats had developed a tumor. Tumor multiplicity (numbers of tumors/tumor-bearing rat) was significantly lower in both generations of whey-fed rats compared to casein-fed rats. There were no other significant differences in tumor parameters. The study authors concluded that soy-rich diets reduce dimethylbenzanthracene-mammary tumor incidence by about 20% in rats and that whey appears to be twice as effective as soy products in reducing tumor incidence and multiplicity.

Table 39. Effect of Diet on Dimethylbenzanthracene-Induced Mammary Tumors in Female Rats

Parameter	Generation	Diet		
		Casein	Soy	Whey
Days after dimethylbenzanthracene				
To first tumor	F ₁	28	42	28
	F ₂	36	36	43
To 50% of rats with ≥ 1 tumor	F ₁	52 ^a	65 ^b	83 ^b
	F ₂	54 ^a	68 ^b	89 ^c
Rats with at least 1 mammary tumor at end of study, %	F ₁	100 ^a	84 ^{a,b}	62 ^b
	F ₂	100 ^a	77 ^b	54 ^b
Rats with at least 1 adenocarcinoma, %	F ₁	95	90	86
	F ₂	85	79	63
Rats with at least 1 mammary tumor with intraductal proliferation, %	F ₁	5	5	5
	F ₂	13	21	32
Tumors/rat, median (range)	F ₁	5 (2–12) ^a	3 (1–12) ^{a,b}	2 (1–22) ^{b,c}
	F ₂	3 (1–7) ^a	3 (1–10) ^a	2 (1–7) ^b
Median tumor volume, cm ³	F ₁	1.9	1.5	0.6
	F ₂	3.0	1.8	0.8

^{a,b,c}Within each row, numbers with different superscripts are statistically different from one another. From Hakkak et al. (174).

Strengths/Weakness: A strength of the study is that rats were fed AIN-93 diets with casein, soy, or whey as the sole protein source for 2 generations. Dietary isoflavone, genistein, and daidzein concentrations were analytically determined. Animals were exposed through diet, a relevant route of exposure. Mammary tissue was examined by a pathologist who was blind to treatment group. In the statistical analyses, day of measurement was included to account for multiple measures per animal and interactions between diet and day were examined. Offspring were exposed to a soy-containing diet across critical windows of development. Dose levels were reportedly similar to those ingested by a 4-month-old infant consuming soy-based infant formula. A weakness is inclusion of only one dietary level of soy, which does not allow for evaluation of dose-response relationships. There were no analytical data presented for the stability of genistein in the diets, and no information as to how often diets were replenished. The authors do not state how many animals were bred in the parental or F₁ generation or how many litters were produced. It is not

3.0 Developmental Toxicity Data

known whether litter effect was controlled (i.e., selection of one male and one female/litter for mating of the next generation and use of litter-based analyses, such that animals from the same litter represented an n of 1). Data for many endpoints (relative organ weights, estrous cycle, fertility, sex ratio, litter sizes) were not presented. The soy effect on lowering tumor incidence was only statistically identified in 1 of the 2 experiments described in this paper. Details were not provided for the assignment of animals into treatment groups (i.e., approximately equal mean body weights and variances across groups). It would have been useful if the authors had reported body weight at the time of puberty onset because a 1-day acceleration in vaginal opening in the soy-fed females likely occurred in animals at lower body weights than in casein controls. Furthermore, the mean age at vaginal opening in the female Sprague Dawley rats (38 days in casein-fed controls) is later than the value reported in many other publications (e.g., 33.4 days, range 31.6–35.1 days (175)).

Utility (Adequacy) for CERHR Evaluative Process: This report is not useful in the CERHR evaluation process.

Rowland et al. (176), funded by the USDA, examined the effects of a soy-protein isolate diet on mammary development in rats. Beginning on GD 4 [**day of plug not specified**], 10 pregnant Sprague Dawley rats/group were randomly assigned to receive 1 of 3 AIN-93G diets containing soy protein isolate, whey, or casein as the protein source. The diets were equivalent in calories, protein, and amino acid content. [**A statement was made that the animals were exposed throughout their lifetimes, and it was therefore assumed that the animals were fed the assigned diets through the remainder of gestation and the entire lactation period.**] Offspring were weaned to the same diets as their mothers. On PND 21, 33, and 50, one female/litter/group (n = 10/time period) was killed for examination of mammary gland morphology. The same diets and exposure protocol were used in a second experiment in which offspring were selected from 5–7 litters and were weaned to the same diets as their mothers. Around PND 48–51, 1 female/litter/group (n = 5–7/group) was gavaged with sesame oil and killed 24 hours later in metestrus. Mammary glands were removed for examination of cell proliferation and expression of ER α , ER β , and progesterone receptor. The litter was considered the statistical unit in analyses that included ANOVA and Student-Newman-Keuls multiple comparison test.

Mammary gland area in rats of the soy diet group was 36–38% larger than in rats of the casein and whey protein groups on PND 50. This effect was not observed on PND 21 or 33. A 4-fold (75%) decrease in terminal end bud density was identified in rats of the soy and whey protein diet groups compared to the casein diet group on PND 50, but the effect did not attain statistical significance. There were no significant differences in alveolar or lobuloalveoli bud density. Terminal end bud cells expressing progesterone receptor were increased by 34% in the soy protein compared to the casein diet group at 48–51 days of age, but this effect was not seen in lobular cells. Soy protein isolate diet had no significant effects on proliferation or expression of ER α or ER β in terminal end buds or lobular cells. The study authors concluded that the soy protein isolate diet stimulated mammary gland differentiation.

Strengths/Weakness: A strength was that rats were fed AIN-93 diets with casein, soy protein isolate or whey protein hydrolysate as the sole protein source. The authors controlled for litter effect, using either one female pup/litter for an endpoint and/or using the litter as the unit of analysis. The methods for scoring mammary whole mounts and immunohistochemistry samples were clearly described. The authors controlled for stage of estrous cycle when proliferation index was determined in mammary glands. A negative control (omitting the primary antibody) was included in immunohistochemistry experiments to verify receptor populations. Animals were exposed in the diet, a relevant route of exposure. The statistical analyses were appropriate.

3.0 Developmental Toxicity Data

Offspring were exposed to a soy-containing diet across several critical windows of development and evaluated at ~50 days of age. A weakness is that the study included only one dietary level of soy, which does not allow for evaluation of dose-response relationships. Blood levels of phytoestrogens (e.g., genistein) were not measured. The authors did not specify whether the sperm-positive day was designated as GD 0 or 1. There were no analytical data presented for the stability of genistein in the diets and no information as to how often diets were replenished. Dietary levels of genistein and daidzein were not specified (perhaps these levels were the same as Hakkak et al. (174)). No data on body weights was presented. Sample sizes were likely insufficient for endpoints such as mean number of terminal end buds/mm², given that a 75% decrease was not statistically different. Details were not provided for the assignment of animals into treatment groups (i.e., approximately equal mean body weights and variances across groups).

Utility (Adequacy) for CERHR Evaluative Process: This report is not useful in the evaluation process.

Simmen et al. (177), supported by USDA, examined the effects of soy diet on chemically induced mammary tumorigenesis. On GD 4 (day of plug not stated), Sprague Dawley rats were randomly assigned to groups fed AIN-93G diets containing either casein or soy-protein isolate as the sole protein source. **[Based on numbers of pups reported later in the study, it appears that 10 dams/group received each diet.]** Dams were allowed to litter, and pups from the same treatment groups were pooled. Five pups/sex/group were randomly placed with dams from the same treatment groups. The 50 female pups/group were weaned on PND 21 (day of birth not defined) and fed the same diets as their dams. On PND 50, female offspring were iv injected with 50 mg/kg bw *N*-methyl-*N*-nitrosourea. Rats were weighed weekly, palpated twice/week for mammary tumors, and killed 115 days following *N*-methyl-*N*-nitrosourea treatment. Mammary tissues were subjected to immunohistochemical analyses to determine proliferative cell nuclear antigen and apoptosis. RNA was isolated from mammary tissues and reverse transcription-polymerase chain reaction was used to determine expression for a number of genes involved in cell proliferation, cell differentiation, and apoptosis. Serum estrogen **[not otherwise identified]** and progesterone levels were measured. MCF-7 breast cancer cells were incubated in sera obtained from each diet group in order to measure apoptosis. Data were analyzed by ANOVA or Student *t*-test.

Compared to rats fed the casein diet, rats fed the soy-protein isolate diet had increased mean \pm SEM tumor latency (93.0 \pm 4.3 vs. 78.0 \pm 3.9 **[unit assumed to be day]**; $P < 0.05$) and decreased incidence of rats with at least 1 tumor (57.4 vs. 83.3%; $P < 0.01$). Rats fed the soy-protein isolate diet had a lower incidence of ductal carcinoma in situ (42 vs. 64% $P = 0.08$) but a higher incidence of infiltrating ductal carcinoma (55 vs. 33%; $P = 0.086$). Diet had no effect on cell proliferation or apoptosis. Progesterone receptor and *HER-2/neu* (involved in cell proliferation) gene expression were increased in rats fed the soy-protein isolate diet. Serum progesterone levels were ~7 times lower in rats fed the soy-protein isolate diet, but there was no effect on serum estrogen level. Apoptotic cells in an MCF-7 culture were modestly but significantly increased following incubation with sera from rats fed the soy-protein isolate compared to the casein diet **[data not shown]**. The study authors concluded that lifetime exposure to soy-protein isolate protected rats from *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis and altered signaling pathways, including progesterone receptor and *HER-2/neu*.

Strengths/Weakness: It is a strength that the amino acid content of the diets was equalized; however, only 1 dose level of soy was used and only female rats were evaluated. Soy was given over a lifetime, making the study difficult to interpret for the current evaluation.

3.0 Developmental Toxicity Data

Utility (Adequacy) for CERHR Evaluative Process: This study is not useful for the evaluation process due to the lifetime exposure to soy and the focus on tumor detection as a primary endpoint.

3.2.3 Brain Structure/Behavior

A number of studies examined the effects of soy diet on nervous system development or behavior of rats. The studies are presented in order of those focusing on brain structure or chemistry followed by those focusing on behavior. There is also a study in rhesus monkeys.

Taylor et al. (178), supported by the National Science Foundation and Brigham Young University, examined the effects of a phytoestrogen-containing diet on fetal calbindin levels in the medial basal hypothalamus and preoptic area of the brain. Pregnant Sprague Dawley rats were fed a phytoestrogen-containing diet (n = 4) or phytoestrogen-free diet (n=2) on GD 0–20 (day of plug = GD 0). The phytoestrogen diet (phyto-200) contained phytoestrogens 200 µg/g diet (genistein 95 µg/g, glycitin 17 µg/g, and daidzein 82 µg/g). **[The protein source of the phyto-200 diet was assumed to be soy; the protein source for the phytoestrogen-free diet was not given.]** Diets contained the same percentages of protein, fat, and carbohydrates. Rats were killed on GD 20. Fetuses were sexed, weighed, and evaluated for anogenital distance (n = 27–30/sex in the phyto-200 group and 15–16/sex in phytoestrogen-free group). The medial basal hypothalamus and preoptic area brain regions were collected from ≥5 fetuses/sex/litter/dietary group and pooled according to sex and dietary group. Medial basal hypothalamus and preoptic area calbindin levels were measured by Western blot. Data were analyzed with ANOVA, followed by pair-wise Tukey comparison. Diets had no significant effect on food or water intake during gestation or on litter size **[data not shown]**. Anogenital distance and fetal body weight were not affected by diet. Calbindin protein expression in the medial basal hypothalamus and preoptic area was significantly higher **[by ~40%]** and more similar to male values in females fed the phytoestrogen-free compared to the phyto-200 diet. Diet had no effect on calbindin protein expression in males. The study authors concluded that the data have far-reaching implications regarding possible influence of dietary phytoestrogens on fetal medial-basal hypothalamus and preoptic-area calbindin levels.

Strengths/Weakness: A strength was that animals were exposed in the diet, which is a relevant route of exposure. Diets were analyzed by HPLC to determine concentrations of the phytoestrogens genistein and daidzein. Western blot data were obtained from six immunoblots. Specificity of the calbindin antibody was verified by demonstrating a lack of binding to other calcium-binding proteins. After the chemiluminescence assay, blots were stained with India ink to ensure equal protein loading and efficient transfer. For immunohistochemistry, a negative control was included using adjacent tissue sections with pre-immune serum or a buffer-only control (without the primary antibody). A weakness was that the study included only one dietary level of soy, which did not allow for a comparison of dose-response relationships. The stability of the phytoestrogen-containing diets was not reported. Sample sizes were too small to be meaningful. Fetuses within the same litter exposed to phytoestrogen-containing or phytoestrogen-free diet in utero were not independent samples. Thus, sample sizes were n = 4 for pregnant dams given the phytoestrogen-containing diet and n = 2 for dams given the phytoestrogen-free diet. These sample sizes were too small to make any conclusions. Furthermore, the authors did not control for litter effects as they reported body weight and anogenital distance measurements in ≥15 animals/group. Medial basal hypothalamus and preoptic area of the brain were collected from ≥5 males and ≥5 females/litter and were pooled by sex and dietary group, thus suggesting that not all litters were equally represented in the samples analyzed. Volumes of the medial basal hypothalamus and preoptic area of the brain were not measured. Blood levels of genistein/daidzein were not

3.0 Developmental Toxicity Data

measured. The authors did not discuss how these data relate to human hypothalamic development.

Utility (Adequacy) for CERHR Evaluative Process: This report is not useful in the CERHR evaluation process.

Lephart et al. (61), supported by the National Science Foundation and Brigham Young University, examined the effects of lifetime phytoestrogen exposure on anteroventral periventricular nucleus structure, aromatase level, and prostate weight in rats. Long Evans rat offspring used in this study were born to dams (at least 8/group) fed a diet containing 600 µg glycosidic phytoestrogens/g diet (phyto-600) or a phytoestrogen-free diet throughout the gestation and lactation period. **[Distribution of fetuses by litter was not discussed.]** According to a reference provided in this study (58), protein sources were soy for the phyto-600 diet and fish meal for the phytoestrogen-free diet. Diets were reported to have equivalent amounts of protein, amino acids, carbohydrates, fats, vitamins, and minerals. F₀ breeders and F₁ offspring were examined in a series of experiments. Data were analyzed by ANOVA followed by Bonferroni post hoc comparison.

Experiment 1 examined effects in male and female breeders that had been exposed only during adulthood. Levels of phytoestrogens in brains of adult male breeders are reported in Section 2.1.2. In experiment 2, offspring were weaned at 30 days of age to the same diets as their mothers. Body and prostate weights were measured at 75 days of age in 7–8 rats/group. Compared to rats fed the phytoestrogen-free diets, rats in the phyto-600 group had significantly lower body weight [**17% lower**], absolute prostate weight [**31% lower**], and relative (to body weight) prostate weight [**17% lower**]. Brain aromatase activity in the anteroventral periventricular nucleus area was measured at 110 days of age (n = 6/sex/group) and reported to be unaffected by diet.

In experiment 3, half of the offspring fed the phyto-600 diets continued to receive that diet and the other half were given the phytoestrogen-free diet for 40 days, beginning at 80 days of age. Both groups were killed at 120 days of age for measurement of the volume of the anteroventral periventricular nucleus (n= 5–7/sex/group). Males and females fed phyto-600 diets throughout their lifetimes displayed the typical pattern of ~2-fold higher anteroventral periventricular nucleus volume in females compared to males. Males switched to the phytoestrogen-free diet displayed a significant increase (nearly 2-fold) in anteroventral periventricular nucleus volume compared to males who remained on the phyto-600 diet. The change in diet did not significantly affect anteroventral periventricular nucleus volume in females.

The study authors concluded that phytoestrogens decrease body and prostate weight and affect anteroventral periventricular nucleus volumes in adult males but have no effect on brain aromatase levels.

Strengths/Weakness: A strength of the study was that brain concentrations of daidzein, genistein, and equol were measured in the medial basal hypothalamus and preoptic area, thus verifying that these phytoestrogens reached the hypothalamus. Animals were exposed to phytoestrogens in the diet, which is a relevant route of exposure. Diets were analyzed by HPLC to determine concentrations of phytoestrogens, genistein, and daidzein (although this determination appears to have been made in previous studies). Validation of the time-resolved fluoroimmunoassay method used to measure brain concentrations of phytoestrogens has been published previously. Ovarian tissues were used as a positive control in the aromatase assay. The anteroventral periventricular nucleus sections were traced by two researchers blind to treatment group, and these traced areas were averaged. A weakness is that this study included only one

3.0 Developmental Toxicity Data

dietary level of soy, which does not allow for evaluation of dose-response relationships. The stability of phytoestrogen-containing diets was not reported. The authors do not provide any litter parameters (e.g., fertility, litter sizes, sex ratio, pup body weights). They also do not provide any body weights at the time of randomization, during gestation and lactation, or for pups at weaning. It is difficult to discern whether the 17% decrease in body weight in the 75-day-old males maintained on the phytoestrogen-containing diet may have contributed to the alterations in prostate weight. Chapin et al. (179) reported a significant (19%) decrease in absolute prostate weights in feed-restricted male rats with a 12% change in body weight. Thus, prostate weight can be influenced by body weight. It is not clear whether the authors controlled for litter effects (i.e., consideration of the litter as the unit of analysis for animals with lifelong phytoestrogen exposures; fetuses within the same litter exposed to phytoestrogen-containing or phytoestrogen-free diet in utero are not independent samples). Equol antiserum showed some cross-reactivity with dihydrogenistein and dihydrodaidzein. There was no phytoestrogen-free control included in the experiment measuring anteroventral periventricular nucleus volume. The experiment compared rats maintained on a phytoestrogen-containing diet with those switched to a phytoestrogen-free diet at 80 days of age. It would have been useful to determine whether the early, in utero/prenatal/prepubertal exposures to phytoestrogens contributed to long-term differences in anteroventral periventricular nucleus area. Protein sources for these diets (per Lund et al. (58)) were soy for the phyto-600 diet and fish meal for the phytoestrogen-free diet. The protein sources likely contributed other differences aside from phytoestrogen levels. Odum et al. (163) reported that different rodent diets with different amounts of phytoestrogens had centrally mediated effects on rodent sexual development due to nutritional differences between the diets rather than ER-mediated effects. The authors do not discuss how these data relate to human hypothalamic development.

Utility (Adequacy) for CERHR Evaluative Process: This report is not useful in the CERHR evaluation process.

Weber et al. (55), supported by the National Science Foundation, examined the effects of low- and high-phytoestrogen diets on aromatase activity on medial basal hypothalamic and preoptic areas of maternal, fetal, and neonatal rats. Sprague Dawley rats were placed on a high-phytoestrogen (n = 10) or a low-phytoestrogen (n = 13) diet. The commercially available high-phytoestrogen diet contained phytoestrogens 603 µg/g consisting mainly of daidzin, genistin, and glycitin. According to Lephart et al. (56), the diet was soy-based. The isoflavone levels were below the detection limits in the low-phytoestrogen diet, which had the same levels of protein, carbohydrates, and fats as the high-phytoestrogen diet. Rats were mated at 80 days of age and remained on their respective diets throughout pregnancy and lactation. At GD 16.5 and 20.5 [**day of plug not specified**] and on PND 3.5 [**day of birth not specified**], brains were collected from dams and offspring. At those same time points, offspring (n = 16–34/sex/group/time period) were sexed, weighed, and evaluated for anogenital distance. The medial basal hypothalamic-preoptic brain area of dams (n = 3–5/time period) and offspring (n = 9–15 sex/group/time period) was incubated in medium with testosterone for determination of aromatase activity by a “tritiated water” method. Isoflavone levels were measured in blood of dams and offspring at each time point, and the results of the analyses are discussed in Section 2.1.2. Data were analyzed by ANOVA, followed by pair-wise comparison with the Newman-Keuls test.

Fetal body weights were significantly higher in the low-phytoestrogen group on PND 20.5; there were no offspring body weight effects at the other time periods. Anogenital distance was significantly higher in low-phytoestrogen males on GD 20.5, but when normalized for body weight, anogenital distance was significantly greater in high-phytoestrogen males and females. On PND 3.5, anogenital distance was significantly longer in high-phytoestrogen females, and no

3.0 Developmental Toxicity Data

statistically significant effects were observed when normalized for body weight. Data indicated that phytoestrogens crossed the placenta and were lactationally transferred to pups; however, the phytoestrogen diets did not significantly alter brain aromatase levels in pregnant dams, fetuses, or neonates. The study authors concluded that dietary estrogens did not alter aromatase activity in the medial basal hypothalamic-preoptic brain area during perinatal development.

Strengths/Weakness: A strength of this study was that plasma phytoestrogen levels were measured in pregnant dams (GD 16.5 and 20.5), fetuses (GD 20.5), and neonates (PND 3.5). Animals were exposed to phytoestrogens in the diet, which is a relevant route of exposure. Diets were analyzed by HPLC to determine concentrations of the phytoestrogens genistein, daidzein and glycitin (although this determination appears to have been made in previous studies). Sex, body weight, and anogenital distance were recorded in the offspring. Analysis of blood samples included internal controls to validate the assay and verify recovery. A coefficient of variation was provided to establish assay precision and, as a comparison, adult male plasma phytoestrogen levels were determined for males on the same diets. A weakness of the study is that it included only one dietary level of soy, which did not allow for evaluation of dose-response relationships. The stability of phytoestrogen-containing diets was not reported. It was not specified whether the day on which the females were sperm-positive was GD 0 or GD 1, nor was the day of delivery specified as either PND 0 or PND 1. The authors did not provide litter parameters (e.g., fertility, litter sizes, sex ratio). They also did not provide body weights at the time of randomization. It did not appear that authors controlled for litter effects (i.e., in Table 2 and Figure 1B of the study, they did not consider the litter as the unit of analysis for animals exposed during gestation and lactation). Blood samples were pooled by treatment (representing 3–5 litters per collection); therefore, variance in blood levels could not be determined (Table 1 of the study). Maternal body weights and body weight gains during gestation were not given. It is puzzling that the increases in relative anogenital distance (normalized to body weight) were transient (not different at PND 3.5). The differences in protein sources (soybeans and fish) likely contributed other differences aside from phytoestrogen levels. Odum et al. (163) reported that different rodent diets with different amounts of phytoestrogens had centrally mediated effects on rodent sexual development due to nutritional differences between the diets rather than ER-mediated effects. The authors assessed plasma phytoestrogen concentrations by ANOVA (Table 1 of the study), despite using pooled samples and not presenting variances. The authors did not discuss how these data relate to human hypothalamic development.

Utility (Adequacy) for CERHR Evaluative Process: This report is not useful in the CERHR evaluation process.

Lund and Lephart (180), supported by the National Science Foundation and Brigham Young University, examined the anxiolytic effects of a phytoestrogen-containing diet on rats. Long Evans male and female rats were fed a diet containing phytoestrogens 600 µg/g diet (phyto-600) or a phytoestrogen-free diet. According to Lund et al. (58), the phyto-600 diet contained soy and the phytoestrogen-free diet contained fish meal. Diets were similar in protein, fat, amino acid, mineral, and vitamin content. Rats were fed the diets prior to mating and throughout pregnancy and lactation. On weaning at 30 days of age, 10–14 F₁ offspring/sex/group received the same diet as their mothers. **[The number of litters from which the offspring were obtained was not stated.]** Body weight and food and water intake were measured in offspring at 50 days of age. At 75 days of age, anxiety was assessed in the offspring using an elevated plus-maze. Data were analyzed with ANOVA and Bonferroni post hoc comparisons. Body weights were significantly lower in males [4%] and females [5%] fed the phyto-600 diet, despite a significant increase in feed intake in the phyto-600 males. Water intake was significantly higher in males and females

3.0 Developmental Toxicity Data

fed the phyto-600 diet. A total of 10–14 rats/sex/ group completed the maze test in all groups except the phyto-600 females. Only 7 females of the phyto-600 group completed the test, because 5 animals fell or jumped off the open arms. The phyto-600 diet was observed to have anxiolytic effects, as noted by an increased percentage of time spent in open arms [**~60 compared to 30% in the phytoestrogen-free group**] and increased numbers of entries into open arms [**~45 compared to 25% of entries**] by both males and females. The study authors concluded that phytoestrogens in soy caused marked anxiolytic effects in rats.

Strengths/Weakness: A strength of this study was that the authors used the dietary route of exposure, which is a relevant route, and the concentration of phytoestrogens was relevant to laboratory animals (commercially available laboratory diet with high phytoestrogen levels; 600 µg/g diet). Rats were exposed to phyto-600 or phytoestrogen-free diets through critical windows of development (pre-mating, mating, gestation, lactation and into adulthood) to monitor the long-term effect of these different diets. Circulating levels of phytoestrogens using this dosing paradigm were reported in previous publications by these authors. Statistical analyses were appropriate. A weakness is that this study used a single dose level of phytoestrogens (phyto-600), which does not allow for an evaluation of dose-response relationships. It is not clear whether the authors controlled for litter effects (i.e., consideration of the litter of origin when assigning pups to different endpoints or use of the litter as the unit for statistical analysis). Fetuses within the same litter exposed to phytoestrogen in utero are not independent samples. Body weights and feed and water consumption were monitored for only 3 days at 50 days of age. Body weights at PND 75, the time of maze testing, were not reported. Rats were tested only once for 5 minutes in the plus-maze.

Utility (Adequacy) for CERHR Evaluative Process: This report is not useful in the CERHR evaluation process.

Lund et al. (60), supported by the National Science Foundation and Brigham Young University, examined the effects of lifelong phytoestrogen exposure on visual spatial memory in rats. In the first 2 parts of the experiment, Long Evans rats were fed either a soy-based diet containing phytoestrogens 600 µg/g diet (phyto-600) or a phytoestrogen-free diet during pregnancy and the lactation period. [**The number of dams treated was not specified.**] According to Lund et al. (58) the protein source for the phytoestrogen-free diet was fish meal. Diets were similar in protein, fat, amino acid, mineral, and vitamin content. Ingestion of the phyto-600 diet was said to result in blood phytoestrogen levels similar to Asians consuming high amounts of soy. The phytoestrogen-free diet was representative of Western populations that consume very little soy. Offspring were weaned 30 days following birth and given the same diets as their mothers. The diet was given ad libitum until 50 days of age when feed was limited to maintain the rats at ~90% of normal body weight. Beginning at 57 days of age, the rats (n = 13–16/sex/group) were tested for their ability to retrieve a food reward from an 8-arm maze. Once the rats mastered the task they were tested for 10 days (1 trial/day). Following completion of the test, rats were killed and brains were collected for measurement of calbindin and cyclooxygenase-2 levels by Western blot. The first part of the experiment examined males, and the second part examined females. The 2 parts of the experiment were conducted independently of one another but at the same time intervals, using the same procedures. Data were analyzed by 1-way ANOVA and multivariate ANOVA.

Males fed the phyto-600 diet required significantly more trials to master the 8-arm maze than males fed the phytoestrogen-free diet (13 compared to 11 trials). In contrast, females fed the phyto-600 diet mastered the maze in significantly fewer trials compared to females fed the phytoestrogen-free diet (11.5 compared to 13.5 trials). Working memory was not affected by diet

3.0 Developmental Toxicity Data

[data not shown]. In the frontal cortex, high levels of phytoestrogens corresponded with decreases in calbindin and increases in cyclooxygenase-2 in males only. Calbindin-protein expression in the frontal cortex but not hippocampus was significantly lower in males fed the phyto-600 compared to the phytoestrogen-free diet. Cyclooxygenase-2 protein expression was significantly lower in the frontal cortex of males fed the phytoestrogen-free compared to the phyto-600 diet. Calbindin and cyclooxygenase-2 protein expression in brain were not affected by diet in female rats. The study authors stated that calbindin protects against neurodegenerative diseases and apoptosis through regulation of intraneuronal calcium. Cyclooxygenase-2 is an inflammatory protein associated with key steps in the development of Alzheimer's disease.

In the third part of the experiment, male and female rats were exposed to the phyto-600 diet from gestation through adulthood, and the 8-arm maze testing (described above) was conducted in 11–13 rats/sex/group, as described for the first and second parts of the experiment. One trial/day was conducted over a 10-day period. Following completion of the maze testing, half the rats were randomly selected to receive the phytoestrogen-free diet and the other half continued to receive the phyto-600 diet ad libitum for 15 days. For the next 10 days, feed was restricted to maintain the rats at ~90% of normal body weight. When rats were reduced to 90% of normal body weight, they were tested for 15 days (1 trial/day) using an 8-arm maze in which only 4 arms contained a food reward (n = 5–7 rats/sex/group).

Males fed the phyto-600 diet mastered the maze in significantly fewer trials compared to females fed the same diet (8 trials in males compared to 12 trials in females). When trial results were averaged over 2-day periods, males had significantly more correct arm entries than females in 3 of the 5 periods. Significant diet-related and sex-related effects were observed in the 4-arm maze test that was conducted after half the animals continued to receive the phyto-600 feed and the other half were switched to the phytoestrogen-free feed. Data were presented as the number of correct arm entries and were averaged over a 3-day period. In general, females maintained on the phyto-600 diet performed better than females switched to the phytoestrogen-free diet. In contrast, performance was better in males switched to the phytoestrogen-free diet compared to males that continued to receive the phyto-600 diet. In rats switched to the phytoestrogen-free diets, males performed better than females. During the last 2 sets of 3 trials, males switched to the phytoestrogen-free diet made significantly fewer reference errors (entering an unbaited arm) than males maintained on the phyto-600 diet. There was no effect on working memory (re-entering a baited arm) or working/reference (re-entering an unbaited arm). Both sexes maintained on the phyto-600 diets completed the task in significantly less time than animals switched to the phytoestrogen-free diet.

Isoflavones were detected in several brain regions of F₀ males fed the phyto-600 diet in adulthood. Concentrations in frontal cortex were about 2 orders of magnitude higher than in hippocampus. According to study authors, both of those brain regions are critical for visual spatial memory. Additional details about brain levels of phytoestrogens are provided in the toxicokinetics discussion in Section 2.

The study authors concluded that dietary phytoestrogens caused a reversal in sexual dimorphic expression of visual spatial memory.

Strengths/Weakness: This study had several strengths and was well designed in that the authors exposed rats to phyto-600 or phytoestrogen-free diets through critical windows of development (pre-mating, mating, gestation, lactation, and into adulthood) to monitor the long-term effect of these different diets. Furthermore, by switching some adults to the alternate diet, the authors could examine the reversibility of diet-related differences in maze performance. Diet is a relevant

3.0 Developmental Toxicity Data

route of exposure, and the dietary concentration of phytoestrogens was relevant to both laboratory animals (commercially available laboratory diet with high phytoestrogen levels; 600 µg/g diet) and humans (reported plasma levels in rats were similar to Asians consuming large amounts of soy protein per day). Maze testing and analysis were conducted blind to treatment group. Measurements were made to determine the levels of phytoestrogens in different brain regions, calbindin in the frontal cortex and hippocampus, and cyclooxygenase-2 on the frontal cortex. Circulating levels of phytoestrogens using this dosing paradigm were reported in previous publications by these authors. A weakness of this study was that it used a single dose level of phytoestrogens (phyto-600), which does not allow for an evaluation of dose-response relationships. It was not clear whether the authors controlled for litter effects (i.e., that they considered the litter of origin when assigning pups to different endpoints or used the litter as the unit for statistical analysis). Fetuses within the same litter exposed to phytoestrogen in utero are not independent samples. Body weight data were not presented in the manuscript, although the authors noted that body weight effects were the same for males and females and should not have accounted for sexually dimorphic differences in maze performance. In the Methods section, the authors stated that the phyto-600 diet contained 600 µg phytoestrogens/g diet, whereas in the legend to Table 1, it was stated that pups were exposed to 300 µg phytoestrogens/g diet from conception until 50 days of age. The reason for this difference was unclear. The authors also mentioned that plasma levels of phytoestrogens were determined but did not present these data. Brain levels of phytoestrogens were only measured in males. Phyto-600 diet caused opposing effects on visual spatial memory in males and females, yet in examining the frontal cortex, there were no diet-related differences in calbindin or cyclooxygenase-2 in females. It would have been useful to measure phytoestrogen levels in brain regions in females to determine if phytoestrogen levels were similar to levels seen in males. The diet change in adulthood from phyto-600 to phytoestrogen-free diet showed reversal of effects (male performance improved, whereas female performance declined); however, the authors did not state whether a complete reversal was achieved. This point is important because a complete reversal of effects would suggest that in utero/lactational exposure did not cause long-term effects on maze performance. Lastly, the authors did not show the data for working memory, which was not altered by diet in either males or females.

Utility (Adequacy) for CERHR Evaluative Process: This report is not useful in the CERHR evaluation process.

Golub et al. (181), supported by the Violence Research Foundation, evaluated neurobehavioral effects of soy formula in rhesus monkeys. The authors explored the hypothesis that manganese content of formulas would lead to neurobehavioral differences, noting that soy formula has a greater manganese content than does cow-milk formula. Infant monkeys were transferred on the day of birth to a primate nursery and assigned to 1 of 3 feeding groups. **[Randomization was not specified, but there were no feeding group differences in dam characteristics or infant birth weights.]** A cow-milk formula group was fed Similac® with Iron, which contains manganese 50 µg/L. A soy-formula group was fed Baby Basics (a private-label soy formula available at Albertson's), which contains manganese 300 µg/L. A third group was given the soy formula with manganese added to give a concentration of 1000 µg/L. Infants were hand-fed for 2 weeks and then progressed to a sipper bottle. At 4 months of age, the monkeys were transitioned to a primate lab diet with supplemental grains, vegetables, and fruit. Behavioral observations and formal neurobehavioral testing occurred over the first 18 months of life. Cerebrospinal fluid was sampled under anesthesia at 4, 10, and 12 months of age and analyzed for homovanillic acid and 5-hydroxyindoleacetic acid. Statistical analyses were performed using ANOVA with post hoc Fisher test. When there was an apparent effect of soy formula, regression analysis was performed

3.0 Developmental Toxicity Data

across the 3 formula groups to evaluate the manganese dose-relatedness of the effect. Manganese dose was calculated based on formula intake during the first 2 weeks of life.

There were no feeding group effects on body weight or length during the first 4 months of life, when the formulas were being given. Between 6 and 9 months of age, the groups that received soy formula lagged “slightly” in height and weight [**data not shown; individuals not weighed or measured after 9 months**]. There were no significant feeding group effects on cerebrospinal fluid levels of homovanillic acid or 5-hydroxyindoleacetic acid. Soy-fed infants initiated more behaviors than did cow milk-fed infants. Wake periods were shorter and sleep periods longer in soy-fed compared to cow milk-fed monkeys at 8 months of age but not at 4 months of age. Initiation and participation in play activity was decreased among soy-fed monkeys compared to cow milk-fed monkeys. This effect showed a significant correlation with manganese intake in the first 2 weeks of life. Behavioral response to apomorphine injection was also correlated with manganese intake. Temperament ratings and frequency of stereotypy were not affected by feeding group status. Infants in the soy-fed group were described as participating less readily in the structured testing, and the authors suggested that testing at an older age would be useful.

The authors concluded that integration of the behavioral findings was difficult but that increased behavioral changes, altered diurnal rhythms, and reduced play behavior may indicate altered regulatory control.

Strengths/Weaknesses: A strength of the study is that the dosing scenario is directly relevant to human exposures. Endpoints were evaluated from birth to 18 months of age to determine longer-term effects of neonatal exposure. The experimental model was the rhesus monkey, which is similar to humans in brain maturation at the time of birth. Infant monkeys were reared under standardized conditions (not specified). In order to assess a variety of behaviors, the authors selected a pre-determined number of sessions during which data for different endpoints was collected. Not all infant monkeys could generate useful data during the limited test sessions, so participation criteria were established for each test to identify data for exclusion. Observer reliability for scoring motor and postural behavior was established prior to the experiments. For social observations, infants were paired with a different infant for each session (one with whom they had not interacted socially) and monitored by a familiar observer. To alleviate inter-observer variability within a test, one observer collected all social dyad observations and another recorded all stereotypy observations. For the cognitive testing, performance criteria were established. Once these criteria were exceeded, a switch or reversal of the reward was introduced. A weakness of this study was that it used a single dose level of soy formula, which does not allow for an evaluation of dose-response relationships for soy formula (although the dose-response relationship for manganese could be evaluated). There was no information provided as to how infants were randomized into the various treatment groups (although pregnancy housing conditions were controlled across treatment groups). The authors stated that body weight and lengths were affected in the soy-fed animals between 6 and 9 months of age, but these data were not shown. Apparently, individual weights were not collected after 9 months of age (monkeys were weighed as a group). Cow-milk and soy formula differ in many other factors aside from soy, including nutritional components related to protein, carbohydrate, and fat. Sample sizes were relatively small for some neurobehavioral endpoints ($n = 8/\text{group}$, although this sample size is good for a primate study). At 4 months of age, the primates were transitioned to commercial nonhuman primate diet with enrichment foods. It is not known whether these dietary components contributed additional phytoestrogen exposures. Because social observations were discontinued at different times, the largest number of sessions completed by all monkeys was used for analysis; thus, some data were discarded. Cerebrospinal fluid metabolites and social interaction endpoints

3.0 Developmental Toxicity Data

were not highly correlated. Infants performed the delayed nonmatch to sample task at chance level, indicating that the young monkeys were unable to learn this task.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful in the CERHR evaluation process.

3.2.4 Other endpoints

3.2.4.1 Rat

Chang and Doerge (59), of the FDA, examined the effects of post-weaning and adult exposure to soy diets on thyroid function in rats. The effects of genistein added to diet were also examined, and that portion of the study is discussed in the CERHR Expert Panel Report on Genistein. On PND 21–190, two groups of male and female Sprague Dawley rats were fed either NIH 31, a soy-based diet reported to contain genistein and daidzein at 30 ppm each or a soy-free 5K96 feed. **[The number of rats treated was listed as 6, and it is assumed that it was 6/sex/dose as in the main part of the study, which looked at genistein effects and is discussed in the CERHR Expert Panel Report on Genistein.]** Microsomal thyroid peroxidase activity was determined using a spectrophotometric method to measure guaiacol oxidation. **[It is assumed that data were analyzed using 2-way ANOVA or Dunnett test as in the main part of this study.]**

Thyroid peroxidase activity was significantly reduced in rats fed the soy-based compared to soy-free diet. Thyroid peroxidase activity in the soy diet group was less than half the activity in the soy-free diet group. The study authors noted that the effect was similar to that observed in rats fed diets containing 100 ppm genistein in the aglycone form, as discussed in greater detail in the CERHR Expert Panel Report on Genistein. Thus, it was noted that the form of genistein did not affect total serum isoflavone concentrations or decrease thyroid peroxidase activity inhibition. Serum levels of genistein and daidzein were also measured and are discussed in Section 2. The study authors noted that consumption of isoflavones by humans could result in uptake by thyroid gland and inactivation of thyroid peroxidase.

Strengths/Weakness: A strength of this study was the measurement of total genistein and daidzein levels in serum using HPLC with electrospray MS, a method that generated very low detection levels (20 nM genistein in serum). Thyroid peroxidase assay procedures were validated for linearity of response versus protein, and steps to prevent loss of thyroid peroxidase activity during isolation were well described. Assuming that statistical analyses were conducted as in the main part of the study examining addition of genistein to feed, the analyses were appropriate, using 2-way ANOVA to examine dose, sex, and dose/sex interactions. A weakness of this study is that stability of genistein in the diet was not verified. Isoflavone dose levels were not reported in mg/kg bw/day, and feed consumption data were not provided. There were no details as to how prepubertal pups were assigned to treatment groups and no indication that the authors controlled for litter effects. There is no indication that the authors controlled for necropsy stress when thyroid hormone samples were collected. Stress can impact the levels of thyroid hormones measured and increase variance (182). It is not clear why rats were killed at 190 days in the diet-comparison study compared to 140 days in the genistein-supplemented diet study. The authors did not include a discussion on the sensitivity of the rat model to thyroid perturbations (e.g., McClain (183)).

Utility (Adequacy) for CERHR Evaluative Process: This report is not useful in the CERHR evaluation process.

3.0 Developmental Toxicity Data

Teichberg et al. (184, 185), supported by the Public Health Service, evaluated the effects of early weaning to artificial diets on gastrointestinal maturation in rats. The marker of gastrointestinal maturation was the development of a barrier to the absorption of horseradish peroxidase, a 40-kD macromolecule. In the normal rat neonate, macromolecular absorption occurs until PND 17, after which absorption decreases and becomes blocked (referred to as closure) by PND 21. In these experiments, Wistar rat pups were distributed among dams to a density of 12 pups (males and females) per dam. On PND 17, the pups were divided into 3 groups of 4 pups each. One group stayed with the dam and was nursed, and 2 groups were given formula as their nutrition source using standard water bottles. The formulas included a protein-hydrolysate formula (Nutramigen®) and a soy formula (Isomil®). Formula group assignments were balanced for sex and weight. In the first study (184), a separate weight-matched control group was constituted of pups that remained with their dams in litters of 12 until PND 21. On PND 21, “several litters” of pups were anesthetized and 15 cm loops of jejunum with intact vasculature were isolated. A solution containing horseradish peroxidase was placed into the isolated loop and left for 30 minutes. The luminal fluid was then collected and evaluated for volume. Horseradish peroxidase concentration was measured in blood to assess absorption. Intestinal loops were processed for histochemical localization of horseradish peroxidase using phase-contrast and electron microscopy. To examine the role of glucocorticoids, the first study (184) administered intraperitoneal (ip) hydrocortisone on PND 14 to some of the pups, and the second study (185) included corticosterone at 2 different concentrations in the formulas of some of the pups. The use of the glucocorticoid was based on the observation that glucocorticoids advanced intestinal closure in rats.

Both artificial diets in both studies were associated with about a 10% decrease in mean offspring weight on PND 21; however, there was no difference in weights of the pups in the first study (184) if control pups were left in litters of 12 through PND 21. Pups fed either formula showed an increase in horseradish peroxidase absorption on PND 21 compared to pups that continued to be nursed by their dams. Histochemical evaluation of jejunal sections showed horseradish peroxidase confined to the brush border of the mucosa in control animals, whereas in animals given formula, horseradish peroxidase was evident within absorptive epithelial cells. The number of horseradish peroxidase-positive cells was greater in the soy formula-fed rats than the protein hydrolysate-fed rats. In the second study (185), extension of the artificial feeding period to PND 26 showed intestinal closure to horseradish peroxidase absorption on PND 26 equivalent to that of control animals on PND 21, suggesting that the artificial diets delayed but did not prevent closure. In both studies, glucocorticoid treatment prevented the delayed closure associated with artificial diets. The authors could not identify the mechanism by which glucocorticoids prevented the delayed closure associated with artificial diets. They believed that soy produced inflammatory epithelial damage, associated with eosinophil infiltration of the lamina propria, but found that low-dose corticosterone, equivalent to concentrations normally present in rat milk, prevented the delay in soy-associated intestinal closure without preventing eosinophil infiltration. They also noted that the effect of protein-hydrolysate formula in delaying closure was similar to that of soy and did not appear to be associated with inflammatory damage to the intestine. The authors pointed out that intestinal absorption of macromolecules is quantitatively less important in the human neonate than in the rat, and that intestinal closure is a less dramatic event in humans.

Strengths/Weaknesses: A strength of this study is that rat pups were fed soy formula, a relevant dosing scenario. The authors tried to balance sex and weight among the treatment groups when assigning rat pups. These studies were conducted on the last 4 days before weaning, a critical time for small intestine epithelial closure and a period when pups are sufficiently mature to feed independently and regulate body temperature. A weight-matched control group was included to account for the decreased body weights in the pups fed protein-hydrolysate and soy

3.0 Developmental Toxicity Data

formula. The authors reported the endogenous peroxidase activity in pups not given horseradish peroxidase. Serum albumin levels were included as an assessment of nutritional status of the pups. The numbers of horseradish-positive crypt cells were assessed blind to treatment group. The low dose of corticosterone was equivalent to corticosterone levels in the maternal milk of unstressed lactating rats, whereas the higher dose was included for dose-response purposes. RIA was used to verify the absence of detectable levels of corticosterone in protein-hydrolysate and soy formulas in Study 2. Intestinal tissue was examined in pups from ≥ 3 experiments performed at different times. These examinations were conducted blind to treatment group. Controls included intestinal loops from animals not exposed to horseradish peroxidase. For these experiments, sample sizes appeared to have been sufficient, although there was no indication that the authors controlled for litter effects. A weakness is that both studies used only one dose level of soy formula, which does not allow for evaluation of dose-response relationships. Data were generated using the formula Isomil®, and it is difficult to determine whether these data apply to all soy infant formulas. There was no evidence that the authors controlled for litter effects or that the litter was used as the unit of analysis. The number of pregnant dams from which the litters originated was not specified. Aside from maternal milk, removing pups from the lactating dam on PND 17 likely introduced other stress factors, which may have contributed to study outcome. The difference in horseradish absorption for pups given soy formula and soy formula + hydrocortisone was not statistically significant. Rat pups and human infants may vary in some aspects of gastrointestinal tract maturation; the authors state that human infants have immunoglobulin absorption across the placenta, which complicates studies on neonatal intestinal absorption. The significance of these data for humans is unclear, as intestinal absorption of macromolecules is less significant in humans. Controls not exposed to horseradish peroxidase showed peroxidase activity in eosinophils, neutrophils, monocytes, and erythrocytes.

Utility/Adequacy for CERHR Evaluation Process: This report is not useful in the CERHR evaluation process.

Churella et al. (186), from Ross Laboratories, examined the effect of trypsin-inhibitor levels in soy formula on development of rats. Rat diets containing varying levels of trypsin inhibitors were prepared by using soy formulas that were not treated, were heat processed, or were heat processed and sterilized to reduce trypsin-inhibitor activity. At 21 days of age, 12 Sprague Dawley rats/group were given feed containing 235, 40, 10.4, 14.7, or 11.5 trypsin-inhibitor units/100 g feed for 3 weeks. A control group was fed a casein-based diet containing 1.5 trypsin-inhibitor units. Diets were fairly similar in protein, fat, carbohydrate, and *d,l*-methionine content. Food intake and body weights were measured over the 3-week period. Animals were then killed for measurement of final body weights and liver and pancreas weights. Pancreases were examined histologically. **[Histologic methods were not discussed, and it was not stated how many rats per group were examined.]** Data were evaluated by ANCOVA. Compared to controls, liver weights relative to body weights were significantly reduced **[by ~10%]** in rats fed diets with 40 or 11.5 trypsin-inhibitor units. **[Absolute organ weights were not reported.]** There were no effects on body weight gain, relative pancreas weight, or pancreatic hypertrophy or hyperplasia. In the analysis of 5 infant soy formulas, 4 were found to have trypsin-inhibitor activity at <15% of the activity of soy-protein isolate used to manufacture the formulas. The study authors concluded that residual trypsin-inhibition activity is low in soy formulas and, at those levels, would be of no nutritional significance to the rat.

Strengths/Weaknesses: A strength of this study is that diets included soy-based infant formulas (at different stages of processing to heat-inactivate soybean trypsin inhibitor, soy isolate (the soy protein used to manufacture formulas), and a casein-based rodent diet. Duration of the treatment period (3 weeks) was determined in a probe study, which demonstrated this as the time of

3.0 Developmental Toxicity Data

maximum pancreatic hypertrophy for rats fed unheated soy protein. Weanling rats were placed into treatment groups such that each group had similar body weight distributions. The authors calibrated the trypsin-inhibition assay, demonstrating that 1 g purified soybean-trypsin inhibitor inhibited 1.64 g trypsin. The impact of processing soy isolate on its soybean-trypsin inhibitor content was evaluated. In deriving the soybean-trypsin inhibitor inhibition curve, extract concentrations were used in a range that inhibited 20–80% of trypsin activity, as values outside this range were not linear. A weakness of this study is that there was no indication that the authors controlled for litter effects. While these experiments are helpful for examining soybean trypsin-inhibitor activity, it is difficult to judge the effects of the different formulas as the authors supplemented the lyophilized formulas with numerous other components (carbohydrate, fat, minerals, vitamins, and methionine) when preparing the diets. The trypsin used to analyze soybean trypsin-inhibitor activity was 52% pure. Dose-response could only be evaluated for soybean trypsin-inhibitor activity. Limited endpoints were examined in the study. Histology procedures were not described. Weanling rats were used in this study, so critical developmental stages during the neonatal period were not evaluated.

Utility/Adequacy for CERHR Evaluation Process: This report is not useful in the CERHR evaluation process.

3.2.4.2 Pig

Li et al. (187), funding not indicated, examined the effects of soy diet on hypersensitivity in early-weaned pigs. Four sows were fed corn-gluten meal from GD 109 through lactation. Piglets (Hampshire × Yorkshire × Duroc) from those 4 litters were randomly assigned to a soy or milk (control) group (n = 16/group). From 7 to 14 days of age, piglets were sensitized by oral gavage with 5 g/day of their respective protein treatment, 48% soybean meal or dried skim milk (control group). Pigs were weaned at 21 days of age and fed a milk-protein or a soybean-meal diet ad libitum until 56 days of age. **[Lactose was listed as the carbohydrate source for the soybean diet but not specified for the milk-protein diet. Lactose was assumed to be the carbohydrate source in the milk-protein diet, but the percentage in diet was not specified. Both diets contained soybean oil.]** One day prior to termination, blood was drawn to obtain lymphocytes and measure IgG titers to soy protein. In addition, dermal soybean hypersensitivity was tested. Half the pigs (2 per litter) were killed at 28 days of age, and the other half were killed at 56 days of age. Duodenum samples were removed to collect lymphocytes and examine villous height and crypt depth by scanning electron and light microscopy. Blastogenic response was determined in lymphocytes. Mitogenic responses were measured in lymphocytes and mononuclear cells. Data were analyzed by ANOVA and Student *t*-test.

Body weight gain was reduced at 28 days of age ($P < 0.01$). Serum concentrations of zinc, selenium, iron, and copper were unaffected by diet at 28 and 56 days of age. No significant differences in skin thickness following intradermal injection of soy or milk proteins were observed between the soybean- and milk-diet groups at 27 and 55 days of age. However, IgG titers to soy were significantly increased in both age groups of piglets fed soybean compared to milk-protein diets. At 28 days of age, intestinal villi of pigs fed soybean diets were shorter and broader than those of pigs fed milk-protein diets, but this difference was not observed at 56 days of age. At 28 and 56 days of age, intestinal crypt depth was slightly higher in pigs fed soybean diets. Neither diet had a significant effect on blastogenic responses of peripheral or intestinal lymphocytes collected at either age and cultured with purified soy proteins, phytohemagglutinin, or pokeweed.

3.0 Developmental Toxicity Data

The study authors concluded that pigs sensitized to soybean protein and fed a soybean-meal diet displayed transient hypersensitivity that was evidenced by decreased villous height and increased IgG titers. The effects coincided with reduced growth from 3 to 4 weeks.

Strengths/Weaknesses: A strength of this study is that sows received a corn-corn gluten diet to prevent the passive transmission of soybean-protein antibodies to the offspring. Pigs in each litter were assigned randomly to treatment groups, and it appears that the authors assigned piglets within the same litter to different treatment groups (i.e., 4 litters were used and at PND 28 necropsy, 8 animals were randomly selected [2 pigs/litter] from each treatment for necropsy). Pigs received soybean orally, which represents a relevant route of exposure. Triplicate intestinal villi samples (10 villi/specimen) were measured for each pig. Villous atrophy has been reported in other species after inducing hypersensitivity to soy proteins. A weakness of the study is that the authors used only one dose level of soy, so dose-response relationships could not be evaluated. Pigs originated from only 4 litters. There was no indication that the authors controlled for litter effect during their data analyses. Aside from soybean meal, there were other differences between the two weaning diets. Abnormal villous morphology and increased serum IgG titers corresponded with a period of decreased growth in soy-fed weanlings. As the authors point out, “low preweaning feed intake, which commonly occurs when weaning at 3 wk or less, results in a transient hypersensitivity to dietary antigens.” It is interesting that skin-fold thickness was doubled in pigs maintained on a milk protein-based diet and given a milk-protein injection on PND 27, particularly given that milk-protein allergies are common in children. There was no increase in serum anti-soy antibody IgG titers in milk protein-fed pigs, but anti-milk-protein IgG was not tested. The contribution of early weaning stress to these effects is not known.

Utility/Adequacy for CERHR Evaluation Process: This report is of limited utility in the evaluation process.

Zijlstra et al. (188), funding not indicated, examined the short-term metabolic effects in neonatal piglets fed soy formula. Two separate experiments were conducted 12 months apart. In the first experiment, a catheter was inserted into the umbilical artery of 1-day-old neonatal pigs. Pigs were fed by gavage dosing with casein-whey protein formula or hydrolyzed-soy formula for 36 hours and then received only water for 12 hours. A catheter was inserted into the portal vein of neonatal piglets (large White × Pig Improvement Company), and baseline blood samples were drawn from the catheters 1 hour later. The piglets were gavaged with formula (20 mL/kg bw) containing cow-milk protein (40:60 casein:whey ratio), hydrolyzed-soy protein, or intact-soy protein. Arterial and portal blood samples were collected at 15, 30, 60, and 120 minutes following dosing. The second experiment was similar to the first except that only an arterial catheter was inserted, and blood sampling was extended to 3 hours. There was also a difference in formula carbohydrate source. In experiment 1, carbohydrate sources were glucose polymers in soy-protein formulas and lactose in cow-milk protein formula. In experiment 2, the soy and cow-milk formulas were standardized to contain equal amounts of reduced lactose and glucose polymers. Soy diets were fortified with *d,l*-methionine and *l*-lysine in both experiments. Parameters measured included plasma amino acid levels using anion-exchange chromatography, insulin and glucagon levels by RIA, and glucose concentrations using a glucose-oxidase method. In experiment 2, piglets were killed following the last sampling time and gastrointestinal contents were weighed to determine gastric emptying. Each experiment was conducted as 4 replicates of 6 littermates. Within each replicate, 2 piglets were randomly assigned to each of the 3 treatment groups. Therefore, there were a total of 8 piglets in each treatment group in experiments 1 and 2. **[Data were analyzed according to a randomized complete block design, but the statistical tests were not specified.]**

3.0 Developmental Toxicity Data

In the 48-hour period prior to formula dosing and blood sampling in both experiments, piglets fed casein-whey formula gained weight, while piglets fed hydrolyzed-soy formula lost weight. Diarrhea was observed in 15 of 16 piglets fed the hydrolyzed-soy formula in experiment 1 and in all piglets fed the casein-whey and hydrolyzed-soy formulas in experiment 2. The study authors noted that concentrations of glucoamylase and maltase are low in the digestive tracts of piglets. Therefore, there may have been impaired digestion and absorption of the carbohydrates used in soy formulas in experiment 1 and all formulas in experiment 2. The authors also cited studies noting that soy proteins were shown to cause small intestine mucosa damage resulting in malabsorption and diarrhea in pigs.

In experiment 1, differences between amino acid concentrations in portal compared to arterial blood following dosing with all formulas indicated absorption from the gut. In piglets fed casein-whey formula, the difference between amino acids in portal compared to arterial blood peaked within 15 minutes, indicating rapid digestion and absorption of proteins. The post-feeding rise in amino acid levels in portal blood was slower and peaked at a lower level with soy formulas, suggesting attenuated digestion and absorption of proteins. Higher concentrations of amino acids in portal compared to arterial blood were maintained for 2 hours following feeding only in the piglets given hydrolyzed soy. In experiment 2, patterns of amino acid concentrations in arterial blood were similar to those in experiment 1. Weight of material remaining in stomach and small intestine did not differ between groups, leading study authors to conclude that differences in gastric-emptying rates were not the reason for altered plasma concentrations between the different feeding groups. In experiment 1, insulin and glucose concentrations were significantly increased at 15–30 minutes after feeding in arterial and portal blood of piglets fed casein-whey compared to either soy formula. There were no significant effects on glucagon levels. In contrast to experiment 1, there were no significant differences in arterial blood concentrations of insulin, glucagon, or glucose at 15–30 minutes following feeding in experiment 2.

The authors concluded that “hydrolyzed soy protein did not result in elevated nutrient concentrations or hormone responses compared with intact soy or [cow] milk protein.” Standardization of carbohydrate sources among formulas in experiment 2 but not experiment 1 was suggested by study authors as a possible reason for differences in results between the first and second experiments.

Strengths/Weaknesses: A strength is that during the experiments, formulas were stirred constantly to ensure homogeneity, and formulas were replaced every 12 hours. Soy hydrolysis was examined using HPLC. The authors controlled for litter effect by using 2 piglets/litter for each treatment group. Treatment groups were equally represented in each replicate. Pigs received formulas orally, which represents a relevant route of exposure. To avoid potential effects on small intestine structure and function prior to sample collection, pigs were maintained on the hydrolyzed soy-protein formula prior to the metabolic study when both hydrolyzed- and intact-soy protein were used. The authors monitored the amount of dry matter in the stomach and 3 segments of the small intestine to confirm that differences in absorption were not related to differences in transit time through these gastrointestinal segments. Statistical analyses included a randomized complete-block design to account for different study replicates, although it is not clear that the authors considered the litter in their analysis. A weakness is that the authors used only 1 dose level of hydrolyzed- and intact-soy protein, so dose-response relationships could not be evaluated. In both experiments, hydrolyzed-soy protein-treated pigs lost weight, whereas control pigs fed casein-whey protein gained weight over the monitoring period prior to formula dosing. In the first experiment, hydrolyzed-soy protein-fed pigs had diarrhea, and both hydrolyzed soy protein- and casein whey-fed groups had diarrhea in experiment 2. Pigs fed casein-whey protein had greater glucose and insulin concentrations in experiment 1, which the

3.0 Developmental Toxicity Data

authors corrected in experiment 2 by normalizing dietary carbohydrate composition. Each of these factors may have contributed to differences in response. The authors report pooled SEM, so it is not possible to determine the variability around individual means. It is unclear why the authors considered $P < 0.05$ to be significant, but noted $P < 0.1$ in figures.

Utility/Adequacy for CERHR Evaluation Process: This report is not useful in the CERHR evaluation process.

3.3 Utility of Data

3.3.1 Human data

There is a single study that was considered of limited utility for evaluation of exposure during pregnancy to soy formula (97). An important limitation is that this study involved vegetarianism in pregnant women and not ingestion of soy formula. There are a large number of papers describing growth or other health parameters in humans exposed to soy infant formula. Thirty-eight papers were judged to have some utility in the evaluation process; however, the utility of these papers was limited. Commonly encountered limitations included the non-random or unspecified method of assignment to feeding groups, the use of self-selected breast- and formula-feeding mothers, failure to control for the reasons for which soy formula was used, and the early and inconsistent introduction of solid foods in some studies. Studies that compared outcomes in children randomized to soy or cow-milk formula were considered the most reliable, particularly when parents and outcome assessors were masked to formula assignment.

3.3.2 Experimental animal data

Experimental animal studies on developmental effects of soy formula or other soy foods have been performed in rats, mice, marmosets, monkeys, and pigs. Ten experimental animal studies were found to have utility for the evaluation process. Of these 10 studies, 1 small study in rats (162) and 1 study in marmosets (170, 171) involved administration of soy milk or infant formula, and only the marmoset study involved direct administration of soy formula to infant animals. A pig study (187) used a 48% soybean meal-based liquid diet for neonatal animals. This soybean-meal content is higher than the 14.6–16% content of soy-protein isolate in marketed formulas (see Section 1.1). Most of the developmental studies performed in rodents examined the effects of dietary soy products or of soy-isoflavone preparations added to soy-free diets, and it is not clear to what extent the effects of soybean meal are similar to those of commercial isolates. The dietary interventions used in the experimental animal studies differ from one another and it is not clear which, if any, are appropriate models for soy infant formula. Dose-response information is available only from the study of McVey et al. (167, 168), in which an isoflavone mixture was added to a soy-free feed giving rise to 5 different isoflavone-intake levels. The use of this study in the assessment of possible toxicity of soy formula assumes that the isoflavone content of soy formula is the toxicologically important component.

3.4 Summary of Developmental Toxicity Data

3.4.1 Human studies

North et al. (97) performed a case-control study of hypospadias using information from questionnaires completed during pregnancy. A questionnaire administered at 32 weeks gestation included dietary items. There were 51 cases of hypospadias identified during the first 3 years of life. Between 5700 and 6600 boys without hypospadias were used as controls for various analyses. A multivariate model showed a significant association between hypospadias and maternal vegetarianism during pregnancy (OR 3.88, 95% CI 1.69–8.92). There was no significant association with intake of soy products during pregnancy; however, few women in the sample

3.0 Developmental Toxicity Data

used such products. The authors hypothesized that phytoestrogens in the diets of vegetarian women may have played a role in the development of hypospadias in male offspring.

Studies on outcomes after feeding soy formula in infancy are summarized in Table 40. Most of the studies on growth have not shown differences between soy and cow-milk formula in term infants (102, 104, 105, 107, 110, 117, 118, 143). Some studies reported that infants given soy formula have transiently lower bone-mineral content than infants fed cow-milk formula (106) or human milk (116); however, other studies reported that serum concentrations of bone accretion-related hormones and minerals in soy formula-fed infants demonstrated the appropriate compensatory response to the vitamin and mineral content of the food source (108, 109, 118). Reports in very low birth-weight and preterm babies suggest that soy formula may not support adequate growth and bone development in these populations (99, 112). Soy infant formula is not marketed for use in preterm infants.

The relationship between exposure to soy formula and hypersensitivity or allergic disorders has been investigated. Intestinal changes similar to those of gluten enteropathy have been described in infants with soy-sensitivity (125); however, infants with intestinal hypersensitivity do not appear to react to soy formula more often than they react to cow-milk formula (126). Studies comparing soy- and cow milk-formula feeding in infants with familial predispositions to allergic disease have not shown an advantage of one formula over another, but have shown that breast feeding was superior to the use of either formula (109, 132, 134-139).

Infants fed soy formula may not absorb thyroxine from the intestine, attributed to fecal wastage with decreased enterohepatic circulation (151-153). Although the addition of iodine and reduction in fiber content of soy formulas is a partial solution, 1 paper noted that the problem may occur if formula intolerance increases stool frequency (152).

A case-control study of premature thelarche used 130 subjects from 552 potentially eligible girls (154). Age-matched controls were recruited, consisting in some but not all instances of friends of the case. Parents were interviewed with regard to family history and possible exposures, including the use of soy formula. Multivariate analysis did not show a significant relationship between premature thelarche and soy-formula feeding. When the analysis was restricted to girls with onset of premature thelarche before 2 years of age, a significant association with soy-formula feeding was identified (OR 2.7, 95% CI 1.1–6.8). Other significant factors included maternal ovarian cysts and consumption of chicken. Consumption of corn was protective.

Strom et al. (15) identified 952 adults who had participated as infants in a controlled, non-randomized formula trial. There were 248 adults who had been given soy formula and 563 who had been given cow-milk formula. The subjects were asked about height, weight, sexual maturation, education level, and reproductive outcomes. There were no formula-related differences in height, weight, body-mass index, or sexual maturation. Women who had been given soy formula had longer menstrual periods (mean 0.37 days, 95% CI 0.06–0.68 days) and there was a soy formula-associated increase in the risk of experiencing severe menstrual discomfort (RR 1.7, 95% CI 1.04–3.00 compared to no or mild discomfort, unadjusted). Another study (156) evaluated 48 children who had been given soy formula and 18 children who had not been given soy formula and found no evidence of precocious puberty or gynecomastia. The sample size may not have been adequate to detect an increase in premature thelarche of the size suggested by Freni-Titulaer et al. (154).

3.0 Developmental Toxicity Data

There are 2 case-control studies of women with breast cancer in which a protective effect of adolescent soy foods was suggested (160, 161). Both studies involved Asian women in either Shanghai or Los Angeles county. Neither study evaluated the use of soy infant formula.

3.4.2 Experimental animal studies

Experimental animal studies are summarized in Table 41. The only study in which exposure to dietary soy was likely limited to dams was that of Masutomi et al. (164). Sprague Dawley dams were given a soy-based or a soy-free diet until PND 10, prior to the time when pups will eat maternal feed. Female offspring in the soy-diet group had an increase in body weight on PND 21 and a decrease in weight-adjusted anogenital distance. Male offspring in the soy-diet group had an increase in body weight on PND 2. The study by Hughes et al. (162) gave soy milk or rice milk instead of water to lactating rats (10–30 mg/kg bw/day) and evaluated offspring at weaning. There were only 3 dams exposed to soy milk and 2 dams exposed to rice milk. There was an increase in female pup body weight, a decrease in weight-adjusted female anogenital distance, a decrease in uterine progesterone receptor, and a decrease in relative epididymal weight; however, the litter was not used as the experimental unit in the analysis, limiting the utility of the study. Although treatment was given to the dam, the pups would have had access to the soy milk during the latter part of the lactation period. The body weight increases in offspring in the Masutomi et al. and Hughes et al. studies were not confirmed by Lund et al. (58) or Atanassova et al. (166), who reported a decrease in offspring body weight associated with developmental exposures to soy-based diets, albeit at a later age of evaluation.

Several rodent studies compared soy-based and soy-free diets during pregnancy and lactation with pups weaned to their dam's diet (58, 79, 163, 167, 168). The effects reported in these studies are inconsistent. Odum et al. (163) reported advancement of pubertal landmarks in males and females, but Lund et al. (58) reported delayed vaginal opening. Testis weight was reported to be decreased by dietary soy (166) and increased by dietary soy (167). These differences may reflect different isoflavone contents of the diets used in different studies or other differences in the composition of the feed.

The study of McVey et al. (167, 168) permits a dose-response evaluation of dietary isoflavone supplementation and developmental effects. These investigators used a soy-free diet to which was added soy protein and soy isoflavones at 5 exposure levels (plus the soy-free diet control). The most sensitive endpoint was an increase in testis weight on PND 28 in rats that were exposed during pregnancy, lactation, and weaning to a dietary-isoflavone intake ≥ 2 mg/kg bw/day. There were no significant alterations at the next lowest dose level, 1.2 mg/kg bw/day.

The study of Sharpe et al. (170), extended by Tan et al. (171) involved the feeding of twin marmosets with either soy infant formula or a cow-milk formula. The authors expected the use of co-twins to permit paired analyses and to decrease variability between animals assigned to different treatments. The study authors reported a decrease in plasma testosterone on PND 35–45 with an increase in testis weight and in numbers of Sertoli and Leydig cells at 120–138 weeks of age. The authors believed the results were consistent with a compensated Leydig cell failure. They expressed concern that because the marmosets were only partially formula-fed, effects in human infants who are exclusively formula-fed may be more severe. Interpretation of this study was limited in part by the lack of information on normal variability in the reported parameters in marmosets and by concern that the marmoset may not be an appropriate model for assessing human risk.

3.0 Developmental Toxicity Data

Conclusions to be developed at the Expert Panel meeting:

Evidence is (sufficient/insufficient) to conclude that soy infant formula (would/would not) produce developmental toxicity with exposure during pregnancy at (dose level) as manifested by (end points).

Evidence is (sufficient/insufficient) to conclude that soy infant formula (produces/does not produce) toxicity with childhood exposure in (girls/boys) at (dose) manifested by (endpoint).

Evidence is (sufficient/insufficient) to conclude that soy infant formula (produces/does not produce) developmental toxicity in (sex, species) at (dose, route, timing of exposure) manifested by (endpoint). The experimental animal data are (assumed relevant/relevant/not relevant) to the assessment of human risk.

Note: The definitions of the term sufficient and the terms assumed relevant, relevant, and not relevant are in the CERHR guidelines at <http://cerhr.niehs.nih.gov/news/guidelines.html>.

3.0 Developmental Toxicity Data

Table 40. Studies in Humans Exposed as Infants to Soy Formula

Comparison groups	Major findings	Reference
	Growth and nutrition	
Infants fed soy formula (n = 14) and infants fed evaporated cow-milk formula (n = 14) from 24 hours to 3 months of age.	Growth in both groups paralleled Iowa Growth charts. Infants on soy formula had lower cholesterol and higher total plasma protein.	Kay et al. (102)
Infants fed soy infant formula (n = 28) and infants fed cow-milk formula (n = 30) for 6 months. The soy formula was not methionine-supplemented as is modern soy formula.	Soy-fed infants, especially girls, lagged in volume consumed and growth from 2 to 4 months of age; cow-milk fed group gained more weight per ounce of formula.	Cherry et al. (103)
Infants fed soy infant formula (n = 26), infants fed cow-milk formula (n = 29), and breast-fed infants (n = 26). Over the 6-month study, many breast-fed infants dropped out or were given cow-milk formula.	Infants in all groups followed their centile growth curves.	Dean (104)
Infants on soy formula (n = 20) and infants on cow-milk formula (n = 20) for 16 weeks.	No difference between the 2 groups in growth parameters, hematology measures, or blood chemistry.	Jung and Carr (105)
Infants on soy formula (n = 13), infants on cow-milk formula (n = 20), and breast-fed infants (n = 26). Infants selected at 6 weeks of age and followed for 1 year.	No growth differences between groups after 6 weeks of age; soy-fed infants showed slower bone mineralization at 3 months but not thereafter.	Köhler et al. (106)
Infants fed soy formula (n = 18) or cow-milk formula (n=17) for 12 months.	No differences in energy intake or growth; bone mineral content less in soy formula-fed infants.	Steichen and Tsang (107)
Infants on soy formula (n = 11), cow-milk formula (n = 11), or human milk (n = 9), followed for 1 year.	No difference in bone width or bone mineral content; differences in serum and urine minerals and bone-related hormones were consistent with homeostatic adjustments to different mineral content in the food sources.	Hillman et al. (108, 109)
Infants on soy formula with (n = 73) or without (n = 73) supplemental nucleotides, and infants breast-fed for 2 months and then given cow-milk formula (n = 67); infants followed to 12 months of age.	Groups were not comparable in weight and length at birth. No group differences in growth were seen after 6 months of age.	Lasekan et al. (110)
Very low birth-weight infants given soy formula, cow-milk formula, or high-calorie cow-milk formula until 3–4 months of age.	After 3 weeks of age, soy-fed infants had lower serum phosphorus and higher serum alkaline phosphatase.	Kulkarni et al. (99)
Preterm infants given soy formula or cow-milk formula (n = 40 overall), with change in soy-formula regimen after 10 infants were enrolled	Infants on soy formula gained less weight than infants on cow-milk formula.	Naude et al. (112)
Infants randomized to 1 of 2 different soy formulas (n = 10/group) and 10 breast-fed infants (for comparisons to 4 months of age).	Bone mineral density and plasma zinc higher in breast-fed than soy formula-fed infants at 4 months of age; no difference between soy formula-fed infants and historical control in these parameters at 6 and 12 months.	Chan et al. (116)

3.0 Developmental Toxicity Data

Comparison groups	Major findings	Reference
Infants exclusively fed soy formula (n = 20), cow-milk formula (n = 19), or breast-fed (n = 17) for 4 months and followed until 6 months of age.	Growth and serum measurements related to bone accretion were not different by group. Bone density was greater in soy formula-fed than breast-fed infants.	Venkatarama n (117)
Infants given 1 of 2 different soy formulas (n = 21/group), infants given cow-milk formula (n = 20), and breast-fed infants (n = 10), followed for 1 year; solid food introduced at 3 months in breast-fed and 4 months in formula-fed groups.	Weight and head circumference unaffected by feeding type; length lower in breast- than formula-fed groups; serum 1,25-dihydroxyvitamin D higher in 1 of the soy-formula groups.	Mimouni et al. (118)
Infants given a soy or cow-milk formula as subjects in 1 of several feeding studies over a 14-year period; contacted as adults 20–32 years of age.	Significant association between adult overweight and having been fed a soy formula as an infant (adjusted OR 1.47; 95% CI 1.01–2.13).	Stettler et al. (119)
Gastrointestinal effects		
Infants with soy protein-induced intestinal villous atrophy (n = 2) and normal infant (n = 1).	Soy-sensitive infants had histologic intestinal changes similar to those seen with gluten enteropathy.	Poley et al. (125)
Infants with enterocolitis suspected due to cow milk- or soy-protein sensitivity, challenged with cow-milk and soy formulas.	Infants did not react more to 1 formula type than the other, and often reacted to both.	Burks et al. (126)
Allergy and immunology		
Infants with a family history of major allergy assigned to soy formula (n = 79) or cow-milk formula (n = 201), followed 17 years; 48 infants with a family history of major allergy were breast-fed.	No difference in development of allergic disease by formula type; breast-fed children were less likely to develop allergy after 3 years of age.	Gruskay (132)
Infants with family history of allergic disease were permitted to be breast fed; an intervention group (n = 238) was told to avoid cow milk and was given soy formula and a non-intervention group (n = 249) was given no instruction; most of the latter infants were exposed to cow-milk formula. 1 year follow-up.	Neither formula offered an advantage in preventing allergic disease; infants breast-fed for some period of time experienced protection from allergic disease compared to infants who were never breast-fed.	Miskelly et al. (134)
Infants with family history of atopy and whose mothers decided not to breast feed were randomized to soy formula (n = 41), cow-milk formula (n = 40), or casein-hydrolysate formula (n = 43) for 6 months; followed up to 5 years with comparison to breast-fed infants.	Breast feeding offered an advantage in protection against atopic disease. Among the formula-fed infants, casein-hydrolysate formula was superior to either soy or cow-milk formula in protection against atopy.	Chandra et al. (135-139)
Children with peanut allergy (n = 49), children with atopy (n = 70), and non-allergic children (n = 140) surveyed for infant feeding history.	Soy consumption was an independent risk factor for peanut allergy (adjusted OR 2.61; 95% CI 1.31–5.20).	Lack et al. (141)
Infants given 1 of 3 soy formulas, each with a different protein content (n = 39 overall) for 4 months.	Blood IgG levels were higher with the high-protein formula.	Zoppi et al. (142)

3.0 Developmental Toxicity Data

Comparison groups	Major findings	Reference
Infants given 1 of 2 soy formulas (n = 13 or 14/formula) or 1 of 2 cow-milk formulas (n = 7/formula), differing in protein content, from birth to 4.5 months of age.	Head circumference and weight were not affected by formula type; serum cholesterol and C3 complement were lower in soy- than cow milk-formula groups. Immunoglobulins were lower in low-protein soy group than other groups.	Zoppi et al. (143)
Infants given soy formula (n = 9), 1 of 3 cow-milk formulas (n = 7–10/formula), or who were breast-fed (n = 27) for 5 months, after which all infants were switched to the same diet for the balance of the first year of life.	Response to immunization was greatest in breast-fed infants and infants given a high-protein cow-milk formula, with the exception of antibody to polio, which was lower in breast-fed children than in formula-fed children. Soy formula-fed infants had more episodes of infection than breast-fed children.	Zoppi et al. (144)
Infants fed cow-milk formula, soy formula, or a mixture of cow-milk and soy formulas (n = 67 overall) and 7 breast-fed infants; follow-up was nearly 4 years.	Serum from soy-fed infants bound soy protein less than serum from cow milk-fed infants bound cow-milk protein; feeding a soy product resulted in comparable or greater antibody response to cow milk than feeding a cow-milk formula.	May et al. (145)
Infants with clinical gastroenteritis that continued after switching to soy formula (n = 18) were challenged with different foods.	Infants with positive challenges to soy and egg had higher anti-soy IgG than infants with a negative response.	McDonald et al. (146)
Children with autoimmune thyroid disease (n = 59), healthy siblings (n = 76), and healthy unrelated controls (n = 54); history of infant feeding was evaluated.	Significantly more children with thyroid disease (31%) received soy formula compared to healthy siblings (12%) and healthy unrelated controls (13%).	Fort et al. (148)
Infants given 1 of 2 different soy formulas (n = 92, 94), one of which contained added nucleotides, and breast-fed children who were weaned to cow-milk formula at 2 months (n = 81); 1-year follow-up.	No difference by feeding group in antibody response to immunizations (except <i>Hemophilus influenza b</i>), parent-reported diarrhea, or otitis media. No consistent differences in immune status, maturation, or level of immunocompetence between soy and cow-milk formula-fed infants.	Ostrom et al. (149), Cordle et al. (150)
	Thyroid function	
Children with goiter related to soy formula-feeding (n = 3).	Improvement with discontinuation of soy formula or addition of iodine.	Shepard et al. (151)
Infant with congenital hypothyroidism who was fed soy formula (n = 1).	Oral thyroxine therapy did not decrease thyroid-stimulating hormone until cow-milk formula was substituted for soy formula.	Chorazy et al. (152)
Infants with congenital hypothyroidism who were fed soy formula (n = 3).	Switching from soy formula to cow-milk formula resulted in increased absorption of thyroid replacement doses.	Jabbar et al. (153)

3.0 Developmental Toxicity Data

Comparison groups	Major findings	Reference
	Reproductive endpoints	
Girls with premature thelarche (n = 130) and age-matched control subjects, retrospective questioning of parents about infant feeding.	No significant association overall between premature thelarche and soy infant formula intake; restriction of multivariate analysis to subjects with thelarche before age 2 years showed significant association (OR 2.7; 95% CI 1.1–6.8).	Freni-Titulaer et al. (154)
Adults who had been fed soy (n = 248) or cow-milk formula (n = 563) during infancy as part of a controlled trial; interviewed at 20–34 years of age	No infant feeding-related differences in adult height, weight, body-mass index, or sexual maturation history; duration of menstrual bleeding was 0.37 days longer and severe menstrual discomfort was more common in women fed with soy formula than with cow-milk formula	Strom et al. (15)
Children age 7–96 months who had been fed soy formula (n = 48) and children age 12–96 months who had not been fed soy formula (n = 18).	There were no group differences in height and weight, no signs of precocious puberty in girls, and no gynecomastia in boys.	Giampietro et al. (156)
	Other endpoints	
Infants on soy formula (n = 16, some with cholesterol added), cow-milk formula (n = 10), or breast-fed with supplemental cow-milk formula (n = 12).	Breast-fed infants had the highest serum cholesterol levels and lowest fractional cholesterol synthesis rate. There was an inverse relationship between cholesterol intake and fractional cholesterol synthesis.	Cruz et al. (157)
Diabetic children (n = 95), non-diabetic siblings (n = 194), and non-diabetic friends (n = 95); infant feeding history obtained.	Almost twice as many diabetic children had been soy formula-fed as had controls [not statistically significant; data were not shown].	Fort et al. (158)
Children age 9–10 years who had been given soy formula without breast feeding (n = 176) and who had received soy formula but also had had some breast feeding (n = 342).	No effect of breast feeding on IQ when controlled for parental education and family income.	Malloy and Berendez (159)
Women age 25–64 years with newly-diagnosed breast cancer (n = 1459) and age-matched women without breast cancer (n = 1556).	Significant inverse association between recalled amount of dietary soy protein eaten during adolescence and breast cancer diagnosis .	Shu et al. (160)
Adult Asian-American women with breast cancer (n = 501) and age- and ethnicity-matched women without breast cancer (n = 594).	Significant inverse association between recalled soy intake during adolescence and adult and breast cancer diagnosis.	Wu et al. (161)

3.0 Developmental Toxicity Data

Table 41. Developmental Effects of Soy Products in Experimental Animals

Model	Experimental groups	Effects of soy product	Reference
Long-Evans rat	Soy milk (n = 3) or rice milk (n = 2) provided to dam instead of water during lactation period (n = 3). Phytoestrogen-free AIN-93G feed was used.	<i>Female offspring</i> ↑Body weight ↓Anogenital distance adjusted for body weight ↓Uterine progesterone receptor <i>Male offspring</i> ↓Relative epididymis weight	Hughes et al. (162)
Wistar derived rat	Dams (n = 12/group) given assigned diet during pregnancy and lactation, and offspring weaned to assigned diet through PND 68. Diet assignments (dam/offspring) were: RM3/RM1 (standard soy-based diets) AIN-76/AIN-76 (soy- and alfalfa-free diet) RM3/AIN-76A Global 2016/Global 2016 (soy- and alfalfa-free diet) Purina 5001/Purina 5001 (based on soybeans and alfalfa)	<i>Dam exposure to RM3 diet</i> ↑Testis and epididymis weights on PND 68 ↑Birth and post-weaning weights <i>Offspring exposure to RM1 diet</i> Advanced puberty landmarks in male and female offspring ↑Uterine weight on PND 26 and 140–144 ↑Male body weight on PND 128 ↑Female body weight on PND 140–144	Odum et al. (163)
Long-Evans rat	Dams (n = 5/group) given phytoestrogen-containing diet (600 µg/g diet) or phytoestrogen-free fish meal-based diet throughout pregnancy and lactation. Offspring weaned to dam's diet.	Delayed vaginal opening ↓Body weight on PND 50 in male and female offspring	Lund et al. (58)
Sprague Dawley rat	Pregnant rats (n = 15/group) given CRF-1, a soy-based diet, or NIH-07, a soy-free diet. On GD 15, seven dams/group also given ethinyl estradiol in the diet.	↑Male pup body weight on PND 2 ↑Female pup body weight on PND 21 ↓Female anogenital distance adjusted for body weight Enhanced ethinyl estradiol effects on female reproductive organ histopathology	Masutomi et al. (164).
Wistar rat	Dams given 15.5% soy-meal diet or soy-free diet prior to and during mating, pregnancy, and lactation (n = 7–29, depending on endpoint).	↑Sertoli cell nuclear volume, PND 18 ↑Spermatocyte/Sertoli cell nuclear volume, PND 18 and 25 ↓Body weight, PND 90–95 ↓Testis weight, PND 90–95 ↑Plasma FSH, PND 90–95	Atanassova et al. (166)

3.0 Developmental Toxicity Data

Model	Experimental groups	Effects of soy product	Reference
Sprague-Dawley rat	Males (n = 6–12/group) from multigeneration study continued on 1 of 6 diets that had been given to their parents: AIN-93G (soy-free, casein-based diet), Control diet + soy protein (isoflavones 31.7 mg/kg feed), or Control diet + soy protein + isoflavone mixture at 36.1, 74.5, 235.8, or 1046.6 mg/kg feed.	↑Testis weight, PND 28 in 3 highest isoflavone groups (total isoflavone intake ≥ 2 mg/kg bw/day) ↑Serum testosterone and dihydrotestosterone, PND 120 in 2 highest isoflavone groups (total isoflavone intake ≥ 6.3 mg/kg bw/day) ↑Testicular 3 β -hydroxysteroid dehydrogenase activity, PND 28, in 2 highest isoflavone groups [Based on PND 28 testis weight: NOAEL 1.2 mg/kg bw/day and LOAEL 2.0 mg/kg bw/day]	McVey et al. (167, 168)
Han-NMRI mouse	Pregnant dams fed 7% soybean meal diet or soy-free diet; half of male offspring were maintained on dam's diet and half were switched to opposite diet (n = 27–29/group)	<i>Effects of offspring exposure</i> ↑Prostate weight in 2-month-old offspring ↑Relative male reproductive organ weight at 1 year	Mäkelä et al. (79)
Mouse	Wild-type male mice fed soy-based or soy-free diet “from birth” (n = 6–12/group; study also included aromatase-knock out mice).	<i>14 weeks of age</i> ↑Seminiferous tubule diameter ↓Seminiferous tubule length ↓Sertoli cell numbers <i>1 year of age</i> ↓Testicular epithelial volume ↑Testicular interstitial volume ↓Seminiferous tubule length	Robertson et al. (169)
Marmoset	Twins fed from 4–5 days of age with soy infant formula or cow-milk formula during part of the day (n = 7–14 twin pairs, depending on age of evaluation).	↓Plasma testosterone PND 35–45 ↑Testis weight, Sertoli cell and Leydig cell numbers at 120–138 weeks of age	Sharpe et al. (170), Tan et al. (171)
Pig	Piglets (n = 16 from 4 litters) randomly assigned 48% soybean meal-based liquid diet or cow-milk based diet; protein source continued after weaning until 56 days of age.	↓Body weight gain at 28 days of age Intestinal villi shortened at 28 but not 56 days of age	Li et al. (187)

↑,↓ Statistically significant increase, decrease in parameter.

4.0 REPRODUCTIVE TOXICITY DATA

4.1 Human data

4.1.1 Female

A number of studies examined the effects of dietary soy intake on hormone levels and/or menstrual cycles of women. The studies are presented in order of publication.

Cassidy et al. (54, 189), in studies funded by NIH and the UK Ministry of Agriculture, Fisheries, and Food, studied the hormonal effects of isoflavone intake in premenopausal women. Subjects included 15 healthy, non-vegetarian women aged 20–29 years. The women tracked their menstrual cycles and measured body temperatures to determine if ovulation was occurring for 4 months before the study. The women were then moved into metabolic suites and fed control diets with no soy products for the duration of 1 menstrual cycle. Beginning on or before the first day of menses and continuing through the next menstrual cycle, the women were fed diets containing isoflavones in various forms. A period of 1–4 months separated the control and soy product-exposed dietary periods. In most cases, diet administration was not randomized. One group of 6 women received 60 g/day textured-vegetable protein, containing 45 mg conjugated isoflavones (mean \pm SD daidzein 25.08 ± 0.31 mg/day and genistein 19.85 ± 0.43 mg/day) in place of beef, chicken, or cod. **[Based on the mean body weight of 61.6 kg, intake of conjugated isoflavones was 0.73 mg/kg bw/day, daidzein was 0.41 mg/kg bw/day, and genistein was 0.32 mg/kg bw/day.]** A group of 6 women received miso 50 g/day, containing 25 mg unconjugated isoflavones **[0.42 mg/kg bw/day isoflavone based on a mean body weight of 59.0 kg]** in place of beef, chicken, or cod. Three of the subjects dropped out of the study because they could not tolerate the high salt levels in miso. A group of 5 subjects received 60 g Arcon F, an isoflavone-free soy product, administered in biscuits or breads. Diets were randomized in study 3. One group of 6 women was given 28 g textured vegetable protein, with 23 mg isoflavone **[0.40 mg/kg bw/day isoflavone based on mean body weight of 57.0 kg]** in place of part of their bread. Isoflavone intake levels were based upon phytoestrogen intakes in Asian diets, reported at 50–100 mg/day. Blood and 24-hour urine samples were collected every 3 days during each dietary period. Blood was also collected on the day of ovulation, as determined by examining urine with an ovulation-detection kit. Plasma sex hormone-binding globulin, progesterone, 17β -estradiol, LH, and FSH levels were measured by RIA in the first study and by time-resolved fluorometry in the remaining 3 studies. Plasma cholesterol levels were measured. Intestinal transit times were determined by measuring radiolabel in feces. Statistical significance of results was analyzed by paired *t*-test. Urinary levels of isoflavones were reported for the earlier study (54) and those results are discussed in Section 2.1.1.3.

Subjects maintained constant body weights during the study. The only dietary-related effect on mean intestinal transit time was an increase during the follicular phase in subjects fed miso. The majority of menstrual and hormonal effects were observed in the 45 mg/day conjugated-isoflavone group, summarized in Table 42. Length of the follicular phase was increased in subjects fed 45 mg/day conjugated isoflavones, mid-cycle levels of LH and FSH were reduced, and 17β -estradiol levels during the follicular phase were increased. However, as noted in the earlier study (54), the reductions in the LH and FSH surge may have been due to sampling time, because no special attempt was made to determine the exact day of ovulation, and peak plasma and urinary concentrations may have been off by 1 day. Time to peak progesterone and peak progesterone concentrations were significantly reduced in the miso group, but the study authors concluded that the group size ($n = 3$) was too small to draw conclusions. In the earlier study (54), overall menstrual cycle length was increased by 3 and 5 days in 2 subjects whose urinary

4.0 Reproductive Toxicity Data

excretion of equol was high (see Section 2.5 on potentially susceptible subpopulations). No other effects were observed on menstrual cycles or plasma hormone levels. Cholesterol levels were reduced with intake of 45 but not 23 mg/day conjugated isoflavones. An increase in cholesterol levels occurred in the subjects fed isoflavone-free soy.

Table 42. Statistically Significant Hormonal Effects in Women Fed Textured Vegetable Protein Containing 45 mg/Day Conjugated Isoflavone

Parameter	Control diet	Isoflavone-enriched diet	
Follicular phase length, days	15.0±0.4	17.5±0.9	[↑17%]
Plasma 17β-estradiol, follicular phase, nM	0.25±0.03	0.35±0.1	[↑40%]
Plasma LH, mid-cycle, IU/mL	22.4±5.3	5.8±1.6	[↓74%]
Plasma FSH, mid-cycle, IU/mL	15.5±2.4	8.9±1.8	[↓43%]

From Cassidy et al. (189). Values are presented as mean ± SEM. Results presented as mean ± SD are available in Cassidy et al. (54), which gives slightly different results for some parameters.

Strengths/Weaknesses: The use of a controlled environment for the diet intervention is a strength of this study, as is the use of subjects as their own controls. The age range of the women was narrow, and the same batches of food and soy protein were used throughout the study. The use of 3 different sources of soy is also a strength. The hormone measures were appropriately selected, and there were good internal quality controls for the assays. Adequate blood, urine, and fecal samples were collected. The paired statistical methods were appropriate, but it is not clear if the hormone data were normally distributed and, if not, if the appropriate adjustments were made. Good data on menstrual history were not collected, and it was not clear that potential confounders such as race, body-mass index, alcohol, and smoking were considered in the hormone evaluations. Past oral contraceptive use was not considered. The small sample size and high drop-out rate on the miso diet are weaknesses and are likely to have limited the ability to control for potential confounders. The lack of randomization of diet order is a problem, and data were not presented in a manner permitting an evaluation of possible effects of diet order. This study at best evaluates acute effects for 1 cycle, and no information is available on whether these effects would have been sustained.

Utility/Adequacy for CERHR Evaluation Process: This study is useful, with the limitations noted.

Lu et al. (190), supported by the Public Health Service, evaluated the effect of soy milk supplementation on menstrual cycle hormones in 6 healthy non-vegetarian women with regular menstrual cycles. The subjects were admitted to a hospital clinical research center for 33 days, during which time they were served a hospital diet with three 12 oz portions of soy milk (1 portion with each meal). The soy milk was assayed periodically for isoflavone content by GC and contained a mean ± SD of 33.49 ± 14.48 mg/12 oz. daidzin/daidzein and 38.39 ± 14.62 mg/12 oz genistin/genistein. **[The aglycones were not separately reported but were estimated as 15–19% of the total isoflavone content.]** Blood was sampled 1 day before and 1 day after the initiation of soy milk, and weekly thereafter for 1 month. Three subjects had blood sampled during the month prior to initiation of soy milk, and all subjects had blood samples taken during at least 3 of the 6 cycles that followed the cessation of soy-milk supplementation. Four subjects were followed up to 10 months. **[Time points reported are baseline, during soy-milk ingestion, 1 month after soy-milk ingestion, and 2–3 months after soy-milk ingestion. Hormone levels from the 1 month prior to soy milk and for the 5–10 months after ingestion were not different and were averaged to produce the “baseline” values.]** Serum assays were performed by RIA for 17β-estradiol, progesterone, and dehydroepiandrosterone sulfate.

4.0 Reproductive Toxicity Data

Menstrual cycle length was recorded, and serum levels of 17 β -estradiol and progesterone were reported by cycle phase (day 5–7, day 12–14, day 20–22). Statistical analysis was performed by Friedman 2-way ANOVA. If duration of treatment affected hormone levels, multiple comparisons using paired *t* tests were performed on unranked data. **[Based on the way the results were presented, it appears that “duration of treatment” refers to the time block with respect to soy ingestion. The paired *t* tests appear to have been performed using the baseline hormone concentration as the control in each pair. There was no control for possible effects of hospitalization on hormone concentrations.]** Results are summarized in Table 43. The authors concluded that 1 month of soy supplementation increased menstrual cycle length and decreased 17 β -estradiol serum levels. They postulated that the decrease in dehydroepiandrosterone sulfate may reflect a decrease in 17 β -estradiol synthesis because dehydroepiandrosterone sulfate is a precursor in 17 β -estradiol synthesis. **[The Expert Panel notes that the sulfate is considered to be an adrenal product rather than a reflection of ovarian steroid synthesis.]** The authors contrasted their results with those of Cassidy et al. (54), noting that women in the Cassidy et al. study had an increase in follicular-phase levels of 17 β -estradiol and no change in this hormone at mid-cycle or in the luteal phase. They speculated that the larger amount of isoflavone supplementation in this study compared to that of Cassidy et al. may have been responsible for the difference in results.

Table 43. Effects of Soy Milk Consumption on Menstrual Cycle Length and Hormones

Parameter	Months after soy milk consumption phase		
	0 (During)	1	2–3
Menstrual cycle length	“↑” (<i>P</i> =0.06)	“↑” (<i>P</i> =0.11)	↔
Serum 17 β -estradiol			
Cycle average	↓	↓	“↓” <i>P</i> =0.11
Cycle days 5–7	“↓” (<i>P</i> =0.09)	↔	↔
12–14	↓	“↓” (<i>P</i> =0.056)	↓
20–22	↓	↓	“↓” (<i>P</i> =0.24)
Serum progesterone, luteal phase	↓	↔	↔
Serum dehydroepiandrosterone sulfate, cycle average	↓	↔	↑” (<i>P</i> =0.08)

n = 4–6 subjects per time period.

↑, ↓, ↔ increase, decrease, or no change compared to baseline value at *P* ≤ 0.05 by paired *t* test.

“↑,” “↓” increase or decrease described by study authors with *P* > 0.05.

From Lu et al. (190).

Strengths/Weaknesses: This study included good information about the components of the soy supplementation and good methods of hormone measurement. The controlled environment for the diet intervention, the use of subjects as their own controls for a portion of the analysis, and the use of at least 2 cycles of follow-up after the intervention are additional strengths. Weaknesses include the small sample size, varying lengths of soy ingestion, varying numbers of cycles for follow-up, missing values at some time points, and lack of control for potential confounders. Women on oral contraceptives should have been excluded or should have had a complete washout period.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Nagata et al. (191) examined the relationship between soy intake and blood levels of 17 β -estradiol and sex hormone-binding globulin. Fifty healthy women (21–42 years old) were

4.0 Reproductive Toxicity Data

questioned about menstrual and reproductive history and dietary intake of 169 foods, including 9 soy foods. A blood sample was taken on days 11 and 22 of the menstrual cycle (first day of menstrual bleeding = day 1) for measurement of serum 17 β -estradiol and sex hormone-binding globulin. Data were logarithmically transformed and adjusted using the Willett method, and Spearman rank correlation coefficients were calculated. After adjusting for age, body mass index, cycle length, and energy intake, a significant negative association was found between soy intake and 17 β -estradiol levels on day 11 ($r = -0.33$) and day 22 ($r = -0.30$) of the cycle; the day-22 association remained significant following additional adjustments for fat and crude-fiber intake. There was no association between total soy intake and sex hormone-binding globulin. However, a significant negative association ($r = -0.36$) was noted between miso intake and sex hormone-binding globulin on day 22, following adjustment for age, body-mass index, cycle length, and energy, fat, and fiber intake.

Strengths/Weaknesses: This study involved healthy subjects with no oral contraceptive or other hormone use. The broad assessment of dietary intake under natural conditions and adjustment for some potential confounders are strengths of the study. The use of detailed questionnaire information and appropriate transformation of hormone data are additional strengths. The sample size was, however, relatively small with a wide range of ages. The food frequency questionnaire covering previous-year average intake may not have accurately represented intake. Isoflavone levels were not measured, only estimated from reported food intake. The use of an exclusively Japanese sample may limit generalizability.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Nagata et al. (192) examined the effects of soy intake on menstrual cycle length and hormone levels in a study conducted at a Japanese university. Study subjects consisted of healthy premenopausal women who were either students or teachers at a nursing school. Subjects were randomly assigned to a soy-milk group ($n=31$) or control group ($n=29$). There were no significant differences in age, weight, height, body-mass index, age at menarche, parity, or smoking status between groups. Subjects in the soy-milk group were instructed to drink 400 mL/day of soy milk that was supplied to them, and only that soy milk. The soy milk contained 109 mg isoflavones. **[Based on actual mean weights of subjects, ~50 kg, isoflavone intake is estimated at 2.2 mg/kg bw/day.]** The study was conducted over 3 menstrual cycles with soy-milk supplementation beginning on day 11 (first day of menstrual bleeding = day 1) of the first cycle and ending on day 11 of the third cycle. Women in the soy-milk and control groups were instructed to continue eating their regular diets. Women were asked to keep dietary records, which were used to estimate intake of soy products, macronutrients, micronutrients, and isoflavones. Blood samples were collected as close as possible to day 11 (actual collection occurred on days 9–12) of cycles 1 and 3 for analysis of estrone, 17 β -estradiol, and sex hormone-binding globulin. Baseline and final values of all parameters were compared in the soy-milk and control groups. Statistical analyses included ANOVA to assess cycle-length changes and Mann-Whitney and Wilcoxon matched pairs signed rank tests to analyze hormone effects.

Diets in the soy-milk and control groups were stated to be almost identical in terms of macronutrient, micronutrient, soy, and isoflavone intakes. **[There was no statistical evaluation of dietary differences between treatment groups.]** Significant dietary changes occurring in both groups over the course of the study included decreased intake of calories, cholesterol, and carbohydrate. Intake of fat and protein was also reduced significantly in the control group. Variable changes in micronutrient intake were noted in both groups. Mean soy-milk intake in the group given soy milk was estimated at 354.8–365 mL. In the soy-milk group, total isoflavone

4.0 Reproductive Toxicity Data

intake [assumed to be daily intake] from all dietary soy sources was about 4.5 times higher at the end (mean = 116.4 mg) compared to the beginning of the study (mean = 25.4 mg). **[The final value of isoflavone intake was made up of isoflavone intake from soy milk: mean \pm SD = 97.0 ± 19.2 and other soy products: mean \pm SD = 19.4 ± 15.0 .]** The authors mentioned that isoflavone intake divided by energy intake was not significantly affected in the control group. **[However, statistical significance was obtained for a slight reduction in isoflavone intake from other soy products at the end (mean \pm SD = 18.4 ± 13.4 mg) compared to the beginning (mean \pm SD = 20.7 ± 12.9 mg) of the study in the control group.]** The third and fourth menstrual cycles were about 2 days longer in the soy-milk group but 1–2 days shorter in the control group compared to the first menstrual cycle; statistical significance was not attained in either group for comparisons of within-group cycle lengths. Differences between groups were also stated to be non-significant. **[It is not clear how this determination was made.]** The only significant effect on hormone level was a ~23% reduction in final compared to initial serum estrone level in the soy-milk group.

Statistical analyses were repeated following restriction to include only data from women providing blood samples no more than 1 day apart in cycles 1 and 3 (e.g., day 11 in cycle 1 and day 10 in cycle 3). The restricted dataset was comprised of data from 21 women in the soy-milk group and 23 women in the control group. Isoflavone intakes for the restricted datasets were similar to intakes obtained for the larger dataset. Compared to the first menstrual cycle, the fourth menstrual cycle was about 3.4 days longer in the restricted soy-milk group and 2.6 days shorter in the restricted-control group. The only significant effect on hormone level in the restricted groups was a ~30% reduction in final compared to initial serum estrone level in the soy-milk group. The study authors concluded that larger studies are needed to confirm the findings of this study.

Strengths/Weaknesses: The random assignment to intervention, the use of subjects as their own controls, and the use of 3 menstrual cycles are strengths of this study. The control and treatment groups were similar at baseline, and the statistical analyses were appropriate. The sample size was small, although the sample size calculation performed by the authors suggested adequate power for the study. Compliance was not assessed objectively, for example, with urine measurements. There were substantial changes in reported dietary composition over the course of the study, and the changes differed in the intervention compared to the control group. These changes were not accounted for in the paired analyses or group analyses. The study would have been strengthened with the use of multiple samples for hormone measurements throughout the cycle.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Xu et al. (87), supported by NIH, evaluated the effects of dietary soy on estrogen metabolism in 12 regularly cycling women aged 26.0 ± 4.9 (SD) years. The subjects were studied on each of 3 different commercial soy-powder supplements in a randomized cross-over design. The 3 supplements were designed to give a mean daily total isoflavone intake of 0.16, 1.01, or 2.01 mg/kg bw. Each supplement period consisted of 3 menstrual cycles plus 9 days of a fourth menstrual cycle, with about a 3-week unsupplemented washout period between treatment periods. The isoflavone content of each supplement consisted of about 55% genistein, 37% daidzein, and 8% glycitein, mostly as their glucoside conjugates. During days 7–9 of the fourth menstrual cycle in each treatment period, 3 consecutive 24-hour urine samples were collected and analyzed for phytoestrogens and for endogenous estrogens and their metabolites. Data were analyzed using ANOVA **[post hoc test not specified]** to compare urinary values between treatments, taking the 0.16 mg/kg bw/day isoflavone treatment as the control. Urinary excretion of genistein increased from a mean of 997 nmol/24 hours in women on the control treatment to 6529 and 14,200

4.0 Reproductive Toxicity Data

nmol/24 hours in women on the higher-isoflavone supplements. Daidzein excretion increased from a mean of 995 nmol/24 hours in women on the control supplement to 4964 and 9528 nmol/24 hours in women on the higher-dose supplements. Other isoflavones and lignans also increased in urine with increasing isoflavone content of the supplement. Urinary estrone and 17β -estradiol and their metabolites decreased with increasing isoflavone supplementation; 17β -estradiol excretion was reduced 15–23% and estrone excretion was reduced 8–15% by the 2 higher-isoflavone soy supplements compared to the control supplement. Metabolites of these estrogens were also reduced by isoflavone supplementation. **[The reduction of some of the hydroxylated estradiol and estrone metabolites was the focus of this report, which dealt with possible mechanisms of breast cancer risk reduction.]** The authors noted that the main study, of which this substudy was a part, did not show alterations in plasma estrogens or in the menstrual cycle (193). They proposed that urinary excretion of endogenous estrogens and their metabolites may be a more sensitive indicator of dietary effects on reproductive hormones than are plasma estrogens.

Strengths/Weaknesses: The randomized cross-over design, clear inclusion and exclusion criteria, range of dose levels, treatment over 3 menstrual cycles, appropriate statistical analyses, and evaluation of other sources of dietary isoflavones were strengths of this study. The control and treatment groups were similar at baseline, and the controls had minimal exposure to soy products. Phytoestrogens were measured in urine for an objective evaluation of soy ingestion. The small sample size, however, made it impossible to adjust for dietary and other differences between groups, and the study may not have been adequately powered to detect important differences between groups. It is not clear if the randomized block design provided random ordering of treatment groups. There was a lot of variability in the urinary levels of phytoestrogens.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Martini et al. (194), supported by the Minnesota Soybean and Research Council and the Minnesota Agricultural Experimental Station, studied menstrual cycle length and serum hormone measurements in 16 regularly cycling women who were not on oral contraceptives and 20 oral contraceptive users. Women were randomized to receive daily treatments consisting of 10 ounces of skim milk with or without a commercial soy supplement for 2 consecutive menstrual cycles. After the 2-month treatment, subjects were crossed over to the other treatment. The 34 g daily serving of the soy supplement contained 38 mg total isoflavones, including 23 mg genistein and 13 mg daidzein. Women who were not on oral contraceptives collected 24-hour urine samples and had fasting blood drawn on 2 consecutive days in the mid-follicular phase and the mid-luteal phase (timed using a urinary LH surge-detection kit). Oral contraceptive users had the same evaluations during 2 consecutive days between cycle days 7 and 9 and cycle days 21 and 23. Serum assays included commercial RIA for 17β -estradiol, estrone, dehydroepiandrosterone sulfate, and sex hormone-binding globulin, and commercial chemiluminescence immunoassays for progesterone and prolactin. Urine enzyme immunoassays were performed for 2- and 16α -hydroxyestrone, estrogen metabolites that have been associated with breast cancer risk modification. Statistical analysis was performed using paired *t*-tests, with each woman serving as her own control. There was no effect of soy supplementation on serum or urine measurements or on lengths of the menstrual cycle or its component phases. The authors called attention to their results differing from those of previous investigators (54, 189, 190), and suggested that hormone measurements may not be very precise unless stable isotope-dilution MS is used. The authors

4.0 Reproductive Toxicity Data

concluded that their results suggested a minimal influence of dietary soy on fertility and no interaction with oral contraceptives.

Strengths/Weaknesses: The randomized cross-over design with subjects serving as their own controls is a strength. The statistical analyses were appropriate; however, an intention-to-treat analysis should also have been performed. The inclusion of an oral contraceptive-using group and the treatment over 2 menstrual cycles are additional strengths. Weaknesses include the small sample size, broad age range, and lack of control for potential confounders. The wide range of ages may be particularly problematic because cycle characteristics in 40-year-old women may be different from those in 18-year-old women. There was large variability in the oral-contraceptive formulations, which may have influenced the results. There does not appear to have been a washout period between treatments. The authors dropped 2 women from the analysis without solid reasons.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Duncan et al. (193), in a study funded by NIH and the National Center for Research Resources, studied the effects of isoflavone intake on hormone action in 14 healthy, non-vegetarian premenopausal women (mean age \pm SD = 26.5 \pm 4.7 years). Using a randomized cross-over design, subjects received 1 of 3 soy-protein powders in addition to consuming their regular diets. They were instructed to avoid foods high in phytoestrogens, alcohol intake, and vitamin and mineral supplementation. Each of the diets provided for total isoflavone intake of 0.15 \pm 0.01 (control), 1.01 \pm 0.04 (low), or 2.01 \pm 0.03 (high) mg/kg bw/day. The dietary isoflavones (expressed as the aglycones) included 55% genistein, 37% daidzein, and 8% glycitein. Ninety-seven percent of genistein and daidzein and 91% of glycitein were present as glucoside conjugates. **[These diets would have resulted in intakes of about 0.08, 0.56, and 1.1 mg/kg bw/day genistin + genistein; 0.06, 0.37, and 0.74 mg/kg bw/day daidzin + daidzein, and 0.012, 0.08, and 0.16 mg/kg bw/day glycerin + glycitein in the control through high dose groups, respectively.]** Doses were selected to represent ranges of isoflavone intakes from typical Asian diets. Beginning on day 2 of the menstrual cycle, subjects consumed each diet through 3 menstrual cycles and an additional 9 days. A washout period of 3 weeks separated each diet. Subjects determined the occurrence of ovulation using home kits. Blood samples were collected during cycles 2 and 3 of each dietary period to determine levels of estrogens, androgens, progesterone, cortisol, thyroid hormones, insulin, prolactin, sex hormone-binding globulin, LH, and FSH. Hormones were measured by RIA, with the exception of LH and FSH, which were measured by immunoradiometric assay. Data were analyzed by ANOVA.

A review of dietary records revealed that soy-protein supplementation significantly increased calorie and protein intake and reduced fiber intake compared to pre-study levels. There were no changes in body weight, body-mass index, or percent body fat. Cycle lengths were recorded for the second and third cycle of each dietary period to allow subjects to adapt to each diet. Isoflavone intake had no significant effect on total menstrual cycle length, follicular phase length, or luteal phase length. There was a significant reduction in estrone **[by 13%]** for the high- compared to the low-isoflavone diet during the mid-follicular stage. Significant reductions in LH **[by 28%]** and FSH **[by 15%]** for the low-isoflavone compared to control diet occurred during the peri-ovulatory period. Consistent with the plasma data for LH, there was a “nonsignificant trend towards decreased excretion with increased isoflavone consumption.” **[Nonsignificant trend was not defined, and data were not shown.]** Also observed with intake of high-isoflavone diet were significant reductions in plasma free triiodothyronine **[by 5.5%]** compared to the control group and dehydroepiandrosterone sulfate **[by 6.7%]** compared to the low-isoflavone group. Due to a

4.0 Reproductive Toxicity Data

lack of effect on thyroxine and thyroid-stimulating hormone, the authors did not expect the change in triiodothyronine levels to be physiologically important. No effects were noted for the other hormones analyzed, including 17 β -estradiol and progesterone. Endometrial biopsies were conducted during the third menstrual cycle of each dietary-exposure period, and a comparison between expected and actual histologic results revealed no dietary effects [**data were not shown**]. According to study authors, the results of this study suggested weak hormonal effects in the absence of dose-response relationships.

Strengths/Weaknesses: The randomized cross-over design and clear exclusion criteria are strengths. There was a range of dose levels, permitting the evaluation of dose-response relationships, and treatment over 3 menstrual cycles was preceded by a washout period. The use of free-living women may improve generalizability. The repeated-measures analysis is an additional strength. Weaknesses include the small sample size, potential selection/recruitment bias, and the discarding of cycle-1 data without a solid rationale. It is not clear that a 3-week washout period between interventions was adequate. The authors did not indicate how many women were excluded based on not having adequate ovulation data. There was also no mention of the normality of the hormone data and whether transformations were used.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Watanabe et al. (195), supported by the Japanese Ministry of Health and Welfare and the Science and Technology Agency, evaluated effects of soy supplementation on Japanese women. [**Ages not given, except that subjects were young premenopausal women and that they were students in a nutrition course.**] The subjects were instructed to consume isoflavones 20 mg/day from food and were observed for at least 2 menstrual cycles [**1 cycle according to the Results section**] before assignment to an isoflavone supplement for 1 menstrual cycle. Diet records showed that actual dietary isoflavone intake averaged 10 mg/day (range 4.1–28.9 mg/day). The isoflavone supplement was given in tablet form with meals. Dose groups included a control group (n = 20), isoflavones 20 mg/day, and isoflavones 40 mg/day (n = 19 or 20 for the supplement groups combined). [**It is not specified whether the control group took a placebo tablet. The isoflavone tablets were described as containing 43.5 mg daidzein, 6.0 mg genistein, and 24.0 mg glycitein, making it difficult to understand how the subjects took daily isoflavone doses of 20 and 40 mg. The authors described the calorie content of the tablets as “per gram tablet.” If they meant also to describe the isoflavone content as per gram tablet, each 200 mg tablet would have contained 8.7 mg daidzein, 1.2 mg genistein, and 4.8 mg glycitein, or 15.7 mg total named isoflavones.**] Subjects were observed for an additional 1 or 2 menstrual cycles after the supplementation period.

Urinary excretion of daidzein, genistein, and glycitein was measured prior to the supplement period and on day 30 of the supplement period. Blood samples were collected “on day 1 of the study and on 11th and 22nd day after the menstruation, and at the end of the experimental period.” [**It cannot be determined if blood samples were collected during each menstrual cycle, which would be a total of 2 prior to the supplemented cycle, 1 during the supplementation period, and 1 or 2 following the supplementation period, or only during the supplementation period. The authors indicated that the 11th- and 22nd-day samples did not correlate well with follicular and luteal phases because “half the students had had rather irregular menstruation cycles.”**] Plasma 17 β -estradiol and progesterone were measured by RIA or by fluorescence immunoassay. An additional 3 students with regular menstrual cycles were evaluated for 1 baseline menstrual cycle and 1 cycle during which they took supplemental isoflavones 50 mg/day. Blood samples were collected every day during the ovulatory period [**not**

4.0 Reproductive Toxicity Data

otherwise specified] and 3–5 times each during the follicular and luteal phases, which were identified using basal body-temperature monitoring. Plasma was used for measurement of estrone, 17 β -estradiol, estriol, sex hormone-binding globulin, LH, FSH, androstenedione, testosterone, triiodothyronine, and thyroxine. **[An additional hormone, identified as “epiandrosterone,” was measured. The authors may have meant dehydroepiandrosterone.]** FSH and LH were determined using time-resolved immunofluorimetric assays, and the other hormones were determined using RIA. **[Statistical methods were not explained except for use of the Pearson correlation to evaluate relationships between isoflavone excretion and plasma hormone levels. It could not be determined whether supplemented cycles were compared to baseline cycles within subjects or supplemented subjects were compared to control subjects.]**

Urinary daidzein was reported to have increased with both supplement levels, and urinary genistein was reported to show a “slight increase” in the group given 40 mg/day supplemental isoflavones. **[Statistical testing was not mentioned.]** Plasma 17 β -estradiol was reported to have increased by 66 pg/mL in the luteal phase and 20 pg/mL in the follicular phase in women given isoflavone supplementation 20 mg/day, and by 97 pg/mL in the luteal phase and 25 pg/mL in the follicular phase in women given isoflavone supplementation 40 mg/day. Plasma progesterone results and comparisons were not given, although the authors wrote, “almost half the subjects showed a low response to progesterone after ovulation.” There was no correlation between isoflavone excretion and follicular or luteal plasma hormone concentrations except for a positive correlation between daidzein excretion and luteal-phase plasma 17 β -estradiol. Among isoflavone-supplemented women, 60% had menstrual cycle lengthening, and 20% had menstrual cycle shortening **[presumably compared to baseline cycles]**. Among the 3 subjects studied in detail before and during the use of 50-mg isoflavone supplements, the cycle was 7 days shorter on supplements in 1 subject and 4 days longer in the other 2 subjects. In the woman with the shorter cycle, plasma 17 β -estradiol, LH, and FSH were decreased. Sex hormone-binding globulin was lower in all 3 women during the supplement period compared to before the supplement period.

The authors concluded that the “observed decreases in ovarian steroid levels” agreed with epidemiology studies suggesting that Asian populations with high-soy intake have lower levels of ovarian steroids and lower incidences of breast cancer than populations with low-soy intake.

Strengths/Weaknesses: The use of isoflavone tablets to control exposures, the use of 2 dose levels, and the use of urinary measurements to estimate exposures are strengths. Some information was provided on genistein half-life. Weaknesses include the small sample size, the lack of age information for subjects, and uncertainty about whether control subjects took a placebo. The method of assignment to groups is unclear but does not appear to have been randomized. There was inadequate attention to other dietary sources of isoflavones. The only measure of ovulation was body temperature, which is not adequate. No information was provided on assay variability. Statistical analysis was poor, and there was no mention of controlling for potential confounders. Cycle data were poor, and hormone values were associated only crudely with a particular phase of the cycle. The use of subjects with irregular cycles makes interpretation difficult. In general, the presentation of the methods and results was confusing.

Utility (Adequacy) for CERHR Evaluation Process: This study is of some utility in the evaluation process.

Lu et al. (53), supported by NIH and the Public Health Service, examined the effects of soy consumption on menstrual cycle lengths and hormone levels. Ten healthy women (ages 23–42 years) with regular menstrual cycles completed the study. Seven of the subjects were Caucasians

4.0 Reproductive Toxicity Data

and 3 were African Americans. Nine of the subjects were nulliparous. During a 3-month baseline period, the women consumed their regular diets. Soy was not a regular part of the usual diets, and the women were instructed to avoid soy intake during the baseline period. Menstrual cycle lengths were monitored throughout the study, and the first day of menstrual bleeding was considered day 1 of the cycle. Subjects were admitted to a metabolic unit for a soy diet. They consumed the soy diet during 1 menstrual cycle, starting on day 2 and continuing through day 2 of the next cycle. Diets consisted of soy and non-soy foods and were designed to maintain body weight. For dinner each evening, the subjects ingested 36 ounces of soy milk under direct supervision. A GC method was used to measure isoflavone levels in soy milk, and it was determined that the soy milk provided daily doses of 68.8 ± 3.7 (SEM) mg daidzein and 85.2 ± 5.4 mg genistein. During the first month of the baseline period, blood was drawn on cycle days 5 (follicular phase), 12 (mid-cycle), and 22 (luteal phase). In the second month of the baseline period and during the soy-diet period, blood was collected on cycle days 5 and 7, and then daily from day 9 of the cycle to day 2 of the next cycle. Serum [**also described as plasma later in study**] was analyzed for ovarian hormones by RIA and for gonadotropins using an immunoradiometric method. Serum isoflavone levels were measured by ELISA. Day of ovulation was determined by body temperature. Two 12-hour urine samples were collected daily from cycle day 2 of the soy cycle to day 2 of the next cycle. Urine was analyzed for daidzein and genistein by GC. Hormone levels were expressed as AUC, mean daily level, peak level, and rates. Within-subject changes were analyzed by paired *t*-tests and Wilcoxon ranked sign tests. Secondary multiple regression analyses were conducted to determine associations between variables.

Compared to regular diets, the soy diets provided significantly more carbohydrate but less protein and fiber. There were no significant changes in body weight or body-mass index. Urinary excretion of isoflavones is summarized in Section 2.1.1.3. Fifteen hours after soy-milk ingestion, mean serum level of genistein was reported at $0.85 \mu\text{g/mL}$ [**3.1 μM**] and daidzein at $2.95 \mu\text{g/mL}$ [**11.6 μM**]. The soy diet had no significant effect on menstrual cycle length, but there was a 6% decrease in length of mean luteal-phase length ($P=0.07$). Decreased luteal-phase length during the soy-diet period was positively and significantly associated with intake and excretion of genistein and daidzein and with decreased protein intake. During the soy-diet period, 17β -estradiol serum AUC was significantly reduced (24%). Reductions in 17β -estradiol levels were positively associated with plasma and urinary levels of daidzein and genistein, an association influenced by age. Reductions in 17β -estradiol levels were inversely related to protein intake. Serum progesterone levels were significantly reduced by about 45% during the soy-diet period. The reduction in serum progesterone was inversely associated with isoflavone intake and positively associated with energy and fiber intake. [**Results of regression analyses were not clearly reported.**] The soy diet had no significant effects on serum LH or FSH. The study authors concluded that soy-induced effects on ovarian hormones were not mediated by gonadotropins; decreases in ovarian hormones may have been related to isoflavones and to intake of energy, protein, and fiber.

Strengths/Weaknesses: The longitudinal study design with clear inclusion and exclusion criteria, use of subjects as their own controls, collection of baseline dietary data, and the control for daily energy and nutritional intakes are strengths. There was, however, no mention of a washout period prior to study entry. Ovulation was monitored using basal body temperature, which is not adequate. There was a high drop-out rate (10 of 26), and different subjects ingested different soy preparations, which could have caused variability. Confounders such as body-mass index, race, smoking, and alcohol, which may have affected hormone levels, were not considered, and given the small sample, a multivariate analysis would not have been possible.

4.0 Reproductive Toxicity Data

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Lu et al. (196), supported by NIH, evaluated the effects of a reduced-isoflavone soy preparation on menstrual cycle hormones in 9 healthy women. Subjects were regularly cycling omnivorous women between the ages of 26 and 42 years with body-mass indices of 25–33. Women were evaluated on their usual diets, with instructions not to consume soy foods, following which they were given a diet containing a 36-ounce serving of a beverage prepared with soy powder from which >99% of isoflavones had been removed by alcohol extraction. The authors indicated that the 37.9 g soy-protein content of this beverage was at the upper end of the dietary soy-protein content of Asian diets. The soy-containing diet was designed to maintain body weight and had the same energy content as the usual diet. The cycle on the usual diet and the cycle on the soy diet were monitored using blood sampling on cycle days 5 and 7 and then daily from cycle day 9 through day 2 of the next menstrual cycle. Serum was used for measurement of 17β -estradiol and progesterone by RIA and FSH and LH by a commercial immunoradiometric assay. Sex hormone-binding globulin was evaluated using a commercial time-resolved fluoroimmunoassay. Hormones were evaluated using AUC, mean concentration, and peak concentration. Cycle lengths were also compared between the 2 diet conditions. Analyses were performed by menstrual cycle phase (follicular and luteal, identified by measurement of the LH surge) and over the whole cycle. Comparisons were made using ANCOVA with a mixed model approach to adjust for body-mass index and for the individual macronutrient components of each diet.

There were no differences in cycle length, body-mass index, or body weight between the 2 diets. On average, the soy diet contained more carbohydrate, less protein, and less fiber than the usual diets. Serum daidzein and genistein concentrations were at the limits of detection of the assays, but daidzein concentrations were slightly higher during the soy-feeding month than the usual-diet month (0.15 ± 0.02 [SEM] $\mu\text{g/mL}$ compared to 0.08 ± 0.05 $\mu\text{g/mL}$, $P = 0.02$, paired t -test). Mean serum genistein did not differ between the 2 feeding conditions. Serum 17β -estradiol concentrations (AUC, mean, peak) were lower on the soy diet during the follicular phase, the luteal phase, and over the whole cycle. The uncorrected decrease in serum 17β -estradiol concentrations was about 20%. The significance of the follicular-phase difference was lost when corrected for each macronutrient in the diets. The significance of the luteal-phase difference in 17β -estradiol was lost when corrected for dietary fiber. Luteal-phase progesterone was lower on the soy diet than the usual diet. The uncorrected decrease in progesterone concentrations was ~30% on the soy diet. During both diets, individual serum progesterone concentrations were correlated with total non-soy-protein intake, and adjustment for protein intake eliminated the statistical difference in luteal-phase progesterone between the diet conditions. Serum concentrations of LH, FSH, and sex hormone-binding globulin were not significantly altered by the soy diet.

The study authors indicated that the effect of the low-isoflavone soy diet on menstrual cycle hormones were similar to hormone changes from an isoflavone-containing diet described by them in a previous study (190). They concluded that the changes in 17β -estradiol and progesterone associated with soy diets were likely to be due to soy components other than isoflavones. They also concluded that alterations in gonadotropins and sex hormone-binding globulin did not explain the steroid hormone alterations. They noted the importance of macronutrients on sex steroid concentrations and postulated that differences between studies in hormonal response to dietary soy might be explained by differences in the macronutrient composition of the diets being studied.

4.0 Reproductive Toxicity Data

Strengths/Weaknesses: The evaluation of non-isoflavone components of soy, the longitudinal study design, the clear inclusion and exclusion criteria, the control for daily energy and nutritional intake, the supervision of meals, and consideration of effect modifiers and confounders are strengths. The sample size was very small, making it difficult to adjust for confounders, and the age range was very wide. There appears to be substantial similarity to the previous study (53), with minimal new information.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Jakes et al. (197), supported by the Singapore National Medical Research Council, conducted a study to determine if dietary soy intake was associated with menstrual cycle length in an “uncontrolled community setting.” Premenopausal women were recruited from a local hospital ($n = 85$) and a nursing school ($n = 115$). Women were menstruating regularly, had no condition that could affect their menstrual cycle or hormone levels, and were not taking medications that could affect hormone levels. The subjects were questioned about soybean intake, menstrual cycle parameters, pregnancies, and use of hormonal drugs. The subjects completed diaries for 3 consecutive menstrual cycles following the interview. Blood samples were taken at the time of the interviews and analyzed for levels of estrone, 17β -estradiol, estriol, LH, and FSH. The subjects’ cycle phase at the time of the blood sample was approximated, assuming ovulation 14 days prior to the start date of the next menstrual cycle. Association between soy intake and cycle length was estimated first by correlation coefficients and then by proportional odds modeling adjusted for age and subject source to account for cycle length being ordered but discrete. Association between soy intake and hormone levels was estimated using correlation coefficients, stratified by cycle phase at the time of sampling.

Exclusions following interviews left 184 subjects in the study, although 12 of these subjects could not be used in analyses using diary information. Estrone was the only hormone found to be significantly correlated with soy-protein intake, with a correlation coefficient of 0.2221 ($P < 0.05$). The researchers found a significant correlation between soy-protein intake and self-reported cycle length, with a correlation coefficient of 0.1728 ($P < 0.05$), but the correlation with diary-recorded cycle length was not significant. A soy-protein intake higher than the median value of 5.7 g/day was found to be significantly associated with a longer diary-recorded cycle length ($P = 0.034$) according to unadjusted ORs. When adjusted for age and parity, this association was no longer statistically significant ($P = 0.06$).

The study authors concluded that they had shown a likely association between soy-protein intake and menstrual cycle length. They noted that subjects in the highest quartile of soy intake had cycle lengths that were 1–2 days longer than those in the lowest quartile of soy intake. This difference would result in 20 fewer cycles over a lifetime for those women in the highest quartile, and thus, a reduction in lifetime ovarian activity. The authors suggested that reduced breast cancer risk could be explained by a reduction in lifetime ovarian activity and with high-soy intake.

Strengths/Weaknesses: This study was relatively large, although the heterogeneous sources of the sample are a weakness of the study. There were clear inclusion and exclusion criteria, and the menstrual cycle data were of good quality. Sample size and power information were provided, and there was control for age and parity, but no mention was made of modeling to examine multiple other potential confounders. The food frequency questionnaire may not have been an accurate measure of soy intake. Other weaknesses include a lack of information on time since recent oral contraceptive use and collection of only 1 blood sample per subject.

4.0 Reproductive Toxicity Data

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

4.1.2 Male

Four studies with reproductive endpoints in men consuming soy are presented below in order of publication.

Habito et al. (198), supported by the Australian Commonwealth Department of Veterans Affairs, evaluated the effects of replacing meat with soy protein on serum sex hormone concentrations. The subjects were 42 omnivorous men between the ages of 35 and 62 years who were assigned in random order to a 4-week period of eating meat 150 g/day and a 4-week period of eating tofu 290 g/day (35 g/day soy protein, provided as prepared meals and biscuits). A 2-week period of usual diet served as a washout between the 2 test diets. The tofu-containing diet was supplemented with butter and lard to make the fat content similar between the 2 experimental diets. Both diets were equivalent in energy and macronutrients, and differed only by the protein source. Blood was collected before the study and on 2 occasions 3 days apart at the end of each diet intervention. Serum was assayed using commercial RIA kits for total testosterone, 17 β -estradiol, dihydrotestosterone, and androstenediol glucuronide. Sex hormone-binding globulin was measured using an immunoradiometric assay. Values for the 2 end-of-diet samples were not different and were averaged. Statistical analyses were performed using the paired *t*-test or Wilcoxon signed-rank test. Because weight change correlated with sex hormone-binding globulin (and subjects were an average of 400 g heavier after the tofu diet than after the meat diet), multiple linear regression was used to adjust for weight.

Mean creatinine-adjusted urinary excretion of genistein and daidzein increased 16–20-fold on the tofu diet compared to the meat diet. There were no significant differences between the diet conditions in mean serum total testosterone, dihydrotestosterone, androstenediol glucuronide, or 17 β -estradiol. Weight-adjusted sex hormone-binding globulin was 9% higher on the tofu diet ($P = 0.01$), and the weight-adjusted testosterone to 17 β -estradiol ratio was 12% lower on the tofu diet ($P = 0.049$). The authors concluded that overall androgenic activity may be reduced by dietary isoflavones, which increase sex hormone-binding globulin with a consequent reduction in free testosterone. They indicated that testosterone is more avidly bound to sex hormone-binding globulin than is 17 β -estradiol, and a reduction in the binding globulin would produce a greater decrease in testosterone than in 17 β -estradiol activity. The authors cautioned that the biologic significance of the small change identified with short-term dietary soy was uncertain, and that the effects of a soy-containing diet over many years required additional study.

Strengths/Weaknesses: The randomized cross-over design with subjects serving as their own controls is a strength. There was a good washout period before study participation, and total dietary intake was controlled. The authors provided sample size and power calculations. Weaknesses include the wide age range of the subjects, the small sample, the lack of adjustment for body-mass index or other potential confounders, and the inadequate assessment of compliance with diet.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Nagata et al. (199), supported by Gifu University and the Second-Term Comprehensive Strategy for Cancer Control in Japan, evaluated the association between estimated dietary-soy

4.0 Reproductive Toxicity Data

consumption and serum androgen and estrogen levels in Japanese men. Study subjects were recruited from participants in a larger study on the association between diet and cancer. There were 256 men randomly selected to be invited to participate in the hormone-level study; of these men, 97 agreed to participate. Blood was collected from participants, and serum was separated and frozen until analysis by RIA for 17 β -estradiol, estrone, total and free testosterone, dihydrotestosterone, and sex hormone-binding globulin. Information on diet, smoking, ethanol use, exercise, and medical history was obtained by questionnaire. Isoflavone intake from soy was estimated from a daily food-frequency questionnaire, administered 12 times over the course of a month, about 3 years prior to the collection of blood samples. **[It is not clear if all participants completed a food frequency questionnaire 12 times or this description applies to a smaller group used in a reproducibility sub-study.]** The association between soy intake and serum hormone concentrations was evaluated using Spearman rank correlation coefficients after regressing hormone and soy-intake values on confounders including age, body-mass index, smoking status, and ethanol. Estimated dietary intake of fat, cholesterol, carbohydrate, and vitamins B₁ and D were significantly correlated with serum androgens and were included in the regression model for serum androgens. The men in the study were a mean \pm SD of 60.5 \pm 10.7 years of age with a mean body-mass index of 22.6 \pm 3.0 kg/m². Serum 17 β -estradiol was inversely correlated with soy-product intake and with isoflavone intake ($r = -0.32$; $P < 0.01$ in the fully adjusted model). None of the other hormone measurements showed a significant correlation with either soy-product intake or isoflavone intake. The authors proposed that soy isoflavones may inhibit enzymes such as aromatase that are involved in estrogen biosynthesis.

Strengths/Weaknesses: The random selection of subjects invited to participate is a strength of this study. Information on exposure was good, with multiple assessments of dietary intake. The sample was relatively large. The control for some potential confounders is an additional strength. Weaknesses include the wide age range, although adjustment was performed, and the collection of dietary data long before blood sampling.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Mitchell et al. (200), supported by UK government agencies, evaluated the effects of a soy-extract food supplement on reproductive parameters in men. Healthy 18–35-year-old non-vegetarian men were given a daily 500-mg tablet of a commercial preparation called RegenTM, supplied by its manufacturer, Novogen Ltd, Australia. This supplement contains 40 mg total isoflavones (genistein, daidzein, and glycitein). Men were asked to take 1 tablet each day for 2 months. Assessments were performed monthly, including 2 assessments prior to the supplement, 2 assessments while on the supplement, and 3 assessments after stopping the supplement. Assessments included an evaluation of testis size, blood sampling for measurement of genistein, daidzein, 17 β -estradiol, testosterone, FSH, and LH, and semen analysis including the use of computer-assisted sperm analysis (CASA). Fifteen men were initially enrolled; 1 subject discontinued himself from the study midway through and was excluded from analysis, and 3 subjects did not appear for the final assessment. Statistical analysis was by ANOVA with adjustment for the covariates alcohol, smoking, technician, season, and sexual abstinence.

Mean plasma genistein peaked in the first month of supplement use at approximately 1 μ M [**270 ng/mL aglycone equivalent**], and mean plasma daidzein peaked at about 0.5 μ M [**127 ng/mL aglycone equivalent**]. There were no significant treatment effects on testicular volume, plasma hormone determinations, or semen analysis parameters. **[Percent of morphologically normal motile sperm appeared to decrease over the course of the study; however, the authors indicated that this apparent effect was attributable to changes in evaluation technique that were introduced during the study period.]** The authors concluded that their study did not

4.0 Reproductive Toxicity Data

identify adverse male reproductive effects at this dose of phytoestrogens but could not exclude an adverse effect of higher intake levels.

Strengths/Weaknesses: The use of younger men, exposure to a standardized tablet, collection of serial blood samples, use of subjects as their own controls, and adjustment for potential confounders are strengths of this study. The authors provided sample size and power calculations. Weaknesses include the small sample and high drop-out rate, which makes multivariate analysis difficult. It is unclear whether the hormone data were normally distributed and, if not, whether the data were transformed before analysis. There was no measure of compliance with the assigned regimen. There was only 1 dose level, precluding dose-response assessment, and the exposure was short-term. It appears that semen was inconsistently evaluated over the course of the study.

Utility (Adequacy) for CERHR Evaluation Process: This study is of minimal utility for the evaluation process.

Nagata et al. (201), supported by the Japanese Ministry of Health and Welfare, examined the effects of soy intake on serum androgen and estrogen levels in healthy men who were either students or teachers at a nursing school. Subjects were randomly assigned to a soy-milk group (n=17) or control group (n=17). During an 8-week period, subjects in the soy-milk group were instructed to drink 400 mL/day soy milk that was supplied to them and no other soy milk. Men from both groups were instructed to continue eating their regular diets and were asked to keep dietary records, which were used to estimate intake of soy products, macronutrients, micronutrients, and isoflavones. Blood was collected just prior to the dietary phase and then every 2 weeks for 12 weeks. Serum was separated for measurement of estrogens and androgens by RIA. Statistical analyses included standard and paired *t*-tests for dietary parameters. Longitudinal hormone data were analyzed using a mixed linear regression model.

Compared to baseline levels, increased intake of vitamin B1, iron, and phosphate and decreased intake of retinol, vitamin A, and salt were observed in the soy-milk group over the course of the study. No significant changes in nutrient intake were noted in the control subjects over the course of the study. Mean intake of soy milk throughout the study was estimated at 360 mL. Mean isoflavone intake from soy milk and other sources in the soy-milk group increased from ~16 mg prior to dietary intervention to ~93 mg at the end of the dietary period. **[Values are assumed to represent daily intakes.]** In the control group, mean isoflavone intake was ~18 mg, similar at the start and end of the study. There were no significant differences in serum levels of 17 β -estradiol, total and free testosterone, or sex hormone-binding globulin between the soy-milk and control groups. A significant difference between the soy-milk and control groups were observed for serum estrone, which tended to decrease in the soy-milk group and increase in the control group. The study authors concluded that soy-milk intake by men may result in modified circulating estrone concentration.

Strengths/Weaknesses: The use of men as their own controls, serial blood collections, randomized assignment, information on doses, sample size calculations, and use of transformed hormone data are strengths of this study. Weaknesses include the small sample size, the wide age range, and the lack of measures of compliance with the assigned regimen. Although the authors adjusted for some potential confounders in multivariate analysis, the small sample size makes it difficult to adjust effectively. The use of only Japanese men may decrease the generalizability of the results.

Utility (Adequacy) for CERHR Evaluation Process: This study is somewhat useful for the evaluation process.

4.2 Experimental animal

4.2.1 Female

Boettger-Tong et al. (202), supported by NIH and the John P. McGovern Foundation, described observations of possible effects of animal feed on the reproductive system of the female Sprague Dawley rat. **[This report provided limited information; e.g., there was no information on animal numbers, experimental procedures, quantitative results, or statistical analysis.]** A series of experiments in ovariectomized, 30-day-old rats demonstrated little response to injected estradiol **[not otherwise specified]** as measured by induction of marker genes such as *c-fos*. It appeared that the lack of response was due to unusually high levels of marker gene expression in control rats. To investigate possible estrogenic activity from exposure to diet or other environmental factors, uterine and vaginal sections were prepared and examined from 30-day-old ovariectomized rats kept at the laboratory in 1995 but not treated with hormones. The sections were compared to those from rats kept at the laboratory 2 years earlier (1993), a time when there were low basal levels of gene expression and robust responses to hormonal treatments. Compared to the rats from 1993, the rats from 1995 displayed an estrogenic response, as indicated by thicker vaginal epithelium, increased height of uterine luminal epithelium cells, and a more edematous uterine stroma. Some signs of estrogenicity were observed in 20-day-old rats obtained from the same vendor, which used diet from the same supplier as the study authors used. No signs of estrogenic response were seen in 20-day-old rats obtained from an alternate vendor. Studies were conducted with rats from the alternate vendor, and it was determined that the laboratory diet, consisting primarily of soy and alfalfa, was the most likely source of the unexpected estrogenic response. Analysis of feed by GC-MS revealed genistein at 21 mg/100 g feed and daidzein at 14 mg/100 g feed. **[The report abstract states that the level of daidzein was 14 mg/kg feed, which would be equivalent to 1.4 mg/100 g feed. Using EPA (84) assumptions for weanling rat body weight and food intake, CERHR estimated intake rates of 31 mg/kg bw/day genistein and 21 mg/kg bw/day daidzein. Daidzein intake would be 2.1 mg/kg bw/day if the number in the abstract is correct.]** Levels of other isoflavones were not analyzed, but the mycoestrogens zearalenone and zearalenol were not detected by a contract laboratory. In earlier study reports, genistein levels were measured in rat feed at 6.8 mg/100 g feed, and daidzein was measured at 0.6 mg/100 g feed. The diet vendor was notified and sent a sample of the feed from the batch in question. Following testing of the feed in an estrogen-sensitive cell culture, the vendor concluded, "It appears that a component of the . . . rat/mouse diet which was used at your facility in the summer of 1995 had a high level of estrogen." To further examine the effects of diet, 20-day-old rats were obtained from the alternate vendor, ovariectomized, and fed either the diet in question or the alternate diet with the same general composition. At 30 days of age, the uteri from animals fed the diet in question were larger and more vascularized than uteri from animals receiving the alternate diet. The study authors concluded that it is necessary to use diets with controlled ingredients and formulations and to monitor the diets for contaminants.

Strengths/Weaknesses: This observation is important for researchers working on estrogenic effects. Strengths are that genistein and daidzein were measured in the actual feed given to the animals, and that the feed was shown to have baseline effects on the rat uterus; however, it is a weakness that uterine weight was not used as an endpoint. A methods section was not provided, and no information was given on the number of animals used. Use of 17 β -estradiol as a positive control would have strengthened the study.

Utility (Adequacy) for CERHR Evaluation Process: This report is useful in showing that dietary soy can have estrogenic effects in rodents.

4.0 Reproductive Toxicity Data

Gallo et al. (203), support not indicated, performed a reproduction study of dietary soy in female Wistar rats. The control diet was a commercial soy-containing diet. **[The soybean meal in the diet was solvent-extracted; isoflavone content was not reported.]** Modified diets were made by adding a soy extract that contained 12% isoflavones. The supplement was added at dietary levels of 0.7, 1.2, and 2.4%. Rats were randomized to 1 of the diet groups at weaning (n = 20/group). The day of vaginal opening was recorded, and daily vaginal smears were examined for estrous cycle stage on PND 63–77. Beginning on PND 77, females were paired with untreated males, and the day of vaginal sperm or a vaginal plug was considered GD 0. Dams were allowed to litter (PND 0). Pups were sexed and examined for external abnormalities on PND 1. Dams and pups were killed on PND 7, and pups were again examined for external abnormalities. Dams were necropsied with histological examination of ovaries, uterus, and vagina. Data were analyzed using ANOVA with post hoc Dunnett multiple comparison test or by the Kruskal-Wallis test followed by the Dunn multiple comparison test.

Results are summarized in Table 44. Unscheduled deaths or litter loss occurred in all treatment groups and was not considered treatment-related. Growth suppression was noted and at least partially attributed to reduced feed consumption **[data not shown]**. In spite of soy-associated alterations in age at vaginal opening and estrous cycle length, reproductive function was not adversely affected. There was no effect of treatment on external abnormalities of pups.

The authors concluded that dietary soy had estrogenic effects on the female reproductive system, either as a direct consequence of phytoestrogen exposure or through effects on ovarian follicles. They indicated that toxicity occurred with a daily isoflavone intake that exceeded human dietary isoflavone intake by more than 100-fold, a fact they recommended considering in interpretation of the results of this study. **[Measurements of isoflavone or feed intakes were not provided. Assuming a female rat ate 0.08 kg feed/kg bw/day (204) and that the middle- and high-dose diets contained isoflavones 0.14 and 0.29 g/100 g feed, daily isoflavone intake would have been 64 mg/kg bw at the low dose, 112 mg/kg bw/day at the middle dose, and 232 mg/kg bw at the high dose. The authors cite an estimate of human isoflavone consumption in Japan as 3 mg/kg bw/day.]**

4.0 Reproductive Toxicity Data

Table 44. Female Reproduction Study of Soy Extract Added to the Diet

Endpoint	Dietary soy (%)			Benchmark dose ^a (% soy in diet)			
	0.7	1.2	2.4	BMD ₁₀	BMDL ₁₀	BMD _{1SD}	BMDL _{1SD}
Body weight							
Prior to mating	↔	↔	↔				
GD 0, 6, and 12	↔	↔	↔				
GD 18	↔	↔	↓8%	2.3	1.9	2.3	1.9
PND 1	↔	↔	↓11%	2.1	1.6	2.1	1.5
PND 4	↔	↔	↓14%	1.9	1.3	1.8	1.3
PND 7 (terminal)	↔	↔	↓12%	2.1	1.5	2.1	1.5
Age at vaginal opening	↔	↔	↓13%	1.9	1.4	1.6	1.2
Estrous cycle duration	↔	↔	↑10%	2.3	Failed	2.2	Failed
Proportion mating (per paired)	↔	↔	↔				
Days until mated	↔	↔	↔				
Proportion pregnant (per mated)	↔	↔	↔				
Proportion with live pups	↔	↔	↔				
Post-implantation loss	↔	“↑”	“↑”				
Gestation length	↔	↔	↔				
Total and live litter size, PND 1, 4, and 7	↔	↔	↔				
Litter weight, PND 1, 4, and 7	↔	↔	↔				
Mean pup weight, PND 1, 4, and 7	↔	↔	↔				
Absolute organ weight (dam)							
Pituitary	↑22%	↔	↔				
Heart	↔	↔	↔				
Liver	↔	↔	↔				
Spleen	↔	↔	↔				
Kidney	↔	↔	↔				
Uterus	↑23%	↑26%	↑24%	1.3	0.7	2.8	1.7
Ovary	↔	↔	↓18%	1.6	0.9	2.2	1.4
Relative organ weight (dam)							
Pituitary	↔	↔	↔				
Heart	↔	↔	↔				
Liver	↔	↔	↔				
Spleen	↔	↔	↔				
Kidney	↔	↔	↔				
Uterus	↔	↑31%	↑42%	0.6	0.4	1.4	1.0
Ovary	↔	↔	↔				
Ovarian follicle types							
Small	↓76%	↓80%	↓78%	0.1 ^b	0.1	0.8	0.5
Medium	↔	↔	↑69%	2.2	0.5	2.4	1.9
Large	↔	↔	↔				
Corpora lutea	↔	↔	↔				
Uterine abnormalities (n, control = 0/16 or 1/16)							
Stromal edema	1/18	6/17	15/17 ^c	0.7 ^d	0.5		
Blood extravasation	0/18	0/17	11/17 ^c	2.2	1.4		
Endothelial hyperplasia	1/18	9/17 ^c	17/17 ^c	0.8	0.6		
Leukocyte infiltration	1/18	0/17	10/17 ^c	1.5	1.1		

4.0 Reproductive Toxicity Data

Endpoint	Dietary soy (%)			Benchmark dose ^a (% soy in diet)			
	0.7	1.2	2.4	BMD ₁₀	BMDL ₁₀	BMD _{1SD}	BMDL _{1SD}
Vaginal abnormalities (n, control = 0/16 or 1/16)							
Inflammation	6/18	9/17 ^c	17/17 ^c	0.4	0.3		
Dyskeratosis	1/18	0/17	17/17 ^c	1.3	1.0		

Data from Gallo et al. (203).

↑,↓,↔ Statistically increased, decreased, or unchanged in comparison to control group according to study authors' analysis of continuous data. "↑" = Described by study authors as increased, although not significantly different from control group.

^aThe BMD₁₀ is the benchmark dose associated with a 10% effect, estimated from a curve fit to the experimental data. The BMDL₁₀ represents the dose associated with the lower 95% confidence interval around this estimate. A 10% alteration in a continuously distributed parameter is an arbitrary benchmark that may not be comparable to a similar alteration in any other endpoint. The BMD_{1SD}, which represents an alteration equivalent to 1 SD of the control distribution, may permit more appropriate comparisons of the responses of continuously distributed parameters. Benchmark doses are used commonly in a regulatory setting; however, they are used in this report when the underlying data permit their calculation, and are only supplied to provide one kind of description of the dose-response relationship in the underlying study. Calculation of a benchmark dose in this report does not mean that regulation based on the underlying data is recommended, or even that the underlying data are suitable for regulatory decision-making. Values were calculated using the power model (unless noted) by CERHR using EPA Benchmark Dose Software version 1.3.2. The program offers models based on homogeneity of variance, and CERHR was guided by the program in this regard.

^bPolynomial model.

^cStatistically different from control group according to study authors' analysis of proportional data.

^dLogistic model used for proportion data.

Strengths/Weaknesses: The use of 3 levels of dietary isoflavones and the examination of female reproductive organ morphology are strengths. Weaknesses include the lack of information on the isoflavone levels in the chow, the failure to determine isoflavone intake, and the lack of detailed information on the offspring. The isoflavone intakes appear to be much higher than adult human exposure levels. The treatment duration from weaning through adulthood makes it difficult to interpret whether alterations would have been developmental, reproductive, or a combination of the two.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful in evaluating the effects of high-dose soy isoflavones on the female reproductive tract and in showing an apparent lack of effect on reproductive function.

Lund et al. (58), supported by the National Science Foundation and Brigham Young University, conducted a series of experiments to examine reproductive and developmental effects in rats fed phytoestrogen-containing feed. From 50 days of age through completion of the study, 5 female Long Evans rats per group were fed a soy-based diet containing 600 µg phytoestrogens/g diet (phyto-600) or a fish meal-based diet that is phytoestrogen free. **[Assuming a female rat ate 0.08 kg feed/kg bw/day (204), isoflavone intake in the phyto-600 group would have been 48 mg/kg bw/day,]** Diets were similar in protein, fat, amino acid, mineral, and vitamin content. From 80 to 85 days of age, the rats were mated with males fed the same diet. The rats were allowed to deliver and nurse their offspring, which were used in experiments described in Section 3.2. When F₀ rats were 150–163 days of age, vaginal cytology was monitored for 14 days. Length and regularity of estrous cycles were normal in rats fed either diet **[data were not shown]**. The authors' conclusions were focused on the developmental effects of the phytoestrogen-containing diet, which are discussed in Section 3.2.

4.0 Reproductive Toxicity Data

Strengths/Weaknesses: Use of a well defined high-phytoestrogen diet, evaluation of multiple endpoints, and use of a 2-generation design are strengths. The measurement of phytoestrogens in serum and demonstration of relevance of these levels to human exposures is helpful. The comparison to a fish-meal diet makes interpretation difficult inasmuch as these diets are likely to differ with respect to many components other than phytoestrogens.

Utility (Adequacy) for CERHR Evaluation Process: The observation that the volume of the SDN-POA in males was decreased by removal from soy for 40 days during adulthood (discussed in Section 3.2) is intriguing; however, the main focus of this paper was developmental effects of dietary soy. The lack of effect of the soy diet on estrous cycles is of use in the evaluation of reproductive effects.

Patisaul et al. (205), supported by the National Science Foundation and Emory University, evaluated the effects of a soy supplement on sexual function in ovariectomized female Long Evans rats. All animals used in the study had been shown to mate after administration of estradiol benzoate. Female rats were fed a phytoestrogen-free diet to which was added a ground commercial-soy supplement at a concentration of 0 or 0.35%. Based on previous studies, the authors estimated that the supplemented diet contained genistein 13 ppm and daidzein 33 ppm, producing plasma phytoestrogen concentrations between those of humans on a typical Western diet and on an Asian diet. **[Neither dietary phytoestrogens nor plasma concentrations were measured in this study, although the phytoestrogen content of the commercial supplement was determined. Assuming a female Long Evans rat consumes 0.08 kg feed/kg bw/day (84), mean genistein intake would have been 1.04 mg/kg bw/day and mean daidzein intake would have been 2.64 mg/kg bw/day.]** Some females were given sc estradiol benzoate 48 hours before mating, and all females were treated with sc progesterone 4–5 hours before mating. There were 4 treatment conditions (n = 8/group) used to evaluate the effects of soy: soy-free diet only, soy diet only, soy-free diet + estradiol benzoate, and soy diet + estradiol benzoate. A sc injection of oil vehicle was given to rats not receiving estradiol benzoate. The experimental procedure consisted of placing each female in a mating chamber with an untreated male retired breeder for 30 minutes. The breeding apparatus included a smaller chamber into which the female could escape. Males were not able to follow the females into the escape chamber due to the size of the tunnel between the 2 compartments. During a 10-minute period beginning 5 seconds before the first attempted mount, counts were made of the number of attempted mounts by the male and the number of lordosis responses by the female. A lordosis quotient was calculated as the percent of mount attempts resulting in lordosis. Data were analyzed using ANOVA with post hoc Fisher least significant difference test.

In a second experiment, ovariectomized female rats were divided into 4 similar diet ± estrogen groups (n = 10 or 11/group), except that estrogen was administered using a sc silastic capsule containing 17 β -estradiol. Measured 17 β -estradiol concentrations in blood were about 4 times those found in ovary-intact animals. An empty capsule was implanted in animals not assigned to estrogen treatment. The implants were inserted 2 days after the dietary intervention was started. Four days after implant insertion, blood was collected for determination of plasma 17 β -estradiol, uteri were removed and weighed, and brains were removed and serial sections were made from the lateral septum to the caudal end of the ventromedial nucleus of the hypothalamus. In situ hybridization was performed in brain sections using a probe for ER β mRNA, and autoradiography was performed for oxytocin receptor, which is up-regulated by ER α agonists. Data were analyzed using ANOVA with post hoc Fisher least significant difference test.

4.0 Reproductive Toxicity Data

Estradiol benzoate treatment resulted in an increase in the lordosis quotient from 6.7 to 91.8% in animals fed a phytoestrogen-free diet. The feeding of the soy diet resulted in a significant decrease of the lordosis quotient to 72.8% in animals also given estradiol benzoate. In the females that were not given estrogen, the number of attempted mounts was decreased at $P < 0.06$ by the soy diet from a mean of ~11 to a mean of ~3 **[estimated from a graph]**. In females given estradiol benzoate, dietary soy had no effect on number of attempted mounts. There was no treatment-related difference in the amount of time the female remained in the same compartment as the male during the mating trial. The in situ hybridization for $ER\beta$ mRNA showed message in the paraventricular nucleus but not the ventromedial nucleus of the hypothalamus. Estrogen treatment decreased the expression of $ER\beta$ by 41%; this decrease was not affected by exposure to the soy diet. In animals not receiving estrogen, $ER\beta$ expression was increased 27% by the soy diet. 17β -Estradiol increased the expression of oxytocin receptor in the ventromedial nucleus. This increase was somewhat attenuated by the soy diet. The soy diet alone had no effect on oxytocin receptor expression. The soy diet neither resulted in increased uterine weight, nor attenuated the uterine hypertrophy produced by 17β -estradiol.

The authors concluded that the soy supplement acted as an anti-estrogen on female sexual behavior. They further proposed that the soy-mediated decrease in hypothalamic oxytocin receptor expression might mediate the suppression of sexual behavior.

Strengths/Weaknesses: This well-designed study reported important observations on the anti-estrogenic effects of soy phytoestrogens in the brain. The use of several estrogen related endpoints and the measurement of circulating 17β -estradiol are strengths. The lack of information on the number of animals is a weakness. There was no uterotrophic effect of dietary soy, but the use of adult animals may be an explanation.

Utility (Adequacy) for CERHR Evaluation Process: This report is useful for the evaluation process.

Patisaul et al. (206), supported by NIH, the National Science Foundation, and Emory University, evaluated the effects of a soy supplement on sexual function in ovariectomized female Long Evans rats. All animals used in the study had been shown to mate after administration of estradiol benzoate. Female rats were fed a phytoestrogen-free diet to which was added a ground commercial soy supplement at a concentration of 0 or 0.35%. Based on previous studies, the authors estimated that the supplemented diet contained genistein 13 ppm and daidzein 33 ppm, producing plasma phytoestrogen concentrations between those of humans on a typical Western diet and those of humans on an Asian diet. **[Neither dietary phytoestrogens nor plasma concentrations were measured. Assuming a female Long Evans rat consumes 0.08 kg feed/kg bw/day (84), mean genistein intake would have been 1.04 mg/kg bw/day and mean daidzein intake would have been 2.64 mg/kg bw/day. The duration of dietary treatment prior to testing sexual function was not given.]** Half of the females were given sc estradiol benzoate 48 hours before mating, and all females were treated with sc progesterone 4–5 hours before mating. There were 4 treatment conditions ($n = 6/\text{group}$) used to evaluate the effects of soy: soy-free diet only, soy diet only, soy-free diet + estradiol benzoate, and soy diet + estradiol benzoate. **[Two additional groups were used to evaluate the effects of tamoxifen with or without estradiol benzoate; all tamoxifen-treated animals received the phytoestrogen-free diet.]** The experimental procedure consisted of placing each female in a mating chamber with an untreated male retired breeder for 10 minutes. The breeding apparatus included a smaller chamber into which the female could escape. Males were tethered and were not able to follow the females into the escape chamber. During the 10-minute cohabitation period, counts were made of the

4.0 Reproductive Toxicity Data

number of attempted mounts by the male and the number of lordosis responses by the female. A lordosis quotient was calculated as the percent of mount attempts resulting in lordosis. Females with a lordosis quotient higher than 50% were evaluated for the number of escapes and the amount of time spent in the escape chamber before returning to the male after mounting, intromission, or ejaculation. Data were analyzed using ANOVA with post hoc Fisher least significant difference test.

Estradiol benzoate treatment resulted in an increase in the lordosis quotient from 1 to 87.9% in animals fed a phytoestrogen-free diet. The feeding of the soy diet resulted in a significant decrease of the lordosis quotient to 62.7% in animals also given estradiol benzoate. This decrease was similar to that produced by tamoxifen. Female hopping and darting behavior was also decreased by dietary soy. The percent of sexual contacts that were followed by escape of the female was decreased by dietary soy, but the latency to return to the male was not affected. The authors noted that only 4 of 6 estradiol benzoate-treated soy-exposed females received an ejaculation, and only 2 of these 4 went into the escape chamber thereafter, limiting the ability to detect a treatment effect. There was no effect of soy supplementation on sexual behaviors in animals that were not given estradiol benzoate.

The authors concluded that the effects of soy supplementation on female sexual behavior are anti-estrogenic.

Strengths/Weaknesses: The use of tamoxifen as a comparator is a strength of this study, which is generally supportive of the first study (205). The number of animals used was too low for definitive conclusions, however, and the endpoints were somewhat subjective.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful in support of the first study (205).

4.2.2 Male

Male reproductive studies of dietary soy or soy isoflavones have been performed in rodents. Studies on rats are presented first followed by a study on mice.

Sharma et al. (207), supported by NIH, the Finnish Cancer Foundation, and the Medical Research Council of the Academy of Finland, examined the effects of soy diet on the rat prostate. For 11 weeks, 5 or 6 adult male Sprague Dawley rats/group were fed 1 of 3 diets: a commercial rat chow with soy as the protein source (control group), a specialty soy-free diet with casein as the protein source, or a specialty diet with soy as the protein source. The 2 specialty diets contained identical ingredients, with the exception of the protein source. At the end of the treatment period, rats were killed and prostates were removed, weighed, and examined histologically. Phytoestrogens and metabolites were measured in urine by GC-MS. Data were analyzed by ANOVA followed by Duncan multiple range test. Urinary excretion of genistein, daidzein, and their metabolites was approximately equal in the control and soy-diet groups, but much lower in the soy-free diet group. Ventral, lateral, and dorsal prostate weights did not differ significantly between diet groups. Morphology of ventral and dorsal prostate was not affected by diet. Severity and incidence of prostatitis was increased in the lateral prostate of rats fed the soy-free diet compared to rats fed the soy or control diets. Incidence of prostatitis was 16% in rats fed the control diet, 80% in rats fed the soy-free diet, and 0 in rats fed the soy diet. The study authors concluded that their results suggest that dietary soy protects against development of prostatitis in rats.

4.0 Reproductive Toxicity Data

Strengths/Weaknesses: It is difficult to reconcile the lack of effect of the different diets on weight of different prostate lobe with the large difference in reported rates of prostatitis. Presumably, the term “prostatitis” refers to a unique inflammatory process in the lateral lobe. There is no evidence that this condition is relevant to human prostatitis, which is due to bacterial infection.

Utility (Adequacy) for CERHR Evaluation Process: This paper is not useful for the evaluation process.

Weber et al. (208), supported by the National Science Foundation, NIH, and Brigham Young University, evaluated the effects of dietary soy on plasma testosterone and brain concentrations of steroid-metabolizing enzymes in male Sprague Dawley rats. The animals were given standard laboratory chow until they were 74 days old, following which they were randomized to a phytoestrogen-free diet or to NIH-07, a diet analyzed as containing a mean concentration of daidzin 81.7 µg/g feed, genistein 95.0 µg/g feed, and glycitin 16.5 µg/g feed (aglycones were not detected). **[Assuming a male rat ate 0.07 kg feed/kg bw/day (204), intakes would have been daidzin 5.7 mg/kg bw/day, genistin 6.6 mg/kg bw/day, and glycitin 1.2 mg/kg bw/day.]** Pooled samples from 18 animals/group showed plasma phytoestrogen concentrations on the NIH-07 diet to be daidzein 202.8 ng/mL (compared to 1.6 ng/mL on the phytoestrogen-free diet), genistein 121.0 ng/mL (compared to 10.5 ng/mL on the phytoestrogen-free diet), and equol 371.7 ng/mL (compared to 7.7 ng/mL on the phytoestrogen-free diet). At 103 days of age, medial-basal hypothalamic/preoptic area and amygdala were dissected from 10 animals/group, and aromatase and 5 α -reductase activities were measured. Trunk blood was collected for plasma testosterone determination by RIA at the same time brains were collected. Statistical comparisons were made by ANOVA with post hoc Tukey test. There were no diet-related alterations in feed consumption, body weight, ventral prostate weight, or plasma testosterone concentration. Brain aromatase activity was not affected by diet in either sampled region. In amygdala, 5 α -reductase was increased, and in medial-basal hypothalamus/preoptic area, 5 α -reductase was decreased by the phytoestrogen-containing diet. **[The magnitude of the changes was 22–28%, estimated from a graph.]** The authors characterized these alterations as surprising but noted that they were consistent with a prior study that had shown phytoestrogen inhibition of 5 α -reductase in skin and prostate tissue (209).

Strengths/Weaknesses: The experimental design appears adequate, and with the exception of some changes in 5 α -reductase activity, dietary phytoestrogens had little effect on most other endpoints (e.g., aromatase, plasma testosterone). It is not clear which isoform of 5 α -reductase was being studied. Type 2 5 α -reductase is highly expressed in male accessory sex organs, hair follicles, and genital skin. Only a small amount of testosterone is aromatized, with most enzymatic activity observed in the brain, liver, and adipose tissues. The current findings do little to add to the understanding of how phytoestrogens affect the male reproductive system.

Utility (Adequacy) for CERHR Evaluation Process: These findings need to be confirmed since the overall effect of various phytoestrogens on steroid-metabolizing enzymes needs further investigation. Presently, these findings are inadequate for interpretation.

Weber et al. (210), supported by the National Science Foundation and NIH, evaluated the effects of a soy-phytoestrogen diet on adult male Sprague Dawley rats. Animals were obtained at 50 days of age and maintained on a standard rat chow containing total phytoestrogens 300 µg/g feed. At 70 days of age, 114 animals were randomly assigned to 1 of 2 commercial diets. One diet was designated phytoestrogen-free and was composed of corn, wheat, fish meal, and wheat middlings. The second diet was phytoestrogen-rich and contained soybean meal in place of fish meal. The

4.0 Reproductive Toxicity Data

nutritional content of the 2 diets was similar. The soy-based diet contained phytoestrogens at about 600 µg/g feed. **[Assuming a male rat ate 0.07 kg feed/kg bw/day (204), isoflavone intake in the soy-fed group would have been 42 mg/kg bw/day.]** Analysis by HPLC of the soy-based diet showed the phytoestrogens to consist (percentage by weight) of genistin (46%), daidzin (33%), glycitin (8%), acetylglucoside daidzein (5%), acetylglucoside glycitin (4%), daidzein (1.7%), genistein (1.6%), and glycitein (0.7%). **[These percentages were derived by CERHR by averaging and rounding duplicate determinations that were reported in the paper.]** Plasma phytoestrogen concentrations were determined by GC-MS in a subset of 31 rats/diet group after 35 days on their respective diets. Mean total circulating isoflavones were 2224 ng/mL in the rats fed the soy-based diet and 63 ng/mL in rats on the phytoestrogen-free diet. On a ng/mL basis, of the circulating isoflavones in the soy-based diet group, about 45% was equol, about 35% daidzein, and about 20% genistein. The authors stated that the plasma phytoestrogen concentrations in rats fed the soy-based diet were similar to those in humans on a typical Asian diet and that phytoestrogen concentrations in the rats fed the phytoestrogen-free diet were similar to those in humans on a typical western diet. After 35 days on the diets, animals were killed. Plasma testosterone, androstenedione, and 17β-estradiol were determined by RIA in 18 rats/diet group. Ventral prostates were isolated in 13 animals/diet group and 5α-reductase activity determined. Testicular tissues from 13 animals/dose group were assayed for steroidogenic acute-regulatory protein by Western analysis. Data, analyzed by ANOVA with post hoc Tukey test, are summarized in Table 45.

The authors attributed the reduction in prostate weight on the soy-based diet to the decreased concentration of androgens. Because there were no effects on LH or steroidogenic acute-regulatory protein (which is involved in cholesterol entry into the steroid pathway), the authors could not identify where in the androgen biosynthetic scheme phytoestrogens might be acting.

Table 45. Effects on Adult Male Rats of Feeding Soy-Based Diet

Parameter	Comparison to phytoestrogen-free diet
Feed intake	↔
Water intake	↑8%
Body weight	↓5%
Prostate weight, absolute	↓7%
Relative to body weight	↓8%
Plasma hormone concentration	
Testosterone	↓50%
LH	↔
Androstenedione	↓85%
Estradiol	↔
Prostatic 5α-reductase activity	↔
Testicular steroidogenic acute regulatory protein	↔

↑, ↓, ↔ Statistically significant increase, decrease, or no change comparing male rats fed a soy-based diet containing total phytoestrogens 600 µg/g feed with male rats fed a phytoestrogen-free diet. From Weber et al. (210).

Strengths/Weaknesses: Most of the endocrine endpoints examined substantiated the estrogen-like effects of the phytoestrogens on the male reproductive system. Reductions in plasma androgens (e.g., testosterone and androstenedione) and prostate weights are consistent with the administration of diets containing genistein and daidzein and are confirmed by the measurements of isoflavones in the two different diets. More studies are needed to understand the action(s) on steroidogenic pathways.

4.0 Reproductive Toxicity Data

Utility (Adequacy) for CERHR Evaluation Process: Overall, these findings contribute little to understanding the action of isoflavones on the male reproductive system.

Yi et al. (211), supported by the Korean Science and Engineering Foundation, evaluated the effect of dietary isoflavones on plasma androgens in adult male Sprague Dawley rats. Rats were fed a basal diet with or without the substitution of soy flour 442.7 g/kg feed for corn starch, corn oil, cellulose, and casein. The soy-flour diet contained isoflavones at a concentration of 67.2 mg/kg feed. The study authors estimated that with a mean feed consumption of 22.4 g/day, the animals consumed 19 mg/day isoflavones. **[This calculation appears to be incorrect. Consumption of 22.4 g of feed containing isoflavones 0.0672 mg/g (67.2 mg/kg) would give a daily isoflavone consumption of 1.5 mg, or about 6 mg/kg bw.]** In a second experiment, animals were randomized to AIN-76 diet or to a diet in which corn starch was replaced with soy extract 20 g/kg feed (isoflavones 3.38 mg/g) or with a semi-purified isoflavone preparation 2 g/kg feed (isoflavones 218 mg/g feed). Estimated daily dietary isoflavone intake was 0.9 mg [**~3.6 mg/kg bw**] for the soy-extract diet and 3.3 mg [**~13.2 mg/kg bw**] for the diet with added semi-purified isoflavones. Animals were placed on their respective diets for 1 week (n = 6 or 8/diet group), after which blood was collected for plasma testosterone and dihydrotestosterone by RIA. Plasma isoflavones were determined by HPLC after hydrolysis of the conjugates. Statistical analysis was by ANOVA with Duncan multiple range test.

In the experiment comparing the basal diet to the soy-flour diet, there were no differences between groups in feed consumption or body weight gain. In the experiment comparing the AIN-76 diet to a diet supplemented with soy extract and a diet supplemented with an isoflavone preparation, feed intake and body weight gain were reduced by the isoflavone preparation (attributed by the authors to taste aversion). The soy-flour diet in the first experiment resulted in a significant reduction in dihydrotestosterone without a change in total androgens (testosterone + dihydrotestosterone). In the second experiment, the soy extract-supplemented diet had no significant effect on total androgens or dihydrotestosterone, although the study authors identified a “trend” toward increased androgens. Total androgens were significantly increased and plasma dihydrotestosterone was significantly decreased by the isoflavone preparation. The group that had received the isoflavone preparation also had significantly higher plasma concentrations of genistein and daidzein than the control group, whereas the group that had received the soy extract had plasma isoflavone concentrations that were not significantly different from the control.

The authors concluded that an isoflavone-associated reduction in dihydrotestosterone might be a mechanism by which diets high in soy prevent prostate cancer. They speculated that an increase in plasma testosterone might be compensatory.

Strengths/Weaknesses: The question about the actual calculated dietary consumption of isoflavones is a weakness. Nevertheless, dietary isoflavones led to a decrease in dihydrotestosterone, yet had varying effects on total androgens. It is not clear how long the animals received dietary isoflavones.

Utility (Adequacy) for CERHR Evaluation Process: This paper is not useful in evaluating a possible relationship between isoflavones and prostate cancer, and it does not contain information that can be used to assess reproductive function in the face of dietary soy.

Faqi et al. (212), supported by the National Cancer Institute and the IIT Research Institute, evaluated the reproductive effects in male Wistar-Unilever rats of dietary soy isoflavones given for 1 year. The basal feed was Teklad 4% Fat Rat/Mouse Diet to which was added a soy-

4.0 Reproductive Toxicity Data

isoflavone mixture at levels of 0, 200, or 2000 mg/kg feed [ppm]. The isoflavone mixture contained 45% genistein, 23% daidzein, and 4% glycitein. Analysis of feed samples was performed monthly and showed the high-dose feed to contain 97.5% of target isoflavone levels and the control feed to contain isoflavones at < 11 mg/kg feed (and often undetectable). Based on feed intake and measured isoflavone levels in the diet, high-dose animals were estimated to have ingested isoflavones 80 mg/kg bw/day during weeks 4–5 and 75 mg/kg bw/day during weeks 29–30. During the same periods, isoflavone ingestion in the control group was estimated to have been 0.11 and 0.17 mg/kg bw/day.

Rats were begun on their assigned diets at 9–10 weeks of age. After 12–13 months, testes and epididymides were removed and weighed. One testis/animal was fixed in Bouin fluid and dehydrated in ethanol. The fixed testis were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin for light microscopy. The other testis was used for counting of homogenization-resistant spermatids. The cauda epididymis was cut into pieces and rinsed with saline to produce a sperm suspension. Sperm morphology was evaluated using eosin-stained samples. Statistical analysis used ANOVA with post hoc Dunnett or Mann-Whitney test. Proportions of morphologically normal sperm were compared using chi-squared test.

There were no treatment effects on mortality, clinical signs, or body weight. Absolute and relative testis weights were unaffected by treatment, and testis histology was comparable in all groups. Testicular spermatid number and morphologically normal epididymal sperm did not differ by treatment group. The authors concluded that soy isoflavones would be unlikely to adversely affect reproductive function at dose levels comparable to those inhibiting prostate cancer.

Strengths/Weaknesses: This well designed study extended over a 1-year period. The endpoints evaluated were appropriate as part of an evaluation of male reproductive effects.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful in showing a lack of adverse effect of dietary soy isoflavones at up to 2000 mg/kg feed.

Mäkelä et al. (83), supported by the Yrjö Jahnsson Foundation, Turku University Foundation, and Emil Aaltonen Foundation, evaluated the effects of a soy diet on the genital tracts of male Han-NMRI mice that had been estrogenized as neonates with sc diethylstilbestrol 2 µg/pup/day for 3 days. Animals were raised on a soy-free diet. At 3–5 months of age, the animals were castrated and either maintained on the soy-free diet or given a diet containing 7% roasted soy meal. Some animals in both diet groups were implanted with 17β-estradiol-containing pellets. After 10 or 21 days, urethroprostatic blocks were sectioned for evaluation by light microscopy. In another experiment, animals on soy diets were treated with 17β-estradiol, coumestrol, or vehicle, and urethroprostatic tissues were isolated at intervals for detection of *c-fos* mRNA by Northern analysis. Soy-feeding did not produce histologic alterations in the urethroprostatic tissues and did not induce *c-fos* mRNA. There was no apparent effect of dietary soy on the response of the male reproductive tract to 17β-estradiol.

The authors concluded that the lack of estrogenic effect of a soy diet in male mice stood in contrast to the apparent estrogenic effect of soy diet in developing female mice.

Strengths/Weaknesses: It is difficult to interpret endocrine effects when the experimental design involved using estrogenized neonates; however, soy-feeding failed to produce histological changes in urethroprostatic tissues or mRNA in male mice.

4.0 Reproductive Toxicity Data

Utility (Adequacy) for CERHR Evaluation Process: This study is not useful given the confounding effect of neonatal estrogenization.

4.3 Utility of Data

4.3.1 Human data

There are 15 studies on possible human reproductive effects of soy products, 11 of which involved women and 5 of which involved men. None of these studies involved the administration of soy infant formula, and the utility of these studies in the current evaluation is limited by the extent to which other soy products represent soy infant formula. Two of the studies in women (192, 197) and 1 study in men (199) involved ascertainment of typical diets and correlation testing with serum hormone levels. These studies do not directly assess reproductive effects of specific dietary components, but could be useful to infer reproductive effects. Most of the studies involved use of soy foods (tofu, soy milk) added to the diet or the use of soy supplements. These studies also used reproductive hormones and, in some cases, menstrual cycle length as endpoints. Only 1 study in men (200), which evaluated semen analysis parameters, had an endpoint directly relevant to the assessment of reproductive toxicity. An important limitation of studies of dietary soy products is the difference between soy food-containing diets and other diets in macronutrient content, which has an independent effect on some hormone levels. Any changes in serum or plasma hormone levels on a soy food- or supplement-containing diet could have been due to these macronutrient changes and not to isoflavones, as suggested by the study of Lu et al. (196).

4.3.2 Experimental animal data

The study of Boettger-Tong et al. (202) amply demonstrated that soy-based diets could exert estrogenic effects in rodents. Additional studies have evaluated estrogenic endpoints in intact and castrated rodents; however, these studies did not necessarily address reproductive function. There are 3 studies in female rats and there is 1 study in male rats that address aspects of reproductive function in animals fed diets containing soy isoflavones.

4.4 Summary of Reproductive Toxicity Data

4.4.1 Human data

Studies in women and men are summarized in Table 46 and Table 47.

Diet-intervention trials included the studies of: Cassidy et al. (54, 189), which used textured vegetable protein, miso, or an isoflavone-free soy product; Lu et al. (53, 190) and Nagata et al. (192), which used soy milk; and Xu et al. (87), Martini et al. (194), Duncan et al. (193), and Watanabe et al. (195), which used a soy supplement. The studies of Nagata et al. (192) and Watanabe et al. (195) used an untreated control group. The other studies used subjects as their own controls, either with a cross-over design or by sampling blood before, during, and after the intervention. Treatment periods were short, on the order of 1 or 2 months.

The results of the intervention studies in women were generally inconsistent. Length of the menstrual cycle or follicular phase of the menstrual cycle was increased in the studies of Cassidy et al. (54, 189), Lu et al. (190), and Nagata et al. (192). Menstrual cycle length was unaffected in the studies of Martini et al. (194) and Duncan et al. (193). Luteal-phase length was decreased in a study by Lu et al. (53). The isoflavone content of the interventions, while differing from 1 study to the next, did not explain the discrepancy in cycle lengths. Sample size may have been limiting in the ability to detect significant changes: the 2 studies showing no effect involved only 14 and 16 regularly cycling subjects. The non-intervention study of Jakes et al. (197) in which dietary

4.0 Reproductive Toxicity Data

intakes were obtained by questioning and menstrual cycle lengths were obtained from diaries, described longer cycle length as associated with soy-protein intake above the group median.

Serum or plasma estrogens were also affected differently in different studies. An increase in 17 β -estradiol or estrone was associated with soy-product intake in the studies of Cassidy et al. (54, 189) and Watanabe et al. (195), and serum estrone correlated with soy-protein intake in the non-intervention study of Jakes et al. (197). A decrease in serum, plasma, or urinary estrogens was identified in the studies of Lu et al. (53, 190, 196), Nagata et al. (192), Xu et al. (87), and Duncan et al. (193). The non-intervention study of Nagata et al. (191) found a negative association between soy products in the diet and serum 17 β -estradiol in the second half of the cycle. One of the studies of Lu et al. (196) found the decrease in serum 17 β -estradiol on a reduced-isoflavone soy supplement to lose statistical significance when adjustments were made for the macronutrient intake of the subjects.

Studies in men used dietary additions of tofu (198), soy milk (201), or a soy supplement (200). Interventions were applied for 1 or 2 months. These studies found no effect of the intervention on serum testosterone or 17 β -estradiol, although 1 of them (201) reported a decrease in serum estrone with soy milk added to the diet. The non-intervention study of Nagata et al. (199) reported an inverse correlation between serum 17 β -estradiol in men and intake of soy products. Habito et al. (198) reported a significant decrease in the testosterone:estradiol ratio and an increase in sex hormone-binding globulin after addition of tofu to the diet for 1 month. Both of these hormone changes might be expected to decrease androgen activity; however, no measures of androgen activity were included in the study. Mitchell et al. (200) found no effect of a daily soy supplement with 40 mg isoflavones on testicular volume or semen analysis parameters after 2 months of treatment. There were only 15 subjects in this study.

4.4.2 Experimental animal data

Gallo et al. (203) added isoflavones to a solvent-extracted commercial-soy diet at levels of 0.7, 1.2, and 2.4% **[estimated to produce daily isoflavone consumption of about 82, 140, and 290 mg/kg bw]**. Female Wistar rats began treatment at weaning and were mated on PND 77. Dams were permitted to litter, and pups were observed until PND 7. Estrogenic changes in the reproductive tract of the dams were apparent at all isoflavone levels. An increase in uterine weight and a decrease in small ovarian follicles were the most sensitive endpoints. In spite of the clear evidence of estrogenic effect in the genital tract, there was no apparent alteration in fertility or the ability to bear young and raise them until PND 7.

Two studies by Patisaul et al. (205) found dietary isoflavones to antagonize the action of estradiol benzoate and 17 β -estradiol in the sexual response of ovariectomized Long Evans rats. The treatment used a soy supplement at a dietary concentration of 0.35%, providing an estimated genistein concentration of 13 ppm (1.04 mg/kg bw/day) and an estimated daidzein concentration of 33 ppm (2.64 mg/kg bw/day). Plasma phytoestrogens, which were not measured in these studies, were estimated to be in the range of human dietary exposures. The anti-estrogenic effect of the isoflavone-containing diet on sexual function was similar to that of tamoxifen.

A study by Faqi et al. (212) used a defined mixture of soy isoflavones administered to male Wistar-Unilever rats in the diet at 200 or 2000 mg/kg feed **[ppm]** for 12–13 months. There were no adverse effects of treatment on body weight, weight of the testis or epididymis, sperm morphology, homogenization-resistant spermatid number, daily sperm production, or testis histopathology. The high-dose group was estimated to have consumed isoflavones 75–80 mg/kg bw/day.

4.0 Reproductive Toxicity Data

Conclusions to be developed at the Expert Panel meeting:

Evidence is (sufficient/insufficient) to conclude that soy infant formula (would/would not) produce reproductive toxicity in men/women at (dose level) as manifested by (endpoints).

Evidence is (sufficient/insufficient) to conclude that soy infant formula (produces/does not produce) reproductive toxicity in (sex, species) at (dose, route) manifested by (endpoint). The experimental animal data are (assumed relevant/relevant/not relevant) to the assessment of human risk.

Note: The definitions of the term sufficient and the terms assumed relevant, relevant, and not relevant are in the CERHR guidelines at <http://cerhr.niehs.nih.gov/news/guidelines.html>.

4.0 Reproductive Toxicity Data

Table 46. Summary of Reproductive Studies in Women

Intervention, n = starting sample	Control	Effects of soy product	Reference
Diet for 1 month containing 1 of: <ul style="list-style-type: none"> • Textured vegetable protein 60 g/day • Textured vegetable protein 28 g/day • Miso 50 g/day • Isoflavone-free soy product, 60 g/day n = 3–6/group	Women received control diet for 1 month and served as their own controls.	Textured vegetable protein 60 g/day (45 mg/day conjugated isoflavones): Follicular-phase length ↑17% Follicular-phase plasma 17β-estradiol ↑40% Mid-cycle plasma LH ↓74% Mid-cycle plasma FSH ↓43%	Cassidy et al. (54, 189)
Soy milk 36 ounces/day for 1 month, n = 6	Blood samples were taken prior to soy milk, during soy-milk consumption, and for a variable number of cycles afterwards, with comparisons made between soy and non-soy time periods.	↑ Menstrual cycle length (<i>P</i> = 0.06) ↓ Serum 17β-estradiol (throughout cycle) ↓ Luteal-phase serum progesterone ↓ Serum dehydroepiandrosterone sulfate (cycle average)	Lu et al. (190)
No intervention: Women were questioned about dietary intakes, n = 50	No control group. Correlation testing was used for analysis of serum 17β-estradiol and sex hormone-binding globulin levels and soy intake. Adjustments were made for age, body-mass index, cycle length, and energy, fat, and fiber intake.	Negative association between soy intake and serum 17β-estradiol on cycle day 22. Negative association between serum sex hormone-binding globulin and miso consumption on cycle day 22.	Nagata et al. (191)
Soy milk, 400 mL/day (109 mg/day isoflavones) for 2 months spanning parts of 3 menstrual cycles, n = 31	Women who were instructed not to consume soy milk, n = 29.	Mid-cycle serum estrone ↓23–30% in soy-milk group Menstrual cycle lengthened over soy-milk treatment interval.	Nagata et al. (192)
Commercial soy supplement providing total isoflavones 0.16, 1.01, or 2.01 mg/kg bw/day, n = 12	Cross-over design with women serving as their own controls	Urinary isoflavones and lignans increased and endogenous estrogens and their metabolites decreased with increasing supplement dose.	Xu et al. (87)

4.0 Reproductive Toxicity Data

Intervention, n = starting sample	Control	Effects of soy product	Reference
Soy supplement containing 38 mg total isoflavones given daily for 2 menstrual cycles, n = 16 regularly cycling women and 20 women on oral contraceptives	Cross-over design using soy-free milk treatment; women served as their own controls.	No effects of soy supplementation on menstrual cycle length or on serum 17 β -estradiol, estrone, dehydroepiandrosterone sulfate, prolactin, or sex hormone-binding globulin; no effect on urinary levels of estrogen metabolites	Martini et al. (194)
Soy supplement resulting in daily isoflavone intake of 0.15, 1.01, or 2.01 mg/kg bw/day, taken for 3 menstrual cycles plus 9 days, followed by 3-week washout, n = 14	Cross-over study with each woman serving as her own control; the 0.15 mg/kg bw/day isoflavone intake was the control condition.	No effect of soy supplement on follicular phase, luteal phase, or total cycle length. ↓Mid-cycle plasma estrone (high versus low dose). ↓Periovulatory plasma LH and FSH (low dose versus control) No change in plasma 17 β -estradiol or progesterone.	Duncan et al. (193)
Isoflavone supplement tablet providing 20 or 40 mg/day for 1 menstrual cycle, n = 19 or 20 for both groups combined	n = 20; unknown whether a placebo tablet was taken.	↑Plasma 17 β -estradiol [Many cycles were irregular and the Methods and Results were confusing.]	Watanabe et al. (195)
Soy milk 36 ounces/day for 1 month, n = 10	Women were monitored during a 3-month baseline period and served as their own controls.	↓Luteal phase length ($P = 0.07$), positively associated with urinary isoflavone excretion and with decreased protein intake. ↓Serum 17 β -estradiol (AUC), positively associated with plasma and urinary isoflavones and inversely related to protein intake. No effect on serum LH or FSH.	Lu et al. (53)

4.0 Reproductive Toxicity Data

Intervention, n = starting sample	Control	Effects of soy product	Reference
Reduced-isoflavone soy supplement, n = 9 for 1 cycle	Women were monitored for a baseline cycle prior to treatment and served as their own controls.	<p>↓Serum 17β-estradiol during each phase of the cycle and overall; significance lost when corrections were made for macronutrient intake.</p> <p>↓Serum progesterone; significance lost when corrected for protein intake.</p> <p>No change in serum LH, FSH, or sex hormone-binding globulin.</p>	Lu et al. (196)
No intervention: Women were questioned about dietary intakes, n = 200	No control group. Correlation testing was used for analysis of serum 17β-estradiol, estrone, estriol, LH, and FSH and for analysis of menstrual cycle parameters from diaries.	Estrone correlated with intake of soy protein Soy-protein intake higher than the median for the sample associated with longer cycle length (P = 0.06 adjusted for age and parity).	Jakes et al. (197)

4.0 Reproductive Toxicity Data

Table 47. Summary of Reproductive Studies in Men

Intervention, n = starting sample	Control	Effects of soy product	Reference
Dietary tofu 290 g/day (35 g/day soy protein) for 1 month, n = 42	Dietary meat 150 g/day for 1 month; cross-over design with each man serving as his own control.	No effect on serum total testosterone, dihydrotestosterone, androstenediol glucuronide, or 17 β -estradiol. Weight-adjusted testosterone:estradiol ratio \downarrow 12%. Weight adjusted sex hormone-binding globulin \uparrow 9%.	Habito et al. (198)
No intervention: Men were questioned about dietary intakes, n = 97	No control group. Correlation analysis was used for analysis of serum 17 β -estradiol, estrone, total and free testosterone, dihydrotestosterone, and sex hormone-binding globulin with adjustment for age, body-mass index, smoking, and ethanol use, and (for androgens) dietary fat, cholesterol, carbohydrate, and vitamins B ₁ and D.	Serum 17 β -estradiol inversely correlated with intake of soy products. No other significant correlations.	Nagata et al. (199)
Soy supplement (40 mg total isoflavones) daily for 2 months, n = 15	Men were evaluated before, during, and after the supplementation period and served as their own controls.	No effect on testicular volume, semen analysis parameters, or plasma levels of 17 β -estradiol, testosterone, FSH, or LH.	Mitchell et al. (200)
Soy milk 400 mL/day for 8 weeks, n = 17	Regular diet without soy milk, n = 17.	\downarrow Serum estrone. No changes in serum 17 β -estradiol, total and free testosterone, or sex hormone-binding globulin.	Nagata et al. (201)

5.0 Summaries, Conclusions, and Critical Data Needs

Section 5.0 will be written at the Expert Panel meeting.

5.1 Summary and Conclusions of Reproductive and Developmental Hazards

5.2 Summary of Human Exposure

5.3 Overall Conclusions

5.4 Critical Data Needs

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