

# Guidance from an NIH Workshop on Designing, Implementing, and Reporting Clinical Studies of Soy Interventions<sup>1–4</sup>

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## Abstract

The NIH sponsored a scientific workshop, “Soy Protein/Isoflavone Research: Challenges in Designing and Evaluating Intervention Studies,” July 28–29, 2009. The workshop goal was to provide guidance for the next generation of soy protein/isoflavone human research. Session topics included population exposure to soy; the variability of the human response to soy; product composition; methods, tools, and resources available to estimate exposure and protocol adherence; and analytical methods to assess soy in foods and supplements and analytes in biologic fluids and other tissues. The intent of the workshop was to address the quality of soy studies, not the efficacy or safety of soy. Prior NIH workshops and an evidence-based review questioned the quality of data from human soy studies. If clinical studies are pursued, investigators need to ensure that the experimental designs are optimal and the studies properly executed. The workshop participants identified methodological issues that may confound study results and interpretation. Scientifically sound and useful options for dealing with these issues were discussed. The resulting guidance is presented in this document with a brief rationale. The guidance is specific to soy clinical research and does not address nonsoy-related factors that should also be considered in designing and reporting clinical studies. This guidance may be used by investigators, journal editors, study sponsors, and protocol reviewers for a variety of purposes, including designing and implementing trials, reporting results, and interpreting published epidemiological and clinical studies. *J. Nutr.* 140: 1192S–1204S, 2010.

## Introduction

The NIH sponsored a scientific workshop, “Soy Protein/Isoflavone Research: Challenges in Designing and Evaluating

Intervention Studies,” July 28–29, 2009. The workshop goal was to provide guidance for the next generation of soy protein/isoflavone human research. Session topics included population

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exposure to soy;<sup>15</sup> the variability of the human response to soy; product composition; methods, tools, and resources available to estimate exposure and protocol adherence; and analytical methods to assess soy in foods and supplements and analytes in biologic fluids and other tissues. The intent of the workshop was to address the quality of soy studies, not the efficacy or safety of soy.

To develop the guidance, the participants identified methodological issues relative to exposures and interventions that may confound study results and interpretation. Scientifically sound and useful options and solutions for dealing with these issues were discussed. This guidance may be used by investigators, journal editors, study sponsors, and protocol reviewers for a variety of purposes, including the design and implementation of trials, reporting results, and interpretation of published epidemiological and clinical studies.

## Background

Two important documents resulting from prior workshops and an evidence-based review served as the background to the current workshop. In 2005 the National Institute of Environmental Health Sciences of NIH held 2 workshops, 1 to review the literature to assess whether phytoestrogens could significantly affect hormonal and other endpoints in animals and the other to develop strategies to address problems caused by batch-to-batch variation in estrogenic compounds in animal diets. The workshop results were reported in early 2008 (1). The key finding of these workshops and report was that research results are often confounded by unanticipated levels of phytoestrogens in laboratory unpurified diets. Even though the protein content of soy-containing animal diets was constant, there was significant batch-to-batch variability in estrogenic content of commercial animal diets and this variability resulted in differences in experimental outcomes. Much of the variability may be eliminated by using phytoestrogen-free diets, such as the AIN 93 semipurified diet, supplemented with a known quantity of phytoestrogens (2,3). Although the National Institute of Environmental Health Sciences workshops focused on animal studies, they concluded that it is clear that variation in diet components may also affect clinical studies. Hence, the NIH questioned the quality of data from human studies.

NIH has supported research on soy in its many forms for a range of outcomes. Results of clinical studies have been inconsistent. Questions concerning which forms of soy might be better for studies of specific health outcomes and at what doses led the National Center for Complementary and Alternative Medicine and the Office of Dietary Supplements, both of NIH, to commission an evidence-based review of the literature, "Effects of Soy on Health Outcomes," through the Agency for Health Care Research and Quality (AHRQ) (4). The review summarized the formulations of soy products and/or soy food used in clinical trials and the current evidence of the health effects of soy and its constituents on the following health outcomes: cardiovascular disease, menopausal symptoms, endocrine function, cancer, bone health, reproductive health, kidney function, cognitive function, and glucose metabolism.

In addition, safety issues and drug interactions associated with using soy were summarized.

The AHRQ evidence-based review, released in 2005 (4), reported that about three-quarters of the 281 included trials studied soy supplements with the remainder being trials of soy foods. Fifty-seven percent of the soy supplement trials used isolated soy protein with isoflavones, 36% used isoflavones alone, and 6% used soy protein without isoflavones. Textured soy protein was evaluated in 25% of soy foods trials and soy flour was evaluated in another 25%. The remaining 50% evaluated soybeans alone, tofu alone, and other soy foods. Isoflavone and soy protein doses varied widely, from 10 to 185<sup>16</sup> mg/d and 14 to 154 g/d, respectively. Only a few studies directly compared doses. In addition, there was a considerable degree of heterogeneity among the studies, including study design, intervention duration, background diet, and controls. A large number of studies suffered from inadequacies of reporting or study design, thereby limiting conclusions.

If clinical studies are to be pursued, then study sponsors, investigators, reviewers, and journal editors need to ensure that the experimental designs are optimal and the studies properly executed. How do we move forward?

## Clinical research guidance

The current NIH workshop resulted in guidance, not requirements. This guidance is presented in the subsequent sections with a brief rationale. The guidance is specific to soy research and does not address nonsoy-related factors that investigators should consider when developing protocols and operations manuals, implementing studies, and reporting results.

### Justification for clinical research

#### Guidance.

Use caution when interpreting past studies; consider study age, source, and quality; historical changes in test agents; background influences; participant characteristics and heterogeneity; and intervention adherence and frequency of its assessment.

Acquire information from previous studies on product characterization, stability, and analytical methods used to determine product integrity.

Determine the quality, sample size, participant characteristics, and heterogeneity of studies that may provide the foundation for new studies.

#### Key points.

The relevance of cell culture and animal studies to human clinical studies requires careful evaluation before translation to clinical trials.

Epidemiological studies of Asian populations provide the background for many clinical studies; the type of soy Asians have traditionally consumed often differs from that consumed by Americans and other Westernized populations and from the majority of what has been studied clinically.

Few dose-response trials have been conducted in humans, so it is difficult to estimate with confidence the threshold amount of soy needed to exert various physiologic effects in vivo.

<sup>15</sup> Unless otherwise noted, the term "soy" [*Glycine max* (L.) Merr.] is used throughout this document to include whole soy in all its chemical complexity, as well as isolated soy constituents (e.g. individual isoflavones or protein). When a distinction needs to be made, specific terminology is used.

<sup>16</sup> Isoflavone values are used as reported in the original literature and not converted to aglycone equivalents unless they are labeled as aglycone equivalents. This document uses the common spelling "aglycone." The alternative is "aglycon," the preferred spelling of the International Union of Pure and Applied Chemistry.

Investigators may use a range of publications to justify their clinical research. Uncertainty exists even among experienced soy researchers who attempt to interpret the hundreds of soy studies published annually. These prior investigations may include epidemiologic studies, human intervention trials, animal studies, and cell culture studies. It is difficult to synthesize results across this wide range of study types and designs. The relative merits and potential public health significance of research reports using different research models are continually debated.

**In vitro studies.** The source of the most confusion and conflicting results is perhaps the use of supraphysiological levels of isolated isoflavones or mixtures of isoflavones in cell cultures. In vitro studies may over- or underestimate in vivo effects. The relevance of these studies to in vivo human situations is uncertain and their results should be interpreted with caution, although they may be helpful in determining how soy may exert effects at cellular and molecular levels (5).

**Animal studies.** Animal studies may also fail to predict outcomes in humans due to a variety of reasons. Animal model systems are not completely comparable to humans when assessing disease progression, metabolism, or health effects of test agents such as soy. Animals may be fed soy at levels exceeding what is typically found in Asian or Western diets or administered to human research participants. Genistein, the primary isoflavone in soybeans, may be administered to animals by injection, which would bypass the gastrointestinal tract and liver and may also exceed the exposure possible by reasonable human consumption. While dietary exposure in preclinical models is preferred, it is critical that blood levels (total and aglycone) be evaluated and be comparable to the blood levels observed in human populations consuming isoflavone-containing products. Physiologically relevant dietary levels should be considered when appropriate. Furthermore, isoflavone metabolism in rodents and nonhuman primates differs markedly from that of humans (6). Finally, the intestinal microflora of rodents and monkeys is more efficient than microflora in humans at producing equol, the metabolite of the isoflavone daidzein and, consequently, results of studies in these species may not predict the effect of soy consumption in humans (5). It is important to note that although the preclinical models have limitations, these studies have provided useful information that has been supported by results from human clinical and epidemiological studies. For example, the 7,12-dimethylbenz[a]anthracene rat and athymic mouse mammary tumor models were critically important in the development of tamoxifen and aromatase inhibitors as frontline therapies for breast cancer. Additionally, the protective effect from early life exposure to soy isoflavones in preclinical models (7) was very useful in demonstrating the protection of early life exposure against breast cancer (8–11).

**Epidemiological studies.** Early epidemiological studies that formed the foundation of our understanding of how to move forward with clinical studies have been based on the foods people in Asia have been eating. A major distinction between epidemiological studies and clinical studies is that the population-based studies are investigating soy intake as whole foods or diets, whereas experimental research is usually based on administration of isolated constituents. For more epidemiologic information, see the “Soy exposure and intervention adherence” section in this document.

**Clinical studies.** Interpreting previous clinical research is difficult, but history may provide guidance. Over time, processing

and development of soy products have evolved, generally for the purpose of improving food functionality (e.g. to promote water retention or for use as a bleaching agent). As a result, different soy constituents or constituents in different proportions may be present in soy products consumed or studied today compared with products from earlier times. Therefore, when evaluating past studies, it is important that the published results contain detailed information on product characterization. It would also be useful to know the content of phytate, selenium, or other possible bioactives (confounders) of intervention products. If the study product is still available, it might be worthwhile to analyze its composition. Furthermore, investigators must keep in mind that many clinical studies involve administering soy in amounts that exceed what is commonly consumed in Western or Asian populations.

Few dose-response trials have been conducted in humans, so it is difficult to estimate with confidence the threshold amount of soy needed to exert physiologic effects in vivo. Most clinical trials, e.g., have used 40–90 mg/d of total isoflavones, with the AHRQ evidence-based review reporting a range of 10–185 mg/d of total isoflavones (4). In addition, the amount needed to exert beneficial effects may vary according to the condition under investigation. It is possible that the amount of soy needed for health benefits when consumed over the course of a lifetime is lower than that needed to produce benefits in short-term clinical trials.

## Product composition and integrity

### Guidance.

- Know and report the product source and supplier.
- Analyze, describe, and report all the potential bioactive constituents relevant to the study.
- Conduct independent analyses of test and placebo agents before, during, and after intervention, and report all analytical methods used to determine product constituents.
- Use appropriate and accurate terminology in describing test agents.
- Express isoflavone values as aglycone equivalents or present isoflavone values with sufficient information so that they can be readily converted to aglycone equivalents for cross-study comparison.
- Know product constituents that may potentially interact within soy or with components in food formulations.
- Refer to the Consolidated Standards of Reporting Trials (CONSORT) statement on reporting herbal interventions (12).

### Key points.

- Soy intervention products differ chemically.
- Published papers often use confusing or incorrect terminology without precisely specifying what has been used in the study.
- Soy provides a variety of potentially biologically active compounds, including protein, various associated peptides, and over 100 other phytochemicals, including isoflavones.
- Interaction can occur among components within a soy product or with other components in the food or supplement formulation.
- The type and proportion of bioactive constituents will vary depending on plant genetics, growing and harvesting conditions, storage before and after processing, plant part used, and processing and extraction methods.
- Differences in the physiochemical composition of soy products may account for differences among clinical and epidemiological studies.

Research articles often use terms such as soy, soy protein, or even soy supplements without specifying what has been used in a

study. Investigators often neglect to provide sufficient details in the methods sections of papers to allow others to understand, interpret, or repeat their work. Consequently, it is possible that the high degree of chemical heterogeneity among isolated soy proteins and soy isoflavone extracts may have contributed to inconsistent results noted in meta-analyses and other compilations. Documenting the chemical composition of the test agent and harmonization of study protocols, including study agents, to enable cross-study comparison has been called for. Ideally, journal editors could require these as a condition of publication. At a minimum, adherence to the CONSORT guidance for reporting randomized controlled trials of herbal interventions (12,13) would provide more consistent reporting quality.

**Chemical composition.** Soy is not a single chemical substance but is a collection of compounds comprising the soybean. Soy provides a variety of compounds that have potential biological activity that cannot be ignored. Components that may have biological activity include but are not limited to: isoflavones, protease inhibitors, phenolic acids, proteins and peptides, saponins, phytic acid, phytosterols, lignans, vitamins, minerals, and other nutrients. This composition is not constant but varies with the soybean strain, where it is grown, when it is harvested, how it is stored before and after processing, the plant part used, and how it is processed and analyzed. Factors affecting composition due to processing include method of isolation (e.g. filtration, precipitation), wash steps (e.g. type and quality of solvents), heat, enzyme treatment, and drying. Compositional changes resulting from processing include protein and amino acid profile, fatty acid profile (e.g. oxidation), phytochemicals (e.g. losses of isoflavones, saponins, phytosterols, oxalates, phytates, etc.), and vitamins and minerals (e.g. losses and gains) (5,14,15). Consequently, soy intervention products often differ chemically. These are absorbed and modified differently in the gastrointestinal tract. Knowledge of the composition of the soy product used in a research project is critical, because the proportion of the test product constituents will vary and may affect study results.

**Protein.** Several different soy proteins with differing phytochemical profiles have been used clinically and in animal studies. These proteins are primarily identified according to their isoflavone content. Frequently, isolated soy protein that has been specially processed to remove essentially all of the isoflavones has been used as a control or negative control to determine the role of isoflavones on health outcomes. However, such processing removes not only isoflavones but other bioactives as well, such as saponins (15,16). Furthermore, the protein may be denatured and the resulting peptides formed upon digestion, which are potentially biologically active, altered. Consequently, conclusions that isoflavones are solely responsible for differences in health outcomes between the effects of differently processed soy proteins based on their isoflavone content should be made very cautiously.

**Isoflavones.** The soybean contains 12 forms of isoflavone isomers. These include the 3 aglycones (hydrolyzed forms of isoflavones) genistein, daidzein, and glycitein; their respective  $\beta$ -glycosides genistin, daidzin, and glycitein; and 3  $\beta$ -glucosides each esterified with either malonic or acetic acid (5,17). The type and concentrations of these isomers in finished food and supplement products will vary by the plant part from which they are derived and the method by which they are processed (5,18,19). Isoflavones in American foods are primarily present as glycoside conjugates. The ratio of these conjugates varies in

different soy foods. The levels of the aglycones in supplements and foods tend to be very low, unless the food is fermented. In addition, fermentation leads to altered chemistry of the isoflavone (19). Reporting only total isoflavones may be inappropriate, because the individual isoflavones have different activities.

Furthermore, isoflavone content of a product is often reported without indicating whether the stated amount refers to aglycone or glycoside. The molecular weight of the aglycone is ~60% that of the glycoside. Considerably different interpretations may result from an unqualified statement of isoflavone content. The term “aglycone isoflavone equivalents” best describes the bioactive form of isoflavones, because cleavage of the glycoside to produce aglycone is probably required before isoflavones can be absorbed. As a result, isoflavone values should be reported in such a way that they can be converted to aglycone equivalents if desired.

## **Soy exposure and intervention adherence**

### **Guidance.**

Identify the food and supplement sources of soy and nonsoy phytoestrogens available to the study population to determine the study population’s baseline exposure and to control and/or monitor nonstudy exposure.

Determine how much nonstudy soy and/or nonsoy phytoestrogen exposure will be allowed to establish adherence criteria.

### **Key points.**

In the United States, mean total isoflavone consumption is 1–2 mg/d, whereas in Asian countries, the range is 25–50 mg/d. Soy and nonsoy phytoestrogens are present in a number of foods and supplements commonly consumed in the United States and may influence study results.

Consumers are not aware of all the soy that they are consuming, because soy is used as an additive/ingredient and the soy content varies by food type, brand, year, and region of the country.

The evaluation of the health effects of soy requires detailed knowledge of its dietary occurrence and thereby of the exposure to human populations.

**Soy intake/exposure.** Data suggest that average consumption of isoflavones in the United States and Europe is 1–2 mg/d (20,21). Intake varies by age, gender, ethnicity, and socioeconomic status (22). Exposure will also vary by geographic region of the country. For example, in Hawaii, although Caucasians have a lower intake of soy compared with Hawaiian Japanese, Chinese, and Native Hawaiian subpopulations, they have a higher intake than the mean intake of U.S. Caucasians (23). When Asians immigrate to Westernized countries, they may acquire many of the eating habits of the new country’s dominant culture. This is exemplified by the dietary patterns, including soy intake, of Japanese women in Hawaii, which are a blend of patterns seen among traditional Japanese and Hawaiian American Caucasian women (24). Lifestyle factors (e.g. vegetarianism, lignan- and flavonoid-containing supplement use) may also influence soy intake (21,25,26).

Most of the epidemiologic research on the relationship between soy consumption and the development of disease in humans has been conducted in Asian populations and examined only the effects of traditional soy foods. Asian populations, in general, are exposed to soy early in life and consume it over a lifetime. The total mean intake of isoflavones in Asian countries

ranges from 25 to 50 mg/d (24,27), with a small proportion (<10%) consuming as much as 100 mg/d (28). Soy intake and sources vary among Asian countries and among regions within countries, by age and gender, and over time (27,29–31). Even at birth, daidzein and genistein are detected in infants' cord blood, and these values are similar to the levels in maternal blood (32). It has been hypothesized that consumption of soy during certain critical stages early in life may be responsible for its health effects later in life.

**Food sources of soy and other phytoestrogens.** Soy contains the highest isoflavone concentration among foods, and dietary isoflavone exposure occurs mainly through intake of soy products. Although Asian diets are becoming Westernized, historically, the type of soy that Asians and Americans eat is quite different. In Asian countries, soyfoods are generally minimally processed and often fermented. Japanese, e.g., consume many soy foods in various forms, such as boiled soybeans, tofu, fried tofu, miso soup, natto, and soy milk (33). Tofu and miso are the major contributors to their soy isoflavone intake. About 30% of total soy foods consumed by Asians is in the form of fermented foods (27). Therefore, consumption of isoflavone aglycones is higher in Asian populations, being ~10–30 mg/d (28).

Americans eat much more processed forms of soy (e.g. soy flour, textured vegetable protein, and isolated soy protein). The primary source of isoflavones in the United States (56%) is legumes, presumably soybeans (21). Soy milk is also a significant source (18%). However, recent analysis of the soy and nonsoy phytoestrogen content (including isoflavones) of a wide variety of foods suggests that phytoestrogen content may be increasing in a number of foods commonly consumed by the U.S. population. Soy protein isolate is the sole source of protein in infant formulas and is a major constituent of many processed foods. Isoflavone intake from these sources may be equal to or greater than that from foods actually identified as soy foods. For example, several meat products and other processed foods, including sausages, pancakes, baked goods, canned tuna, and hot dogs, have been reported to contain isoflavones because of their soy additives (16,34). These hidden sources of soy will vary by brand and the same branded product will change composition over time and by region of the country (35). In addition, the number of new products introduced into the market annually is substantial (36).

The importance of the hidden sources is tempered by the fact that many of these foods contain isoflavone levels below our limit of reliable detection. Nonetheless, given the comparatively high levels of isoflavone excretion observed among some nonAsian women who generally do not consume traditional soy-based products, there is a need to examine a wide variety of foods when evaluating the relationship between isoflavone exposure and a health outcome (26,37,38). Foods other than the traditional soy-based ones may be major contributors to isoflavone exposure in most Western populations. Accurately quantifying isoflavone intake when exposure to easily identifiable soy products is low is extremely difficult.

Nonsoy foods may contribute to phytoestrogen intake and may influence study results. In many Western diets in which soybeans do not contribute substantially to the diet, lignans are the phytoestrogens that are primarily consumed and provide the major source of phytoestrogens (34,39). Some foods that contain low concentrations of lignans, such as fruits, vegetables, and beverages, are consumed in large amounts and can contribute significantly to lignan intake. Major sources are

coffee and orange juice, not because these foods are high in lignans, but because they are frequently consumed (37). Nonsoy isoflavone intake from other foods, including cereals and breads, eggs, dairy products, meat, fish, nuts, and vegetables, has been documented (40–45).

**Dietary supplement sources of soy and other phytoestrogens.** In addition to foods, soy dietary supplements, especially in certain populations, may contribute significantly to isoflavone intake and other nonsoy phytoestrogen intake, in general. The soy supplement market is not as large as the overall soy food industry and has been declining (46) but is nonetheless substantial with >50 different preparations available. Some supplement products also have soy-like isoflavones. Kudzu contains very high levels of daidzein and puerarin, which is a methoxylated derivative of daidzein. Red clover is also found in supplements and includes formononetin and biochanin A, which are methoxylated forms of genistein and daidzein. In the absence of a comprehensive assessment, studies (particularly in non-Asian populations) could suffer from the effects of uncontrolled confounding by unrecognized and unmeasured sources of phytoestrogen exposure potentially leading to biased estimates of effect and misinterpretation of findings.

## Assessing exposure and adherence

### Guidance.

Validate dietary assessment methods specific to soy and/or nonsoy phytoestrogen intake in relationship to timing, frequency, study population characteristics, and geographic region.

Assess isoflavone metabolites in biologic fluids (optimally both blood and urine), and target tissues, as appropriate, to improve study and nonstudy exposure assessment.

### Key points.

Dietary assessment methods can be used to determine prior or lifetime exposure to soy and nonsoy phytoestrogens, distinguish high from low consumption, capture dietary supplement use, and monitor adherence.

Dietary assessment requires food composition databases that are a challenge to keep up-to-date and which contain isoflavone values for only a select number of foods.

Food composition databases can be useful in identifying soy food and soy and nonsoy phytoestrogen (including isoflavone) sources.

Urine can be a good predictor of systemic availability of soy isoflavones.

Control and/or monitor nonstudy exposure to soy and nonsoy phytoestrogens using study-appropriate assessment methods.

**Dietary assessment.** Dietary assessment methods can be used in intervention studies to determine prior or lifetime exposure to soy, distinguish high from low consumption, and monitor adherence. Dietary assessment methods include FFQ, 24-h recalls, and dietary records. Each method has strengths and limitations and may be used differently depending on whether the assessment determines long- or short-term intake, is for individuals or groups, distinguishes very high from very low soy consumption, is for very accurate or general consumption, identifies soy supplement or soy food intake, etc. In addition, the foods commonly available and consumed in the study geographic region may be an important variable in determining which method is appropriate. Choosing a method with repro-

ducible data, especially in a population similar to the study population, is important.

Validation of assessment instruments is difficult because, despite the availability of urinary and plasma biomarkers, the short half-life of isoflavones and their metabolites limits the use of these biomarkers for validation if consumption of soy is only occasional. In addition, urinary isoflavones may be less frequently correlated with a soy questionnaire among low consumers, because isoflavones from soy additives, such as those in breads, meats, or canned foods, are detected in urine but are not captured in FFQ or recalls. Thus, carefully designed assessment methods may be required to obtain such information.

For dietary assessment, a sufficient number of data collection points is needed. This will be influenced by the dietary assessment method used. Which instrument is used will depend on the study population and the inclusion of the relevant foods and supplements. One must keep in mind that a gram of a soybean or soy food is not a unit of amount of isoflavones; that is, the amount of isoflavones does not parallel the amount of soy food. This calls for the augmentation of assessment instruments by analysis of what is being eaten by the study participants. Both the frequency of consumption and the soy content have measurement issues.

**Assessment in biologic fluids.** Isoflavones in urine and plasma have been used in a number of studies as a biomarker of soy food or isoflavone intake (32,47). There is a good correlation between peak concentrations of plasma daidzein and genistein and their concentrations in the first 24-h urine following soy consumption (48). In populations that consume small to moderate amounts of soy only occasionally, different methods of adherence assessment may be necessary, because urinary isoflavones reflect primarily the intake within the past 48 h. Multiple urine collections could correct this drawback but may not be feasible.

The use of spot urine samples for isoflavone measurements may be problematic. There is a poor correlation between spot urine isoflavone levels and peak plasma concentrations of isoflavones. An example of this problem with spot urine collection is illustrated by a study of women in Gifu, Japan (24). Although urinary isoflavone assessment confirmed the validity of soy consumption measured by the FFQ, the difference in mean urinary isoflavone excretion between groups was considerably greater than the difference in self-reported soy intake. This was most likely a result of the women donating urine samples each morning, typically after a breakfast of tofu and/or miso soup, when isoflavone excretion peaked.

The advantages and disadvantages of using plasma, urine, and/or target tissue for assessment of isoflavone bioavailability need to be considered and include the invasiveness of the method, ability to identify equal producers, ability to collect a more concentrated matrix (e.g. overnight urine), participant control over collection, privacy of collection, and how well the method reflects exposure over the course of the study. For more information on isoflavone bioavailability, see the “Pharmacokinetics” section in this document.

**Food composition databases.** Analysis of the dietary intake will rely on a food composition database that should contain a representative sample of soy foods. Conflicting results from epidemiologic or clinical studies may in part be related to food sources or databases used in estimating phytoestrogen intake. Studies illustrate large variability in the isoflavone content of isolated soy proteins and reinforce the need to accurately determine the isoflavone content of foods and dietary supplements

used in dietary intervention studies while exposing the limitations of food composition databases for estimating daily isoflavone intakes (35).

Food composition databases can be useful in identifying some soy and nonsoy phytoestrogen sources. Consideration should be given to analyses of major sources. To do this, these sources should be specifically defined and the analytical methods well described. Databases can be used for quantifying the minor phytoestrogen sources without conducting further analyses. Despite the usefulness of databases, the variability in isoflavone values in foods limits the usefulness of a database for intervention studies or even for intake assessment studies. One must keep in mind that each country’s food composition tables is based on different chemical analyses, conversion factors, products, and definitions (49,50). The food composition databases developed and maintained by the USDA report values for genistein, daidzein, glycitein, coumestrol, formononetin, and biochanin for selected foods and with well-documented methodology (51). Foods that may contain small amounts of these constituents, but that may be consumed frequently, are not included.

## **Analytical methods**

### **Guidance.**

Identify constituents in the study product that should be and can be analyzed.

Consider constituent characteristics, both biochemical and physical, when choosing appropriate analytical methods.

Choose analytical methods that are precise, accurate, reliable, valid, and validated for the analyte and matrix.

Establish specifications for laboratory performance of chosen analytical methods.

Include the use of internal and external standards and reference materials for instrument calibration and method accuracy.

### **Key points.**

Investigators in the past provided insufficient details on product composition in papers to allow others to understand, interpret, or repeat their work.

Analyzing or reporting only total isoflavone content of the study agent does not provide a complete characterization of the product.

When evaluating past studies, if the study product is still available, consider analyzing the study product for critical constituents.

Sample preparation and conservation is particularly important, because some isoflavones are relatively unstable and adequate storage conditions are necessary.

There is considerable variability across laboratories using reliable, valid analytical methods.

A good understanding of the absorption, distribution, metabolism, elimination, and bioavailability of soy is needed and demands reliable, precise, accurate, valid, and validated analytical techniques applicable to a wide variety of food, supplement, and biological matrices.

**Foods and dietary supplements.** Soy isoflavone and protein products should be characterized by clearly defined attributes that allow comparison among studies. The provision of accurate data on the phytoestrogen/isoflavone content of different foods, soy protein isolates, and supplements has been hampered until recently by the availability of suitable validated analytical methods. Several reviews have assessed currently available methods (15,52–55).

Accurate estimation of isoflavones and/or protein in soybeans, soy foods, and dietary supplements, as well as in other samples, will enable researchers to correctly evaluate the influence of these phytochemicals on health as well as provide precise dietary and safety guidelines on the consumption of these compounds. Most importantly, the scientific community, including academics, industry, journal editors, and reviewers, must recognize that the validation, preservation, and extraction of samples are critical steps in describing the content of isoflavones and/or protein used in research (15,49).

Challenges to getting good numbers exist and laboratories may poorly perform otherwise reliable, valid analytical methods. First and foremost, sample conservation is particularly important and adequate storage conditions are necessary to preserve the original compositional profile of soybeans and soy products. Inadequate conservation and storage are major contributors to the misreporting of product composition despite the adequate performance of appropriate analytical methods. Additional challenges include extraction methods, sample preparation, and use of internal standards (49,55). Isoflavone degradation can occur during the processing of the sample, because some isoflavones are relatively unstable. The isoflavones most susceptible to degradation may be the malonyl forms. Glucoside and aglycone concentrations may increase (49). Internal standards are critical to correct for the dilution volume and also to determine the repeatability of the method. Without internal standards, high accuracy of isoflavone analysis is not achievable. Each step of an analytical method must be carefully observed and documented. Otherwise, the results may not be reproducible by others and will introduce misinformation in the scientific literature (49). Clinical investigators should include analysts and/or natural products chemists on their study team to determine appropriate storage conditions, including temperature and humidity. Prior to conducting the study, they should consider performing stability studies on the study agents, monitoring stability throughout the study, and developing specifications for the amount of degradation allowed.

**Biologic fluids.** Conflicting results arising from the use of only 1 method of assessment underscore the importance of having multiple adherence or exposure measures, each with a different source of error. Optimally, collection of both blood and urine collection may be considered for future clinical studies. Isoflavones and their metabolites occur in relatively high concentrations in plasma, urine, and to a smaller extent in feces, and can be measured.

Many epidemiological studies rely almost entirely on spot urines. Even when 24-h urine collections are obtained, an exogenous marker, e.g. para-aminobenzoic acid, is lacking. Internal standards are critical to standardizing methods, because there are many variables that must be considered. An isotopically labeled internal standard for each analyte allows traceability. An external standard is necessary to ensure that the method consistently provides the same answer, i.e. the method when performed correctly is precise. Calibration materials will provide assurance of accuracy. An extensive review of techniques applied to phytoestrogen (including isoflavone) analysis that focuses primarily on the analysis of biologic fluids has been published (56).

**Reference materials.** Reference materials are substances with 1 or more values that are sufficiently homogeneous and well established to be used for the calibration of equipment, the assessment of a measurement method, or the assignment of values to materials. A reference material accompanied by a certificate of

analysis, which establishes analytical values with a stated level of confidence, is referred to as a certified reference material. Both types are available commercially. A standard reference material is a very specific type of certified reference material and is only produced by the National Institutes of Standards and Technology. These standard reference materials may be used as calibration solutions and internal standards, for method development/quality control, and to provide traceability. National Institutes of Standards and Technology is currently developing natural matrix standard reference materials for soy products. The soy suite will include soy flour, soy milk, soy protein isolate, soy protein concentrate, and soy-containing solid oral dosage form, and will be certified for isoflavones, fatty acids, vitamins, elements (minerals), amino acids, and proximates.

## Pharmacokinetics

### Guidance.

- Determine bioavailability as part of each intervention study to assist in interpretation of variability in results.
- Determine dose, dose schedule, and intervention duration based on quality preliminary human data.
- Stratify for equol-producer status with sufficient statistical power to reduce variability in clinical outcomes.
- When collecting biologic fluids, allow enough time to lapse after initial soy intake to ensure that measurements will reflect the intake.

### Key points.

- Understanding bioavailability may help optimize the health effects and interpret variability in study results.
- Fractional absorption of isoflavones decreases with increasing dose.
- Several doses throughout the day may help maintain optimum steady-state isoflavone levels.
- Isoflavones from different foods and supplements have different rates of absorption.
- The behavior of isoflavone aglycones and glycosides differs, as does their metabolism.
- About 30% of individuals have the capacity to produce equol and such individuals will contribute variability in study results.
- Most soy ingredients act by different modes of action, may act by more than 1 mechanism, and may significantly interact.

Many papers, often with conflicting results, have been published about the relationship between soy and health-related endpoints. Investigators often apply a reductionist approach to studying soy, which might contribute to conflicting results. They assume that: 1) the effects of feeding a soy food reflect the activity of 1 or a small number of related soy components; 2) the activity of a purified soy component reflects the effects of eating whole soy foods; and 3) soy foods or supplements equal isoflavones that are estrogenic or antiestrogenic, which explains the biological effects of soy consumption. Animal studies demonstrate these assumptions are false (57–61). Bioavailability and potential modes of action of various soy constituents differ. Most ingredients act via more than 1 mechanism (62). In addition, soy constituents may potentially interact synergistically in maintaining/obtaining study endpoints.

It is very important to understand factors that influence bioavailability and modes of action. A homogeneous study group based on phenotypic expression of a health characteristic will be quite heterogeneous genotypically. This may explain why the outcomes of studies are sometimes conflicting. Moreover, differences in individual lifestyles, nutrition habits, and specific

gut flora also contribute to significant heterogeneity in target groups, which leads to debatable efficacies of the constituents tested. Other factors affecting bioavailability and mode of action include the chemical composition and matrix of the soy product, dose, interaction with other components in product formulation, oral antibiotic use, participant exposure history, and additional participant characteristics, such as age, gender, and ability to produce equol, a biologically active metabolite. An understanding of these factors is required to predict the risk of adverse effects and to optimize the potential health effects of these compounds by ensuring that sufficient concentrations are reaching the target tissue. It may also help in the interpretation of the variability and results that we see among clinical trials.

**Soy protein isolate.** Soy protein or peptide components may, in addition to isoflavones, be responsible for soy's purported health effects (57,63,64). There are several mechanisms by which protein/peptide components ( $\beta$  conglycinin, small water-soluble peptides, amino acid profiles, phytochemical components other than isoflavones) may be biologically active. The protein  $\beta$  conglycinin (7S globulin) constitutes 40–60% of the total protein in soy. Hydrolysates and  $\beta$  conglycinin-derived peptides have been reported to have biological effects *in vitro*. However, very few studies of this protein have been conducted in animals (65–67) or humans (68) and their results have shown equivocal effects. The problem is that it is unclear whether or not these peptides are actually formed by digestion by the gut *in vivo* and whether or not they are bioavailable and at what concentrations.

There are a number of small water-soluble peptides (e.g. Bowman-Burke inhibitor, Kunitz inhibitor, Lunasin) that are associated with soy protein isolate (69,71). However, their bioavailability is questioned and their bioactivity cannot be realized without extremely large doses of soy protein isolate. The same pattern of amino acids in plasma is likely to be different from the pattern shown after *ex vivo* hydrolysis of the proteins because of differences in amino acid absorption and hepatic metabolism and transport *in vivo* (71).

Phytochemical components of soy protein isolate other than isoflavones may have biological activity. Intact soy protein isolate induces cytochromes P450 *CYP3A1* and *CYP1A1* and carnitine palmitoyltransferase, whereas the stripped soy protein isolate, which is processed to have negligible levels of phytochemicals, including isoflavones, has a lesser or no ability to induce these proteins. Levels of genistein or daidzein in amounts equivalent to that in intact soy protein isolate also do not induce these proteins, indicating that something other than the isoflavone in the soy protein isolate is responsible (60,72,73).

**Isoflavones.** Isoflavones from different foods and supplements have different rates of absorption. The differences are attributable to differences in gastric emptying related to the food or supplement matrix and differences in intraluminal dissolution of the isoflavone from the matrix (74–76). In addition, fermented foods, with greater aglycone content, may be absorbed more rapidly than foods composed primarily of glycosides (75,76); however, more recent data shows this not to be the case (48). In addition, current data are equivocal as to whether the bioavailability of isoflavones from soy foods differs from that of supplements (76–79).

Controversy exists regarding the extent of bioavailability of isoflavone glycosides and the mechanism of intestinal absorption of isoflavones in humans is unclear. Isoflavone glycosides are basically biologically inactive and are not absorbed intact across the enterocyte of healthy adults, and their bioavailability

requires initial hydrolysis of the sugar moiety by intestinal  $\beta$ -glucosidases for uptake in the peripheral circulation (80). The aglycones are bioavailable and biologically active.

A biphasic isoflavone appearance in urine and plasma when humans consume soy or purified isoflavones has been observed (81). Patterns in plasma and urine are correlated. There is an initial rapid surge uptake of isoflavones, at 1–2 h and a second peak at 4–8 h. The initial peak representing ~10% of the overall uptake probably reflects absorption of aglycones without hydrolysis in the proximal intestine, although recent data challenges this explanation (81). Uptake in the proximal small intestine may indicate a saturation limit, because even bioavailable aglycones are absorbed only partially in the small intestine. The initial hydrolysis of the sugar moiety may not be the rate-limiting step in initiating plasma levels. The majority of isoflavones is absorbed after hydrolysis in more significant amounts in the more distal intestine, predominantly the large intestine. Fractional absorption decreases with increasing dose. There may be no advantage to larger doses. In addition, studies of high and low doses indicate that steady-state serum levels could be maintained if doses are divided or taken several times throughout the day rather than via a single bolus dose (74).

Isoflavone half-life in humans is 7–9 h. In general, plasma genistein levels are consistently higher than plasma daidzein levels for equimolar doses, because the volume of distribution of genistein is less than the volume of distribution of daidzein. The clearance rate of daidzein is much faster than the clearance rate of genistein. The clearance rate for equol is even slower, and equol levels for the same concentration attain much higher values. Equol is far more bioavailable.

**Equol.** Equol production may provide a clue to the inconsistency among some clinical study results and disparity between observation and clinical findings. Daidzin and daidzein are the precursors of equol, which is exclusively a product of intestinal bacterial metabolism. It is not produced in germ-free animals, newborn infants whose gut is not yet colonized by microflora, and many adults. Following ingestion of a defined dose of daidzein or soy foods, there is a wide interindividual variability in the ability to produce equol. There is little difference between males and females, although there is a tendency for males to have a higher propensity to make equol than females (82). Infants with immature gut flora (83) and older adults whose gut flora may be compromised or with alterations in intestinal morphology (84) may have altered absorption or conversion to equol. There may be greater absorption in children compared with adults due to more efficient hydrolysis or slower degradation (85). However, the greater absorption could be due to the “grazing” nature of children's eating behaviors. There appear to be no differences in pharmacokinetics between pre- and postmenopausal women (74,75).

In the United States and other Western populations, the overall frequency of equol producers is ~30%. Around 50–60% of Asians make equol and ~60% of vegetarians make equol when consuming soy (86). The reason for these differences is not understood. One reason may be that dietary factors influence equol production. Clinical studies may benefit from a pretest for equol producers, which would consist of repeated administration of a soy challenge for several days before actually collecting samples to determine whether equol is present or not. Furthermore, studies could stratify by equol-producer status, which may reduce variability in clinical studies.

There is a time-dependent conversion from daidzein to equol. Equol production occurs somewhat distally in the intestine,



**TABLE 1** Summary guidance from an NIH workshop on designing, implementing, and reporting clinical studies of soy interventions

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Summary

I. Introduction/background

Use caution when interpreting past studies; consider study age, source, and quality, historical changes in test agents, background influences, participant characteristics and heterogeneity, and intervention adherence and frequency of its assessment.

Acquire information from previous studies on product characterization, stability, and analytical methods used to determine product integrity.

Use preclinical and epidemiological data only if they are predictive of human response in the proposed study population.

Use data from studies that investigated products relevant to the proposed health outcome of interest and to the proposed soy intervention.

Determine if pharmacokinetic and bioavailability studies support the proposed study hypothesis.

Document that Phase 1 studies have been completed or whether the safety of the test agent has been established.

II. Methods

A. Participants

Consider the appropriate homogeneity or heterogeneity of the proposed study population.

During screening, identify subgroups, e.g., equal producers, that may have greater or lesser response to soy.

Take advantage of special homogeneous populations with higher and/or constant exposure to understand the gene-soy interaction.

Consider establishing eligibility criteria based on recruits' previous exposure to soy (when and for how long this occurred, how much, and type of soy).

Consider the requirement that study recruits be subjected to a washout period or discontinue foods or supplements containing soy prior to randomization.

Develop eligibility criteria relevant to intake of foods or supplements containing soy, its constituents, or other ingredients (e.g. other phytoestrogens) with similar mechanisms of action for the duration of the study.

Consider how to address oral antibiotic use, other prescription drugs, or over-the-counter preparations that may confound study results.

B. Intervention

Refer to the CONSORT statement on reporting herbal interventions (13).

Do not test purified constituents to answer questions related to food or complex product intake.

Determine study purpose, hypothesized or actual mechanism(s) of action of soy constituents on study endpoints before selecting the test agent.

Understand how soy processing impacts the study outcome and choice of test agent

Consider both biochemical and physical properties in choosing a test agent.

1. Product name

Use appropriate and accurate terminology in describing test agents.

2. Product characteristics

Know and report the product source and supplier.

Analyze, describe, and report all the potential bioactive constituents relevant to the study.

Express isoflavone values as aglycone equivalents or present isoflavone values with sufficient information so that they can be converted to aglycone equivalents for cross-study comparison.

3. Dosage regimen and quantitative description

Determine dose, dose schedule, and duration of administration based on quality preliminary human data, including dosing or dose ranging studies, sufficient to achieve a steady state and appropriate for the study hypothesis.

Consider the clearance rate of active constituents or their metabolites in selecting the dose.

Determine if the matrix in which the test agent is administered (e.g. in or with food, as a supplement) influences the frequency and timing of administration.

Determine habitual diet and/or gut microflora in selecting dose and mode of administration.

Evaluate the acceptability of the dose and administration schedule.

4. Qualitative/quantitative testing

Identify constituents in the study product that should be and can be analyzed.

Consider proximate composition (protein, carbohydrate, fiber, and fat).

Choose analytical methods that are precise, accurate, reliable, valid, and validated for the analyte and matrix.

Establish specifications for laboratory performance of chosen analytical methods.

Include the use of internal and external standards and reference materials for instrument calibration and method accuracy.

Conduct independent analyses of test and placebo agents before, during, and after intervention, and report all analytical methods used to determine product constituents.

Store samples of the test product appropriately for future analysis if new analytical methods are developed, new constituents discovered, or questions arise that require additional analyses.

5. Intervention adherence

Consider the impact of nonstudy soy and/or nonsoy phytoestrogen exposure on the study outcome.

Identify the food and supplement sources of soy and nonsoy phytoestrogens available to the study population to determine the study population's baseline exposure and to control and/or monitor nonstudy exposure.

Determine how much nonstudy soy and/or nonsoy phytoestrogen exposure will be allowed to establish adherence criteria, taking into consideration measurement error and variability.

Validate dietary assessment methods specific to soy and/or nonsoy phytoestrogen intake in relationship to timing, frequency, study population characteristics, and geographic region.

Assess isoflavone metabolites in biologic fluids (optimally both blood and urine) and target tissues, as appropriate, to improve study and nonstudy exposure assessment.

When collecting biologic fluids, allow enough time to lapse after initial soy intake to ensure that measurements will reflect the intake.

Understand how dietary assessment and analytical methods compare with those used in previous clinical studies.

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*(Continued)*

**TABLE 1 Continued**


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	Save biologic tissues for future analysis when methods for metabolites are developed or new metabolites discovered.
	When choosing methods to assess adherence, balance acceptability, feasibility, frequency or number of measurements, and appropriateness and validity of the method.
C. Outcomes	Assess safety, in particular address the effects of high levels of soy intake following long-term soy exposure. Simultaneously investigate gene-gene, gene-diet, and diet-diet interactions. Determine bioavailability as part of each intervention study to assist in interpretation of variability in results. Determine the influence of gut microflora on bioavailability and metabolism of soy or its constituents, and the influence of soy on gut microflora.
D. Sample size	Increase sample size to understand gene-soy interaction. Consider variability of response when determining sample size and expected effect size.
E. Statistical methods	Analyze health outcomes in relationship to serum isoflavone level because serum levels of daidzein and genistein are directly related to efficacy. Stratify for equol-producer status with sufficient statistical power to reduce variability in clinical outcomes Power the study to analyze predefined subgroups separately.
III. Reporting results	Refer to CONSORT statement on reporting clinical studies, which serves as a useful tool in designing the trial as well as reporting results (12). Address potential confounding factors such as the isoflavone profile of the intervention product, isoflavone dose, and participant characteristics (e.g. genotype).
IV. Investigators	Compose the multidisciplinary investigative team to include sufficient expertise to address study design and statistical issues, interpretation of past studies upon which the current study is based, product composition and integrity, assessment of exposure or protocol adherence, as well as the disease or health conditions being studied.

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probably the colon. Consequently, equol appears in the urine on d 2 and 3 following ingestion. This is important for intervention and epidemiological studies. Sampling of biologic fluids needs to occur far enough after initial intake to ensure that measurements will reflect the intake (74–76).

### Genetic polymorphisms

#### Guidance.

Increase sample size to understand gene-soy interaction.  
Simultaneously investigate gene-gene, gene-diet, and diet-diet interactions.

#### Key points.

Homogeneous study groups based on phenotypic expression of a health characteristic will be quite heterogeneous genotypically.

Genetic makeup may play a major role in the inconsistent findings from human studies, including clinical and epidemiologic studies. Studying the genetic polymorphisms may help us to understand why the variations in results exist. The human body has >25,000 genes and each gene has many polymorphisms. Genotypes, which consist of single nucleotide polymorphisms, are functional and influence nutrient-gene interactions that determine the internal dose of exposure for the response. For example, people with different genetic profiles may have higher or lower metabolic capabilities, which leads to the differential dietary requirement for the same effects. It is generally accepted that genotypes are not directly related to social status or lifestyle factors. However, some of the single nucleotide polymorphisms are known to be associated with the utilization and/or metabolism of dietary components, as well as the development of various diseases. Randomization to different soy exposure levels by genotypes may provide us with insights into the susceptible population to soy-related disease risk.

The following provides an example. Isoflavones have both antiestrogenic and estrogen-like effects depending on the endogenous estrogen level. Endometrial cancer is a sex hormone-related disease. Isoflavones may reduce endometrial cancer risk by interfering with the synthesis, metabolism, and signal trans-

duction of steroid hormones. Soy interacts with several estrogen-related genes, such as *UGT1*, *HSD17B1*, and *CYP19A1* in endometrial cancer. In vitro studies have consistently found that isoflavones inhibit 17 $\beta$ -hydroxysteroid dehydrogenase type I activity, the key enzyme in the last step of estrogen synthesis, catalyzing estrone to the biologically more active estradiol in estrogen-susceptible tissues. Recently, a large-scale, population-based, case-control study demonstrated that isoflavones interact with the sex hormone-binding globulin polymorphisms in the development of endometrial cancer (87,88). The investigators found that the *ASP<sup>327</sup>ASN* polymorphism modified associations of soy isoflavones with endometrial cancer, with the inverse association of soy intake, especially in women with low endogenous estrogen and low soy intake (87). Isoflavones may act through multiple mechanisms to reduce carcinogenesis and it is possible that the effect of this polymorphism is obscured by the competing pathways under conditions of high estrogen or isoflavone exposure (88).

### Guidance summarized

Study design characteristics may account for conflicting results among soy clinical studies. A major challenge is the need to have sufficient statistical power to make firm conclusions. In studies with relatively small sample sizes, variation could easily contribute to inconsistent clinical results. Contributors to variability have been addressed in previous sections and include product composition, whole soy compared with isolated constituents, dose, duration of intervention, previous exposure to soy, nonstudy exposure to soy and other nonsoy phytoestrogens, equol producers compared with nonproducers, interactions with drugs (e.g. antibiotics) or food and supplement components, isoflavone metabolism, genetics, and measurement error. Workshop participants made many suggestions and recommendations for the design, implementation, and reporting of soy clinical studies, all of which cannot be addressed in depth in this report. **Table 1** consolidates the previously identified suggestions and the remaining suggestions identified at the workshop. These are formatted similarly to the CONSORT recommendations on

reporting clinical studies (12). Workshop participants emphasized that investigators should follow Good Clinical Practices (89) in designing and implementing soy studies and the CONSORT recommendations (12,13) when reporting clinical investigations of soy. This guidance is intended to improve the quality of clinical studies of soy interventions and may be used as a tool by study investigators, protocol reviewers, sponsors, and journal editors.

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