

# **Soy Isoflavone Variability in Postmenopausal Women**

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## **Dedication**

I would like to dedicate this thesis to my husband, Nicholas Patrick Meehan.

## Abstract

The purpose of this Master's thesis was to examine the variability related to isoflavone metabolism in postmenopausal women.

The study involved 124 postmenopausal women who consumed 80 or 120 mg of isoflavones per day during a three year time period. The isoflavones genistein, glycitein, daidzein, dihydrodaidzein (DHD), *O*-desmethylangolensin (ODMA), and equol were extracted and characterized from 24 h urinary samples.

Overall, there was little effect of time on the excretion of isoflavones. There was a significant effect of treatment on the excretion of genistein, glycitein, and ODMA where daily excretion was higher for the 120 mg/d group compared to the 80 mg/d group. Intra-individual variability was high, with percent coefficients of variation (%CVs) ranging from 32-64%. Inter-individual variability was found to be higher than intra-individual variability, with %CVs ranging from 40-256%. Approximately 36% of the subjects were defined as equol producers. In addition, approximately 16% of the women experienced a change in equol-producing status which was found to be significantly related to reported antibiotic use. Finally, equol was the only metabolite for which excretion significantly decreased with reported antibiotic use.

In conclusion, isoflavone excretion was found to be highly variable both within and between women. Results from this study also indicate that antibiotic use has a profound effect on the ability to produce equol.

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## **Chapter 1: Literature Review**

## **I. Isoflavones**

### **A. Introduction**

#### **1. Chemical Structure, Properties, and Types of Isoflavones**

The interest in the physiological effects and dietary role of isoflavones began in the 1950s when sheep grazing on subterranean clover became infertile. Upon investigation, the high isoflavone content of the ingested clover was found to be the cause of infertility (1-5). Isoflavones belong to a class of compounds known as phytoestrogens. There are three major types of isoflavones, genistein, daidzein, and glycitein. Isoflavones can exist in four chemically similar forms which include the glucosides, aglycones, acetylglucosides, and malonylglucosides (6).

Phytoestrogens have heterocyclic diphenolic properties making them structurally similar to 17-beta estradiol in humans. The presence of an aromatic A ring with a hydroxyl group in the same plane as estradiol, allows them to bind to estrogen receptors within the body exerting both estrogenic and antiestrogenic effects. As a result, isoflavones are best classified as naturally occurring selective estrogen receptor modulators (SERMs) (7, 8). The biological effects of SERMs vary depending on the type of receptor present in the targeted tissue. Isoflavones have a stronger binding affinity for estrogen receptor  $\beta$  found in bone cells compared to estrogen receptor  $\alpha$  found predominantly in reproductive cells of the breast and uterus (9).

### **B. Dietary Sources**

#### **1. Types of Food that Contain Isoflavones**

Soy foods (tofu, tempeh, miso, natto, soymilk, soy-based yogurts and desserts) contain the highest concentration of isoflavones found in the human diet (10). The

predominant chemical form of isoflavones found in soy foods varies with the level of processing. For example, 95% of the isoflavones in cooked soybeans, texturized vegetable protein, and soy milk powders are in the glucoside form. Fermenting soy products decreases the amount of isoflavones in the glucoside form, while increasing the amount in the aglycone form. For instance, the percentage of isoflavones in the glucoside form drops to 60 and 80% for tempeh and tofu, respectively (11).

## **2. Factors Contributing to the Amount of Isoflavones in Food**

There are a number of factors that influence the concentration of isoflavones in soy foods. Soybean variety, soil type, environmental exposures (sun light, temperature, precipitation), and sowing date can all greatly affect the isoflavone content in foods (12), as well as a multitude of stressors the plant endures such as viral, bacterial, or fungal exposures (11).

A review by Reinli et al. (13) indicates that the average isoflavone content in soy containing foods ranges from 100-400 mg/100g depending on the food type. However, a more recent review reported the total isoflavone content for the following soy containing foods: 60-265 mg/100 g of soy flour, 5.1-64 mg/100 g of tofu, 1.3-21 mg/100 g of soy milk, 23-126 mg/100 g of miso, 20-124 mg/100 g of natto, 6.9-63 mg/100 g of tempeh, 0.1-26 mg/100 g of a soy burger, 1.6-11.8 mg/100 g of soy yogurt, and 2.3-33 mg/100 g of soy cheese (12).

## **C. Isoflavone Consumption**

### **1. Intake Levels in Westernized versus Asian Countries**

Isoflavone intake in Asian countries has been estimated to be around 25-50 mg/day with the highest reports being 100 mg/day among elderly Japanese men (14). On

the other hand, European and Western diets contain very few milligrams of isoflavones per day because soy based foods are not widely consumed (15).

#### **a. Importance of Intake Difference**

Inequalities in the prevalence of numerous cancers between Asian and Western populations have been attributed to differences in soy isoflavone intake levels. For example, people from Asian countries have been shown to have a lower risk for developing hormone dependent cancers of the breast, ovary, and prostate compared to people from Westernized countries (16, 17). In addition, studies have shown that Asian people, who migrate to countries where they adopt Westernized dietary habits and lifestyle practices, are at an increased risk for developing breast cancer in women (18) and prostate cancer in men (18-20).

### **D. Isoflavone Metabolism and Bioavailability**

#### **1. Hydrolysis of Glucosides into Aglycones**

Most naturally occurring isoflavones exist in the glucoside form which is characterized by the attachment of a sugar molecule. The sugar moiety usually consists of glucose; however, 6'-O-malonyl- and 6'-O-acetylglucose can also attach to isoflavones, but to a lesser extent (21). The glucosides daidzein, genistein, and glycitein maintain their structural integrity through most food processing exposures except fermentation, in which case, glucosides can be converted to aglycones (22, 23). After ingestion, the glucosides enter the small intestine where they are poorly absorbed due to their high hydrophilicity and large molecular weight (24, 25). As a result, glucosides remain in the small intestine to be hydrolyzed by beta glucosidase, an enzyme produced by colonizing bacteria. Upon hydrolysis, the sugar molecule is removed, and the isoflavones are then

considered to be free aglycones. This is an important step in metabolism because aglycones are more biologically active as they have a higher permeability and can diffuse passively across the intestinal brush border of the small intestine allowing for uptake into the peripheral circulation (25, 26).

## **2. Transport and Further Metabolism of Aglycones**

The transport and further metabolism of aglycones is complex and not fully understood. Isoflavones can be detected in the plasma as early as thirty minutes post consumption of soy foods. This is likely due to the rapid absorption of any aglycones present in the food (27). However, the first major peak in the concentration of isoflavones in the plasma does not occur until approximately one to two hours post-consumption. This reflects the time needed for hydrolysis of glucosides into aglycones (28, 29). A second peak in the plasma concentration of genistein and daidzein has been shown to occur four to eight hours post consumption (27, 30), suggesting that isoflavones undergo enterohepatic circulation (30) as well as enteric recycling (31).

During enterohepatic recycling, free aglycones are absorbed and transported to the liver where they are hydroxylated and conjugated into sulfonic or glucuronic acid conjugates. This process makes them more hydrophilic in preparation for urinary excretion (32-34). From the liver, the conjugates can either enter the systemic circulation where they are transported to various tissues and will eventually be excreted via the kidneys, or they can be secreted in the bile where they will return to the small intestine (24). Upon return to the small intestine, the conjugates are de-conjugated by intestinal bacteria. The free aglycones are then reabsorbed and transported back to the liver via the portal vein to be re-conjugated for release into the circulation or bile (35).

Enteric recycling is a process that also occurs and consists of conjugating aglycones within the cells of the small intestine. The conjugates are then secreted into the lumen of the small intestine where they can be reabsorbed and re-conjugated (25, 36). Enteric recycling along with enterohepatic recycling greatly prolongs systemic exposure to isoflavones (31).

Isoflavone glucosides that escape hydrolysis and absorption in the small intestine pass into the large intestine along with any unabsorbed aglycones and liver conjugates (25). Further metabolism can occur as colonic microbiota degrade the isoflavones into daughter compounds (26). For example, daidzein and genistein are metabolized into the secondary metabolites dihydrodaidzein and dihydrogenistein, respectively. From there, daidzein can be reduced to the metabolite equol, or it can undergo ring cleavage to become *O*-desmethylangolensin (ODMA). Genistein can also be metabolized into the secondary compounds *p*-ethyl phenol or 6'-hydroxy-*O*-desmethylangolensin (6'ODMA). However, equol is the only secondary metabolite reported to be biologically active thus far (37, 38).

Isoflavones are predominately excreted from the body via the urine and to a lesser extent in the feces. In the urine, isoflavones are found predominately as conjugated glucuronides followed by sulphates (39). Studies have reported peak urinary excretion levels of genistein and daidzein to occur between 6 and 12 hours post-consumption (27, 30, 40). By 24 hours post-consumption, the majority of isoflavones are excreted from the body (40, 41); however, Watanabe et al. (30) found small excretion peaks up to 48 hours post-consumption, suggestive of enterohepatic circulation.

Urinary recovery of isoflavones has been reported to be between 10 and 50% relative to the amount ingested (42, 43). Several studies have reported a higher concentration of daidzein in the urine compared to genistein, in contrast to concentrations found in the plasma (28, 44). The lower molecular weight and greater water solubility of daidzein likely contributes to the increased concentration excreted in the urine (27, 30, 44). On the other hand, the lower hydrophilicity and higher molecular weight of genistein likely contributes to its increased excretion in the bile (27).

The relatively low percentage of isoflavones recovered in the urine after soy intake may be a result of biliary excretion (43) and/or the colonic degradation of isoflavones into unidentified metabolites (44, 45). Kulling et al. (33) have also suggested that the cytochrome p450 enzymes may be responsible for oxidizing isoflavones. This in turn, would decrease the amount available for urinary recovery. Fecal isoflavone recovery has also been reported to be quite low. Several studies found 5% or less of the total isoflavones consumed to be excreted in the feces (24, 30, 44).

Isoflavones have been quantified in a variety of biological fluids and tissues. Plasma, urine, feces, bile, saliva, breast aspirate, prostate fluid (46), breast tissue, and breast milk have all been shown to accumulate isoflavones post consumption (47-50). The use of 24 hour urinary collections following the consumption of soy foods has been shown to be a reliable biomarker of isoflavone exposure (51) and has been previously validated (52). Thus human intervention studies frequently use urine to measure isoflavone metabolism and bioavailability following the intake of soy isoflavones.

### **3. Equol**

#### **a. Discovery**

Equol was first discovered in 1932 by Marrian and Haslewood (53) when they isolated it from the urine of pregnant mares. It was thus named after its equine origins. Initially, equol was thought to be associated with pregnancy but that was soon rejected when it was found in non-pregnant mares and stallions. In addition, the urinary concentration of equol was found to be high during the summer months and almost non-detectable during the winter months. It was later discovered that the isoflavone concentration was high in the pastures that were fed during the summer and very low in the hay that was fed during the winter (53-55).

After the discovery of equol, there was little interest in researching it any further until the 1940s when it was linked to devastating infertility problems in sheep grazing on pastures in South Western Australia. The pastures contained a large amount of the *Trifolium* clover which was found to have a very high concentration of isoflavones. The estrogenic nature of the isoflavones led to reproductive complications that later became known as the “Clover Disease” (56, 57).

In 1982, equol was the first isoflavone to be discovered in human blood and urine. Soy foods were later identified as the primary source of isoflavones in the human diet (58). Furthermore, isoflavones were proposed to have a preventative role in the development of hormone dependent cancers after high levels were found in subjects regularly consuming soy foods. In addition, the production of equol has been correlated with numerous health benefits among populations that widely consume soy isoflavones (37).

## **b. Production**

### **i. Reduction of Daidzein**

Once daidzein has been ingested, it is transported to the small intestine where it can be hydrolyzed into an aglycone for absorption and uptake into the systemic circulation; however, if it escapes hydrolysis and/or absorption it will travel to the colon where it can be metabolized by the microflora into the secondary metabolites equol or ODMA (59).

## **ii. Transport of Equol Throughout the Body**

After equol is produced in the colon, it is efficiently absorbed (60) and transported to the liver to be conjugated into a glucuronic acid (61). From the liver, it can either undergo enterohepatic recycling or enter the systemic circulation. Equol remains in the plasma longer than daidzein or genistein and reaches its maximal plasma concentration approximately 24 hours post-ingestion. Plasma concentration begins to decline after 24 hours; however, it can be detected up to 48 hours post-consumption (37, 62).

Some studies suggest that the formation of equol may depend on initial intake levels of daidzein. Additionally, it has been speculated that the conversion of daidzein to equol may be greater after the consumption of glucosides versus aglycones due to the longer transit time needed for hydrolysis and microflora exposure. For example, Zubik et al. (59) reported subjects to have significantly higher plasma concentrations of equol during a 48 hour period following the ingestion of daidzein in the glucoside form compared to the aglycone form. Similarly, Setchell et al. (58) found that two out of four women who were given daidzein in the glucoside form had detectable levels of equol in their plasma whereas when the same women were given daidzein in the aglycone form, none of them had detectable levels of equol in their plasma. This phenomenon seems biologically plausible since the aglycone form of daidzein could be passively absorbed in

the proximal small intestine and therefore distributed throughout the body without exposure to the gut microflora capable of producing equol. The glucosides, on the other hand, would have to undergo hydrolysis in the small intestine and would therefore have a chance of passing into the large intestine where they could be exposed to equol-producing bacteria.

### **c. Equol Producers versus Equol Non-Producers**

Studies have shown that animals including mice, rats, and monkeys produce high amounts of equol accounting for 70-90% of circulating isoflavones (63). This is likely due to a large cecum and the abundance of microflora responsible for metabolizing daidzein into equol (37, 64). In contrast, humans produce relatively small amounts of equol following isoflavone consumption compared to many animal species (37, 63). Moreover, equol is not produced by all humans, only 30-50% of the population has the ability to convert daidzein into equol (38, 45, 60, 65-71). Thus people are often classified based on their daidzein metabolizing phenotype. People, who have the ability to convert a substantial amount of daidzein into equol, as determined by measuring the concentration found in their plasma or urine, are defined as equol producers, and those who do not are defined as equol non-producers (37, 58, 71, 72). Generally, researchers have defined people as equol producers if their plasma concentration is at least 80 nmol/L or their urinary concentration is at least 1000 nmol/d; individuals with lower plasma or urinary equol concentrations have been defined as equol non-producers (37).

ODMA is also a secondary metabolite of daidzein degradation in the large intestine and it has been approximated that 80-90% of the population is capable of producing it (69, 70, 73, 74). Although the ability to produce ODMA is more widespread

than equol, it is not always the predominant metabolic pathway. In fact, observational studies have shown an inverse relationship between the urinary excretion of equol and ODMA suggesting that the human body has a preference for the production of one metabolite over the other (75). For example, equol producers have been shown to favor the production of equol whereas equol non-producers have been shown to favor the production of ODMA (71, 74, 76). The ability to favor the production of one metabolite over the other is thought to be related to genetic and environmental factors (76, 77).

#### **d. Biological Importance of Equol**

Isoflavones have been shown to exert an estrogenic response within the body. In particular, genistein, daidzein, and equol have been shown to have a stronger binding affinity for estrogen receptors compared to other metabolites (78). Furthermore, animal and *in vitro* studies suggest that equol is more estrogenic (37, 77, 79) and has a greater antioxidant capacity (80-83) than other isoflavones creating an interest in further investigating its effects on human health. Thus far, the ability to produce equol has been associated with a number of health favoring outcomes such as a decreased risk for developing breast cancer, prostate cancer, and cardiovascular disease (73, 76, 84-86).

A case control study performed by Ingram et al. (87) reported a substantial decrease in breast cancer risk among equol producers compared to equol non-producers. They found that subjects in the highest quartile of urinary equol excretion had a much lower breast cancer risk compared to subjects in the lowest quartile, with an odds ratio of 0.27. Another study involving premenopausal women reported a more favorable plasma hormone profile with respect to breast cancer risk among equol producers; this included higher concentrations of SHBG and lower concentrations of estrone, estrone-sulfate,

testosterone, androstenedione, dehydroepiandrosterone (DHEA), DHEA-sulfate, and midluteal progesterone (88). Results reported by Frankenfeld et al. (73) also support the notion that equol producers may have a reduced risk of developing breast cancer as they found postmenopausal equol producers to have a 39% lower mammographic density, a measure used to assess breast cancer risk, compared to equol non-producers.

Equol production has also been associated with a decreased risk for developing prostate cancer in men. Akaza et al. (86) conducted a case control study involving Japanese and Korean men and reported a 1.5-2 fold higher proportion of equol producers in the control group than in the prostate cancer group.

#### **4. Variation in Isoflavone Metabolism**

##### **a. Inter-Individual Variability**

The literature suggests that inter-individual variability in isoflavone metabolism is quite high (17, 24, 27, 40, 44-46, 48, 70, 89-94). Several studies have found considerable variation with respect to isoflavone concentrations found in plasma, urine, and feces, as well as in the ability to produce secondary metabolites. Rowland et al. (71) conducted a crossover designed feeding study that required 24 subjects to consume a high (21.2 mg of daidzein and 34.8 mg of genistein) or low (0.9 mg of daidzein and 1.0 mg of genistein) isoflavone soy protein burger daily for 17 days with a 25 day washout period between treatments. Their results indicated substantial inter-individual variability in the urinary excretion of equol and ODMA with 664- and 76-fold differences between subjects, respectively. Similarly, Lampe et al. (45) found equol excretion to vary 967-fold between subjects and Kelly et al. (45, 70) reported it to vary 1527-fold between subjects.

The literature has reported lower inter-individual variations in the urinary excretion of genistein, daidzein, and glycitein. After subjects were challenged with 40 g of soy flour, Kelly et al. (75) found the excretion of daidzein, genistein, and glycitein to vary 4-, 6-, and 12-fold between subjects, respectively. Additionally, Karr et al. (95) found daidzein and genistein excretion to vary 15- and 12-fold between subjects, respectively. Finally, variation in total urinary isoflavone excretion has been reported to be 16-fold between subjects (71).

#### **b. Intra-Individual Variability**

The literature contains little information regarding the intra-individual variability in isoflavone metabolism and/or excretion. Many of the studies that discuss intra-individual variability do so with respect to changes in the ability to produce equol. Frankenfeld et al. (96) conducted a study involving 92 subjects. They calculated the number of equol and ODMA producers at baseline (T1) and 3 years later (T2). The frequency of equol producers at T1 and T2 was 41 and 45%, respectively and the frequency of ODMA producers at T1 and T1 was 90 and 95%, respectively. The percentage of agreement between producers at T1 and T2 was 82 and 89% for equol and ODMA, respectively. Other studies have also reported equol producing status to be relatively stable over time (86, 97). Therefore based on these data, it has been suggested that the intra-individual variability with respect to equol-producing status is low; however, there have not been any publications to date that have reported on how the excretion of isoflavones varies within a subject.

#### **c. Factors that Influence Variation**

The variability related to isoflavone metabolism and bioavailability is complex and multifaceted. Numerous factors are thought to influence isoflavone metabolism and subsequent production of secondary metabolites. Ethnic background and/or genetics, bacterial composition of the intestine, antibiotic therapy, and dietary habits may collectively affect the metabolism of these compounds. Since equol is not produced by everyone, researchers have compared many of these lifestyle factors between equol producers and equol non-producers to better understand variability related to equol production.

**i. Genetics**

The physiological differences between equol producers and equol non-producers are not fully understood despite numerous attempts. Some researchers believe there is a genetic component responsible for the differences seen in the ability to produce equol in Asian and Western populations. Song et al. (7) found Korean American women to have a higher prevalence of equol producers compared to Caucasian American women living in the same geographical area, 51% versus 36% of the women, respectively. Additionally, Morton et al. (98) found a significant difference in the ability to produce equol among Japanese men and women compared to Ukrainian men and women. Fifty-eight percent of the Japanese men and 38% of the Japanese women produced equol whereas none of the Ukrainian men and only 2.2% of the Ukrainian women produced equol. Moreover, Akaza et al. (86) found similar results when they compared the prevalence of equol producers among Japanese, Korean, and American study participants. The Japanese and Korean subjects had the highest prevalence with 46 and 59% of the subjects being equol producers, respectively. In contrast, only 14% of the American subjects were classified as

equol producers. These results along with others, support the notion that Asian populations have a higher prevalence of equol producers compared to Western populations (7, 65, 86, 99), suggesting a possible genetic component.

## **ii. Gut Microflora**

Another source of variation related to the ability to produce equol might be the composition of the gut microflora (37, 66, 78, 88, 100-105). The human gastrointestinal tract has the densest microbial population of any other organ with approximately  $10^{12}$  organisms per gram of tissue (106); an amount that outnumbers somatic cells and likely has a profound impact on overall health (107). The gut microflora has been thought of as an organ in itself because it has several vital functions including but not limited to: nutrient absorption, epithelial protection from injury, and regulation of fat storage (106-108). The species of bacterium varies along the length of the small and large intestine and has been shown to be influenced by age and environmental exposures (106). Furthermore, analyses of fecal and mucosal composition have indicated significant inter-individual variability in the microbial population suggesting a genetic component (108).

The absence and/or presence of specific intestinal bacteria have been speculated to be influential in determining whether or not daidzein will be metabolized into equol or ODMA (37, 103). Atkinson et al. (105) confirmed this *in vitro* when they inoculated daidzein with fecal flora from equol producers and equol non-producers. Their results showed that the fecal flora from equol producers was capable of converting daidzein into equol whereas the fecal flora from equol non-producers was not. Thus efforts are being made to specifically identify which bacterial strains are responsible for equol production (65).

Numerous *in vitro* studies have been conducted in attempts to characterize the microflora of equol producers. Three specific strains have been identified after culturing fecal flora from equol-producing Japanese adults consuming 70g of tofu. The gram negative *Bacteroides ovatus* spp., the gram positive *Streptococcus intermedius* spp., and the *Ruminococcus productus* spp. were all reported to convert daidzein into equol *in vitro* (109). Decroos et al. (60) also successfully identified and isolated a microbial mixture capable of producing equol *in vitro* which included the following bacterial strains: *Enterococcus faecium*, *Lactobacillus mucosae*, *Fingoldia magna*, and *Veillonella*. Furthermore, they found that when this microbial mixture was transferred and inoculated with fecal samples from equol non-producers, it was capable of producing equol. Thus the ability to produce equol *in vitro* by adding microbial mixtures to equol non-producer fecal samples supports the theory that equol producers and equol non-producers differ with respect to microflora composition.

### iii. **Antibiotics**

Antibiotic treatment has been shown to deplete microbial inhabitation in the gastrointestinal tract. This has been proposed to influence isoflavone metabolism, equol production in particular. Blair et al. (104) examined the effects of antibiotics on equol production in monkeys. They found that plasma equol concentrations decreased by varying amounts depending on the type of antibiotic administered. Treatment with metronidazole, kanamycin, vancomycin, and kanamycin plus vancomycin decreased equol concentrations by 80, 93, 98, and 99%, respectively. In addition, the antibiotics deoxycycline, kanamycin, and kanamycin plus vancomycin increased the plasma concentration of daidzein. The plasma concentration of dihydrodaidzein increased after

treatment with kanamycin and metronidazole. Furthermore, a human study conducted by Atkinson et al. (110) found that the antibiotics metronidazole and kanamycin inhibited equol production. They also found that some antibiotics interfered with equol production but had little effect on the conversion of daidzein to dihydrodaidzein. Results from this antibiotic data suggest that there are different microbes responsible for the conversion of daidzein to dihydrodaidzein compared to the conversion of dihydrodaidzein to equol.

Some microbes responsible for these conversions have been identified. Hur et al. (101, 111) isolated a gram positive bacterial strain named HGH6 which specifically converts daidzein to dihydrodaidzein. On the other hand, Wanget al. (112) identified a specific strain named Julong 732, a gram negative anaerobic bacterium, specifically capable of converting dihydrodaidzein into equol. Thus, the *in vitro* identification of bacterial species capable of converting daidzein into equol has been promising considering the vast amount of bacterium that resides in the human gastrointestinal tract (37, 113).

#### **iv. Dietary Habits**

Dietary habits have been reported to influence the bacterial composition of the gut. Thus it is believed that dramatic alterations could potentially alter isoflavone metabolism and the ability to produce equol (65, 99, 114). Setchell et al. (115) found vegetarian subjects to have a higher frequency of equol producers, one that is more similar to Japanese populations, compared to non-vegetarian subjects, 59 and 25%, respectively. In addition, low dietary fat intake and high dietary carbohydrate, fiber, and plant protein intake have been associated with a higher frequency of equol producers in female subjects (45). Furthermore, equol producers have been shown to consume a higher

percentage of their total energy in the form of carbohydrates compared to equol non-producers (71). Therefore, carbohydrate consumption has been thought to stimulate the colonization of equol producing bacteria (60, 114). However, even though this notion seems biologically plausible, dietary intervention studies feeding soy protein have not demonstrated a substantial ability to change equol-producing status within a person, suggesting that intestinal microflora is relatively stable despite dietary alterations (113).

#### **d. Implications**

Recent evidence suggesting beneficial health outcomes related to isoflavone consumption has driven researchers to understand the metabolic response they have. Clinical measures used to assess isoflavone bioavailability have been highly variable. The inconsistencies in the literature related to isoflavone intake and health outcomes are likely due to the inter-individual variability in how these compounds are metabolized. The processes by which isoflavones are transported, absorbed, distributed, and excreted from the body are thought to be influenced by genetics, gut microflora, antibiotic use, and habitual diet. In addition, the conversion of daidzein to its more biologically active metabolite equol is highly variable among people. Therefore, it is crucial to solidify the evidence regarding the effects of isoflavone consumption on health outcomes. In doing so, isoflavones could be more effectively used in a clinical setting.

## **II. The Relationship Between Isoflavones and Disease**

### **A. Epidemiological Studies**

Much of the epidemiological data has compared disease prevalence in Asian populations where consumption of soy containing foods is high, to Westernized populations where consumption of soy containing foods is low. In doing so,

epidemiological studies have associated high dietary isoflavone intake with a reduced risk for developing certain diseases including breast cancer (116), prostate cancer (117), cardiovascular disease (118, 119), and bone fractures (120). Furthermore, Asian migration studies have reported an increased risk for developing hormone dependent cancers among immigrants who have relocated to Westernized countries (121, 122). Additionally, Kim et al. (123) reported an increased incidence in cardiovascular disease among Japanese immigrants adopting a Westernized diet; a finding that concurs with results from a cross sectional study in Japan revealing an inverse relationship between serum total cholesterol and dietary soy intake (124). Therefore, dietary intake of soy isoflavones has been implicated as being protective against many chronic diseases that are characteristic of a Westernized life style. As a result, numerous human intervention studies have been conducted to elucidate the mechanistic role of isoflavones with respect to hormone dependent cancers, cardiovascular disease, and bone loss related to menopause.

## **B. Intervention Studies**

### **1. Breast and Prostate Cancer**

Isoflavones bear structural similarity to  $17\beta$ -estradiol (125) and are able to exert an estrogenic effect that is either agonistic or antagonistic depending on the level of circulating estrogens (126), the target tissue affected, and the type of estrogen receptor (ER) available for binding (9, 114). Isoflavones and to a greater extent equol, have a stronger binding affinity for ER- $\beta$  versus ER- $\alpha$  (127, 128). Thus the ER expression within a tissue may influence the response isoflavones have within the body following intake

(129, 130). These properties are believed to be responsible for the potential role isoflavones have in reducing cancer risk (130).

Human intervention studies involving isoflavone intake and cancer outcomes are few in number and have been inconclusive. A review of 3 large double-blinded randomized controlled trials supplementing soy isoflavones to pre- and postmenopausal women for 1-2 years, resulted in no significant changes in mammographic density, a marker used to predict breast cancer risk (131). On the other hand, Ide et al. conducted a clinical trial to investigate whether the supplementation of soy isoflavones in combination with curcumin would reduce prostate-specific antigen (PSA) levels, a marker of inflammation in the prostate. They randomized 85 men into a placebo group or a treatment group supplementing 40 mg of isoflavones in combination with 100 mg of curcumin. The subjects were required to take the supplement once a day for 6 months. Their results indicated a significant decrease ( $P = 0.01$ ) in serum PSA levels after treatment with isoflavones and curcumin in men with a baseline PSA level  $> 10$  ng/ml.

Human intervention studies measuring the effect of isoflavone intake on cancer outcomes are difficult to conduct. In order to effectively evaluate results, it is imperative to consider the variability between studies related to experimental design, dose and duration of isoflavone intake, age, gender, diet, ethnicity of the population being studied, the family history of cancer, the previous exposure to soy isoflavones, and the biological marker used to assess cancer risk (15).

## **2. Heart Disease**

Plasma lipid profiles and low density lipoproteins (LDL) in particular, are biomarkers commonly used to assess and/or predict the development of heart disease

(132). The effects isoflavones have on lipid profiles and LDL levels have not been fully elucidated. The literature thus far has reported decreases in lipid profiles and LDL oxidation following isoflavone interventions (133). These effects are likely associated with the demonstrated anti-oxidative capabilities of isoflavones (83) in both aqueous and lipophilic solutions (134, 135). Additionally, isoflavones have been shown to influence the hepatic metabolism of lipoproteins (136) and alter LDL levels via modifications to receptor uptake (137).

A meta-analysis including 23 randomized controlled trials revealed a beneficial effect of soy consumption of lipid profiles. The results showed a significant lowering effect on serum total cholesterol, LDL cholesterol, and triglycerides along with a significant increase in the protective high density lipoprotein (HDL) cholesterol. The changes observed were related to the amount and duration of the intake as well as gender (reductions in total and LDL cholesterol were greater in men) and initial serum lipid concentrations (138). However, Kreijkamp-Kaspers et al. (139) compared a 12 month supplementation of a soy protein to a milk protein placebo in 202 postmenopausal women and found no significant effects on blood pressure and endothelial function.

### **3. Bone Loss Related to Menopause**

Menopause is the cessation of the menstrual cycle in women and is accompanied by a decrease in estrogen production. Bone loss and possible development of osteoporosis are unfavorable outcomes related to menopause (140). Estrogen therapy has been used to prevent bone loss (141) in postmenopausal women; however, it has been shown to increase the risk for developing heart disease (142) and breast cancer (143). As a result, the estrogenic properties of soy isoflavones have been hypothesized to alleviate

bone loss and other menopausal related symptoms; however, human intervention studies supplementing isoflavones in postmenopausal women have reported inconsistent results related to the effects on bone mineral density (BMD) (114).

Taku et al. (144) conducted a meta-analysis containing 1240 menopausal women and found an average daily intake of 82 mg of isoflavones for 6-12 months resulted in a significant increase in spinal BMD. When compared to controls, there was a 2.38% increase in BMD ( $P$  value = 0.001). The analysis also reported varying treatment effects across the trials that were related to the duration of the intervention (6 vs. 12 months), the geographical region (Asian vs. Western), and the baseline BMD (normal vs. osteopenia vs. osteoporosis).

On the contrary, Liu et al (145) conducted a meta-analysis including 10 randomized controlled trials containing 896 women consuming an average of 87 mg of isoflavones per day for at least 1 year. They found no significant effect on BMD; however, when they compared larger (>80 mg/day) and smaller (<80 mg/day) dose interventions, they found a weak beneficial effect ( $P = 0.08$ ) on spinal BMD for women in the larger dose interventions.

### **III. Master's Thesis Rationale**

The purpose of the research included in this Master's thesis was to examine the variability in urinary isoflavone excretion in postmenopausal women. It was hypothesized that the variability would be high, but that the within person variation would be less than the between person variation.

Chapter one presents a review of the literature concerning isoflavone metabolism and bioavailability. In addition, there is a brief description regarding the relationship between isoflavones and human health.

Chapter two presents the clinical trial involving 124 postmenopausal women consuming either 80 or 120 mg of extracted isoflavones per day during a 3 year period. The urinary isoflavones extracted for this study included: genistein, glycitein, daidzein, dihydrodaidzein, *O*-desmethylangolensin (ODMA), and equol.

The appendices section of this thesis includes supplementary results corresponding to the study in chapter two.

**Chapter 2: Urinary Isoflavone Variability in Postmenopausal Women  
during a Three-Year Isoflavone Intervention Study**

## I. Chapter Summary

Isoflavone metabolism in response to isoflavone supplements has been shown to be highly variable but has not been evaluated in long-term studies. Given the likelihood that variability in isoflavone bioavailability and metabolism influences exposure and thus potential effectiveness, this study examined the effects of two isoflavone doses on urinary isoflavone excretion during a 3 year period in healthy postmenopausal women (N=124). Fifty-eight and 66 women, respectively, consumed tablets containing 80 and 120 mg isoflavones (expressed as aglycones) /day. Urine (24-h) collections at 0, 6, 12, 24, and 36 months were analyzed for genistein, glycitein, daidzein, dihydrodaidzein (DHD), *O*-desmethylangolensin (ODMA), and equol. Overall, the 120 mg/d group excreted higher ( $P = 0.012$ ,  $P = 0.022$ , and  $P = 0.034$ ) amounts of genistein, glycitein, and ODMA compared to the 80 mg/d group. Equol producers excreted lower ( $P \leq 0.0001$ ,  $P = 0.0034$ ) amounts of DHD and ODMA compared to equol non-producers. Approximately 30% of women were equol producers, and this percentage tended to increase over time. Intra-individual variability (30-149 percent coefficient of variation (%CVs)) in the excretion of each metabolite was generally lower than inter-individual variability (40-212 %CVs). Equol producers tended to have lower inter- and intra-individual variability compared to equol non-producers, suggesting that equol-producers exhibited a more stable profile of intestinal microflora. Some subjects (10.5%) were inconsistent with respect to their equol-producing status, and this appeared to be related to antibiotic use, suggesting that antibiotics may well have a long-term effect on the ability to produce equol. Reported antibiotic use significantly ( $P = 0.0042$ ) decreased equol excretion,

with corresponding non-significant increases in genistein, daidzein, DHD, and ODMA excretion. In conclusion, from a clinical perspective, isoflavone intake may exert a more pronounced and predictable response in equol producers, which is likely dependent upon antibiotic use. This concept is worth investigating further because of implications with respect to biological responsiveness to isoflavone treatment.

## II. Introduction

Isoflavones are non-steroidal plant estrogens that are structurally similar to 17-beta estradiol, the most biologically active endogenous form of estrogen in humans (8). Soy foods contain the highest concentration of the three major isoflavones genistein, daidzein, and glycitein (10). Epidemiological studies have shown that Asian populations consuming high amounts of soy have a lower risk for developing hormone-dependent cancers of the breast (116) and prostate (146). Additionally, dietary intake of soy protein isolate has been shown to reduce total and LDL cholesterol in normal and hypercholesterolemic individuals (147), as well as attenuate lumbar spine bone loss in menopausal women (144).

Metabolism of isoflavones is complex and likely influences their *in vivo* biological effects. Once ingested, isoflavones present in their naturally occurring glycoside forms are hydrolyzed into more biologically active aglycones by intestinal microflora (26). Aglycones then follow one of three metabolic pathways: they can be rapidly absorbed as free aglycones within the first hour post consumption (30, 58, 148), they can be transported to the liver where they are conjugated and undergo enterohepatic circulation (35), or they can be transported to the colon where the microflora can degrade them into secondary metabolites, such as equol and *O*-desmethylangolensin (ODMA) (25, 26).

The use of 24-h urine collections as biomarkers of isoflavone intake has been shown to be reliable (51) and has been previously validated (52). Human intervention studies, however, have reported substantial variability with respect to phytoestrogen excretion. Kelly et al. (75) found 4-, 6-, and 12-fold variation between subjects with

respect to the urinary excretion of daidzein, genistein, and glycitein, respectively. Several studies have reported even higher inter-individual variability for urinary excretion of equol and ODMA, ranging from 54 to 922- and 17 to 180-fold, respectively (75, 95, 113). Gut microflora are thought to be largely responsible for urinary isoflavone variability, as they have been shown to influence the bioavailability of isoflavones and production of secondary metabolites such as equol (103, 149, 150). Numerous factors are thought to exert an impact on the profile of gastrointestinal microflora, including genetics, dietary habits, and antibiotic use (26, 35, 89, 114). Soy isoflavone intake has been shown to induce changes in gut microflora after short-term exposure (113, 151); however, relatively little is known about the biological response to long-term isoflavone intake.

The overall objective of this study was to characterize the urinary excretion of soy isoflavones in response to long-term intake, including four specific objectives: 1) to evaluate the change in urinary excretion of isoflavones during a 3 yr time period, considering treatment dose and equol-producing status; 2) to determine the intra- and inter-individual variation in urinary isoflavone excretion during a 3 yr time period; 3) to characterize the distribution of equol producers over time; and 4) to assess the effects of antibiotic use on isoflavone excretion.

### **III. Methods**

**Experimental Design.** This ancillary project used data from the Soy Isoflavones for Reducing Bone Loss (SIRBL) study, a randomized double-blind, placebo controlled, multi-center clinical trial that took place at Iowa State University (ISU) and the University of California at Davis (UCD) to evaluate the effects of long-term isoflavone consumption on bone density in postmenopausal women. The study design has been

described previously (152). In brief, the SIRBL study examined the effects of two doses (80 vs. 120 mg/d) of soy isoflavones on bone loss during a 36-mo period in healthy postmenopausal women aged 45 to 65 years.

**Subjects.** A total of 255 women were randomized to treatment in the SIRBL study: 87 in the 80 mg isoflavone group, 85 in the 120 mg isoflavone group, and 83 women in the placebo group. For the purpose of this ancillary study, data collected from the women in the placebo group were excluded from analyses because their isoflavone excretion was near zero. Of the 172 women in the 80 mg/d and 120 mg/d treatment groups, 25 were excluded from these analyses because they dropped out of the study and had insufficient data available (data collected at fewer than two time points); 9 women were excluded from analyses because their isoflavone tablet compliance was less than 80% at all time points; and one woman was excluded for lack of baseline data. Because of the known effects of antibiotics on isoflavone metabolism, women were asked about antibiotic use at each time point; if they reported using an antibiotic anytime since the previous data (urine) collection, that time point was defined as an antibiotic time point. Women were classified as antibiotic users if they took antibiotics at least once (as defined by one prescription period) during the study or antibiotic non-users if they never took any antibiotics. Because reported antibiotic use was considered in our analyses, 8 women were excluded because they had fewer than 2 antibiotic-free time points, and 5 women were excluded because their reported antibiotic use was irregular due to prescription on an “as needed basis”. A final total of 124 women were included in the current isoflavone analyses. The 80 mg/d group contained 58 women, 32 of whom reported some antibiotic use; the 120 mg/d group contained 66 women, 31 of whom reported some antibiotic use.

The analyses in this paper included both groups of women: those who reported use and those who did not report use of antibiotics during the study.

**Isoflavone Tablets.** Women in the SIRBL study were instructed to take 3 dry compressed tablets per day totaling 80 or 120 mg of isoflavones (expressed as aglycones). The tablets contained Novasoy® isoflavone extract (James R. Randall Research Center, Archer Daniels Midland Company [ADM]; Decatur, IL) with a genistein-to-daidzein-to-glycitein ratio of 1.3-to-1-to-0.3, a proportion similar to that found in soybeans. Women followed their usual dietary practices but were instructed with a specific list of soy-containing foods to avoid.

**Study Procedures.** Characteristics evaluated at baseline pertaining to these analyses included age, time since last menstrual period (TLMP, years), body weight (kg), body mass index (BMI, kg/m<sup>2</sup>), macronutrient intake (g), and dietary fiber intake (g). Nutrient intake was assessed using a Semi-quantitative Food Frequency Questionnaire (FFQ) developed by Block et al. (153). Urine samples (24 h) were collected at each visit (6, 12, 24, and 36 mo) in polypropylene containers and kept cold (4°C) until each subject's sample was processed and volume recorded. Aliquots were frozen for isoflavone, creatinine, and mineral analyses, with the isoflavone aliquot preserved with 1 mL 5% vol:vol of sodium azide (Fisher Biotech; Fair Lawn, NJ) and stored frozen at -70°C until analysis.

**Analytical Methods.** All urinary samples for genistein, glycitein, daidzein, dihydrodaidzein (DHD), ODMA, and equol were analyzed for a given subject in batch using a modified high pressure liquid chromatography-mass spectrometry (LC-MS/MS) method previously described (154). To summarize, the preserved frozen urine samples

were thawed, vortexed, and centrifuged at 5°C for 5 min. From each centrifuged urine sample, duplicate 5 mL aliquots were transferred into glass test tubes. Next, 25 µl of a 10 ppm solution of formononetin (Sigma, St. Louis, MO) was added to each test tube to serve as an internal standard. At the same time, 100 µl of a 0.5 M sodium acetate buffer at pH 7.0 was added to each test tube along with 8 µl of a β-glucuronidase/sulfatase (type H-2 from Helix Pomatia, Sigma; St. Louis, MO) hydrolyzing solution. The samples were vortexed for 1 min and incubated at 37°C overnight to allow hydrolysis. The following day, 3 mL of ethyl ether (Fisher Scientific; Fair Lawn, NJ) was added to the samples that were then vortexed for 1 min and centrifuged for 5 min. The supernatant containing the extracted isoflavones was transferred into a clean glass test tube. This process was repeated two more times. The 3 supernatant extractions were combined and evaporated to dryness under nitrogen. Once dry, the extracted isoflavones were reconstituted using 0.25 ml of methanol and 0.25 ml of a 0.2 M sodium acetate buffer at pH 5.0. Samples then underwent chromatographic separation using a 150 x 2.0 (ID) mm Phenomenex Synergi Max-RP 80A column and LC/MS-MS analysis was achieved using a triple quadrupole linear ion trap system (Qtrap 2000, Applied Biosystems; Foster City, CA). Proprietary software by Applied Biosystems Analyst was used to quantitatively analyze isoflavone excretion. Quality control urine samples were hydrolyzed, extracted, and reconstituted using the same protocol.

**Statistical Analysis.** Statistical analyses were performed using SAS (version 9.2; Cary, NC). Results were considered statistically significant (two-sided) at  $P \leq 0.01$ , whereas  $P$  values ranging from 0.01 to 0.05 were interpreted as moderate to weak evidence. The chosen (reduced) levels of significance address the concern of conducting multiple

comparisons throughout the statistical analyses, which, if ignored, can increase the Type I error rate. For the N=124 sample size, there were 63 women who reported antibiotic use at 2 or fewer time points and, unless stated otherwise, those time points in which antibiotic use was reported were removed from the analyses. Analyses were also performed on the sub-group of women (N=61) who indicated they were non-antibiotic users throughout the entire duration of the study. Because the results and conclusions for the N=61 and N=124 data sets were similar, the data and results presented correspond to the larger (N=124) sample size, unless stated otherwise. Women who excreted more than 1000 nmol of equol per day were defined as equol producers, women who excreted less were defined as equol non-producers (71, 113), and women who experienced at least one change in their equol-producing status were defined as inconsistent equol producers. For variables that were approximately normally distributed, we reported descriptive statistics for baseline characteristics using mean +/- SD; otherwise, we reported median and range values. Statistically significant differences between groups were established through 2 sample t-tests (based on independent samples or, when appropriate, matched pairs). For comparisons of 3 or more groups, we used Analysis of Variance (ANOVA; PROC GLM in SAS). We verified statistical assumptions before conducting corresponding analyses and conducted adequate nonparametric procedures when necessary. Conclusions based on nonparametric procedures did, however, not deviate from conclusions of the corresponding parametric procedures. For this reason, we reported results of the more familiar parametric procedures. We conducted repeated-measures ANOVA using the PROC MIXED procedure in SAS to examine the effects of treatment over time for each of the circulating metabolites (genistein, glycitein, daidzein, dihydrodaidzein, ODMA,

and equol). We used restricted maximum likelihood estimation to obtain estimates of variances and correlations between repeated measures (default in SAS for PROC MIXED). For all models the most appropriate covariance model for the dependence structure in the repeated measurements was an unstructured (unrestricted) covariance matrix. These particular model selections were guided by model diagnostic statistics (Akaike's information criteria and Schwarz's information criterion) available in SAS. In addition to the independent variables allowing us to explore the effects of treatment over time, the analyses also included a variable describing the equol producer status of a woman throughout the duration of the study and site (ISU vs. UCD). Each model included site (ISU and UCD) as obligatory explanatory variables, to account for possible site differences. To assess between- and within-person variability of excreted isoflavone concentrations, we calculated the percent coefficient of variation (%CV). To explore the effect of antibiotic use on isoflavone excretion, paired t-tests were used to assess the difference in isoflavone excretion at each time point a woman reported antibiotic use versus no antibiotic use. The difference was calculated by averaging the isoflavone excretion for the time points at which a woman reported no antibiotic use, and then subtracting that from the average excretion at which a woman reported antibiotic use.

#### **IV. Results**

There were no significant differences in baseline characteristics between the women in the 80 mg/d and 120 mg/d treatment groups, nor between equol producers and equol non-producers (**Table 2.1**). Total isoflavone excretion in nmol/d by equol-producing status within treatment group is shown in **Table 2.2**. These excretion values

refer to the 36-mo time point reflecting the end of the study, thus reflecting maximal, continuous treatment exposure.

### **Urinary Isoflavone Excretion in Response to Isoflavone Treatment, Accounting for Equol Producer Status**

The 36-mo treatment with soy isoflavones did not significantly affect average excretion values for genistein, glycitein, daidzein, DHD, and ODMA. Treatment-by-time point interaction did not reach statistical significance for any of these models (**Table 2.3**). For equol, the treatment-by-time point interaction term approaches significance ( $P=0.014$ ) suggesting a treatment effect over time. For genistein, glycitein, and ODMA, average excretion values appear to vary according to treatment dose (80 mg/d vs. 120 mg/d); corresponding  $P$  values were  $P=0.012$ ,  $P=0.022$ , and  $P=0.034$ , respectively. For women in the 120 mg/d group, excretion values increased, on average, by 1521 nmol/d (genistein), 856 nmol/d (glycitein), and 2957 nmol/d (ODMA), respectively, after accounting for all other factors. Equol-producing status did not exert an effect on average excretion of genistein, glycitein, and daidzein. A significant effect was, however, noted for DHD ( $P\leq 0.0001$ ), ODMA ( $P=0.0034$ ), and equol ( $P\leq 0.0001$ ). Holding all other model terms constant, these women showed, on average, lower excretion concentrations of DHD (-2099 nmol/d) and ODMA (-3036 nmol/d) and higher excretion of equol (8279 nmol/d) over time. In addition to equol-producing status, a significant interaction between equol-producing status and treatment was noted for equol ( $P=0.0012$ ), implying that the effect of equol-producing status on the average equol excretion concentration depended on the administered treatment (80 vs. 120 mg/d). On average, given that a

woman was an equol producer, she excreted 4454 nmol/d more on the 80 mg/d than a woman on the 120 mg/d treatment.

### **Variability in Isoflavone Excretion**

**Within person variability:** The mean within person %CV for genistein, glycitein, daidzein, DHD, ODMA, and equol according to treatment group and equol-producing status is shown in **Table 2.4**. The mean %CV for each metabolite was generally high and there were no significant differences between the 80 mg and 120 mg/d treatment groups. The mean within person %CV for each metabolite according to equol-producing status (consistent equol producers, consistent equol non-producers, inconsistent equol producers) was also high. However, equol producers tended to have a lower %CV for each metabolite compared to equol non-producers, although significance was only achieved for equol ( $P \leq 0.0001$ ). Genistein showed a  $P$  value = 0.049, suggesting a trend for an equol producing status effect. For glycitein and daidzein, mean within person %CV were comparable for equol producers and inconsistent equol producers.

**Between person variability:** Between person %CVs were calculated for each metabolite at each time point for all subjects (**Table 2.5**). The between person %CVs for each metabolite at each time point were generally higher than the observed within person %CVs. An analysis of the between person %CVs according to treatment group and according to equol-producing status was performed (results not shown). The analysis did not yield any consistent patterns over time in distinguishing between treatment groups, except in the case of equol. In this case, the between person %CV was consistently higher for women in the 120 mg/d group in comparison to women in the 80 mg/d group. Analysis of the between person %CVs according to equol-producing status yielded

similar tendencies to those noted in the within person %CV analysis. Equol producers generally have lower %CVs for most metabolites than equol non-producers.

### **Frequency of Equol Producers**

In combining subjects from both treatments, the percentage of equol producers ranged from 33-39% across all time points, consequently yielding a percentage of equol non-producers ranging from 61-67% (data not shown). Furthermore, there was a tendency (albeit not significant;  $P$  for trend = 0.057) for the frequency of equol producers to increase and the frequency of equol non-producers to decrease from the 6- to 36-mo time point.

### **Antibiotics and Isoflavone Excretion**

In analyzing the effect of antibiotics on equol-producing status, the number of women who changed equol-producing status who reported taking antibiotics was significantly higher ( $P=0.0022$ ) compared to women who reported not taking antibiotics (**Table 2.6**). Among the 20 women who experienced a change in equol-producing status, 16 of them reported antibiotic use and 4 of them reported no antibiotic use. For the 16 women who reported antibiotic use, 10 of them were equol producers who then became equol non-producers after reported antibiotic use. The remaining 6 were equol-non producers who then became equol producers at a time point following reported antibiotic use. For the 4 women who experienced a change in equol producing status without reported antibiotic use, 3 of them began the study as equol non-producers and then became equol producers at the 36-mo time point. There was one woman who had a very unpredictable pattern of change with no apparent explanation. We performed a pair-wise analysis to assess the differences in isoflavone excretion between antibiotic and non-

antibiotic time points among women with reported antibiotic use, consequently excluding 4 women because of missing data, resulting in a sample size of n=59 (data not shown).

The results indicated that equol was the only metabolite for which excretion significantly decreased ( $P=0.0042$ ) at time points with reported antibiotic use. Contrary to this, there was a non-significant increase ( $P$  values ranged from 0.32-0.99) in the excretion of genistein, daidzein, DHD, and ODMA at time points with reported antibiotic use.

## **V. Discussion**

To our knowledge, this is the longest soy isoflavone intervention study to date. Results from the repeated measures ANOVA analysis indicated that there was no treatment effect over time on the excretion of genistein, daidzein, glycitein, DHD, and/or ODMA. These results concur with findings from two intervention studies lasting longer than one mo (113, 155), suggesting that for these isoflavones and their metabolites, excretion neither increases nor decreases significantly over time. However, for equol producers, the excretion of equol tended to increase over time in the 120 mg/d group, whereas it tended to decrease over time in the 80 mg/d group (**Figure 2.1**). These results suggest that the dose of isoflavones consumed may have an influential role on the activity of equol producing bacteria during a 3-year period. Additionally, the effect of equol-producing status varied depending on the metabolite. Furthermore, equol-producing status had a significant effect on the excretion of equol, DHD, and ODMA. As expected and according to our definition, the equol producers excreted significantly more equol, whereas the equol non-producers excreted significantly more DHD and ODMA, which is biologically plausible considering these are secondary metabolites resulting from alternative metabolic pathways of daidzein degradation. These findings are in agreement

with the suggestion by Kelly et al. (70) that equol producers are poor producers of ODMA compared to equol non-producers. Our models suggest an increase in the average urinary excretion of genistein, glycitein, and ODMA with an increase in dose; this is in agreement with findings published by Wiseman et al. (113).

Our %CV data showing marked intra-individual variation in urinary isoflavone excretion are consistent with the findings of Kwak et al. (156). The within person %CVs were generally high with no significant differences noted between treatment groups. However, when the data were analyzed by equol-producing status, the %CVs for all metabolites tended to be lower for equol producers compared to equol non-producers, although significance was only achieved for equol and genistein. These observations suggested that equol producers exhibited a more stable profile of intestinal microflora than equol non-producers. Numerous publications have also reported on the high inter-individual variation in isoflavone metabolism (24, 38, 44, 75, 95, 113, 155-157) and the results from our %CV data reflected these findings as well. As expected, the %CVs were much higher between than within women. Our results indicated few differences in the %CVs according to treatment group. Additionally, although inter-individual variability was found to be substantially high, the urinary excretion of most metabolites tended to be less variable for equol producers compared to equol non-producers. Overall, our %CV data suggested that isoflavone metabolism is highly variable both between and within individuals, with a tendency for equol producers to have lower %CVs for most metabolites compared to equol non-producers.

In this study, the frequency of equol producers tended to increase from 6 mo to 36 mo, although the trend was non-significant. The mean frequency of equol producers

across all time points was 36%, which is in accordance with other human intervention trials (45, 68, 71, 88, 105). These results suggested that even after a 3-year isoflavone intervention study, the frequency of equol producers remained relatively stable. The ability to produce equol within an individual has been considered to be relatively stable. Frankenfeld et al. (96) and Akaza et al. (86) evaluated concordance of equol-producing phenotypes over time and found that 82 and 85% of individuals, respectively, retained their equol-producing phenotypes when measured at two time points separated by more than 1 year. Thus, the discordance of 16% found in our study is similar to the 15-18% reported in the literature. Moreover, we found a significant increase in the number of women who experienced a change in equol-producing status with reported antibiotic use. In addition, our antibiotic analysis that compared isoflavone excretion when women were on and off antibiotics demonstrated a significant decrease in equol excretion with reported antibiotic use. Given the long-term nature of this study and that antibiotic use was retrospectively self-reported, this finding is rather remarkable. Yet, these data support similar findings in monkeys (104), *in vitro* (110), and in children (158), illustrating the importance of the gut microflora in the production of equol. On the other hand, we found that genistein, daidzein, DHD, and ODMA increased after reported antibiotic use. Similar results have been reported in adults who consumed soy while taking antibiotics (159), suggesting an increased bioavailability of genistein, daidzein, DHD, and ODMA during antibiotic use. In interpreting these results, it is important to take into consideration the inequality related to the time elapsed between each time point and how that may have influenced the impact of antibiotic use. Reported use at 6 and 12 mo may have had a more pronounced effect, depending on when the antibiotic was taken,

because there was less time between data collection points compared to the 24- and 36-mo time points. Further, this crude analysis examining the effect of reported antibiotic use on isoflavone excretion did not take into account the precise timing or duration of antibiotic use, which might have occurred one month or nine months (for example) prior to the actual urinary measurement. Nonetheless, these analyses were initiated because a substantial number of women could not avoid antibiotic use during the course of the study. Consequently, this ancillary study has provided very intriguing data on the potential impact of self-reported antibiotic use on the urinary excretion of isoflavones, with implications related to bioavailability and hence bioactivity of soy isoflavones.

Among the few (n=4) women who experienced a change in equol-producing status with no reported use of antibiotics during the study, 3 of them were equol non-producers at 6, 12, and 24 mo and then became equol producers at 36 mo. The ability to become an equol producer after chronic soy consumption has been reported in some women (160), suggesting that daily exposure to soy isoflavones may induce the ability to metabolize daidzein into equol over time in some individuals. Alternatively, the 4 women who experienced a change in equol-producing status with no reported antibiotic use may have inadvertently not recorded their antibiotic use between appointments. Although the interviewers asked women about medication use since their last visit, retrospective reporting is never perfect. Approximately 44% of the antibiotic users who changed equol-producing status did so only at the time point with reported antibiotic use, providing some degree of confidence in our antibiotic use data gathering. However, for the remaining antibiotic users and one antibiotic non-user who experienced a change in

equol-producing status, the change was less predictable with no apparent pattern or explanation.

## **VI. Conclusion**

The greatest strength of this study was the provision of extracted soy isoflavones to a large number of women from whom we collected health and medication use data during a 3-year period. Overall, our results indicated that urinary isoflavone excretion was highly variable, both within and between individuals. Nevertheless, the intra-individual %CVs for all metabolites were generally lower than the inter-individual %CVs. Moreover, isoflavone excretion in equol producers tended to be less variable compared to equol non-producers, both within and between individuals. Therefore, from a clinical perspective, the effect of isoflavone intake may be more pronounced and predictable for equol producers, a concept that is worth investigating further because of implications with respect to biological responsiveness to isoflavone treatment.

Approximately 16% of women experienced a change in equol-producing status, but upon further analysis we found that this change was significantly related to antibiotic use. For the antibiotic non-users who experienced a change in equol-producing status, 3 of the 4 became equol producers at 36 mo, suggesting that certain individuals may have inducible gut microflora with an ability to produce equol after chronic isoflavone intake. It is important to keep in mind that collecting medication use data from human subjects is memory-dependent and hence fallible. Nevertheless, further characterization of the factors responsible for the variation in isoflavone excretion presented in this study (particularly antibiotic use) and others may prove advantageous in determining to what

extent and/or for whom these compounds might provide the most health promoting effects.

**Table 2.1** Baseline Characteristics and Dietary Intake of the Participants (Mean±SD)  
According to Soy Isoflavone Treatment Group and Equol-Producing Status

Characteristic	80 mg/d <sup>a</sup> (n=58) <sup>c</sup>	120 mg/d <sup>a</sup> (n=66) <sup>c</sup>	ENP <sup>b</sup> (n=72) <sup>c</sup>	EP <sup>b</sup> (n=39) <sup>c</sup>
Age (yr)	54.6 ± 3.4	54.4 ± 3.4	54.5 ± 3.0	54.3 ± 4.2
Time since last menstrual period (yr) <sup>d</sup>	3.0 (1.1-10.0)	2.8 (1.0- 8.0)	2.4 (1.0-10.0)	3.2 (1.1-8. 2)
Body weight (kg)	67.8 ± 10.4	67.0 ± 9.6	68.4 ± 9.9	66.5 ± 9.9
BMI (kg/m <sup>2</sup> )	25.2 ± 3.0	24.6 ± 3.1	25.0 ± 3.1	24.7 ± 3.3
Energy intake (kcal)	1535 ± 543	1557 ± 489	1551 ± 535	1525 ± 505
Carbohydrate intake (g)	178 ± 71	174 ± 61	176 ± 69	175 ± 63
Protein intake (g)	61.6 ± 24.9	63.5 ± 23.0	63.2 ± 24.2	59.1 ± 23.2
Fat intake (g)	63.8 ± 28.5	67.3 ± 27.1	66.4 ± 30.0	64.7 ± 25.3
Dietary fiber intake (g)	17.4 ± 7.2	17.9 ± 7.9	17.6 ± 7.0	16.8 ± 8.0

Abbreviations: body mass index (BMI), equol non-producer (ENP), equol producer (EP)

<sup>a</sup>There were no significant differences in these characteristics between the 80 and 120 mg/d treatment groups (*P* values ranged from 0.30-0.84)

<sup>b</sup>There were no significant differences in these characteristics between EP and ENP (*P* values ranged from 0.30-0.99)

<sup>c</sup>All sample sizes reflected the women who remained in the analysis after the exclusion of women who dropped out, were non-compliant, or had fewer than 2 time points at which they were not taking antibiotics.

<sup>d</sup>Values for time since last menstrual period reflect the median and range

**Table 2.2** Isoflavone Excretion (nmol/day) According to Isoflavone Treatment and Equol-Producing Status at 36 Months<sup>a</sup>

Metabolite	80 mg/d		120 mg/d	
	ENP (n=26)	EP (n=17)	ENP (n=34)	EP (n=21)
Genistein	6208 ± 5115	4055 ± 1717	6953 ± 4250	8366 ± 5494
Glycitein	3662 ± 2671	2808 ± 1824	4007 ± 3009	5307 ± 2883
Daidzein	10645 ± 8136	7499 ± 4208	11736 ± 9233	13016 ± 6606
ODMA	5935 ± 4417	4896 ± 2840	9095 ± 6346	5476 ± 2983
Dihydrodaidzein	5475 ± 5180	2320 ± 1808	5699 ± 5078	4391 ± 2531
Equol	40.7 ± 37.5	11772 ± 6228	53.2 ± 82.8	15435 ± 16233

Abbreviations: equol producer (EP), equol non-producer (ENP), *O*-desmethylangolensin (ODMA)

<sup>a</sup>Mean ± SD

**Table 2.3** Repeated Measures ANOVA: Urinary Isoflavone Excretion (nmol/day) in Response to Isoflavone Treatment, Accounting for Equol Producer Status (N=124)

Model Variable <sup>a</sup>	Parameter	95% CI for PE	DF	F	P value
	Estimate		Num/De	value	
<b>Genistein</b>					
Site (ISU vs. UCD)			1/121	3.78	0.054 <sup>b</sup>
Treatment overall	-1521	-3058 to 16	1/121	6.44	0.012 <sup>c</sup>
Time point			3/121	1.25	0.29 <sup>d</sup>
Treatment x Time			3/121	0.30	0.83 <sup>e</sup>
point					
EP status			1/121	0.00	0.96 <sup>f</sup>
<b>Glycitein</b>					
Site (ISU vs. UCD)			1/121	0.01	0.93 <sup>b</sup>
Treatment overall	-856	-1773 to 61	1/121	5.38	0.022 <sup>c</sup>
Time point			3/121	2.47	0.065 <sup>d</sup>
Treatment x Time			3/121	0.09	0.97 <sup>e</sup>
point					
EP status			1/121	0.84	0.36 <sup>f</sup>
<b>Daidzein</b>					
Site (ISU vs. UCD)			1/121	0.01	0.92 <sup>b</sup>
Treatment overall			1/121	1.24	0.27 <sup>c</sup>
Time point			3/121	2.89	0.038 <sup>d</sup>

Treatment x Time			3/121	0.52	0.67 <sup>e</sup>
point					
EP status			1/121	0.16	0.69 <sup>f</sup>
<b>Dihydrodaidzein</b>					
Site (ISU vs. UCD)			1/121	1.89	0.17 <sup>b</sup>
Treatment overall			1/121	0.05	0.83 <sup>c</sup>
Time point			3/121	1.16	0.33 <sup>d</sup>
Treatment x Time			3/121	1.47	0.23 <sup>e</sup>
point					
EP status	-2099	-3147 to -1050	1/121	15.7	≤0.0001 f
<b>ODMA</b>					
Site (ISU vs. UCD)			1/121	1.11	0.29 <sup>b</sup>
Treatment overall	-2957	-5000 to -907	1/121	4.62	0.034 <sup>c</sup>
Time point			3/121	0.88	0.46 <sup>d</sup>
Treatment x Time			3/121	1.97	0.12 <sup>e</sup>
point					
EP status	-3036	-4815 to -1256	1/121	8.94	0.0034 <sup>f</sup>
EP status x Treatment			1/121	3.25	0.074 <sup>g</sup>
<b>Equol</b>					
Site (ISU vs. UCD)			1/121	2.01	0.16 <sup>b</sup>
Treatment overall			1/121	1.80	0.18 <sup>c</sup>
Time point			3/121	0.07	0.97 <sup>d</sup>

Treatment x Time			3/121	3.67	0.014 <sup>e</sup>
point					
EP status	8279	6077 to 10482	1/121	232	≤0.0001
					f
EP status x Treatment	4454	1799 to 7109	1/121	11.0	0.0012 <sup>g</sup>
EP status x Time			3/121	2.81	0.042 <sup>h</sup>
point					

Abbreviations: parameter estimate (PE), Iowa State University (ISU), University of California, Davis (UCD), *O*-desmethylangolensin (ODMA), equol producer (EP), degrees of freedom (df), numerator (num), denominator (den)

<sup>a</sup>Independent variables for each isoflavone metabolite outcome model included: site (ISU and UCD), treatment, time point, treatment-by-time point interaction, EP status, EP status-by-treatment interaction, and EP status-by-time point interaction

<sup>b</sup>*P* value for F-test to assess association between covariates and outcome variable

<sup>c</sup>*P* value for F-test to assess difference in outcome variable between treatments

<sup>d</sup>*P* value for F-test to assess a change in outcome variable with respect to time point

<sup>e</sup>*P* value for F-test to assess a treatment-by-time point interaction (existence of parallel treatment profiles with respect to time point)

<sup>f</sup>*P* value for F-test to assess difference in outcome variable between equol-producing statuses

<sup>g</sup>*P* value for F-test to assess an equol producer status-by-treatment interaction

<sup>h</sup>*P* value for F-test to assess an equol producer status-by-time point interaction

**Table 2.4** Within Person %CVs (Mean) for Each Metabolite According to Treatment and Equol-Producing Status<sup>a,b</sup>

<b>Metabolite</b>	<b>80 mg/d<sup>c</sup></b>	<b>120</b>	<b>IEP<sup>d</sup></b>	<b>ENP<sup>d</sup></b>	<b>EP<sup>d</sup></b>
	<b>(n=58)</b>	<b>mg/d<sup>c</sup></b>	<b>(n=13)</b>	<b>(n=72)</b>	<b>(n=39)</b>
Genistein	43	40	34 <sup>e</sup>	48 <sup>e</sup>	33 <sup>e</sup>
Glycitein	46	43	32	50	37
Daidzein	39	39	30	44	32
Dihydrodaidzein	54	52	55	57	45
ODMA	44	48	46	50	39
Equol	53	64	149 <sup>f</sup>	55 <sup>f</sup>	36 <sup>f</sup>

Abbreviations: inconsistent equol producers (IEP), equol non-producers (ENP), equol producers (EP), *O*-desmethylangolensin (ODMA)

<sup>a</sup>Percent coefficient of variation (%CV) = (standard deviation/mean)\*100

<sup>b</sup>Analysis reflects the exclusion of time points at which women reported use of antibiotics

<sup>c</sup>There were no significant differences (*P* values ranged from 0.19-0.99) between the 80 and 120 mg/d treatment groups for each metabolite

<sup>d</sup>There were no significant differences (*P* values ranged from 0.086-0.29) between IEP, ENP, and EP for glycitein, daidzein, dihydrodaidzein, and ODMA

<sup>e</sup>There was a significant difference between the mean %CV for ENP and EP (F value = 3.10, degrees of freedom = 2, *P*=0.049)

<sup>f</sup>There was a significant difference between the mean %CV for IEP, ENP, and EP (F value = 73.16, degrees of freedom = 2, *P*≤0.0001)

**Table 2.5** Between Person %CVs (Mean) for each Urinary Isoflavone Metabolite at Each Time Point<sup>a,b</sup>

	<b>6 months</b>	<b>12 months</b>	<b>24 months</b>	<b>36 months</b>
<b>Metabolite</b>	<b>(n=107)</b>	<b>(n=108)</b>	<b>(n=102)</b>	<b>(n=98)</b>
Genistein	60	55	62	71
Glycitein	59	53	59	70
Daidzein	57	59	61	71
Dihydrodaidzein	81	81	82	92
ODMA	76	68	73	74
Equol	196	174	172	193

Abbreviations: *O*-desmethylangolensin (ODMA)

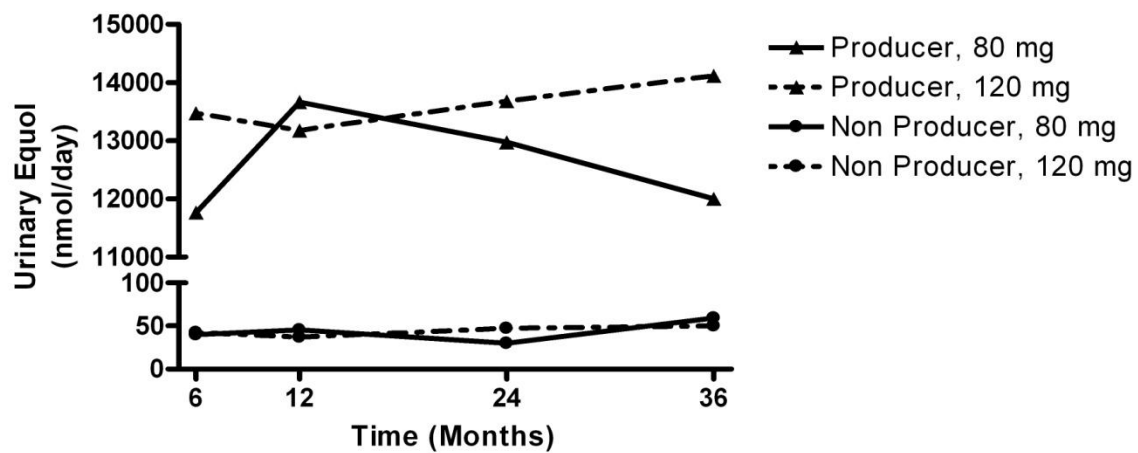
<sup>a</sup>Percent coefficient of variation (%CV) = (standard deviation/mean)\*100

<sup>b</sup>Analysis reflects the exclusion of time points at which women reported use of antibiotics

**Table 2.6** Number of Subjects who Experienced a Change in Equol-Producing Status According to Antibiotic Use<sup>a</sup>

	<b>Non Antibiotic Users</b>	<b>Antibiotic Users</b>	<b>Total</b>
Consistent Producers	57	47	104
Changed Producer Status	4	16	20
<b>Total</b>	61	63	124
<b>Statistic</b>	<b>DF</b>	<b>Test Statistic Value</b>	<b>P Value</b>
<b>Chi-Square</b>	1	8.13	0.0044

<sup>a</sup>Analysis reflects the inclusion of time points at which women reported use of antibiotics



**Figure 2.1** Parallel Profile Plot Characterizing Equol Excretion According to Isoflavone Treatment Group and Equol-Producing Status

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## **Appendices**

**Appendix 2.1** Frequency of Equol Producers at each Time Point<sup>a, b, c, d</sup>

	<b>6 mo</b> (n=107)	<b>12 mo</b> (n=108)	<b>24 mo</b> (n=102)	<b>36 mo</b> (n=98)	<b><i>P</i></b>
ENP	67.3	65.7	61.8	61.2	0.18
EP	32.7	34.3	38.2	38.8	0.18

Abbreviations: equol non-producers (ENP), equol producers (EP)

<sup>a</sup>Values reflect a percentage

<sup>b</sup>All sample sizes reflected the women who remained in the analysis after the exclusion of women who dropped out, were non-compliant, or had fewer than 2 time points at which they were not taking antibiotics

<sup>c</sup>Analysis reflects the exclusion of time points at which women reported use of antibiotics

<sup>d</sup>Equol producers were defined as women excreting more than 1000 nmol/day

**Appendix 2.2** Between Person %CVs (Mean) at each Time Point According to Isoflavone Treatment Group<sup>a, b, c</sup>

<b>6 months</b>			
	<b>80 mg/d (n=49)</b>	<b>120 mg/d (n=58)</b>	<b>P</b>
Genistein	58	61	0.62
Glycitein	54	62	0.24
Daidzein	54	59	0.48
Dihydrodaidzein	87	75	0.47
ODMA	75	77	0.79
Equol	148	256	0.011
<b>12 months</b>			
	<b>80 mg/d (n=50)</b>	<b>120 mg/d (n=58)</b>	<b>P</b>
Genistein	56	53	0.76
Glycitein	51	53	0.75
Daidzein	56	60	0.60
Dihydrodaidzein	86	77	0.42
ODMA	72	64	0.49
Equol	133	231	0.042
<b>24 months</b>			
	<b>80 mg/d (n=49)</b>	<b>120 mg/d (n=53)</b>	<b>P</b>
Genistein	48	67	0.036
Glycitein	51	62	0.18
Daidzein	56	64	0.41
Dihydrodaidzein	83	81	0.87
ODMA	72	72	0.96
Equol	132	227	0.061
<b>36 months</b>			
	<b>80 mg/d (n=43)</b>	<b>120 mg/d (n=55)</b>	<b>P</b>
Genistein	79	64	0.23
Glycitein	72	67	0.68

Daidzein	74	68	0.069
Dihydrodaidzein	105	83	0.064
ODMA	70	72	0.87
Equol	149	210	0.28

Abbreviations: *O*-desmethylangolensin (ODMA)

<sup>a</sup>Percent coefficient of variation (%CV), all values for the respective metabolites reflect the mean.  $\%CV = (\text{standard deviation}/\text{mean}) * 100$

<sup>b</sup>All sample sizes reflected the women who remained in the analysis after the exclusion of women who dropped out, were non-compliant, or had fewer than 2 time points at which they were not taking antibiotics

<sup>c</sup>Analysis reflects the exclusion of time points at which women reported use of antibiotics

**Appendix 2.3** Between Person %CVs (Mean) According to Equol-Producing Status at each Time Point<sup>a, b, c</sup>

<b>6 months</b>			
	<b>ENP (n=62)</b>	<b>EP (n=33)</b>	<b>P</b>
Genistein	57	68	0.31
Glycitein	61	59	0.66
Daidzein	58	52	0.37
Dihydrodaidzein	81	70	0.22
ODMA	72	55	0.090
Equol	212	76	0.027
<b>12 months</b>			
	<b>ENP (n=65)</b>	<b>EP (n=34)</b>	<b>P</b>
Genistein	55	58	0.84
Glycitein	55	48	0.31
Daidzein	61	50	0.17
Dihydrodaidzein	81	55	0.034
ODMA	67	57	0.21
Equol	88	63	0.23
<b>24 months</b>			
	<b>ENP (n=59)</b>	<b>EP (n=33)</b>	<b>P</b>
Genistein	68	57	0.57
Glycitein	72	40	0.0020
Daidzein	73	41	0.0020
Dihydrodaidzein	89	68	0.066
ODMA	75	49	0.016
Equol	99	74	0.49
<b>36 months</b>			
	<b>ENP (n=57)</b>	<b>EP (n=30)</b>	<b>P</b>
Genistein	72	78	0.73
Glycitein	76	68	0.62

Daidzein	80	59	0.22
Dihydrodaidzein	92	75	0.21
ODMA	74	52	0.033
Equol	146	86	0.37

Abbreviations: equol non-producers (ENP), equol producers (EP), *O*-desmethylangolensin (ODMA)

<sup>a</sup>Percent coefficient of variation (%CV) = (standard deviation/mean)\*100

<sup>b</sup>All sample sizes reflected the women who remained in the analysis after the exclusion of women who dropped out, were non-compliant, or had fewer than 2 time points at which they were not taking antibiotics

<sup>c</sup>Analysis reflects the exclusion of time points at which women reported use of antibiotics

**Appendix 2.4** Mean Difference in Urinary Isoflavone Excretion within Subjects between Time Points in which Antibiotic Use was Reported and Time Points in which Antibiotic Use was not Reported<sup>a</sup>

<b>Metabolite</b>	<b>Mean Difference</b>	<b>DF</b>	<b>t Value</b>	<b>P</b>
	<b>(n=59)</b>			
Genistein	506.4	58	1.26	0.21
Glycitein	-2.69	58	-0.02	0.99
Daidzein	539.5	58	1.01	0.32
Dihydrodaidzein	218.8	58	0.56	0.58
ODMA	400.8	58	0.8	0.43
Equol	-2361.9	58	-2.98	0.0042

Abbreviations: *O*-desmethylangolensin (ODMA)

<sup>a</sup>The difference was calculated by averaging the isoflavone excretion for the time points in which a woman reported she was not taking antibiotics and subtracting that from the average isoflavone excretion from the time points in which a woman reported she was taking antibiotics

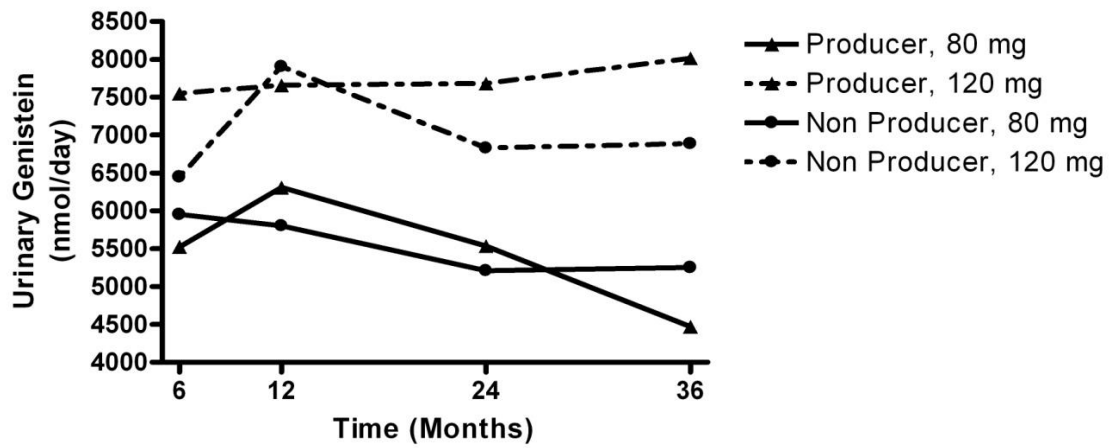
**Appendix 2.5** Number of Subjects who Experienced a Change in Equol-Producing Status According to Reported Antibiotic Use<sup>a, b</sup>

	<b>Non Antibiotic Users</b>	<b>Antibiotic Users</b>	<b>Total</b>
<b>Consistent Producers<sup>c</sup></b>	57	54	111
<b>Inconsistent Producers<sup>c</sup></b>	4	9	13
<b>Total</b>	61	63	124
<b>Statistic</b>	<b>DF</b>	<b>Test Statistic Value</b>	<b>P Value</b>
<b>Chi-Square</b>	1	1.9724	0.16

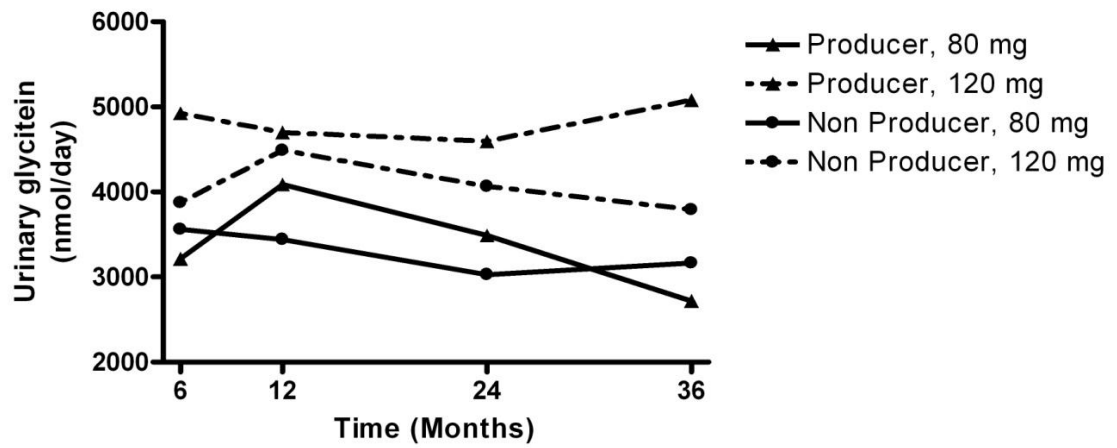
<sup>a</sup>All sample sizes reflected the women who remained in the analysis after the exclusion of women who dropped out, were non-compliant, or had fewer than 2 time points at which they were not taking antibiotics

<sup>b</sup>Analysis reflects the exclusion of time points at which women reported use of antibiotics

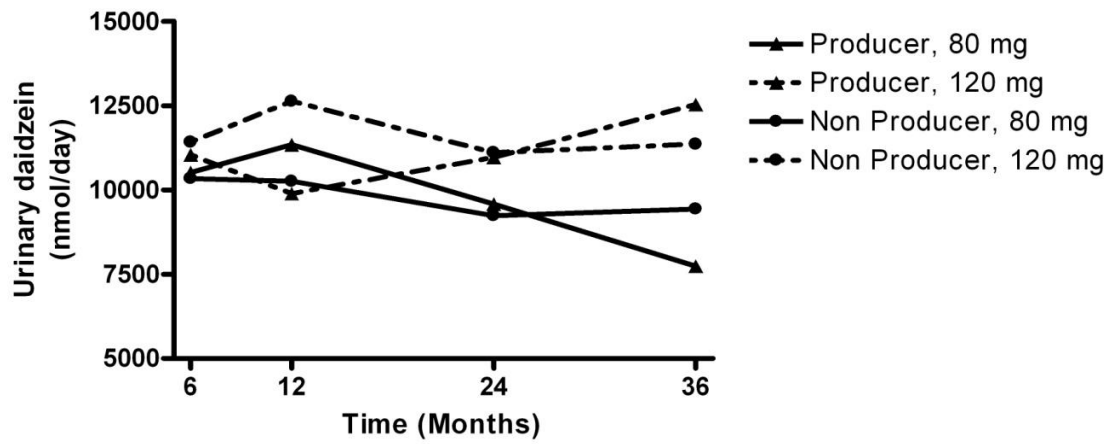
<sup>c</sup>Women who maintained their equol status as either equol producers or equol non-producers for the entire duration of the study were classified as consistent producers and those who experienced a change in equol status were classified as inconsistent producers



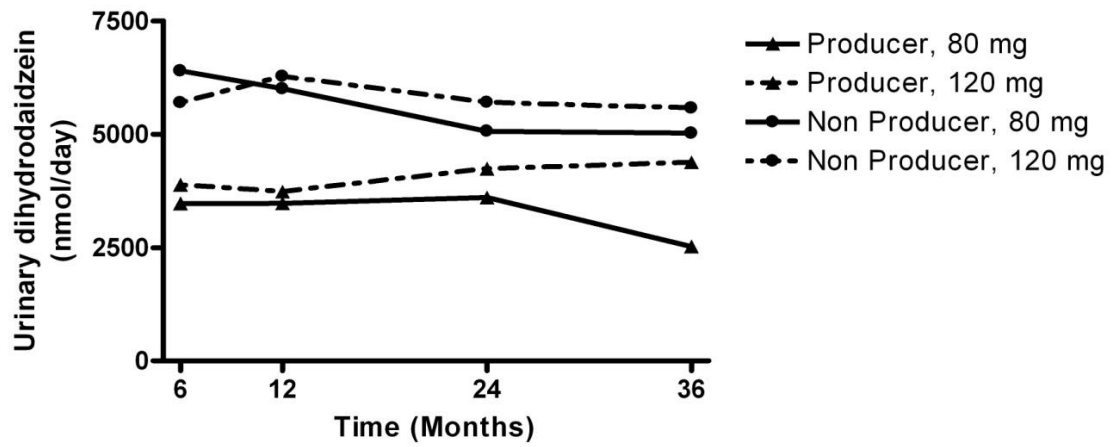
**Appendix 2.6** Parallel Profile Plot Characterizing Genistein Excretion According to Isoflavone Treatment Group and Equol-Producing Status



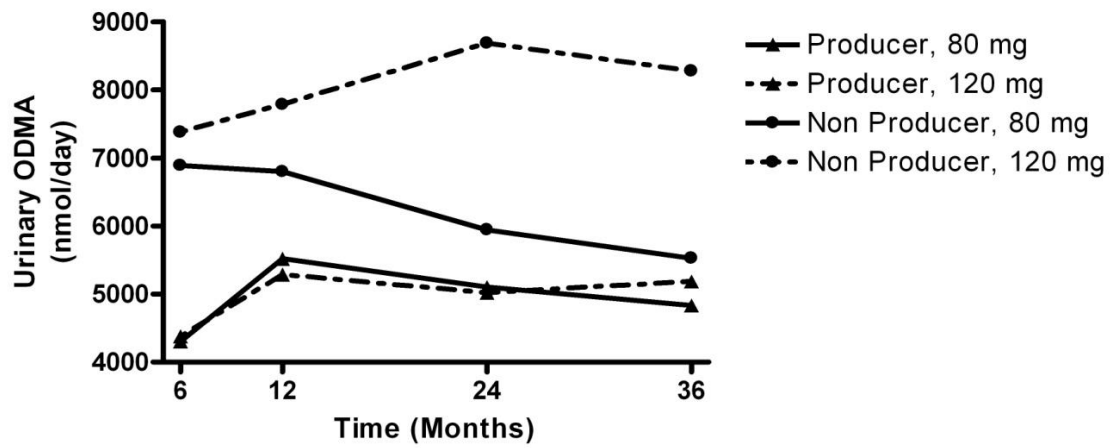
**Appendix 2.7** Parallel Profile Plot Characterizing Glycitein Excretion According to Isoflavone Treatment Group and Equol-Producing Status



**Appendix 2.8** Parallel Profile Plot Characterizing Daidzein Excretion According to Isoflavone Treatment Group and Equol-Producing Status



**Appendix 2.9** Parallel Profile Plot Characterizing Dihydrodaidzein Excretion According to Isoflavone Treatment Group and Equol-Producing Status



**Appendix 2.10** Parallel Profile Plot Characterizing *O*-desmethylangolensin (ODMA)

Excretion According to Isoflavone Treatment Group and Equol-Producing Status