Special Article

Emerging evidence of the health benefits of S-equol, an estrogen receptor β agonist

Richard L Jackson, Jeffrey S Greiwe, and Richard J Schwen

Many clinical studies have been carried out to determine the health benefits of soy protein and the isoflavones contained in soy. S-equol is not present in soybeans but is produced naturally in the gut of certain individuals, particularly Asians, by the bacterial biotransformation of daidzein, a soy isoflavone. In those intervention studies in which plasma S-equol levels were determined, a concentration of >5–10 ng/mL has been associated with a positive outcome for vasomotor symptoms, osteoporosis (as measured by an increase in bone mineral density), prostate cancer, and the cardiovascular risk biomarkers low-density lipoprotein cholesterol and C-reactive protein. These studies suggest that S-equol may provide therapeutic benefits for a number of medical needs.

INTRODUCTION

Soy foods are rich sources of protein, minerals, vitamins, polyunsaturated fatty acids, and isoflavonoids, also referred to as phytoestrogens. Many health benefits have been observed in populations that consume >50 g of soybeans per day, including a lower incidence of cardiovascular disease, fewer bone fractures, and lower rates of prostate and breast cancers. The component(s) in soy responsible for these health benefits are not known with certainty, but there is much evidence suggesting an association with the isoflavones contained in soy. The Cardiovascular Diseases and Alimentary Comparison (CARDIAC) Study was coordinated by the World Health Organization and carried out in 61 communities in 25 countries around the world.[1] Age-adjusted mortality rates related to coronary artery disease, prostate cancer, and breast cancer were inversely related to 24 h urinary isoflavone excretion. The populations with the lowest mortality rates were Asian communities that excreted >20 µmol isoflavones/day. In contrast, those populations with the highest mortality rates excreted <1 µmol isoflavones per day. The two major isoflavones in soybeans are the conjugated derivatives of genistein and daidzein. Another isoflavone, called equol, is not present in soybeans but is a metabolic product of the biotransformation of daidzein. In 2002, Setchell et al.[2] proposed the "Equol Hypothesis" as another explanation for the health benefits of soy isoflavones. In this hypothesis, daidzein is converted to equol by bacteria in the gut of certain individuals, and it is the equol produced by these individuals that accounts for some of the health benefits of soy isoflavones. This review traces the nearly 70-year history of equol, with the focus placed on those intervention studies in which the plasma or serum concentration of equol was determined with the goal of elucidating the relationship between equol exposure and the related health benefits. The potential mechanism(s) of action of equol is also discussed.

EQUOL DISCOVERY AND EARLY HISTORY

Urine from pregnant mares was the first source of a dihydroxyphenolic compound [$C_{15}H_{12}O(OH)_2$] that Marrian and Haslewood[3] called "equol" in 1932. Subsequently, equol was isolated from an ether-soluble phenolic fraction of toluene extracts of horse urine and crystallized from chloroform.[4] It was not until 1982, through structural-elucidation studies using gas chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy, that the structure of equol was established.[5] In

Affiliation: *RL Jackson*, *JS Greiwe,* and *RJ Schwen* are with Ausio Pharmaceuticals, LLC, Cincinnati, Ohio, USA.

Correspondence: *RL Jackson*, Ausio Pharmaceuticals, LLC, 1776 Mentor Avenue, Cincinnati, OH 45212, USA. E-mail: richard@ausiopharma.com.

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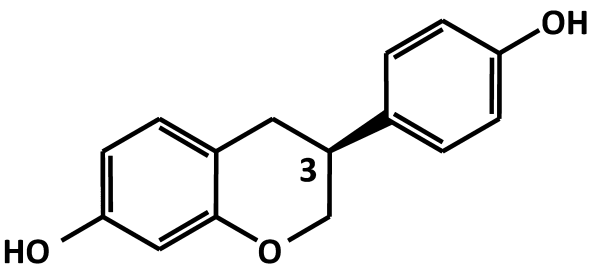

(S)-3,4-Dihydro-3-(4-hydroxyphenyl)-2H-1-benzopyran-7-ol

Figure 1 **Structure of S-equol.**

this study, equol was shown to be excreted in urine predominately as the monoglucuronide conjugate. Equol has a chiral center at carbon 3 (Figure 1) and, thus, could exist as either the S- or R-enantiomer. In 2005, Setchell et al.[6] showed that equol is present in humans and animals only as the S-enantiomer.

The first study showing that excreted equol was derived from dietary soy was reported by Axelson et al.[7] These investigators measured equol in rat urine after adding various food constituents and food extracts to a semisynthetic diet. Of the foods tested, including wheat, rye, oat, millet, barley, buckwheat, corn, alfalfa, white and brown beans, and soy flour, only soy flour generated equol. The precursor of equol was isolated from soy flour and shown to be daidzein.[7] It was then demonstrated that daidzein, when given orally to rats, produced equol in the urine. The conversion of daidzein to S-equol is shown in Figure 2.

BIOTRANSFORMATION OF DAIDZEIN TO S-EQUOL BY GUT BACTERIA

Setchell et al.[8] demonstrated that bacteria are responsible for the biotransformation of daidzein to equol. Six subjects were given a diet of soy meal. After 5 days, only four subjects excreted equol, indicating that the biotransformation of daidzein does not occur in all individuals. In vitro incubation of soy germ with fecal bacteria obtained from one of the equol producers resulted in the production of equol. Further evidence for the role of bacteria in the production of equol in animals was provided by the lack of equol in the urine of germ-free rats given soy.[9]

Atkinson et al.[10] investigated the conditions required for the biotransformation of daidzein to equol. Fecal samples were obtained from 13 subjects, 7 of whom were equol producers and 6 of whom were not. Fecal inoculates from equol nonproducers did not produce equol from daidzein, whereas those from the equol producers did. The equol producers were consistent producers over a 2-year period of sampling. Equol was formed only when the incubations were carried out under anaerobic conditions. The effects of various antibiotics on the in vitro metabolism of daidzein revealed interindividual differences. In addition, some antibiotics inhibited the conversion of daidzein to dihydrodaidzein but not the conversion of dihydrodaidzein to equol, suggesting that multiple bacteria are responsible for the biotransformation. Interindividual variability in the metabolism of daidzein to equol was also reported by Rafii et al.[11] Feces were collected at various times over a 2-year period from individuals between the ages of 3 and 56 years and then incubated with daidzein. In some subjects, equol was not produced over the 2-year sampling period. In other subjects, equol was produced at one time but not consistently over time, a finding that differs from that reported by Atkinson et al.[10] It is possible that changes in the bacterial population as a result of diet, antibiotic use, and other unknown factors, may affect an individual's ability to be a consistent equol producer. Interestingly, two subjects in the study by Rafii et al.[11] were not equol producers, but after they began consuming fruits and vegetables, they were able to produce equol in vitro.

Various bacteria have been isolated from the feces of rat, pig, monkey, and mouse and from the rumen of cow and shown to convert daidzein to equol (Table 1). Table 2 lists the bacteria isolated from human feces that convert daidzein or dihydrodaidzein to equol. These bacteria are anaerobes, rod shaped, and gram positive. In most of

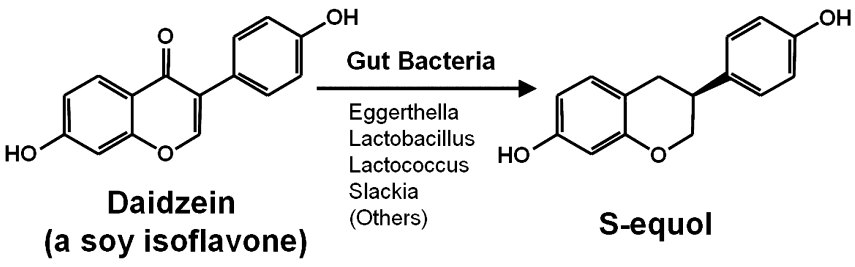

Figure 2 **Biotransformation of daidzein to S-equol. Various bacteria have been shown to carry out this process.**

Table 1 **Bacteria isolated from animals that convert daidzein (D) and/or dihyrodaidzein (Dihydro D) to equol (E).**

Animal	Bacterium strain	Characteristics	Reference
Rat	*Asaccharobacter celatus* do 03	D → E	Minamida et al. (2006, 2008)[100,101]
Erhualian pig	*Eubacterium* sp. strains D1 and D2	D → E	Yu et al. (2008)[102]
Bovine	*Lactobacillus* sp. Niu-O16	D → Dihydro D	Wang et al. (2005)[103]
Rhesus monkey	Fecal bacteria	D → E	Rafii et al. (2004)[104]
Mouse	*Coriobacteriaceae* strain Mt1B8	D → E	Matthies et al. (2008)[105]

these reports, the stereoselectivity of equol was not determined. However, from the information available to date, it can be concluded that only S-equol is formed in humans and animals. Two strains of *Clostridium* have been isolated that convert daidzein to dihydroequol but do not convert dihydroequol to equol.[12,13] 16S RNA gene sequences have revealed that nine strains isolated from human feces are related to the genus *Eggerthella*.[14] Maruo et al.[14] proposed a new genus, *Adlercreutzia equolifaciens* gen. nov. Two of the strains isolated by these researchers were not able to convert daidzein to dihydrodaidzein but did convert dihydrodaidzein to equol; the other strains converted daidzein to equol.

A rod-shaped anaerobe that was isolated from human feces and designated *Eggerthella* strain Julong 732 has a 92.8% 16S RNA sequence similarity to *Eggerthella hongkongenis* HKU10.[15] In studies of this anaerobe, S- and R-equol were prepared and used as reference standards. Dihydrodaidzein was prepared as a racemic mixture, but when incubated with Julong 732, only S-equol was produced. Since all of the dihydrodaidzein was metabolized, these results show that the bacterium has a racemase that transforms R-dihydrodaidzein to the S form, i.e., a form that is further metabolized to S-equol. Tetrahydrodaidzein was also prepared chemically and was converted to S-equol by Julong 732. In contrast, dehydroequol was not converted to S-equol. On the basis of these results, Wang et al.[16] proposed a pathway for the conversion of daidzein to S-equol (Figure 3). Kim et al.[17] further refined the stereoselectivity for

Table 2 **Human intestinal bacteria that convert daidzein (D) and/or dihydrodaidzein (Dihydro D) to equol (E).**

Bacterium strain	Characteristics	Reference
Clostridium sp, Strain HGH6	D → Dihydro D	Hur et al. (2000)[12]
Clostridium sp, Strain TM-40	D → Dihydro D	Tamura et al. (2007)[13]
Adlercreutzia equolifaciens	Dihydro D → E	Maruo et al. (2008)[14]
Strain FJC-A10	D → E	
Strain FJC-A161		
Eggerthella SNU	Dihydro D → S-equol	Wang et al. (2005, 2007)[15,16]
Strain Julong 732		Kim et al. (2009)[17]
Eggerthella sp, Strain YY98	D → E	Yokoyama and Suzuki (2008)[106]
Slackia isoflavoniconvertens Strain HE8	D → E	Matthies et al. (2009)[107]
Slackia equolifaciens sp. nov.	D → E	Jin et al. (2008)[108]
Strain DZE		Jin et al. (2010)[109]
Strain DSM 22006		
Slackia sp. Strain NATTS	D → E	Tsuji et al. (2010)[110]
Lactococcus garvieae Strain 20-92	D → E	Uchiyama et al. (2007)[18]
Bacteroides ovatus Strain E-23-15	D → E	Uchiyama et al. (2004)[111]
Streptococcus intermedius Strain E-23-17	D → E	Uchiyama et al. (2004)[111]
Streptococcus constellatus Strain Abg225	D → E	Uchiyama et al. (2004)[111]
Mixed bacterial culture	D → E	Decroos et al. (2005)[112]
Lactobacillus mucosae		
Enterococcus faecium		Decroos et al. (2006)[113]
Finegoldia magna		

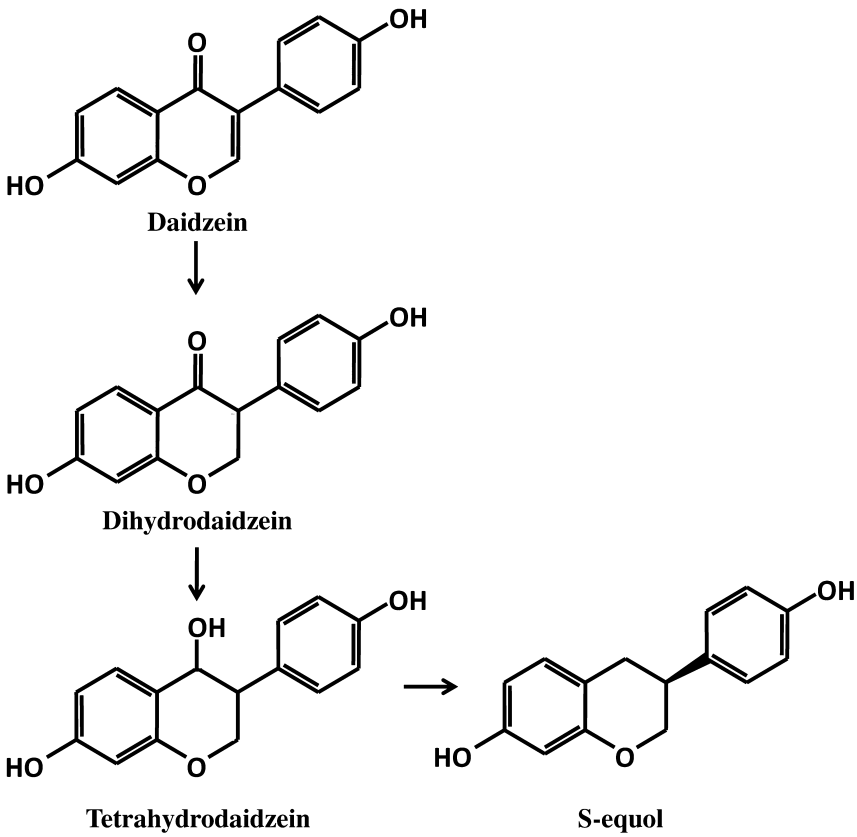

Figure 3 **Biotransformation of daidzein to S-equol, as modified from Wang et al.[16]**

tetrahydrodaidzein and demonstrated that the 3-R, 4-S enantiomer of tetrahydrodaidzein was converted to S-equol; however, no R-equol was noted.

Uchiyama et al.[18] isolated a strain of lactic-acid-producing bacteria from human feces that they called *Lactococcus* 20-92. This bacterium is not normally present in human feces but it is found as a fish pathogen. In order to make an equol supplement with this bacteria, soy germ was fermented with *Lactococcus* 20-92 such that 50% of the daidzin/daidzein was converted to equol. The resulting nutritional supplement has been shown to relieve menopausal symptoms in a placebo-controlled trial of Japanese women.[19]

FACTORS THAT DETERMINE EQUOL PRODUCER STATUS

In Japan, Korea, and China up to 80% of individuals are equol producers.[20,21] In contrast, as few as 25% of individuals in North America and Europe make S-equol.[22] Messina et al.[23] summarized the various reports on soybean intake in Japan, China, Hong Kong, and Singapore. The Japanese National Nutritional Survey showed that soybean intake has remained fairly constant from 1960 to 2004, at about 65 g/day. It was estimated that the average daily soy protein and isoflavone intakes of older Japanese adults in 2004 were approximately 6–11 g and 25–50 mg, respectively.[23] If one assumes that daidzin/daidzein constitutes 30% of the isoflavones in soy, then older Japanese people are consuming 8–15 mg daidzin/daidzein per day. Furthermore, if all of the daidzein in the diet were converted to S-equol, this would correspond to a daily dose of 8–15 mg S-equol. Those individuals who are not equol producers may consume sufficient soy, but they lack the appropriate bacteria, diet, or other factors to carry out the biotransformation of daidzein to S-equol. Interestingly, the percentage of equol producers in younger men in Japan and Korea today is significantly lower than that of older men.[20] In Japanese men 20–29 years of age, the percentage of equol producers is 24%, while 59% of men in the 70–79 year age range produce equol. Fujimoto et al.[20] suggest that the younger generations are consuming fewer soy products as a result of Western influence. This may lead to reduced levels of equol and, furthermore, to an increased risk of prostate cancer and other diseases of the aging population in the years to come.

One of the first studies to address the role of diet in the production of equol was by Adlercreutz et al.,[24] who measured urinary excretion of equol in 20 postmenopausal women in the Boston area; 10 were omnivores and 10 were vegetarians. The study revealed no differences between the two groups in the amount of equol excreted

(0.14 µmol/24 h). Setchell and Cole[25] measured equol in serum and urine collected from a cohort of 41 healthy Australian men and women (29 vegetarians and 12 nonvegetarians) after consuming 500 mL/day soy milk containing 14 mg daidzein for 3 consecutive days. A wide variation in serum S-equol concentration was noted, with some individuals having levels of equol below 5 ng/mL while others had levels as high as 35 ng/mL. On the basis of these findings, an equol producer was defined as an individual with fasting serum levels of equol of >20 nM (>5 ng/mL) or a 24-h urine concentration of >82 nM (>20 ng/mL). Using these criteria, 25 (61%) of the 41 subjects were defined as equol producers, with 4 (33%) of the 12 nonvegetarians being equol producers, and 21 (72%) of the 29 vegetarians producing equol. This suggests a vegetarian diet may influence an individual's ability to produce equol.

Atkinson et al.[26] evaluated the relationship between equol-producing status and demographic, anthropometric, lifestyle, and dietary factors among premenopausal women in the United States and found that 28% (55/200) of the women were equol producers. An equol producer was defined as an individual with an equol concentration of >362 nM (>87.5 ng/mL) in a 24-h urine collection. Hispanic and Latino women were more likely to be equol producers. Using a 3-day food record and a food frequency questionnaire, a positive association between equol production and servings per day of vegetables and eggs was observed.

The relationship between diet and the ability to produce equol has also been assessed in an Italian population of 90 subjects.[27] Fecal samples from 37% of the men and 25% of the women produced detectable levels of equol from daidzein, which was the criterion for being an equol producer. Thirty-one percent of the individuals produced dihydrodaidzein but did not produce equol, suggesting that in some individuals more than one bacterial species is involved in the biotransformation of daidzein to equol. Using a chiral column for enantiomeric separations, dihydrodaidzein was present as both the S- and R- enantiomers. Since only S-equol was produced, this finding shows that bacteria selectively reduce the S-enantiomer of dihydrodaidzein to S-equol, which is consistent with a previous finding.[15]

The following important question remains: How and at what age does one become an equol producer? In adults, consumption of isoflavones does not convert a nonproducer of equol to a producer.[28,29] However, if one is an equol producer, the consumption of isoflavones can change the bacterial flora in the gut. Clavel et al.[30] gave postmenopausal French women (mean age, 60.4 ± 7.1 years) 100 mg of isoflavones per day for 1 month. Fecal samples were taken at the beginning and end of the study. Among the equol producers, 12 of 24 had at least a 50% increase in the percentage of the *Clostridium coccoides-Eubacterium rectale* cluster of bacteria, whereas no change in the microflora was observed in nonproducers. An increase in this cluster of bacteria following consumption of isoflavones has also been reported by Possemiers et al.[31] While it is not clear when one becomes an equol producer, it may be at an early age. Hoey et al.[32] determined the equol status of 60 infants and children from Northern Ireland who were between the ages of 4 months and 7 years. The subjects were divided into four age groups: 4–6 months, 7–12 months, 1–3 years, and 3–7 years. The study recruited infants and children ($n = 30$) who had been fed a soy-based infant formula (Soy Group) in early infancy and continued to use soy products after weaning and compared them to a group ($n = 30$) who had consumed only cow's milk. Each group was given a 2-day soy challenge of yogurt containing 4.8 g soy protein and 22 mg total isoflavones; urine samples were collected prior to and after the soy challenge, and total equol was determined. Equol excretion in urine was detected in only 6 (20%) of the 30 soy-fed infants and children; this low percentage of equol producers is consistent with other findings for non-Asian populations. Nonetheless, equol was detected in only one subject in the milk-fed group, indicating that exposure to soy early in life may be required if one is to become an equol producer.

HEALTH BENEFITS OF S-EQUOL

Setchell et al.[2] and Atkinson et al.[33] reviewed the early literature on the relationship between soy consumption and daidzein-metabolizing phenotype to determine the potential health benefits. In general, these relationships are variable, and it is difficult to conclude to what the health benefits, if any, are attributable. More recent studies suggest that soy isoflavones may provide a clinical benefit for breast cancer,[34] neurological health,[35] diabetes,[36] and obesity,[36] but again, the results are variable and in most of the studies the equol producer status was not determined.

In studies in which the equol producer status was reported, the equol status was determined, in most cases, by the total amount or concentration of equol produced in a 24-h urine collection. In some studies, plasma equol concentrations were determined, and it is these studies that are most relevant for determining the potential health benefits of exposure to S-equol.

VASOMOTOR SYMPTOMS

Hot flashes are the most common vasomotor symptom (VMS) the world over.[37] More than 70% of women experience hot flashes with menopause, with some having >10

severe episodes per day. Hot flashes also occur in women treated with estrogen receptor antagonists and in men being treated for prostate cancer with a luteinizing hormone-releasing hormone.[38] There is no agreement as to what causes hot flashes, but changes in the levels of luteinizing hormone and follicle-stimulating hormone are thought to be important. Since hot flashes occur in women at life stage in which estrogen production decreases, it is generally assumed that estrogens play a role in the disorder. However, no relationship has been found between plasma estrogen levels and the frequency or severity of hot flashes. While estrogen therapy is very effective in reducing hot flashes, the Women's Health Initiative showed an increased risk of cancer and cardiovascular events associated with estrogen therapy.[39]

The efficacy of isoflavones in relieving menopausal symptoms has been reviewed.[40–42] Table 3 summarizes those intervention studies where the equol studies were determined. The plasma levels of equol were determined in only one study.[43] These investigators gave a daidzein-rich isoflavone (DRI) supplement (Effisoy™, a dietary supplement containing 70% daidzein) to postmenopausal women (mean age, 53 years) living in the Boston area; the women were randomized into a placebo group ($n = 45$), a group receiving 40 mg DRI daily ($n = 48$), and a group receiving 60 mg DRI daily ($n = 49$). After 12 weeks of supplementation, the frequency of hot flashes in the 40 mg DRI and 60 mg DRI groups was reduced by 52% and 51%, respectively; the reduction in the placebo group was 39%. While the differences between the 40 mg and 60 mg DRI groups and placebo were not significant ($P = 0.07$ and 0.09, respectively), combining the two DRI treatment groups did show a significant reduction in hot flashes. The plasma levels of equol in the 40 mg and 60 mg DRI groups were 8.89 $\pm$ 11.69 ng/mL and 13.19 $\pm$ 20.55 ng/mL, respectively. The equol concentration in the placebo group was 3.7 $\pm$ 6.41 ng/mL. DRI supplementation did not produce any significant changes in the serum levels of thyroid-stimulating hormone, thyroxine, lutenizing hormone, or estradiol.

The clinical effectiveness of a supplement (SE5-OH) containing 10 mg of equol per 1 gram capsule has been evaluated for menopausal symptoms and mood states in 127 peri- and post-menopausal Japanese women between the ages of 40 and 59.[19] Although it was not stated in the report, it can be assumed that the supplement contains S-equol. Prior to treatment, women were stratified into equol producers and nonproducers. Equol producers were defined as those subjects who excreted more than 10 ng/mL of equol in a 24-h urine sample after consuming a single dose of 50 mg of isoflavones at dinner; plasma levels of equol were not determined. The subjects consumed the 10 mg equol supplement once a day ($n = 28$) or three times a day ($n = 29$). In addition, study

Table 3 **Summary of studies investigating the effects of dietary isoflavones on vasomotor symptoms.**

Treatment	Content of daidzein	Location of population	Clinical endpoint (duration of treatment)	Equol producer status	Reference
Effisoy™ (40 mg/day)	28 mg	Boston, USA	52% ↓ in HF (12 weeks)	Plasma S-equol 8.89 ng/mL	Khaodhiar et al. (2008)[43]
Effisoy™ (60 mg/day)	43 mg	Boston, USA	51% ↓ in HF (12 weeks)	Plasma S-equol 13.19 ng/mL	Khaodhiar et al. (2008)[43]
Isoflavone tablet (42 mg/day)	13.5 mg	Tokyo, Japan	65% ↓ in HF (8 weeks)	>0.01 nM equol/mM Cr in urine	Uesugi et al. (2004)[114]
Soy germ extract powder (135 mg/day)	32.7 mg	Taipei, Taiwan	68% ↓ in HF (6 months)	Positive chromatographic peak for equol in urine	Jou et al. (2008)[44]
Soy flour (45 g/day)	ND	Australia	40% ↓ in HF (12 weeks)	3.6 $\pm$ 1.6 µmol S-equol in a 24-h urine collection	Murkies et al. (1995)[115]

Abbreviations: HF, hot flashes; ND, not determined.

participants were allowed to consume 20 mg of isoflavones daily from their meals. Menopausal symptoms and mood states were evaluated by a questionnaire at the beginning and at the end of the 12-week study. In those subjects who were equol producers based on an isoflavone challenge, neither the once-daily nor the thrice daily supplement group showed any significant improvement from baseline values for menopausal or mood symptoms after 12 weeks of treatment. In contrast, the subjects who were not equol producers after the isoflavone challenge showed a significant clinical benefit, but only in the thrice daily consumption group. The vasomotor score, which consisted of hot flashes, sweating, and chilliness, decreased from 2.4 ± 1.9 to 1.4 ± 1.3 ($P < 0.05$), and the total menopausal score decreased from 20.2 ± 7.5 to 13.6 ± 6.2 ($P < 0.01$).

As noted, the plasma levels of S-equol were not determined in the supplement study described above.[19] However, Setchell et al.[116] recently described the pharmacokinetics of S-equol after the administration of a single dose of SE5-OH. The supplement was formulated into 250 mg capsules containing 5 mg of S-equol. The subjects ($n = 12$) were Caucasian postmenopausal women between 45 and 65 years of age and were randomized into one of two treatment arms, either the 10-mg dose (2 capsules) followed 48 h later by the 30-mg dose (6 capsules), or the 30-mg dose followed by the 10-mg dose. The serum concentrations of total S-equol (conjugated plus unconjugated) at C_{max} for the 10-mg and 30-mg doses were 1,907 ± 693 nM (470 ng/mL) and 4,953 ± 747 nM (1,270 ng/mL), respectively. The plasma concentration of total S-equol at 24 h for the single dose of 30 mg was approximately 40 ng/mL, whereas it was 10 ng/mL for the 10-mg dose. Although this study is not directly relevant to that of Ishiwata et al.,[19] it does show that a steady-state plasma level of S-equol between 10 ng/mL and 40 ng/mL can be achieved by consuming 10–30 mg S-equol per day as SE5-OH.

The mechanism(s) by which S-equol improves VMS is speculative, since it is not even known how hormone replacement therapy alleviates hot flashes. The estrogenic activity of equol was first reported by Thompson et al.[45] Binding studies using rat uterine cytosol showed that equol and estrogen are competitive, but that equol's binding activity was 1% of that of 17β estradiol. 17β estradiol is nonselective for estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) and binds to and activates both estrogen receptors equally. In contrast, S-equol binds with greater affinity for ERβ.[6,46–48] In the study by Setchell et al.,[6] S-equol binds to human ERβ with a $K_i = 0.73$ nM, whereas its affinity for ERα is 6.41 nM. A major unanswered question is whether the improvement in VMS is due to S-equol binding to ERα, to ERβ, or to both estrogen receptors. Animal studies have failed to show efficacy of ERβ-selective ligands in a rat hot flash model, while the ERα-selective compound 1,3,5-tris-4hydroxyphenyl-4propyl-1H-pyrazole blunted the temperature rise induced by naloxone treatment of opiate-dependent animals, suggesting that hot flashes could be prevented by ERα- but not ERβ-selective ligands.[49] However, another study reported that estradiol cypionate was effective in suppressing ovariectomy-induced rises in tail skin temperature in ERα knockout animals,[50] indicating that ERβ is sufficient to maintain control of tail skin temperature. No data have been presented on S-equol in estrogen receptor knockout animals. Another finding demonstrating the importance of ERβ and the control of hot flashes is related to a selective ERβ agonist. Opas et al.[51] showed that ERβ-19, a highly selective ERβ compound, suppressed the elevation in basal rat tail skin temperature caused by ovariectomy. ERβ-19 is a tetrahydrofluorenone with an IC_{50} of 1.8 nM for ERβ and 141 nM for ERα. After 16 days of treatment, ERβ-19 (1 mg/kg) was as effective as estradiol cypionate (0.1 mg/kg) in reducing tail temperature, from 28.7°C to 26.4°C. The selectivity of ERβ-19 for ERβ and not ERα was reflected by the compound's lack of effect on the uterus and the fact that it did not stimulate the expression of uterocalin, which is dependent on the activation of ERα.

Grady et al.[52] are developing an ERβ agonist, MF101 (Menerba®), that has been shown to decrease the frequency of hot flashes. MF101 has been evaluated in preclinical studies and, based on the results of these studies, three classes of ERβ agonists have been proposed.[53] The first class is represented by diarylpropionitrile, which has a 70-fold higher in vitro binding efficacy for ERβ versus ERα and a 170-fold higher potency in transcriptional assays.[54] The Wyeth compound ERB-041 is representative of the second class and has even higher binding affinity (>200-fold) than the Class 1 compounds for ERβ versus ERα and regulates only ERβ genes.[49] MF101 belongs to the third class of ERβ agonists. MF101 is a mixture of compounds from the extracts of 22 different plants. MF101 binds with equal affinity to ERα and ERβ but preferentially regulates genes by ERβ.

In an attempt to determine the selectivity of the genes regulated by S-equol, gene profiling was carried out in lymphocytes isolated from postmenopausal women who were either equol producers or not.[55] Subjects were given isoflavone capsules containing genistein (558 mg/day), daidzein (296 mg/day), and glycitein (44 mg/day). Blood was collected on day 0 and day 84 (the last day of dosing), lymphocytes were collected, and RNA was prepared for microarray hybridization. Of the 5,629 genes examined, there was a change in the expression of 322 genes in the equol nonproducers versus 319 in the equol producers. Of particular interest were those genes that contain estrogen-response elements. Twenty-seven of

these genes were responsive to soy isoflavone treatment, with 10 being overexpressed in the equol producers and only three overexpressed in the nonproducers. In the equol producers, estrogen-related receptor beta and four orphan nuclear receptors (NROB2, NR2C1, NR2F2, and NR113) were the most overexpressed genes. These findings demonstrate that status as an equol producer is associated with selective changes in gene expression. How these changes in gene expression relate to a therapeutic advantage, however, needs more research.

OSTEOPOROSIS

A number of review articles describe the effects of dietary soy and isoflavones on bone.[56–60] In general, controlled studies in humans have shown that soy isoflavone supplements have a positive effect on bone mineral density (BMD). However, the results are variable, and the equol producer status in many studies was not determined. The largest study to show a relationship between soy consumption and the risk of bone fractures is the Shanghai Women's Health Study, in which 75,000 Chinese women aged 40–70 years were monitored for 4.5 years.[61] Soy intake was determined by a questionnaire, and based on the information gathered, the subjects were divided into either low or high soy consumption groups. The mean daily intake of soy isoflavones in the two groups was 8.5 mg (low) and 38.0 mg (high). During the study, 1,770 incident fractures were reported. After adjustment for age and total calorie intake, higher isoflavone consumption was associated with lower risk of bone fractures. After the data were stratified by time since menopause, there was a pronounced positive benefit associated with women taking isoflavones in early menopause. In this study, plasma equol levels were not determined. Thus, it is not possible to relate equol status and incidence of bone fractures. However, it is interesting to speculate, and consistent with other findings discussed below, that women treated during the perimenopausal phase of their life may have a better clinical outcome as opposed to those who wait until after menopause to begin treatment.

Kreijkamp-Kaspers et al.[62] carried out a 12-month double-blind, randomized, placebo-controlled trial in 202 postmenopausal (18 years past menopause) Dutch women receiving 99 mg isoflavones/day (41 mg daidzein) versus placebo. Equol producer status was defined as subjects having a plasma equol concentration of > 83 nmol/L (approximately 20 ng/mL). The proportion of equol producers in the isoflavone group was 29.9%. Both the treated and placebo groups showed a decrease in BMD after 1 year. There were no significant differences in hip and lumbar spine BMD between the two groups. However, the BMD in the intertrochanteric region of the hip was significantly higher in the isoflavone-treated group. Subgroup analysis for the number of years since menopause showed that women in the lowest tertile, with <14 years since menopause, had the largest improvement in BMD, whereas those individuals with >22 years since menopause had a BMD no better than those in the placebo group. Plasma equol levels in these groups were not reported. This study is important because it considered the amount of time since the start of menopause as a factor in the clinical outcome.

Four intervention studies have evaluated the effects of isoflavones on bone, and in these reports plasma equol levels were determined (Table 4). A 2-year, double-blind study in postmenopausal Danish women (mean age, 58.2 years) examined the effects of isoflavones on bone markers of osteoporosis.[63] The isoflavone diet contained soy milk supplemented with isoflavone (76 mg total isoflavones/day), which was compared with alcohol-washed soy milk (control group) that provided only 1.0 mg isoflavones per day. In the control group, there was a greater than 4% decrease in BMD and bone mineral composition (BMC) in the lumbar spine over the 2-year period, which is typical for bone loss in women after menopause. However, the isoflavone-supplemented group showed an overall mean increase of 1.1% and 2.0% for lumbar spine BMD and BMC, respectively. After the data for the isoflavone group were stratified into equol producers (serum equol > 10 ng/mL) and nonproducers, there was an increase of 2.4% in BMD and 2.8% in BMC in the equol producers. Serum equol levels were 44.0 ± 25.2 ng/mL in the equol producers and 3.2 ± 0.74 ng/mL in the nonproducers. In contrast, increases of only 0.6% and 0.3% were observed in lumbar spine BMD and BMC of nonproducers. In this study, there were no differences in the isoflavone-treated group between baseline values and post-treatment values in BMD and BMC of the hip. This is an important study because it is the first to measure serum equol concentrations in a controlled clinical study, but more importantly, the data suggest that equol has bone-building activity in younger postmenopausal women.

Wu et al.[64] carried out a 24-week placebo-controlled study in postmenopausal Japanese women who were within 5 years of their natural menopause. The isoflavone group (33 subjects) received a daily morning dose of 75 mg isoflavone conjugates (38.9 mg daidzin/daidzein) in two capsules; the placebo group (33 subjects) received capsules containing only dextrin. All subjects were allowed to consume their normal diet of soy, equivalent to 40–45 mg isoflavones per day. No significant differences in BMD between the isoflavone and placebo groups were noted in any of the bone areas measured after 24 weeks of treatment. However, when the subjects were stratified into equol producers (defined as >10% conversion of daidzein to equol after a 96-h incubation in fecal

Table 4 **Summary of studies investigating the effects of dietary isoflavones on bone.**

Treatment	Content of daidzin/ daidzein	Population	Clinical endpoint	Plasma S-equol concentration	Reference
Soy milk containing isoflavones (76 mg/day)	38 mg	Danish women	↑ BMD of lumbar spine ↑ BMC of lumbar spine	44 ng/mL plasma S-equol	Lydeking-Olsen et al. (2004)[63]
Isoflavone capsules (75 mg/day)	38.9 mg	Japanese women	↑ BMD of whole body hip	81 ng/mL plasma S-equol	Wu et al. (2006)[64]
Isoflavone capsules (75 mg/day)	38.9 mg	Japanese women	↑ BMD of Total hip and inter-trochanter	150 ng/mL plasma S-equol	Wu et al. (2007)[65]
Isoflavone tablets (67 mg/day)	39 mg	American women (USA)	No effect	>35 ng/mL plasma S-equol	Kenny et al. (2009)[66]

Abbreviations: BMC, bone mineral composition; BMD, bone mineral density.

culture) versus nonproducers (0% conversion of daidzein to equol in fecal culture), there was a significant positive effect on whole-body and hip BMD in the equol producer group. After 6 months of intervention, the mean serum equol concentration in the isoflavone-supplemented subjects who were equol producers was approximately 350 nmol/L or 81 ng/mL, whereas in the isoflavone-supplemented subjects who were not equol producers, the mean equol concentration was <2 ng/mL. In this study, the subjects were allowed to consume their daily diet of soy products. In the placebo group of equol producers who were not given the supplement but were allowed to consume their normal diet, the mean plasma concentration of equol was approximately 60 nmol/L or 14 ng/mL. Thus, one estimate for the average serum concentration of S-equol in Japanese women who are equol producers is 14 ng/mL.

In another study from the same group of investigators, Wu et al.[65] gave 75 mg of isoflavone conjugates to 25 postmenopausal Japanese women for one year and compared the effect on BMD to that in 29 placebo subjects; all subjects were allowed to consume their normal diet. The baseline mean serum concentration of equol in both the placebo group and in the isoflavone-supplemented group was approximately 150 nmol/L or 36 ng/mL. After 1 year of treatment, the equol concentrations were 215 nmol/L (50 ng/mL) and 645 nmol/L (156 ng/mL) for equol producers in the placebo group and in the isoflavone group, respectively; the nonproducers had barely detectable levels of S-equol. Among the equol producers in the isoflavone group, there was a significant increase in total hip and intertrochanteric BMD as compared with the nonproducers.

Kenny et al.[66] conducted a randomized, double-blind, placebo-controlled clinical trial with an isoflavone-supplemented diet in healthy women aged 60 years and older (age range, 60–93 years). The women received either a soy protein isolate (containing 18 g protein) or the soy protein isolate plus three isoflavone tablets containing 22.5 mg isoflavones/tablet or approximately 13 mg/daidzein tablet. Thus, the daily consumption of daidzein was 39 mg. Of the 51 subjects supplemented with isoflavones, 25 were equol producers. The serum levels of equol in the equol producers (defined as >5 ng/mL) was 145 ± 46 nmol/L or 35 ng/mL. After 1 year of treatment, the isoflavone-supplemented group showed no significant improvement in BMD or in markers of bone turnover. Furthermore, there were no significant differences in BMD between equol producers and nonproducers in any of the bones evaluated. It is not obvious as to why there was no effect on BMD. The isoflavone tablet provided sufficient daidzein, and the criteria for being an equol producer was >5 ng/mL. However, it should be pointed out that the women in the study were

60 years of age and older (mean age, 73.1 ± 5.9 years), and there is growing evidence that women become less responsive to treatment after menopause.

The mechanism for S-equol's effect on bone is not known. It is known that 17β estradiol plays a critical role in the osteoblast to build bone and is bone sparing in the osteoclast. ERβ is expressed in both osteoblasts and osteoclasts.[67] Thus, S-equol may activate ERβ in order to regulate the expression of bone-specific genes. One preliminary report has described the effects of S-equol on bone in a rat model of osteoporosis.[68] Animals were given S-equol (30 or 100 mg/kg) for 3 months, and the results were compared with those obtained with 17β estradiol benzoate (0.4 mg/kg) and placebo. Vertebral compression strength and femoral three-point bending strength in the rats treated with high-dose S-equol (100 mg/kg) were comparable with results found in the estrogen-treated and sham-treated groups and were significantly higher than values obtained in the ovariectomized placebo group or the low-dose (30 mg/kg) S-equol group. Quantitative histomorphometry demonstrated that high-dose S-equol preserved vertebral bone volume and femoral cortical thickness. There were no differences in osteoclast numbers, and peripheral quantitative computed tomography revealed no effect on BMD. These findings show that S-equol (100 mg/kg) preserved vertebral and femoral bone strength and bone volume comparable to that in sham-operated rats or in those with estrogen replacement.

DISEASES OF THE PROSTATE

It is well known that both the incidence of and mortality due to prostate cancer are much lower in Asians compared with Western populations. Although there may be many reasons for this difference, one consistent finding is that the intake of phytoestrogens, plant-origin isoflavones, and lignans is higher in Asian populations. Yan and Spitznagel[69] reviewed the epidemiological studies that relate soy consumption to the risk of prostate cancer. An overall risk estimate of 0.74 ($P < 0.01$) was determined, suggesting that the consumption of soy is associated with a lower risk of prostate cancer. For isoflavone consumption, the risk was 0.88 ($P = 0.09$), but further analysis showed that in Asian populations the risk was 0.55 ($P = 0.001$). Not all the studies are positive for this relationship, however. The European Prospective Investigation into Cancer and Nutrition Study,[70] the European Prospective into Cancer – Norfolk Study,[71] and a nested case-control study in the Multiethnic Cohort[72] found no evidence to support an inverse association between isoflavone exposure and prostate cancer risk. It is important to note that the negative studies were carried out in non-Asians, and in the eight European countries where plasma equol levels were reported, they were <1.7 ng/mL, which is below the level of >5 ng/mL considered to indicate an equol producer.[25]

In the studies carried out in Japan, Akaza et al.[73] found no significant difference in serum equol concentrations in 141 subjects with prostate cancer and 112 cancer-free controls. However, a subgroup analysis based on the severity of the disease indicated that the percentage of equol producers (plasma levels of equol > 10 ng/mL) in the subjects with a poor histological grade was 25% versus 44% in subjects with lower-severity disease. In other studies, Akaza et al.[74] and Fujimoto et al.[20] compared the equol producer status in Japanese, Korean, and American men with prostate cancer and in control subjects; the equol producer status was defined as those subjects with a serum equol concentration above the lower limit of detection of the assay system (>0.5 ng/mL). Both the Japanese and the Korean men showed a significantly lower percentage of equol producers in the subjects with prostate cancer. The percentage of equol producers in the American men was less than 17% in both groups. Interestingly, the percentage of equol producers in younger men in Japan and Korea is significantly less than that in older men.[20]

In a case-control study among Japanese subjects (Japan Collaborative Cohort Study),[75] serum equol was measured in 35 men with prostate cancer and the results were compared with those obtained from 113 control subjects. The mean serum equol concentration in the patients was 33.6 nM (8.1 ng/mL) versus 55.4 nM (13.4 ng/mL) ($P < 0.04$) in the control subjects. The proportion of equol producers (defined as serum equol > 1.9 nM) did not differ between the two groups (67.3% for cases and 75.3% for controls). Moreover, in this study there were no differences in serum total testosterone or sex hormone-binding globulin.

Kurahashi et al.[76] conducted a nested case-control study (Japan Public Health Center-Based Prospective Study) in 14,203 men between the ages of 40 and 69 years. Observations were made between 1990 and 2005. After follow-up (mean period of 12.8 years), 201 patients with prostate cancer were identified. The soy food intake for the cancer patients and 402 control subjects was not significantly different, 61.4 g/day and 60.5 g/day, respectively. The plasma equol concentration in the cases was 3.7 ng/mL versus 4.7 ng/mL in the control group. When plasma equol concentrations were stratified into low (<1 ng/mL), middle (1–15 ng/mL), and high (>15 ng/mL) levels, there was a dose-dependent decrease in the risk of prostate cancer ($P < 0.04$). This relationship was strongest in the patients with localized disease. Those patients with advanced prostatic cancer did not show this relationship. While these epidemiological studies are interesting and argue for a relationship between plasma levels of equol

and the incidence of prostate cancer, placebo-controlled studies using pure S-equol are needed to prove that the compound is effective for the treatment or prevention of prostate cancer. Nonetheless, the data suggest that a plasma equol concentration of >10–15 ng/mL may have a therapeutic benefit.

Only one preclinical study has examined the in vitro activity of S-equol on the growth of cancer cells[77]; all the other studies used racemic equol. Magee et al.[77] determined the effects of S-equol on the growth of human breast and prostate cell lines in culture. S-equol inhibited growth of the ER-negative cell line MDA-MB (21% inhibition at 10 μM); S-equol had no effect on MCF-7 (estrogen receptor positive) or MCF-10A (normal, nontumorigenic) cells. These results suggest that S-equol should not stimulate the growth of ER-positive breast cancer cells, which is not unexpected since the compound is ERβ selective. S-equol was also evaluated in prostate cancer cell lines having a wild-type androgen receptor (AR) (LAPC-4), a mutated but functional AR (LNCaP), and a cell line with no AR (PC-3). S-equol had no effect on the growth of the AR-negative cell line. However, the compound inhibited growth of the normal and mutated AR cell lines, with 40% and 25% inhibition, respectively, at an S-equol concentration of 20 μM. Although this is a high concentration, the concentration of S-equol in the prostate tissue of two men with prostate cancer who were given daidzein and were equol producers was reported to be 5 μM and 10 μM.[78] Hedlund et al.[78,79] showed a 30% inhibition of growth of LNCaP and LAPC-4 cells with racemic equol (10 μM). Equol also had a significant inhibitory effect on the growth of a benign prostatic cell line (PrEC). At 10 μM, equol caused an 80% inhibition of cell growth. The IC_{50} was 1.8 μM. This concentration of equol is within the range of concentrations observed in the prostate of equol producers and may explain why Asians have a lower incidence of prostate cancer and benign prostatic hyperplasia compared with men from Western countries.

One possible mechanism by which S-equol inhibits prostate cell growth is by downregulation of the AR. Lund et al.[80] fed male Long-Evans rats a phytoestrogen-free diet or one containing 600 μg of phytoestrogens per gram of diet. Compared to the phytoestrogen-free diet, there was a significant decrease in prostate weight after 55 days in the animals on the phytoestrogen-supplemented diet. The concentration of equol in the prostate of animals on the phytoestrogen diet was 100 ng/g tissue or approximately 0.4 μM. In situ hybridization was used to quantify the amount of the AR in the prostate of animals fed the phytoestrogen-containing diet versus the phytoestrogen-free diet. Animals fed the phytoestrogen diet showed a 50% reduction in the expression of the AR. Hamilton-Reeves et al.[81] carried out a similar experiment in men at high risk of prostate cancer. Fifty-eight men were divided into three groups and were assigned to consume one of three diets: soy protein diet (107 mg isoflavones/day); alcohol-washed soy protein diet (<6 mg isoflavones/day); or milk protein isolate diet (0 mg isoflavones/day). After 6 months on these diets, the isoflavone-supplemented diet was associated with a significant reduction in the expression of the AR in the prostate; there was no effect on the expression of ERβ, and plasma levels of testosterone and dihydrotestosterone were unchanged.

Another selective ERβ agonist that has been shown to decrease prostate weight is the benzopyran SERB-1.[82] This compound has a structure and ER-binding activity similar to that of S-equol (K_i = 0.19 nM for ERβ versus a K_i = 2.68 nM for ERα). SERB-1 was given orally to male mice for 7 days, and prostate weight was determined. The compound showed a dose-dependent (0.01–0.05 mg/kg) reduction in prostate weight, with no effect on the testes, seminal vesicle, or plasma levels of testosterone or dihydrotestosterone. These data provide further evidence that an ERβ-selective agonist has therapeutic usefulness for diseases of the prostate, including benign prostatic hyperplasia and prostate cancer.[83]

CARDIOVASCULAR DISEASES

The WHO CARDIAC study determined the relationship between the mortality rate of coronary heart disease and the consumption of soy products and isoflavones.[84] The amount of isoflavones excreted in a 24-h collection was used as an indicator of soy consumption. A study of the age-adjusted mortality rate in individuals in Japan and China with >10 μmol isoflavones excreted per day showed that these individuals had a much lower rate of coronary heart disease (<100 events per 100,000 people) than populations with <1 μmol/day (>500 events per 100,000 people). The potential benefits of soy and isoflavones for the treatment and prevention of cardiovascular diseases have been reviewed.[85] In 1999 the US Food and Drug Administration approved a health claim for the cholesterol-lowering effects of soy protein. With respect to equol producer status and cardiovascular disease, Thorp et al.[86] and Liu et al.[87] reported no differences in plasma total cholesterol, low-density lipoprotein (LDL) cholesterol, or high-density lipoprotein (HDL) cholesterol between equol producers and nonproducers. Likewise, Kreijkamp-Kaspers et al.[62] found no difference in diastolic or systolic blood pressure, and Hall et al.[88] reported no difference in inflammatory biomarkers. In contrast, Clerici et al.[89] tested a novel, soy-germ pasta naturally enriched with isoflavones that contained only aglycones; the glycosides were removed by the manufacturing process. In this randomized, controlled study, 62 adults with hypercholesterolemia consumed a diet that

included an 80-g serving per day of pasta containing 33 mg of isoflavones, as aglycones. After 4 weeks of consumption, the enriched pasta had a significant effect on lowering serum total cholesterol and LDL-cholesterol; HDL-cholesterol and triglycerides were not changed. C-reactive protein was also significantly reduced compared with levels in the group that consumed pasta without the isoflavones, suggesting that equol has an anti-inflammatory effect. Twenty of the subjects consuming the isoflavone-enriched soy pasta were equol producers and nine were not. The serum total equol concentration in the equol producers was 105 ± 17 nmol/L (25.5 ng/mL), compared with 9.5 ± 1.6 nmol/L (2.3 ng/mL) in the nonproducers. The equol producers showed a significant 15 ± 7 mg/dL decrease in LDL-cholesterol and a 0.9 ± 0.5 mg/L decrease in C-reactive protein. These data provide the most convincing evidence that equol-producing individuals may have cardiovascular benefits.

The mechanism(s) of equol's effect on cardiovascular disease is not known with certainty. Equol is a potent antioxidant, and with this activity it could prevent the oxidation of LDL and uptake by macrophages. Free unconjugated S-equol contains two phenolic groups that function to scavenge free radicals in solution. Using an in vitro system for the oxidation of LDL after the addition of copper, Rüfer and Kulling[90] compared the antioxidant properties of racemic equol to other isoflavones and to the positive control, ascorbic acid; the EC_{50} for equol was approximately 6 µM compared to 19 µM for daidzein and 10 µM for ascorbic acid. Hwang et al.[91] reported that ascorbic acid and equol were synergistic in inhibiting the metal-induced oxidation of LDL. These investigators postulated that the antioxidant activity of equol and ascorbic acid cannot account for all of the synergism. They proposed that the primary mechanism by which equol contributes to the prevention of LDL oxidation is by stabilizing the structure of apolipoprotein B, the major protein of LDL, in order to suppress the propagation of free-radical chain reactions, thus increasing the efficacy of vitamin E and other fat-soluble antioxidants present in LDL.

The importance of cell-associated equol in the prevention of cellular damage due to oxidized LDL has been studied in the human macrophage cell line, J774.[92] In these experiments, J774 cells were activated with lipopolysaccharide and the cells were then incubated with freshly prepared LDL. In the absence of equol, LDL is oxidatively modified to produce an electronegative, modified LDL^-. Preincubation of the J774 cells with 0.5 µM racemic equol reduced the amount of oxidized LDL formed from 4.1% to 2.0% after 8 h; without pretreatment, equol had no effect on the amount of LDL^- formed. Equol-pretreated J774 cells also produced less superoxide (O_2^-). The O_2^- formation rates were 2.01, 1.97, and 1.52 nmol O_2^-/min/mg protein for control cells, for control cells to which equol was added, and for equol-pretreated cells, respectively. The investigators proposed that the equol in the pretreated cells prevented the inactivation of the reduced nicotinamide adenine dinucleotide phosphate oxidase complex[92]. With decreased O_2^- production, the levels of nitric oxide (NO) increased, and the investigators suggested that the increased level of NO prevents LDL modification.

Prevention of the cytotoxicity of oxidized LDL by equol in human umbilical vein endothelial cells has been examined by Kamiyama et al.[93] Treatment of the cells with copper caused oxidization of LDL and a >3-fold increase in cell death for up to 48 h post treatment. Pretreatment of the endothelial cells with 1 µM racemic equol suppressed the induction of apoptosis by oxidized LDL. To understand the mechanism for the cytotoxicity of oxidized LDL, Kamiyama et al.[93] used real-time polymerase chain reaction and showed that oxidized LDL increased the expression of NAD(P)H oxidase, the enzyme that produces superoxide. Equol pretreatment prevented the increase in NAD(P)H oxidase. Since equol prevents LDL oxidation, the authors concluded that equol may be protective against atherosclerosis and coronary heart disease.

Equol may also protect against cardiovascular diseases by increasing the transcription of endothelial nitric oxide synthase (eNOS) and redox-sensitive genes.[94,95] The target gene eNOS contains an estrogen-response element, and it can be reasoned that the binding of S-equol to ERβ may be responsible for enhanced eNOS expression. NO can react with superoxide anions to form peroxynitrite. NO and peroxynitrite then, in turn, enhance the nuclear accumulation of Nrf2, nuclear factor (erythroid-derived 2)-like 2, which binds to an antioxidant response element in target genes to enhance the transcription of the phase II antioxidant defense enzymes, such as superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase, and heme oxygenase-1.

CONCLUSION

This review summarizes those studies that have shown an effect of isoflavone supplements on menopausal symptoms, bone, prostate cancer, and cardiovascular biomarkers. The review has been further refined to examine those studies in which the equol producer status was determined, particularly the serum or plasma levels of S-equol. The criteria for being an equol producer is not consistent across all the studies, with some reports using 24-h urine collection and others using total plasma S-equol (unconjugated plus conjugated equol). Nonetheless, the emerging evidence to date suggests that being an equol producer has a clinical benefit in some people. A

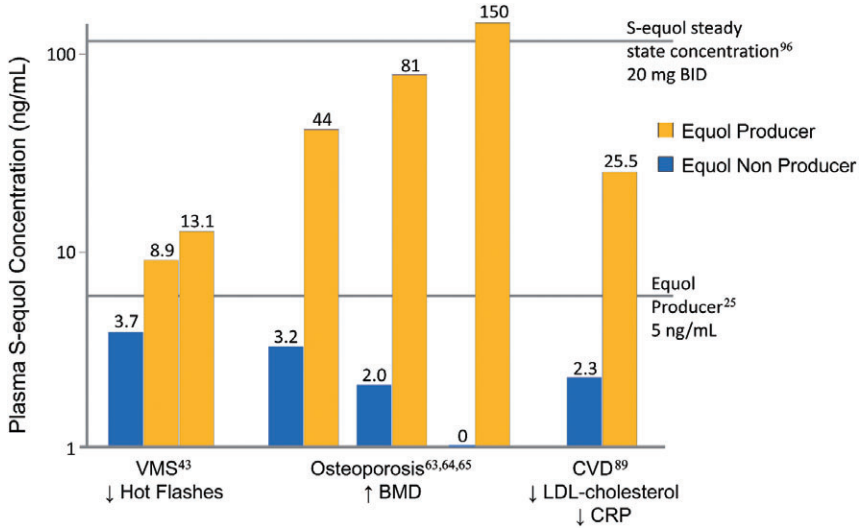

Figure 4 **Plasma S-equol concentrations in intervention studies that showed a clinically beneficial effect on vasomotor symptoms (VMS), osteoporosis, and cardiovascular diseases (CVD). The numbers above each bar are the reported plasma concentrations of S-equol (ng/mL). BMD, bone mineral density; CRP, C-reactive protein.**

major limitation of all the studies performed to date is that total equol, i.e., unconjugated plus conjugated equol, was determined. Since only free, unconjugated S-equol binds to ERβ with high affinity, total equol does not provide a measurement of the active form of the compound. Nonetheless, a number of conclusions can be reached from this review. The precursor of S-equol is daidzein. Thus, to be an equol producer, one has to consume sufficient soy, isoflavone supplements, or daidzein itself; the amount of daidzein needed to show a clinical benefit ranges from 20 mg to 40 mg daily. Secondly, one must be able to biotransform daidzein to S-equol and to achieve a clinically beneficial plasma level of S-equol. Subjects in some studies consumed sufficient daidzein to produce equol but were unable to efficiently metabolize daidzein to equol at plasma levels high enough to provide a therapeutic benefit. Other, much less clear requirements for producing equol are the role of diet and other environmental factors. Figure 4 shows the plasma levels of total S-equol in those intervention studies in which a positive clinical effect was noted for menopausal symptoms (decrease in the frequency of hot flashes), osteoporosis (increase in BMD, BMC), and cardiovascular biomarkers (decrease in LDL-cholesterol and C-reactive protein). The plasma levels of S-equol in these positive studies are all >5 ng/mL, the concentration that defines an equol producer.[25]

Also shown in Figure 4 is the steady-state plasma concentration of total S-equol (113 ng/mL) in male and female subjects who were given 20 mg of S-equol twice a day for 14 days (Jackson et al. [2011]).[96] Thus, based on a person's ability to produce S-equol from dietary daidzein and the observed clinical outcome, a pharmaceutical dose of S-equol of 20 mg given twice daily should provide appropriate blood levels of S-equol for a clinical benefit. However, only controlled clinical studies with pure S-equol will confirm these predictions. In the only study to date in which S-equol was given as a nutraceutical supplement,[19] a positive effect on menopausal symptoms in Japanese women was observed when 10 mg of S-equol was given three times a day, consistent with the above prediction.

An important question for future research is an understanding of the mechanism(s) of S-equol's actions for multiple therapeutic indications. The most likely mechanism is that S-equol activates ERβ to regulate the expression of specific genes. Takahashi et al.[97] attempted to establish a molecular signature for equol using the human androgen-responsive prostate cancer cell line LNCaP. Exposure of these cells to racemic equol led to downregulation of a number of androgen-responsive genes, including prostate-specific antigen and transmembrane protease serine 2; cell cycle-related genes, including ASK, a regulatory unit of CDC7 kinase involved in the G/S transition; and the MAP kinase pathway gene $DUSP_4$ that dephosphorylates the MAP kinases ERK1 and ERK2. Thus, for diseases of the prostate, it is proposed that S-equol activates ERβ to downregulate the AR, prostate-specific antigen, and transmembrane protease serine 2, resulting in inhibition of cell growth.

S-equol's selectivity for ERβ may provide a better safety profile than those compounds that are selective for ERα. In the menopausal study by Cheng et al.,[98] equol producers showed no increase in endometrial thickness as measured by ultrasound after 12 weeks of treatment with 60 mg isoflavones. In this study, mammography was

also performed before and after treatment; no sign of cell proliferation in the breast was noted. Furthermore, the expression of ERα was not stimulated by treatment. For both the endometrium and breast tissue, no stimulation of proliferation by the isoflavone supplement was found using the proliferation marker K_i67. While long-term safety studies in women will be required, the evidence to date suggests that S-equol is safe at doses that exceed those associated with a therapeutic benefit.

Another important finding from this review relates to the age of the subjects at the time of treatment and the clinical benefit. For the treatment of menopausal symptoms and osteoporosis, the relationship between equol producer status and positive clinical outcome appears to be strongest in women younger than 60 years of age. Recent subanalysis of the Women's Health Initiative trials also suggests that women who initiated hormone replacement treatment less than 10 years post menopause had a lower risk of future cardiovascular events.[99] Women with VMS who were not given hormone therapy until 10 years after menopause had the greatest risk of developing cardiovascular disease.

In summary, S-equol represents a novel compound for further development as a therapeutic agent for the aging population. Additional research is needed to evaluate the safety and efficacy of S-equol in controlled clinical trials.

Acknowledgments

Declaration of interest. All authors are employed by and RLJ and RJS hold equity in Ausio Pharmaceuticals, LLC, a clinical-stage biotechnology development company whose lead product is a nonsteroidal, nonhormonal, small molecule (S-equol) estrogen receptor β agonist.

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