Toxicological Assessment of the Phytoestrogen Equol

Equol is a metabolite of daidzein (see Fig. 1). Both compounds belong to the chemical class of isoflavones. Whereas daidzein is present in soybean or red clover, equol is not present in plants, but is formed by bacterial metabolism in the gut of the host.

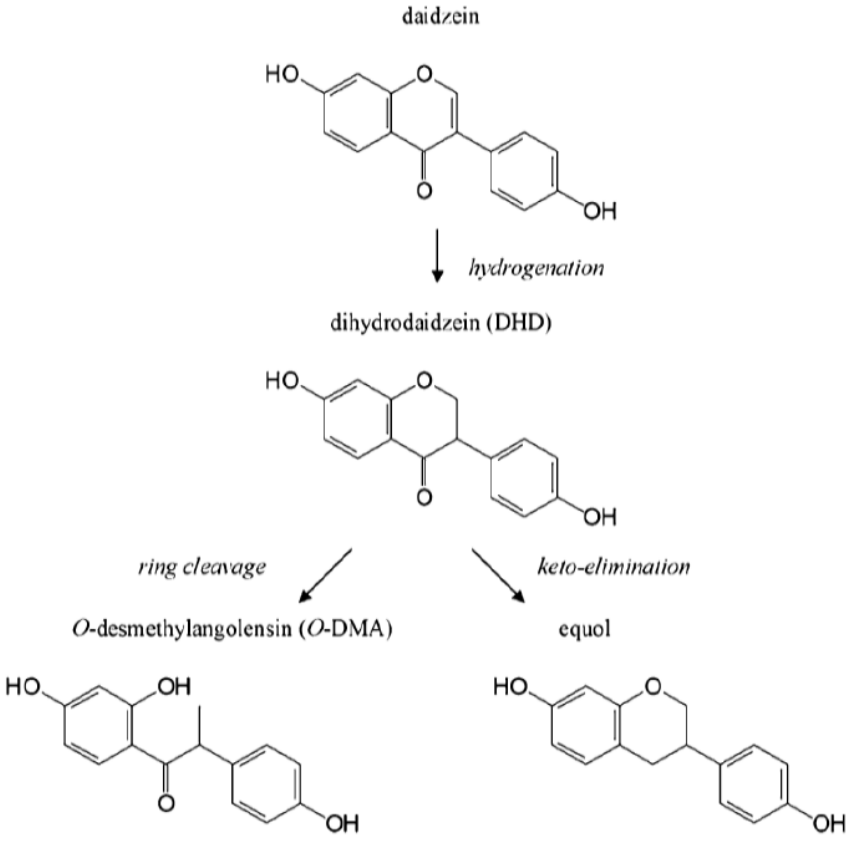

Fig. 1: Proposed pathway for the reductive metabolism of daidzein by intestinal bacteria

Due to its structural similarity with estradiol (see Fig 2), equol binds to the estrogen receptor β with high affinity (K(i) = 0.73 nmol/l [1].

Fig. 2: Structural similarity between Estradiol and Equol [2]

Equol is planned to be administered to women in order to improve menopausal symptoms in a controlled study at a dose level of up to 80 mg/day (which corresponds to 1.6 mg/kg/day if a bodyweight of 50 kg/person is assumed).

However, the binding of isoflavones to estrogen receptors is not only the reason for their postulated beneficial effects, but there is also a risk of adverse effects due to this binding.

Therefore, although equol is not considered to be a drug substance, but to be a food additive, a toxicological assessment of equol is presented here, applying similar principles as normally used in recent drug development.

The following toxicological issues should be covered by preclinical studies: Genotoxicity, single dose toxicity, repeated dose toxicity, immunotoxicity (including sensitizing potential) and (if women of childbearing potential are intended to be treated) reproduction toxicity.

In the present assessment, the available literature on these topics is reviewed in order to assess the safety of equol administration and to identify potential gaps in the available information.

In some cases, it is also useful to investigate toxic effects of daidzein, as it is metabolized to equol in the gut of several species. Rat studies of daidzain are only of limited value, however, as it is discussed controversely, if rats are able to convert daidzain to equol [3, 4, 5, 6]. Therefore, only rat studies in which exposure to equol was confirmed are reported here.

Genotoxicity

A functional food ingredient relying on bacterial conversion of daidzein to equol called SE5-OH has been studied for its potential genotoxicity. SE5-OH was negative in *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and in *Escherichia coli* tester strain WP2uvrA with and without metabolic activation at concentrations of up to 5,000 µg SE5-OH per plate (corresponding to approx. 26 µg of equol per plate). SE5-OH was negative for chromosome aberrations in chinese hamster lung cells up to 3,000 µg/ml (corresponding to approx. 16 µg/ml equol) with and without metabolic activation and did not induce increases in micronucleated polychromatic erythrocytes taken from Sprague-Dawley rats administered (via gavage) up to 4,000 mg/kg SE5-OH (corresponding to approx. 21 mg/kg equol) twice daily for two consecutive days [7].

The genotoxicity of some daidzein metabolites was assessed in vitro by analyzing effects on cell cycle distribution and cell morphology as well as the induction of micronuclei in cultured human Ishikawa cells. Equol caused a slight increase in G1 and decrease in S phase of the cell cycle, and slightly but significantly induced kinetochore-positive as well as kinetochore-negative micronuclei and an elevated proportion of abnormal mitotic spindles [8].

In an *in vitro* micronucleus assay in V79 cells equol caused an increase in the number of micronuclei up to 25 µM with no further increase at higher concentrations. Additional staining with anti-kinetochore (CREST) antibodies revealed that Equol induced mostly

CREST(+) micronuclei indicative of an aneugenic action rather than acentric fragments [9].

In an *in vitro* micronucleus assay in L5178Y mouse lymphoma cells, equol induced micronuclei in a concentration-dependent manner (2.3-fold induction at 100 µM) [10].

Sierens *et al*. report that treatment of human lymphocytes with equol, as well as with genistein, offered protection against hydrogen peroxide induced DNA strand breaks as demonstrated by a comet assay [11].

So, in conclusion it can be stated that equol produces genotoxic effects in *in vitro* tests. There are, however, damaging effects reported as well as protective effects. The only available *in vivo* study, however, could not confirm these effects.

As the equol content in this *in vivo* study, was, however, only 5.24 mg/g, the total amount of equol administered to the rats was only about 21 mg/kg body weight. This is far too low to satisfy the requirements of current guidelines to genotoxic testing and is furthermore far below the dose intended to administer to humans.

Therefore, it is recommended to perform an *in vivo* test for chromosomal aberrations with isolated equol at doses that match current guidelines. For the same reason, it is recommended to repeat the Ames test with isolated equol at concentrations that match current guidelines.

Single dose toxicity

SE5-OH, a functional food ingredient with an equol content of 5.24 mg/g was administered via gavage to five male and five female rats in two oral gavage doses of 2000 mg/kg, separated by four hours, for a total acute dose of 4000 mg/kg. Mortalities did not occur in treated rats or control rats, and there were no treatment-related changes in clinical signs, food consumption and body weights. Necropsy findings

showed no treatment-related effects Thus, the rat oral LD_{50} was >4,000 mg/kg for of SE5-OH or > 21 mg/kg for equol [7].

Repeated dose toxicity

SE5-OH, a functional food ingredient with an equol content of 5.97 mg/g, was administered via gavage to groups of ten or fifteen male and female rats at dose levels of 1000 and 2000 mg/kg (corresponding to 5.97mg/kg/day or 11.94 mg/kg/day, respectively) for 92 days.

There were no deaths in either the control or treated rats throughout the study. Treated male and female rats gained body weight throughout the study at the same rates as did control rats. There were no remarkable pathologies observed following microscopic or macroscopic examination from any group of rats. Water consumption rates, organ weights, and behavioral observations revealed no significant differences between groups of animals. Overall, there were no significant differences observed between animals treated with SE5-OH and vehicle-treated control animals. Therefore, the NOAEL for the 91-day study was 2,000 mg/kg/day SE5-OH, corresponding to 11.94 mg/kg/day equol. At this dose level, an equol serum level of 8.21 to 14.20 ng/ml was achieved [7].

In a study on reproduction toxicity of daidzein [5], virgin female rats were administered 0, 250, or 1000 mg daidzein/kg diet, starting 2 weeks prior to breeding (10 females/group). This treatment resulted in blood concentrations of 529 $\pm$ 201 nM (corresponding to 128 ng/ml, for the 250 mg/kg diet,) or 4462 $\pm$ 1551 nM (corresponding to 1080 ng/ml, for the 1000 mg/kg diet).

As in this study equol was not administered to the rats, but was metabolized from daidzein, the equol blood concentrations are used to compare equol exposures to the study of Yee et al [7]. In the 250 mg/kg diet group, the blood concentration is approx. tenfold if compared to the 2000 mg/kg/d SE5-OH. It seems reasonable to conclude that

the exposure in the Lamartiniere study [5] is that which would be expected for an oral equol administration of 120 mg/kg/day (linearity in dose/bioavailability is presumed).

Reproduction Toxicity

Equol is developed for treatment of menopausal symptoms. Therefore, it is not intended to be adminstered to women of childbearing potential. Reproduction toxicity studies therefore are not considered necessary.

Immunotoxicity

Gredel *et al*. investigated the effects of genistein, daidzein, matairesinol, and secoisolariciresinol, including metabolites such as equol, O-desmethylangolensin, enterodiol, and enterolactone on natural killer cell activity, proliferation, cytokine secretion, as well as apoptotic and necrotic rate of human leukocytes. Genistein, daidzein, and its metabolite equol were the most potent inhibitors of leukocyte functions. Ten µM of genistein decreased proliferation, lytic activity of natural killer cells, and cytokine secretions. The latter proved to be the most sensitive marker of immune functions.

Carcinogenicity

Due to their well-established estrogenic properties in cell culture and rodent models, soy isoflavonoids are suspected to promote development of uterine and breast cancers. Equol is intended to be administered to women for a long period after menopause. Therefore a review of the available literature on carcinogenicity of equol is presented in the following section.

Preclinical data

Thirty adult female ovariectomized Cynomolgus monkeys (*Macaca fascicularis*) were randomized to receive a control diet 1) alone, 2) with 509 mg/day of the soy isoflavones genistein and daidzein (IF), or 3) with 1020 mg/day of racemic equol (EQ), an isoflavan, for approximately 1 month. Total serum isoflavonoid levels 4 h postfeeding were <20 nmol/l, 2570.7 nmol/l, and 6944.8 nmol/l for control, IF, and EQ groups, respectively. Equol was the predominant serum isoflavonoid in both IF (72.5%) and EQ (99.7%) groups. Uterine weight, endometrial thickness, glandular area, and epithelial proliferation in the uterus were not significantly different among treatment groups. Endometrial progesterone receptor gene expression was significantly increased in the IF group, while protein expression was not altered. Within the mammary gland, proliferation and indicators of estrogen exposure did not differ among treatment groups. These findings indicate that high doses of dietary soy isoflavonoids have minimal uterotrophic or mammotrophic effects in an established primate model [2].

In a study in the hairless mouse, the authors examined the potential of the isoflavone to protect from skin carcinogenesis. Daily topical applications of equol lotions significantly protected against skin carcinogenesis induced by chronic exposure to solar-simulated UV radiation (SSUV) or by topical treatment with the chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) or by the combined cocarcinogenic treatment of DMBA followed by chronic SSUV. Monitoring of tumor development for 40 weeks showed significantly delayed tumor appearance and reduced tumor multiplicity in all equol-treated groups. In mice treated with either SSUV or DMBA + SSUV, equol significantly reduced the proportion of tumors progressing from benign papillomas to malignant squamous cell carcinoma (SCC) by 33-58% and reduced the average diameter of SCC by 71-82%. In a short-term study, equol dose dependently inhibited the SSUV induction of the tumor promotion biomarker enzyme, ornithine decarboxylase, in the skin, suggesting the anticarcinogenic activity of equol may be attributed to its inhibition of the tumor promotion phase of carcinogenesis [13].

Daidzein and (+/-)-equol were reported to have proliferative effects on MCF-7 cell growth *in vitro*. In athymic mice, dietary daidzein had a slight but significant stimulatory effect on tumor growth, whereas (+/-)-equol did not stimulate the growth of estrogen-dependent breast tumor growth, increase the cell proliferation in tumors, or induce an estrogen-responsive pS2 expression, with plasma concentrations being at the same concentration level as in the in vitro experiments. These results suggest that pharmacokinetic and/or metabolic factors attenuate the estrogenic effects of daidzein and equol *in vivo* [14].

Clinical data

Adlercreutz *et al.* concluded that lignans and isoflavonoids may influence sex hormone metabolism and cancer by influencing plasma sex hormone binding globulin levels resulting in lower uptake and less biological activity of these steroids and by inhibiting growth and proliferation of hormone-dependent cancer cells [15].

In a recent case-control study in 950 cancer prostate cases and 1042 matched control participants, Travis *et al*. found that higher concentrations of circulating genistein may reduce the risk of prostate cancer but elevated equol concentrations do not [16].

This is, in the view on equol, confirmed by Venkitaraman *et al*, who did not find a significant association between time to disease progression, adverse histopathology on repeat biopsy or PSA velocity and urinalry levels of daidzein, genistein, enterolactone or equol [17].

In the European Prospective into Cancer-Norfolk study, no evidence to support an inverse association between phytoestrogen exposure and prostate or colorectal cancer risk was found [18]. (Ward et al, 2008)

Pendleton *et al*., however, found in a Phase II study with prostate cancer patients a decreased rise in serum PSA when treated with isoflavones if compared to values before treatment [19].

This is confirmed by a Japanese nested case-control study with 14,203 men, where the highest tertile for plasma equol was significantly associated with a decreased risk of total prostate cancer (odd ratio = 0.60; 95% confidence interval, 0.36 to 0.99; P(trend) = .04). The authors concluded that Isoflavones may prevent the development of prostate cancer [20].

Mammographic density, an established marker of breast cancer risk is not influenced by daidzein metabolizing phenotype (equol producing capacity) as shown in clinical studies with postmenopausal women [21, 22].

In a nested case-control study, breast cancer odds ratios were calculated for tertiles of phytoestrogen plasma levels using conditional logistic regression analysis. For genistein, the risk estimate for the highest versus the lowest tertile was 0.68 (95% CI, 0.47 to 0.98). Similar protective effects, although not statistically significant, were seen for the other isoflavones (daidzein, glycitein, O-desmethylangolensin, and equol) [23].

In contrast to these studies is the outcome of the Cancer-Norfolk cohort study. There, breast cancer risk was marginally increased with higher levels of total urinary isoflavones; among those with oestrogen receptor-positive tumours, the risk of breast cancer was increased with higher levels of urinary equol [24].

In conclusion, it can be stated that evidence for a protective effect of equol against hormone related cancers like mammal cancer or prostate cancer is equivocal. Although in the Cancer-Norfolk cohort study hints to an increased risk of breast cancer are found in a subgroup, there are other studies claiming a protective effect for equol. At the present state of science, it is, therefore, reasonable to assume that the risk for developing breast cancer for equol treated women is not increased by this treatment.

Stereochemical considerations

S-equol is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora as demonstrated with the use of chiral-phase HPLC and mass spectrometry. Both enantiomers are bioavailable after oral administration. S-equol has a high affinity for estrogen receptor beta (K(i) = 0.73 nmol/L), whereas R-equol is relatively inactive [1].

It has been postulated that the R- and S-equol enantiomers have different biological properties given these different binding affinities for the estrogen receptor. Magee *et al*. have compared the biological effects of purified S-equol to that of racemic (R and S) equol on breast and prostate cancer cells of varying receptor status *in vitro*. Both racemic and S-equol inhibited the growth of the breast cancer cell line (> or = 10 µM) and two prostate cancer cell lines (> or = 5 µM and > or = 2.5 µM). The compounds also showed equipotent effects in inhibiting the invasion of two cancer cell lines through matrigel.

S-equol (1, 10, 30 µM) was unable to prevent DNA damage in breast cells following exposure to 2-hydroxy-4-nonenal, menadione, or benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide. In contrast, racemic equol (10, 30 µM) prevented DNA damage in MCF-10A cells following exposure to 2-hydroxy-4-nonenal or menadione. These findings suggest that racemic equol has strong antigenotoxic activity in contrast to the purified S-equol enantiomer implicating the R-, rather than the S-enantiomer as being responsible for the antioxidant effects of equol, a finding that may have implications for the *in vivo* chemoprotective properties of equol [25].

So it seems that the estrogen receptor mediated effects are due to S-equol, whereas the antioxidant effects can be assigned to the R-form.

Conclusions

The genotoxic potential of equol is unclear. In *in vitro* experiments protective effects against chromosomal damage as well as damaging effects are reported. The only available *in vivo* study is of limited value, as the equol concentrations achieved are far below the requiements of current guidelines. It is therefore recommended to perform an Ames test and an *in vivo* chromosomal aberration study with isolated equol at sufficient high concentrations/doses.

The single dose toxicity study shows that the LD_{50} in rats is higher than 21 mg/kg. This is by a factor of 13 higher than the intended dose in humans. If a correction factor of 10 for the species difference is applied, the safety margin is only 1.3. It has to be taken into account, however, that in the rat study there was no toxic effect found at all and that the highest dose tested was limited by technical reasons only. It is therefore not expected to induce any risk to humans if single doses of up to 80 mg/person are administered.

In a 90 day oral rat toxicity study, the NOAEL was found to be 11.94 mg/kg/day, which is more than sevenfold the intended human dose. Again it has to be highlighted, that in this study the highest dose was limited due to technical reasons. In a reproduction toxicity study, 10-fold higher blood concentrations of equol could be achieved. Therefore, it can be concluded that up to an oral dose of 120 mg/kg/day, no adverse effects were observed in rats. It is therefore not expected, that repeated adminstration of up to 1.6 mg/kg/day induces a risk for the test persons.

In vitro immunotoxicity data indicate that there may be a risk for immunosupression after equol treatment. Tests on leucocyte and natural killer cell function should therefore be included in a human study.

At the moment it is unclear if there is a carcinogenicity risk after the prolonged exposure to equol. If the genotoxicity studies proposed above reveal no genotoxic potential, the administration of equol for a short study period is not expected to result in a significant risk for the volunteers.

Seelze, 07.06.2009

Prof. Dr. Stefan Hockertz
European reg. Toxicologist

References

[1] Setchell K DR, Clerici C, Lephart E D, Cole S J, Heenan C, Castellani D, Wolfe B E, Nechemias-Zimmer L, Brown N M, Lund T D, Handa Rt J, and Heubi J E (2005): S-Equol, a potent ligand for estrogen receptor, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora
Am J Clin Nutr 2005;81:1072–9.

[2] Wood C E, Appt S E, Clarkson T B, Franke A A, Lees C J, Doerge D R, and Cline J M (2006): Effects of High-Dose Soy Isoflavones and Equol on Reproductive Tissues in Female Cynomolgus Monkeys
BIOLOGY OF REPRODUCTION 75, 477–486 (2006)

[3] Rafii F, Jackson LD, Ross I, Heinze TM, Lewis SM, Aidoo A et al.(2007): Metabolism of daidzein by fecal bacteria in rats.
Comp Med 2007, 57: 282-286.

[4] Bayer T, Colnot T, Dekant W (2001): Disposition and biotransformation of the estrogenic isoflavone daidzein in rats.
Toxicol Sci 2001, 62: 205-211

[5] Lamartiniere CA, Wang J, Smith-Johnson M, Eltoum IE: Daidzein (2002): bioavailability, potential for reproductive toxicity, and breast cancer chemoprevention in female rats.
Toxicol Sci 2002, 65: 228-238.

[6] Minamida K, Tanaka M, Abe A, Sone T, Tomita F, Hara H and Asano K (2006): Production of Equol from Daidzein by Gram-Positive Rod-Shaped Bacterium Isolated from Rat Intestine
Journal of Bioscience and Bioengeneering Vol. 102, No. 3, 247-250, 2006

[7] Yee S, Burdock GA, Kurata Y, Enomoto Y, Narumi K, Hamada S et al. (2008): Acute and subchronic toxicity and genotoxicity of SE5-OH, an equol-rich product produced by Lactococcus garvieae.
Food Chem Toxicol 2008, 46: 2713-2720.

[8] Lehmann L, Esch HL, Wagner J, Rohnstock L, Metzler M (2005): Estrogenic and genotoxic potential of equol and two hydroxylated metabolites of Daidzein in cultured human Ishikawa cells.
Toxicol Lett 2005, 158: 72-86

[9] Di Virgilio A L, Iwami K, Watjen W, Kahl R, Degen G H (2004): Genotoxicity of the isoflavones genistein, daidzein and equol in V79 cells.
Toxicol Lett 2004, 151: 151-162.

[10] Schmitt E, Metzler M, Jonas R, Dekant W, Stopper H (2003): Genotoxic activity of four metabolites of the soy isoflavone daidzein.
Mutat Res 2003, 542: 43-48.

[11] Sierens J, Hartley JA, Campbell MJ, Leathem AJ, Woodside JV (2001): Effect of phytoestrogen and antioxidant supplementation on oxidative DNA damage assessed using the comet assay.
Mutat Res 2001, 485: 169-176.

[12] Gredel S, Grad C, Rechkemmer G and Watzl B (2008): Phytoestrogens and phytoestrogen metabolites differentially modulate immune parameters in human leukocytes
Food and Chemical Toxicology 46 (2008) 3691–3696

[13] Widyarini S, Husband A J and Reeve V E (2005): Protective Effect of the Isoflavonoid Equol Against Hairless Mouse Skin Carcinogenesis Induced by UV Radiation Alone or with a Chemical Cocarcinogen
Photochemistry and Photobiology, 2005, 81: 32-37

[14] Ju Y H, Fultz J, Allred K F, Doerge D R and Helferich W G (2006): Effects of dietary daidzein and its metabolite, equol, at physiological concentrations on the growth of estrogen-dependent human breast cancer (MCF-7) tumors implanted in ovariectomized athymic mice
Carcinogenesis vol.27 no.4 pp.856–863, 2006

[15] Adlercreutz H, Mousavi Y, Clark J, Höckerstedt K, Hämäläinen E, Wähälä K, Mäkälä T and Hase T (1992) Dietary Phytoestrogens and Cancer: *In vitro* and *In vivo* Studies
J. Steroid Biochem. Molec. BioL Vol. 41, No. 3-8, pp. 331-337, 1992

[16] Travis R C, Spencer E A, Allen N E, Appleby P N, Roddam A W, Overvad K, Johnsen NF, Olsen A, Kaaks R, Linseisen J, Boeing H, Nöthlings U, Bueno-de-Mesquita H B, Ros M M, Sacerdote C, Palli D, Tumino R, Berrino F, Trichopoulou A, Dilis V, Trichopoulos D, Chirlaque M-D, Ardanaz E, Larranaga N, Gonzalez C, Suárez LR, Sánchez M-J, Bingham S, Khaw K-T, Hallmans G, Stattin P, Rinaldi S, Slimani N, Jenab M, Riboli E and Key T J (2009): Plasma phyto-oestrogens and prostate cancer in the European Prospective Investigation into Cancer and Nutrition
British Journal of Cancer (2009) 100, 1817 – 1823

[17] Venkitaraman R, Thomas K, Grace P, Dearnaley D, Horwich A, Huddart R, Parker CC (2008): Baseline urinary phytoestrogen levels and the natural history of untreated, localised prostate cancer in a British population.
Int J Biol Markers. 2008 Jul-Sep;23(3):192-7

[18] Ward H, Chapelais G, Kuhnle G G C, Luben R, Khaw K-T, and Bingham S (2008): Lack of Prospective Associations between Plasma and Urinary Phytoestrogens and Risk of Prostate or Colorectal Cancer in the European Prospective into Cancer-Norfolk Study
Cancer Epidemiol Biomarkers Prev 2008;17(10). October 2008

[19] Pendleton J M, Tan W W, Anai S, Chang M, Hou3W, Shiverick K T and Rosser C J (2008): Phase II trial of isoflavone in prostate-specific antigen recurrent prostate cancer after previous local therapy

BMC Cancer 2008, 8:132

[20] Kurahashi N, Iwasaki M, Inoue M, Sasazuki S, Tsugane S (2008): Plasma isoflavones and subsequent risk of prostate cancer in a nested case-control study: the Japan Public Health Center.
J Clin Oncol. 2008 Dec 20;26(36):5923-9.

[21] Atkinson C, Newton K M, Aiello Bowles E J, Lehman C D, Stanczyk F Z, Westerlind K C, Li L and Lampe J W (2008):
Daidzein-metabolizing phenotypes in relation to mammographic
breast density among premenopausal women in the United States
Breast Cancer Res Treat

[22] Verheus M, van Gils CH, Kreijkamp-Kaspers S, Kok L, Peeters PH, Grobbee DE, van der Schouw YT (2008). Soy protein containing isoflavones and mammographic density in a randomized controlled trial in postmenopausal women.
Cancer Epidemiol Biomarkers Prev. 2008 Oct;17(10):2632-8.

[23] Verheus M, van Gils C H, Keinan-Boker L, Grace P B, Bingham S A, Peeters P H (2007): Plasma phytoestrogens and subsequent breast cancer risk.
J Clin Oncol. 2007 Feb 20;25(6):648-55. Epub 2007 Jan 2.

[24] Ward H, Chapelais G, Kuhnle G G C, Luben R, Khaw K-T and Bingham S (2008): Breast cancer risk in relation to urinary and serum biomarkers of phytoestrogen exposure in the European Prospective into Cancer-Norfolk cohort study
Breast Cancer Research Vol 10 No 2

[25] Magee P J, Raschke M, Steiner C, Duffin J G, Pool-Zobel B L, Jokela T, Wahala K and Rowland I R (2006): Equol: A Comparison of the Effects of the Racemic Compound With That of the Purified S-Enantiomer on the Growth, Invasion, and DNA Integrity of Breast and Prostate Cells In Vitro
NUTRITION AND CANCER, 54(2), 232–242