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Session 3: Influences of food constituents on gut health
Is equol production beneficial to health?

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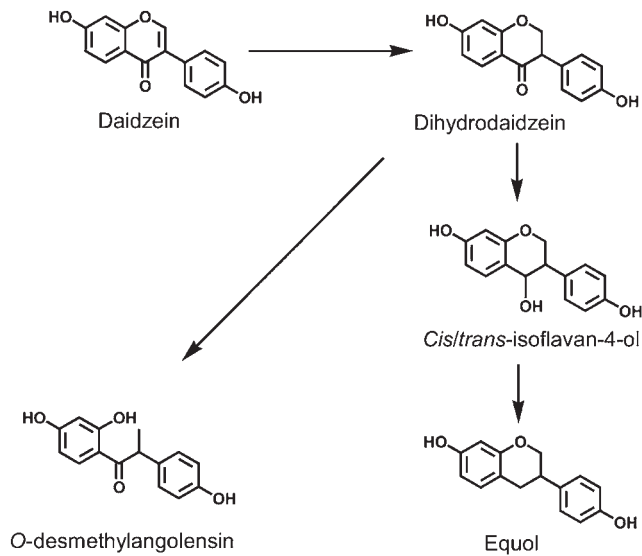
The health benefits associated with soya food consumption have been widely studied, with soya isoflavones and soya protein implicated in the protection of CVD, osteoporosis and cancers such as those of the breast and prostate. Equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman), a metabolite of the soya isoflavone daidzein, is produced via the formation of the intermediate dihydrodaidzein, by human intestinal bacteria, with only approximately 30–40 % of the adult population having the ability to perform this transformation following a soya challenge. Inter-individual variation in conversion of daidzein to equol has been attributed, in part, to differences in the diet and in gut microflora composition, although the specific bacteria responsible for the colonic biotransformation of daidzein to equol are yet to be identified. Equol is a unique compound in that it can exert oestrogenic effects, but is also a potent antagonist of dihydrotestosterone *in vivo*. Furthermore, *in vitro* studies suggest that equol is more biologically active than its parent compound, daidzein, with a higher affinity for the oestrogen receptor and a more potent antioxidant activity. Although some observational and intervention studies suggest that the ability to produce equol is associated with reduced risk of breast and prostate cancer, CVD, improved bone health and reduced incidence of hot flushes, others have reported null or adverse effects. Studies to date have been limited and well-designed studies that are sufficiently powered to investigate the relationship between equol production and disease risk are warranted before the clinical relevance of the equol phenotype can be fully elucidated.

Soya: Isoflavones: Equol: S-equol

Equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman) is a non-steroidal oestrogen that is formed exclusively via the bacterial metabolism of the soya isoflavone daidzein in the intestine. Equol was first identified in the urine of pregnant mares in 1932[1], but was not discovered in human urine until the 1980s[2]. The oestrogenic effects of equol first became apparent in the 1940s when sheep grazing on pastures containing clover in South Western Australia became infertile[3]. This 'clover disease' was later attributed to the rich quantities of formononetin present in the clover, which is converted to daidzein in the rumen[4]. The isoflavone intake associated with clover disease (20–100 g/d) was, however, far in excess to that of human subjects who consume a soya-rich diet (isoflavone intake among older adults in Japan is about 40 mg/d)[5].

Daidzein, one of the major soya isoflavones, is present in most soya foods as the glycoside daidzin. Upon consumption, daidzin is hydrolysed by intestinal β-glycosidases to form the bioavailable aglycone, daidzein. Daidzein is further metabolised by colonic bacteria to form equol or *O*-desmethylangolensin via a pathway that involves the formation of the intermediate dihydrodaidzein[6,7] (Fig. 1). The importance of the gut microflora in the biotransformation of daidzein to equol is evident from studies that have demonstrated that germ-free animals[9] and infants below the age of 4 months[10-12] who have a poorly

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Abbreviations: BMC, bone mineral content; BMD, bone mineral density; DHT, dihydrotestosterone; ER, oestrogen receptor.
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Fig 1. Metabolism of the soya isoflavone daidzein to *O*-desmethyl-angolensin and equol. (From Lampe[8]; reproduced with permission.)

Fig 2. Comparison of the conformational structures of the diastereoisomers of equol, showing the site position of the chiral carbon centre. (From Brown *et al*.[13]; reproduced with permission.)

developed gut microflora, do not produce equol when fed a soya diet.

In comparison with other isoflavones, equol is unique in structure, having a chiral carbon atom at position C-3 of the furan ring. Equol can therefore exist in two distinct enantiomeric forms, S-equol and R-equol (Fig. 2). It has recently been shown that human intestinal bacteria exclusively synthesise the S-equol enantiomer from daidzein[14]. Not every individual can, however, produce equol; approximately 30% of adults among western populations[15–17] and 60% of Asian adults[18,19] have the ability to produce equol from daidzein following a soya challenge. Inter-individual variation in the ability to produce equol has been attributed to a number of factors, primarily inter-individual variation in gut microflora composition. Bacterial species that have been identified as being capable of converting daidzein to equol *in vitro* include *Streptococcus intermedius*, *Ruminococcus products* and *Bacteriodes ovatus*[20]. It is evident that different bacteria are involved in different stages of the biotransformation of daidzein to equol and that these bacteria may differ between individuals[7]. *Lactococcus garvieae* can efficiently convert daidzein to S-equol and has recently been used to produce the natural S-equol-containing supplement SE5-OH[21]. Diet has also been associated with the 'equol producer' phenotype, with equol producers reported to consume higher amounts of carbohydrate[15,16] and lower amounts of fat[16] as a percentage of energy than non-producers. Fibre[15], meat[22], PUFA and alcohol intake[23] have also been positively associated with the ability to produce equol among western populations. The frequency of equol producers among vegetarian adults has been reported to be much higher than non-vegetarians, with levels comparable to those of Asian adults[17]. Dietary modification may therefore potentially influence equol producer status. Consequently, prebiotics and probiotics have been studied as an aid to induce equol production in adults;
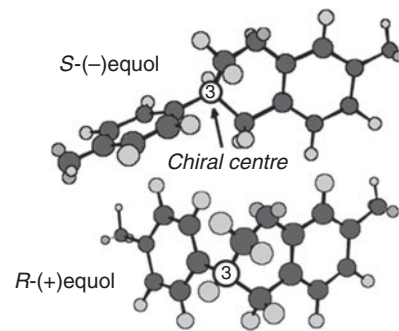
however, studies to date have been unsuccessful[24–26]. Familial correlation and segregation analysis has suggested that the ability to produce equol is under a certain degree of genetic control with other non-genetic factors also involved[27]. The involvement of host genetics is credible given that the ability to produce equol appears to remain stable over time[28].

Individuals with a plasma equol concentration of <40 nmol/l (10 μg/l) can be classified as 'equol non-producers' and those with concentrations >83 nmol/l (20 μg/l) classified as 'equol producers'[6]. Cut-offs can also be applied to urinary concentrations with an equol producer being defined as excreting >1000 nmol/l[15,16]. Defining thresholds for equol producer status is complicated by variations in the methodologies used to detect S-equol. Indeed, with sensitive methods, small quantities of equol can be detected in the urine of most individuals, as small amounts of equol can be obtained via animal foods in the diet e.g. cow's milk[29]. Many studies have defined equol producers as those in whom equol is detectable according to the assay system used. Professor Ken Setchell has developed a more accurate approach to define an 'equol producer' that is independent of isoflavone intake and the analytical technique used. Thus, a threshold value of $\log_{10}$-transformed urinary S-equol : daidzein ratio of greater than $-1\cdot75$ can be used to define an equol producer[17].

Biological properties of equol

Approximately 50% of equol circulates in the 'free' form, unbound to serum protein[30] in comparison with only 18·7% daidzein and 4·6% oestradiol[6]. Equol can be classified as a selective oestrogen receptor (ER) modulator, having a selective affinity for ERβ. S-equol binds to ERβ with approximately 20% as much affinity as 17β-oestradiol and has a much stronger affinity for ERβ compared to R-equol[14]. Equol also has stronger *in vitro* antioxidant activity compared to other isoflavones[31–33]. S-equol is somewhat unique in that in addition to its oestrogenic properties, it potently antagonizes dihydrotestosterone (DHT) *in vivo*[34]. In addition, equol inhibits binding of oestradiol, testosterone and sex hormone binding globulin in a dose-dependent manner[35]. Due to the biological properties of equol, it has been postulated that the ability to

produce equol may be beneficial to health, in particular, in relation to hormone-dependent diseases.

A wealth of clinical studies investigating the effects of soya and isoflavones on health has been conducted to date, yielding somewhat inconsistent findings. In 2002, Professor Ken Setchell postulated that the beneficial effects of soya/isoflavones may be dependent on equol producer status; a theory now known as the 'equol hypothesis'[6]. This paper will outline research to date that has examined the effects of equol on the risk and progression of a number of chronic diseases including breast and prostate cancer and CVD. Effects on bone health and menopause will also be discussed.

Equol and breast cancer

Breast cancer is the most common cancer in women worldwide, accounting for about 23 % of all female cancers[36]. Incidence in western countries, including the UK and Ireland, is between two- and four-fold higher compared to Asian countries[37]. In the 1980s, Setchell and Adlercreutz were the first to postulate that soya isoflavones may confer protection against breast cancer[38] and Adlercreutz et al. have demonstrated that individuals living in countries with high rates of breast cancer excrete low amounts of isoflavones[39–41] in comparison with those living in areas at low risk[42–46].

Few epidemiological studies have investigated the association between equol and breast cancer risk, albeit two studies have reported positive findings. In a case–control study conducted in women (144 pairs, aged 30–84 year) resident in Perth, western Australia, Ingram et al.[47] reported that equol excretion was associated with a substantial reduction in risk of breast cancer (OR 0·27, 95 % CI 0·1, 0·69 highest v. lowest quartile equol excretion). Furthermore, a nested case–control study conducted within the Multiethnic Cohort Study in Hawaii and Los Angeles reported similar findings for white post-menopausal women, with the highest quartile of equol excretion associated with a reduced risk of breast cancer compared to the lowest quartile (OR 0·27 (95 % CI 0·08, 0·95))[48]. This finding, however, was not statistically significant ($P = 0·07$) possibly due to the small sample size (51 white cases and 96 controls). In contrast to the above findings, a prospective study conducted within the UK arm of the European Prospective Investigation of Cancer and Nutrition study, analysed 114 spot urines and 97 serum samples from women (aged 41–76 years) who developed breast cancer 4–8 years after sampling[49]. Samples were matched to 219 urine and 187 serum controls. Exposure to daidzein and equol was significantly associated with increased breast cancer risk ($\log_2$ OR = 1·34 (95 % CI 1·06, 1·7)), $P = 0·013$ for urinary equol). It was noted, however, that dietary intake was low, with only 3 % of the study population consuming soya foods and mean urinary equol excretion was 0·5 (SD 2·1) µg/mmol creatine. A follow-up to this study that included 237 cases and 952 controls (aged 45–75 years), however, reported no association between serum or urinary equol and breast cancer risk, although urinary equol excretion was associated with an increased risk of cancer in those women with ER-positive

tumours[50]. It is plausible that inconsistent findings from epidemiological studies conducted in western populations investigating the relationship between equol and breast cancer risk are due to the low isoflavone intakes among these groups. Isoflavone intake in Europe and USA is about 3 mg/d[51] and the European Prospective Investigation of Cancer and Nutrition study outlined earlier[49] reported mean dietary intakes of only 0·2 mg/d daidzein and 0·2 mg/d genistein.

Breast cancer biomarkers have also been studied, to a limited extent, in relation to equol excretion in human subjects. Duncan et al.[52] supplemented the diet of fourteen premenopausal women with 0·15, 1·0 and 2·0 mg isoflavones/kg bodyweight per day and reported that for all doses tested, equol producers had a more favourable hormonal profile compared to the equol non-producers. Equol producers generally had lower concentrations of oestrone, oestrone-sulphate, testosterone, androstenedione, dehydroepiandrosterone, dehydroepiandrosterone-sulphate and cortisol and higher levels of sex hormone binding globulin, a profile associated with lower risk of breast cancer. In addition, Nettleton et al.[53] have shown that in post-menopausal women, equol producer status is associated with increased concentrations of urinary 2-hydroxyestrogens and an increased 2-hydroxyestrogen: 16α-hydroxyestrone ratio. 2-hydroxyestrogen: 16α-hydroxyestrone has previously been reported to be inversely associated with breast cancer risk[54–56] although not all studies support this finding[57,58]. Equol producer status may also confer protection on mammographic density, an established marker positively associated with breast cancer risk. Frankenfeld et al.[59] studied fifty-five overweight post-menopausal women given a soya challenge (soya protein containing >10 mg daidzein/d for 3 d) to determine equol status and reported that mammographic density was 39 % lower in equol producers compared to non-producers ($P = 0·04$). Although Fuhrman et al.[60] found no independent association of equol status or soya intake with percent mammographic density, among post-menopausal equol producers (n 75), those who consumed soya on a weekly basis had a significantly lower breast density. In contrast, for the equol non-producers (n 157), weekly soya intake was associated with increased mammographic density, suggesting that soya may exert differential effects on breast tissue depending on equol producer status. In a recent study among premenopausal women in USA (n 200), no differences were found in mammographic breast density between equol producers and non-producers[61].

Although the mechanisms of action of soya isoflavones in breast and prostate cancer have been well studied[62], little research has focused on equol specifically. In vitro studies have demonstrated that equol, at concentrations ≤10 µM, stimulates the proliferation of ER positive breast cancer cells[63–65], whereas at higher concentrations (≥10 µM), proliferation of the oestrogen-insensitive breast cancer cell line MDA-MB-231 is inhibited[64,65]. We have also demonstrated that racemic equol and S-equol inhibit the invasion of MDA-MB-231 breast cancer cells through matrigel and that racemic, but not S-equol, protects MCF-10A breast cells against DNA damage induced by 2-hydroxy-4-nonenal or menadione[65]. This latter finding

is of particular interest as it implicates the R-equol rather than the S-equol enantiomer as being responsible for this antigenotoxic effect and warrants further investigation. In support of our findings, another recent study[13] has highlighted the potential beneficial effects of the unnatural R-equol enantiomer in breast cancer. In this study, the effects of a diet supplemented with 250 mg/kg of S- or R-equol on dimethylbenz(a)anthracene-induced mammary tumours in rats were investigated. While S-equol was found to have no chemopreventive effects, R-equol significantly reduced the number of tumours detected, increased tumour latency and reduced the invasive capacity of the tumours. Both R- and S-equol enantiomers therefore deserve further investigation as potential anti-carcinogenic agents in the breast.

There have been concerns over the safety of isoflavones due to their oestrogen-like properties, particularly in relation to breast cancer. The suggestion that isoflavones may have adverse effects in women with breast cancer or at increased risk of the disease has arisen mainly from animal studies and is generally not supported by clinical and epidemiological studies. However, little is known regarding the effects of isoflavones on breast tissue and so these women should perhaps be cautious with regard to their isoflavone intake[66]. The European Food Safety Authority is currently conducting a safety evaluation and will assess the hazards to human health and also the potential benefits of isoflavones, foods rich in isoflavones and isoflavone supplements.

Equol and prostate cancer

Prostate cancer is the second most common cancer in men worldwide[36]. Similar to breast cancer, prostate cancer incidence is much higher (3–30-fold) in the western world in comparison with Asian countries[37]. A case–control study[67] that examined the equol producer status among residents with prostate cancer and controls in Japan, Korea and USA reported that the number of equol producers was significantly lower in the prostate cancer group compared to controls for both the Japanese (29% cases, 46% controls) and Korean (30% cases, 59% controls) cohorts, suggesting that the ability to produce equol is protective against prostate cancer. As expected, the number of equol producers in USA cohort (17% cases, 14% controls) was lower compared to the Asian groups. In another case–control study nested in a community-based cohort in Japan[68], equol status was examined in relation to prostate cancer risk in 52 cases and 151 controls aged ≥40 years from 45 areas of Japan. Serum equol was inversely associated with prostate cancer risk, with the highest tertile of serum equol associated with a 60% reduction in disease risk.

As first reported in 2004, equol is a potent antagonist of DHT[34], one of the key sex hormones implicated in prostate cancer development. Unlike other 5α-reductase inhibitors, equol does not affect the synthesis of DHT. Instead equol appears to bind directly to DHT, thus preventing DHT binding to the androgen receptor. In a recent Japanese intervention trial[18], twenty-eight healthy volunteers were given 60 mg soya isoflavones (containing 19·1 mg daidzein) daily for 3 months and the effects on sex hormones implicated in prostate cancer development was investigated. Following supplementation, serum sex hormone binding globulin increased and serum-free testosterone and DHT decreased significantly compared to baseline concentrations. When subjects were stratified according to equol status, these beneficial effects were only significant in those men who were equol producers (64%), further implicating equol as a chemopreventive agent in prostate cancer. Of interest, in this study, two of the volunteers who had no detectable serum equol (<0·5 µg/l) at baseline, had detectable concentrations of equol in their serum (1·1 µg/l and 24·1 µg/l) following three months of soya supplementation, suggesting that equol producer status can be manipulated with prolonged and consistent soya isoflavone consumption. This theory is further supported by Hedlund et al.[22] who reported that Caucasian men who consumed ≥30 mg soya isoflavones/d for at least 2 years were 5·3 times more likely to produce equol than men who consumed ≤5 mg/d.

In vitro studies have demonstrated that equol inhibits the proliferation of benign human prostatic epithelial cells, causing an accumulation of cells in G0/G1 of the cell cycle[69], with the effects of equol being 10-fold more potent than that of daidzein. Equol has also been shown to inhibit the proliferation of the prostate cancer cell lines 22Rv1[69], DU145[69], LAPC-4[65,69], LNCaP[65,70] and PC-3[70]. Inhibition of growth occurred at physiologically relevant equol concentrations (≤10 µM) given that equol concentrations in the prostatic fluid of men consuming a soya-rich diet can be as high as 13·5 µM[69,71]. Equol has also been shown to inhibit the invasion of PC-3 prostate cancer cells through matrigel[65], an effect that was only significant at an equol concentration of 50 µM. Although tissue concentrations of equol are unknown, daidzein concentrations in prostate tissue have been reported to be ten-fold higher in comparison with serum levels[72] and therefore the possibility of a concentration of 50 µM equol being physiologically relevant in the tissue cannot be excluded.

Equol and CVD

CVD is one of the leading causes of premature death in the UK[73]. High soya intake has been associated with a lower incidence of CVD[74,75] and in 1999 the Food and Drug Administration approved a health claim for CHD based on the cholesterol-lowering effects of soya protein[76], a claim supported by the Joint Health Claims Initiative in the UK in 2002. Recent meta-analyses, however, demonstrate that the cholesterol-lowering effects of soya protein, though still relevant, are much smaller (about 3–5%)[77–80] than initially reported (12·9%)[81]. European Food Safety Authority recently concluded that a cause and effect relationship has not been established between the consumption of soya protein and the reduction of LDL-cholesterol concentrations[82]. In addition, the Food and Drug Administration is currently re-evaluating its soya protein health claim.

Although a wealth of studies has investigated the effects of soya on heart health, few have stratified subjects
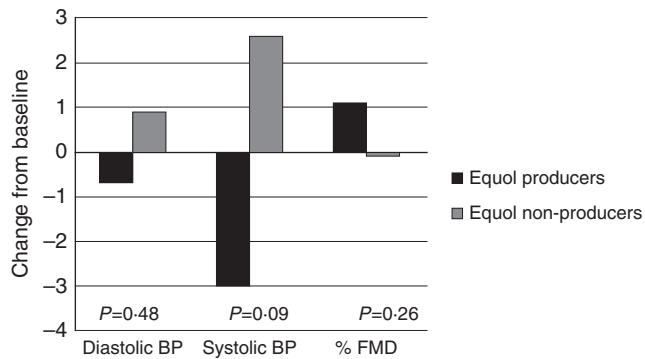
Fig 3. The effects of a soya supplement (99 mg isoflavones/d for 12 months) on blood pressure (BP) and flow-mediated dilation (FMD) in healthy postmenopausal women[85]. (*P* values represent repeated-measures ANOVA for time × equol-status interaction.)

according to equol producer status. In a randomized, placebo-controlled crossover trial in which twenty-six mildly hypercholesterolaemic subjects consumed soya foods containing 80 mg isoflavones/d or placebo for 5 weeks, no significant differences in plasma lipids, blood pressure or arterial compliance were observed[83]. Retrospective analysis, however, showed that in the equol producers (35% of cohort), significant reductions in total cholesterol (8·5%), LDL cholesterol (10%), LDL:HDL (13·5%), plasma TAG (21%) and lipoprotein(a) (11%) were observed with the soya diet, suggesting that it may only be those capable of producing equol that benefit from the lipid-lowering effects of soya. In contrast, in another crossover study, Hall *et al.*[84] found no effect of an isoflavone-enriched cereal bar (providing 50 mg isoflavones/d) on plasma lipids or blood pressure in post-menopausal women, with no differences observed between equol producers (28%) and non-producers. This study, however, involved healthy post-menopausal women (aged 45–70 years) who likely had a low risk of CVD at baseline which may explain why no significant effects were detected. Furthermore, in addition to the fact that a lower isoflavone dose was used in this study, in contrast to the previous study[83], isolated isoflavones added to a cereal bar were used rather than a whole soya food, which highlights the potential importance of the food matrix used in these studies.

In a 12-month double-blind, randomized trial, Kreijkamp-Kaspers *et al.*[85] compared the effects of soya protein (providing 99 mg isoflavones/d) to milk protein on blood pressure and endothelial function in 202 post-menopausal women aged 60–75 years. For the equol producers within the soya group (28%), both systolic and diastolic blood pressure decreased and endothelial function improved following intervention, whereas blood pressure increased and endothelial function deteriorated in the non-producers (Fig. 3). These beneficial outcomes in the equol producers were not significantly different from the non-producers possibly due to the small number of equol producers within the study.

In one of the first studies to examine the relationship between equol production and CVD risk markers within a Chinese population[86], it was observed that equol producers, who comprised 50% of the cohort of 202 adults

aged 20–69 years, had lower serum uric acid, TAG and waist:hip ratio and tended to have higher LDL cholesterol compared to non-producers, suggesting that equol phenotype may influence CVD risk.

Equol and bone health

Due to the oestrogenic properties of isoflavones, their effects on bone have been widely investigated[87]; however, the European Food Safety Authority has recently concluded that there is insufficient evidence to establish a cause and effect relationship between the consumption of soya isoflavones and the maintenance of bone mineral density (BMD) in post-menopausal women[88]. Few studies have stratified subjects according to equol producer status. In a two-year randomized, placebo-controlled trial, Lydeking-Olsen *et al.*[89] investigated the effects of soya-milk (providing 76 mg isoflavones/d), natural transdermal progesterone or the combination, on BMD and bone mineral content (BMC) in the lumbar hip and spine in post-menopausal women who either had established osteoporosis or had three or more risk-factors for the disease. The percentage change in BMD and BMC in the lumbar spine was not significantly different from baseline in the soya isoflavone or transdermal progesterone group, whereas BMD and BMC significantly decreased in the groups receiving the combined treatment (−2·8% BMD loss, −2·4% BMC loss) or the placebo (soyamilk without isoflavones) (−4·2% BMD loss, −4·3% BMC loss). Equol producers (*n* 10) showed a 2·4% and 2·8% increase in BMD and BMC, respectively, in comparison with equol non-producers (*n* 12) who had corresponding increases of 0·6% and 0·3%. Although these findings were not statistically significant, doubtless due to the small numbers involved, they highlight the potentially important role of equol in bone health.

In contrast to the above study, in exploratory analyses, Frankenfeld *et al.*[90] reported that among post-menopausal women who regularly consumed soya (>1 serving of tofu or soyamilk per week, *n* 10), spinal BMD was 20% lower among equol producers than non-producers. Numbers investigated in this study were, however, low (five equol producers and five non-producers) and clearly further studies that are appropriately designed and adequately powered are needed to investigate this association sufficiently.

Equol and menopausal symptoms

In 1992, Adlercreutz postulated that the oestrogen-like effects of soya isoflavones may account for the low prevalence of hot flushes and other menopausal symptoms in Japanese women[43]. Subsequently, studies have shown that soya isoflavones reduce menopausal symptoms, in particular hot flushes, by up to 50%[91]. In a study of 180 Japanese women[92] in which the severity of menopausal symptoms was assessed by questionnaire, it was found that only 5% of these women suffered from hot flushes. Of interest was the finding that the equol producers (53·5% of the group) reported the least severe menopausal symptoms. More recently, Ishiwata *et al.*[93] investigated the effect of

a natural S-equol-containing supplement on menopausal symptoms in a randomized placebo-controlled trial in Japanese women. The supplement used in this study, as mentioned previously, was developed by a pharmaceutical company in Japan, and was prepared by the fermentation of soya germ with the bacteria *L. garvieae*[21]. In Ishiwata's study, 134 Japanese women were randomly assigned to receive either placebo, 10 mg equol/d or 30 mg equol/d (10 mg three times daily) for 12 weeks, with habitual isoflavone intake limited to 20 mg/d. Menopausal symptom and Profile of Mood States questionnaires were completed at baseline and post intervention. Mood-related symptom scores were significantly improved following supplementation with 30 mg equol/d in perimenopausal/post-menopausal equol non-producers, the group most likely to benefit from equol supplementation. Follow-up studies have demonstrated that 10 mg S-equol significantly improved shoulder stiffness and reduced the frequency and severity of hot flushes in Japanese post-menopausal equol non-producing women[94]. Thus, S-equol supplementation may be a potential alternative treatment for the management of menopausal symptoms.

Conclusions

Despite the abundance of studies that have investigated the effects of soya and isoflavones on health, a few studies have been adequately designed and appropriately powered to examine the influence of equol producer status. Although research to date suggests that the ability to produce equol may confer protection against breast and prostate cancer, CVD, bone disease and menopausal symptoms, clinical intervention trials with S-equol are needed to confirm or refute the 'equol hypothesis'. Such trials are now possible with the development of the equol-rich soya product SE5-OH, a new functional food ingredient that relies on the bacterial conversion of daidzein to S-equol[21]. The effects of this supplement on menopausal symptoms have been investigated as outlined above. SE5-OH is not genotoxic and the no-observed-adverse-effect-level was 2000 mg/kg per d (the highest dose tested) in a subchronic feeding study in rats[21]. In addition, reproductive and developmental toxicity studies in rats have defined no-observed-adverse-effect-levels of 1000 mg/kg per d (6·5 mg equol/kg per d) and 2000 mg/kg per d (13 mg equol/kg per d), respectively[95]. The pharmacokinetics of SE5-OH has also been investigated in healthy post-menopausal women[96]. Following oral ingestion, S-equol was reported to be rapidly absorbed from the supplement, was highly bioavailable and had a terminal elimination half-life of 8 h.

Limited *in vitro* research has examined the mechanisms of action of equol specifically and it is important that more research is conducted in this area so that, together with clinical intervention studies, the precise role of equol in human health can be elucidated.

Acknowledgements

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