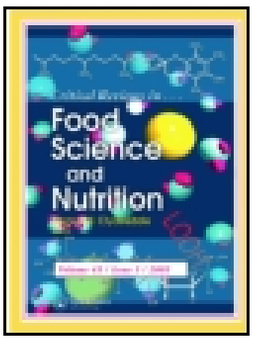

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Metabolism and health effects of phyto-estrogens

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Abstract

Phyto-estrogens are plant derived compounds that can exert various estrogenic and antiestrogenic effects and usually used as a natural alternative to estrogen replacement due to their health benefits including a lowered risk of osteoporosis, heart disease, breast cancer, and menopausal symptoms. Phyto-estrogens are also considered as endocrine disruptors due to their structure similar to human female hormone 17-β oestradiol. However, the issue of whether phyto-estrogens are beneficial or harmful to human health remains unknown, by which, may depends on the dose, form, level and duration of administered phyto-estrogens and influence by genetics, metabolism, gut physiology, age, diet and health status of individuals. Clarification on this issue is necessary, for the sake of their two-side effects on human health and rapidly increasing of global consumption in phyto-estrogens. This review mainly

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includes the metabolism of phyto-estrogens and weighs the evidence for and against the purported health benefits and adverse effects of phyto-estrogens.

Keywords

phyto-estrogens, metabolism, menopausal syndrome, cardiovascular health, breast cancer

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1. Introduction

Phyto-estrogens are naturally-occurring nonsteroidal plant compounds that are structurally and/or functionally similar to the human female hormone 17-β estradiol (D'Mello, 1997), and they have the ability to bind to estrogen receptors (ER) (Setchell, 2001), with a greater affinity for ERβ than ERα (Kuiper et al., 1998). Phyto-estrogens have been shown to have both estrogenic and antiestrogenic properties, and thus can act as estrogen agonists and antagonists competing for oestradiol at the receptor complex (Bingham et al., 1998). There are three main classes of phyto-estrogens: isoflavones, coumestans and lignans (Tham et al., 1998), in which isoflavones primarily attract the current interest from nutritional and health perspective. Isoflavones, the most frequently studied, are present in large amounts in soybeans, and soy products such as miso and tofu, as well as red clover. The most important isoflavones are genistein and daidzein, which can be produced by their precursors biochanin A and formononetin(that are present in some legumes), respectively (Hur and Rafii, 2006). Coumestans occur in bean sprouts and fodder crops, and they have received little scientific attention so far (Pillow et al., 1999). Lignans occur as prelignans and are more widespread in plants where they form the building block in cell walls including cereals, seeds, nuts, fruits, vegetables, and beverages such as tea and coffee (Bateman et al., 2008; Touré et al., 2010).

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Phyto-estrogens have been received widespread concern because of the potential health effects like lowering risks of osteoporosis, heart disease, breast cancer, and menopausal symptoms (Cassidy et al., 2006). Especially, breast cancer is the leading cause of cancer-related deaths in women and it becomes a serious public health concern across the world. Phyto-oestrogens also have a direct effect on the following processes: antiangiogenesis, antioxidative effects, decrease in serums levels of cholesterol, antitumoral effects on breast carcinoma cell lines, protection against osteoporosis, lengthening of the menstrual cycle, and suppression of gonadotropins in the middle of the cycle and reduction of free and active oestrogen in the plasma (Briese V., 2000). Menopausal women suffered from deleterious effects of estrogen deficiency and Peri- and postmenopausal women seeking an alternative solution to Hormone Replacement Therapy could possibly benefit from treatment with phyto-estrogens in regard to menopausal symptoms and lipids. Breast cancer is resulted in 1.68 million cases and 522,000 deaths in 2012 that reported by World Health Organization. It is more common on the world and is more than 100 times more common in women than in men. Fortunately, despite the present ambiguity, current data do suggest a potential benefit from use of phyto-oestrogens in breast cancer chemoprevention and therapy (Limer and Speirs, 2004). Epidemiological evidence from human subjects also suggests that high soyabean consumption, the main dietary source of isoflavones, is cardioprotective (Aedin et al., 2006) and woman also showed higher bone mineral density (BMD) and reduced bone turnover

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(Branca F, 2003). So the therapeutic effect of phyto-estrogens exerting estrogenic and antiestrogenic effects on disease from estrogen disorder should be paid more attention in the future. However, the results were controversial in the past decades for the reason that there are several variable agents serves as key points, including dose and form of administering phyto-estrogens (Barnes, 2003), individual difference, gene diversity (Brössneret al., 2006), gut physiology and the bioavailability of phyto-estrogens is dependent on the some of these factors to realize biotransformations (Rowland et al., 2003), therefore, the evidence was suggestion, not conclusion that phyto-estrogens have health benefits on human being. There are also exist lot's of absorption and metabolism pathway of dietary phyto-estrogens but not well clarified and may influenced by many reasons (Schwanck et al., 2011; Gee et al., 2000). So their metabolism should be explored in order to understand the potential health effects of these nonnutrient food components.

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In current review, we compared the previous results to illustrate whether or not phyto-estrogens are beneficial to humans including phyto-estrogen and menopausal syndrome (hot flash and osteoporosis), cardiovascular (serum, body weight, glucose, blood pressure and endothelial function) and estrogen-sensitive tumor (breast cancer); we also summarized the possible metabolism mechanism of phyto-estrogens which may help to evaluate the benefits and the risk.

2. The metabolism of phyto-estrogens

The metabolism of xenobiotics is often divided into three phases: modification, conjugation, and excretion. In Phase I, a variety of enzymes (including cytochrome P450 (Guengerich, 2003), which are found abundantly in the liver, gastrointestinal tract, lung and kidney) act to introduce reactive and polar groups into their substrates through oxidation, reduction and hydrolysis reactions. In subsequent Phase II reactions, these activated xenobiotic metabolites are conjugated with charged species such as glutathione (GSH), sulfate, glycine, or glucuronic acid. Sites on drugs where conjugation reactions occur include hydroxyl (-OH), carboxyl (-COOH), amino (NH_2), and sulfhydryl (-SH) groups. We focus on metabolism of the two classes of phyto-estrogens, isoflavones and lignans in the first section based on recent publications.

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Isoflavones

Isoflavones are naturally glycosylated to the aglycone. Interesting in soy isoflavones has exploded in the past decades after a wealth of scientific data showing that these compounds possess potent and wide-ranging biological activities. The intestinal absorption and metabolism of dietary isoflavones are not well clarified and may influenced by many reasons. Generally, it is recognized that intact isoflavones glycosides are hardly absorbed from the small intestine because sugar moieties elevate their hydrophilicity. Isoflavones glycosides from diet are hydrolyzed to their aglycone by enterobacteria or lactase phlorizin hydrolase (LPH) (Setchell et al., 2002). Due to its lipophilicity, isoflavones aglycone are believed easily to pass through phospholipid bilayer of cytomembrane and can be easily absorbed into epithelial cells in the small intestine. Thereafter,

the absorbed aglycones undergoes extensive (40% maximum) phase II metabolism via O-methylation, glucuronidation, and/or sulfation in the small intestine epithelial cells or in the liver after entering the circulation (Liu and Hu, 2002). After the distribution throughout the body, a substantial portion of those metabolites are excreted in urine, and part of metabolites were excreted in the bile and returns to the intestinal lumen again, where they may be hydrolyzed and reabsorbed by intestinal cells or excreted into the feces.

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Many *in vitro and in vivo* studies suggested that aglycones were transported at a much higher rate than their corresponding glycosides (Liu and Hu, 2002; Piskula et al., 1999; Setchell et al., 2001). But human studies showed contradictory results on the bioavailability of isoflavones in the aglycone and glucoside forms (Kano et al., 2006; Rüfer et al., 2008). The evidence showed the different transport mechanisms of aglycones across Caco-2 cell monolayers. Foti et al. (2006) found that daidzein ((25, 50, and 80 μM) was absorbed by passive, unsaturable transport in the small intestine of rats. But some reports suggested that the absorption of aglycones via other mechanisms other than simple diffusion (Zhou et al., 2008) and another study reported that flavanone aglycones were absorbed through human intestinal epithelium via both passive diffusion and an active transport mechanism (Kobayashi et al., 2012).

However, the glycoside form of flavonoids are the most common form in plants. A body of evidence has shown that the natural flavonoid glycosides can be absorbed by the

gastrointestinal tract *in vivo* (Liu and Hu, 2012; Tew et al., 1996). If so how does it occur? Two hypotheses on absorption mechanisms of flavonoid glucosides across the small intestine have been proposed. One is active uptake of the flavonoid glucoside via the sodium-dependent glucose transporter (SGLT1) with subsequent deglycosylation by cytosolic β-glucosidase (CBG) (Day et al., 1998; Hu et al., 2003) within intestinal epithelial cells. The other is luminal hydrolysis of the glucoside by LPH that resides on the brush border membrane of enterocytes (Day et al., 2000) or by gut microbiota residing in the large intestine as well as the small intestine (Yuan et al., 2007), then the released aglycones are easily absorbed as previously described. Thus, it has been accepted that there are two possible mechanisms by which the glucoside conjugates are absorbed by the epithelial cells, namely “LPH/diffusion” and “transport/CBG.”

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The deglycosylation of isoflavone glucosides to release the aglycones byendogenous (LPH and CBG) and exogenous enzyme (gut microbiota) is a critical first step in their disposition because it acts as an initiator of all subsequent disposition processes (Schwanck et al., 2011; Gee et al., 1998; Gee et al., 2000). and enzyme polymorphism and gut microflora population may have crucial repercussions on the release of active aglycones. There are also high inter individual variability due to the diversity and variability of gut microflora.

Prior to passage into the blood stream, the aglycones undergo phase αmetabolism and form glucuronide, sulfate and/or methylated metabolites via the action of uridine-5’-diphosphate glucuronosyltransferases (UGTs), sulfotransferases (SULT), and

catechol-O-methyltransferases (COMT) in intestine epithelial cells, respectively (Crespy et al., 1999; Piskula and Terao, 1998). Moreover,

efflux of the metabolites slightly back into the lumen of the small intestine and this is thought to involve some transporters, such as multidrug resistance protein (MRP), P-glycoprotein (P-gp) and organic anion transporters (OATs) (Chen et al., 2005). Once in the peripheral blood circulation, the metabolites rapidly reach the liver where they can be subjected to further phase II metabolism before distribution into tissues (such as brain, breast, uterus, ovary, prostate, etc. (de Boer et al., 2005; Takumi et al., 2011)) and being excreted in urine in substantial quantities. Moreover, some metabolites in the liver are recycled back to the intestine tract through bile excretion which is also called enterohepatic recirculation (Donovan et al., 2007). Aglycones that are not reabsorbed, along with any conjugates from liver or intestinal biotransformation that are not deconjugated, together with the fraction of isoflavone that has not been hydrolyzed or absorbed in the small intestine (Decroos et al., 2005) will reach the large intestine. The colonic microbiota will then cleave conjugating moieties and the aglycones will undergo ring fission leading to the production of smaller molecules (Jaganath and Crozier, 2010). These secondary metabolites can then be absorbed and enter the circulatory system via the large intestine (Stalmach et al., 2010), which indicates that the colon may be an active site of isoflavones metabolism and deserves further attention.

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Daidzein was bioconverted to its secondary metabolite equol through a very specific type of intestinal microbe (Setchell et al., 2002), and it seems that, at best, only 30--50% of individuals are capable of making that conversion (20--30% of adults from western populations and 50% of Japanese women) with vegetarians and Asian being most likely (Setchell et al., 2003; Lampe et al., 1998). Another alternative metabolite is O-desmethylangiolensin (O-DMA), which is produced by 80--90% of the population. Thus, isoflavone consumers could be systematically classified as “equol producers” or “equol non-producer” as well as “O-DMA producers” or “O-DMA non-producers” (This et al., 2001). However, it has not yet been clearly elucidated what factors influence the ability to produce equol and O-DMA, but the interindividual differences purportedly lie in gut physiology, genetics, age, and diet.

Fig.1 summarises the proposed absorption and metabolism pathways of dietary isoflavones glucosides and their distribution in the body. Hydrolysis to its aglycone by LPH, enterobacteria or enterocytes CBG is crucial for the effective absorption of glucosides in the intestinal tract. Isoflavone and other flavonoids absorbed from the intestinal lumen are mostly converted to conjugated metabolites before entering circulation (Murota and Terao, 2003).

In the circulation, aglycones represent only a small fraction (generally <5%) of the total plasma isoflavones (Setchell et al., 2001), and the main metabolites are glucuronateconjugates, with small proportions of sulphate conjugates (Morand et al., 1998). Blood isoflavone levels vary widely between individuals, part of this variation results from local and/or seasonal

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differences in content of food phyto-estrogens, and differences in age, dietary preferences, previous and current health condition, absorption and metabolism of individual (Markiewicz et al., 1993). Phyto-estrogens consumption is different among different regions and populations, and the content of soy-enriched foods consumed may have influence on varying blood levels of isoflavones (Verkasalo et al., 2001).

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Lignan

In contrast to isoflavonoids, plant lignans do not have inherent estrogenic activity. The major lignan in flaxseed is called secoisolariciresinol diglucoside (SDG). Once ingested, SDG is converted into active mammalian lignans, enterodiol (END) and enterolactone (ENL) in the colon, which have weak estrogenic properties (Kilkkinen et al., 2001). Animal and human studies have found that the urinary excretion of the lignans END and ENL were significantly increased after consumption of flaxseed or berries (Axelson et al., 1982; Mazur et al., 2000; Wang, 2002) (**Table 1**) and men excreted more enterolactone and less enterodiol than women (Kirkman et al., 1995). The sugar moiety of SDG was hydrolyzed by gastrointestinal (GI) bacteria and secoisolariciresinol (SECO) was released in gastrointestinal (GI) (Thompson, 1999; Muir et al., 1997; Clavel T et al., 2006). Following dehydroxylation and demethylation by the colonic microflora, the mammalian lignan END is formed in the human body. END is presumed to be oxidized by the GI microbial flora to produce ENL, which may also be formed directly from matairesinol, but this is likely to be a minor metabolic route if other lignans are present in the

diet (Muir et al., 2000). Hutchins AM et al. (2000) also reported excretion of matairesinol was not significantly altered by flaxseed consumption and urinary excretion of lignan metabolites is a dose-dependent biomarker of flaxseed intake within the context of a habitual diet (Nesbitt PD et al., 1999). In western populations, the daily intake of lignans ranged from 0.4 to 13.8 mg/d and Caucasian women had the highest intake of 13.8mg/d (French et al., 2007; Cotterchio et al., 2008), but its daily intake level in Asians was relatively low (Adlercreutz, 1998).

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3. Heath effects of phyto-estrogens

Numerous symptomatic and asymptomatic manifestations (such as vasomotor symptoms, osteoporosis, cardiovascular disease, bladder and vaginal symptoms) can occur in postmenopausal women due to estrogen deficiency. These have been treated by estrogen or hormone replacement therapy (HRT; estrogen combined with progestin) for decades, but evidences accumulated in the recent past have shown that HRT can increase the risk of endometrial and breast cancers, coronary heart disease, stroke, and pulmonary embolism, so HRT is currently a source of considerable controversy and debate (Chen et al., 2002; Lambe et al., 2010). Thus, the selective estrogen-receptor modulators (SERMs) (Frasor et al., 2004 ； Riggs and Hartmann, 2003) amoxifene (TAM) and Raloxifene (RAL) are also used to exert their estrogenic and antiestrogenic effects, but side effects still existed to reduce the quality of life in postmenopausal woman (Shang and Brown, 2002; Khovidhunkit and Shoback, 1999).

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Therefore, increasing research on alternatives and natural strategies for menopausal oestrogen deficiency is necessary for prevention and treatment of postmenopausal conditions. In this light, phyto-estrogens, such as soy isoflavones, are of particular interest because of their potential health benefits in a range of hormonal conditions, such as menopausal symptoms, postmenopausal osteoporosis, cardiovascular disease, and breast cancers. Especially, breast cancer has become a serious public health problem across the world, so, considerable researches have been conducted to explore health effects of phyto-estrogens on breast cancer. But many studies have shown that phyto-estrogens are also considered as endocrine disruptors because of their estrogenic activity, indicating that they may have the potential to cause adverse health effects as well. Numerous epidemiological and clinical studies have now evaluated the relationship between phyto-estrogens consumption and human health effects, but the results are difficult to compare because dose, form, dietary composition, level and duration of administered phyto-estrogens and methods varied considerably across these studies, so, it remains unknown whether or not these compounds have therapeutic potential (Sirtori et al., 2005). The potential role in health effects (menopausal syndrome, cardiovascular health, breast cancer) of phyto-estrogens were reviewed as follows. In which, association between phyto-estrogens and breast cancer risk was described largely because breast cancer is the leading cause of cancer-related deaths in women.

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3.1 Phyto-estrogens and menopausal syndrome

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Menopausal women suffer from deleterious effects of estrogen deficiency including dysfunction of autonomic nerve system (hot flashes, sweats and palpitations etc.), the changes of metabolism (osteoporosis), the nervous and mental symptoms (insomnia, anxiety and memory deterioration etc.) and hypercholesterolemia during early postmenopause and tend to become attenuated during late postmenopause. Phyto-estrogens are of particular interest because they can potentially alleviate estrogen deficiency related deleterious effects. Menopause is a special problem for women in developing countries and leads to a considerable degree of clinical and psychological as well as social problems.

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3.1.1 hot flashes

Relief from vasomotor symptoms of perimenopause (including hot flashes and night sweats) is the first widely attributed health benefit of phyto-estrogens consumption. And these symptoms can markedly affect the quality of life and interfere with daily activities for some women. The efficacy of HRT on the reduction of hot flashes has been well established: estrogen alone and combined HRT reduced vasomotor symptoms by 65% and 90% respectively (MacLennan et al., 2004). However, as it could increase the risk of developing breast cancer, this option has fallen out of favor (Schairer et al., 2000; Beral, 2007). Incidence of vasomotor symptoms was higher in Western countries (70--80% of women) than in Asian countries (10--20%) (Kurzer, 2008), which leads to the now popularly held belief that soy phyto-estrogens may bring relief. For example, Crisafulli et al. (2004) found that soy isoflavone may help to

reduce the frequency and severity of hot flushes in climacteric women without stimulating their endometrium by multicenter, double-blind, randomized, placebo-controlled studies; van de Weijer et al. (2002) reported that there was a significant decrease in hot flushes (44%) between the 80 mg isoflavones and placebo group; and another study reported that there was a significant improvement in the menopausal symptom scores, with a decrease in severity of hot flashes, less headaches and less joint pains in postmenopausal women supplemented with pumpkin seed oil which is rich in phyto-estrogens (Gossell et al., 2011). The mechanism of this action of phyto-estrogens is unclear but is thought to be primarily through their estrogen-like effects. However, demonstrable evidence for such an association is weak and most clinical trials have shown no or minimal relief (Quella et al., 2000; Simbalista et al., 2010; Matvienko et al., 2010). A recent meta-analysis reported by Bolaños et al (2010) suggested that it was difficult to establish conclusive results with the minimum heterogeneity, which may be because of the uncertainties, such as the forms and contents of phyto-estrogens as well as the gut microflora and metabolism of different individuals. Despite this, dietary phyto-estrogens intake continues to be popular, particularly among postmenopausal women who are subjected to vasomotor symptoms of menopause and are seeking a natural alternative to HRT.

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3.1.2 osteoporosis

Osteoporosis is a systemic disease characterized by reduced bone mass, increased fracture risk and structural deterioration of bone tissue. Osteoporosis is a major public health concern

around the world, especially in countries with aging populations (Levine, 2007). Osteoblastic bone formation and osteoclastic bone resorption are balanced such that there is little net bone loss prior to menopause, but estrogen deficiency disrupts this equilibrium by increasing bone turnover and skewing bone remodeling in favor of resorption in the first 5 or 10 years after menopause. Except HRT, postmenopausal women are typically prescribed bisphosphonates to prevent bone loss, however, it is also accompanied by several complications including acute phase response and osteonecrosis of the jaw (Cole et al., 2008). Therefore, other potential therapeutic interventions have been examined including phyto-estrogens.

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Although inconsistent and negative in some cases, results from animal studies are nonetheless encouraging. Numerous animal studies reported that many phyto-estrogens including genistein, coumestrol and daidzein have bone beneficial effects (Wu et al., 2001; Cano et al., 2008; Wang et al., 2005), but the efficacy seems to depend on dose, form, route and duration of administration and the animal model used. As the characteristics of skeletal physiology in ovariectomized (OVX) rats are very similar to those of early postmenopausal women, a large number of studies have examined the effects of phyto-estrogens on bone in OVX rats (Hertrampf et al., 2009; Kim et al., 2011; Filipović et al., 2010).

Evidence for measurable effects in humans is equally controversial. Some epidemiological studies and intervention studies suggest that phyto-estrogens-enriched diets may be associated with a more robust skeleton and have found their beneficial effects on aging-related osteoporosis

already observed in the experimental models (Atkinson et al., 2004; Chen et al., 2004). Published evidences showed that the effects of phyto-estrogens on bones were predominantly mediated through ERβ (Li et al., 2012). On one hand, phyto-estrogens were reported to increase bone formation by enhancing osteoblast differentiation into osteocytes and bone lining cells (Guo et al., 2011), increasing levels of alkaline phosphatase (ALP), osteocalcin (OC), osteoprotegerin (OPG) and osteopontin, inducing the synthesis of nitric oxide (NO) and bone morphogenetic protein-2 (BMP-2), as well as by stimulating bone matrix protein synthesis, bone-specific runt-related transcription factor 2 (Runx2, a bone marker) and Type I collagen which can increase the formation of calcium deposits (Li et al., 2012; Alcantara et al., 2011; Sheu et al., 2012). Moreover, these compounds can upregulate vascular endothelial growth factor-α (VEGF-α) and promote angiogenesis by activation of early growth response factor-1 (Egr-1) genes, Srckinase, p38 mitogen-activated protein kinases (MAPK) and the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in osteoblasts (Faure et al., 2008; Huh et al., 2011; Liao et al., 2014). On the other hand, phyto-estrogens can also inhibit osteoclast formation (Gao and Yamaguchi, 1999; Bandyopadhyay et al., 2006) by directly suppressing the response of monocytes to osteoclast-inductive stimuli and reducing osteoblastic nuclear factor-kappa B ligand (RANKL) expression (Yin et al., 2010; Palacios et al., 2005), and some of them (such as genistein, coumestrol and daidzein) can suppress tumour necrosis factor α (TNF-α)-induced osteoclast differentiation and bone resorption by inhibiting c-fos-induced NFATc1 (nuclear factor of activated T-cells) expression

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in an ER dependent manner (Karieb and Fox, 2011), and the inhibitory action may be related to cyclic AMP signaling (Gao and Yamaguchi, 1999).

However, not all studies have found beneficial actions of phyto-estrogens on the skeleton. For example, Alekel et al. (2010) reported that soy isoflavones did not have a bone-sparing effect in nonosteoporotic women with either the intent-to-treat or compliant analysis and could not conclude that soy isoflavones hold potential promise in the prevention of postmenopausal osteoporosis; Wangen et al. (2000) reported that the effects of soy isoflavones on markers of bone turnover in premenopausal and postmenopausal women were of small magnitude and not likely to be clinically relevant; a randomized, double-blind, placebo controlled studies reported by Brink et al. (2008) found that long-term consumption of isoflavone-enriched foods did not affect BMD, bone metabolism, or hormonal status in early postmenopausal women. These controversial results may lie in differences in study design, type and dose of phyto-estrogens used, as well as age, menopausal status, gut bacteria and other lifestyle factors of participants, in which gut bacteria metabolism of isoflavones to produce the more potent estrogenic metabolite, equol (the equol-producing phenotype) might partly explain these discrepancies (Setchell et al., 2002).

Equol and O-DMA are differently produced from daidzein due to variation in the intestinal bacterial flora in humans, but the main bacteria responsible for daidzein metabolism have not been definitely identified (Atkinson et al., 2005). Equol is a chiral molecule with two natural enantiomers, S-equol and R-equol. In humans, the metabolism of daidzein can only produce S-equol, which has a

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13-fold higher relative binding affinity for ERβ than ERα, in contrast, the R-equol has a stronger affinity for ERα. Equol has been found to have greater estrogenic activity than its parent compound, daidzein and other isoflavones (Muthyala et al., 2004). Emerging data from several animal and clinical studies suggest that equol potentiates the estrogenic action and has potent bone sparing effects. However, there was very few evidence for a direct effect of O-DMA on bone loss in estrogen-deficient subjects (Fujioka et al., 2004; Ohtomo et al., 2008).

Few clinical trials have considered equol production as a potentially important variable. Lydeking-Olsen (2002) reported that the lumbar spine BMD of equol producers increased by 2.4% compared with the control group, whereas there were no changes in BMD in the non-producers after a 2-year intervention with isoflavone-rich soymilk. Wu et al. (2006) found that the positive effect of isoflavones (75 mg/kg/d) on bone loss depended on equol production though individual's intestinal flora in postmenopausal Japanese women. However, the total and site-specific BMD did not differ between equol producers and non-producers (Frankenfeld et al., 2006). In addition, Kenny et al. (2009) reported that there were no significant differences in BMD between equol and non-equol producer after 1-year intervention. These results conflict with previous studies, and the incongruous results may be due to dose, duration of therapy and subject age and more further studies are needed to understand the mechanism by which equol and other phytoestrogens act to enhance BMD. Thus, adding soy to diets may be an appealing choice to help relieve bone loss for many women in their mid-life.

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3.2. Phyto-estrogens and cardiovascular health

There are numerous risk factors for cardiovascular disease (CVD) including systemic arterial compliance, obesity, high blood pressure, glucose intolerance, uric acid, C-reactive protein (CRP) levels, triglycerides (TG), total cholesterol (TC) and the ratio of high density lipoprotein cholesterol (HDL-c) to low density lipoprotein cholesterol (LDL-c) (Anderson et al., 1991; Feinleib et al., 1977). Coronary heart diseases(CHD) and stroke are a major cause of morbidity and mortality worldwide and these diseases substantially increased social health care budget (Go et al., 2013). Many epidemiological studies showed that the daily consumption of phyto-estrogens had negative correlation with the incidence of CVD, and many basic and clinical researches suggested that phyto-estrogens had beneficial effects on cardiovascular function by mechanisms that include improving blood lipid metabolism, inhibition of LDL oxidation, protection and maintenance of intact endothelium, anti-atherosclerotic properties, promotion of vasorelaxation, suppression of platelet aggregation, and estrogen-like actions (Zhang et al., 2003; Goodman and Kritz, 2001). Therefore, this healthy benefit has arguably received the greatest attention and stimulated the rapid proliferation and adoption of soy foods in Western countries for the reason that Food and Drug Administration (FDA) has claimed that soy protein included in a diet low in saturated fat and cholesterol may reduce the risk of CHD (Labeling, 1999).

Serum lipids

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Many studies examined the effect of phyto-estrogens or soy products on lipoprotein status. For instance, Nagata et al. (1998) found a significant trend (p = 0.0001) for decreasing TC concentration with an increasing intake of soy products in 1242 men and 3596 women after controlling for confounding factors. Zhang et al. (2003) reported that high consumption of soy food reduced the risk of CHD in a population-based prospective cohort study of 75,000 Chinese women aged 40--70 years. Pipe et al. (2009) found that the 57-d consumption of soy protein (80 mg/d aglycone isoflavones) modulated some serum lipids in a direction beneficial for CVD risk through reducing serum LDL-c, the ratio of LDL-c to HDL-c, and the ratio of apolipoprotein B to apolipoprotein A-I. Furthermore, numerous reports also suggest the hypocholesterolemic effects of soy protein or isoflavones (Anderson et al., 1995; Zhan and Ho, 2005; Zhuo and Melby, 2004). All these results indicated that soy protein or isoflavones could be an important non-pharmacological cholesterol-reducing agent.

Animal studies showed that the mechanism of hypocholesterolemic effect of phyto-estrogens were likely related to reducing the gene expression of hepatic sterol regulatory element binding protein (SREBP)-1 and 2, malic enzyme, fatty acid synthase (FAS), downregulating hydroxymethylglutaryl-coenzyme A reductase (HMGCR) and LDL receptor (LDLr), differentially regulating liver X receptor isoforms (LXR), as well as promoting the antioxidant enzyme activity of superoxide dismutase (SOD) and catalase (CAT) (Tovar et al., 2002; González et al., 2012; Ronis et al., 2009).

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However, a cascade of evidence that show such a claim by FDA might be spurious (Hall et al., 2006). For example, Campbell et al. (2010) reported that 1-year soy protein supplementation did not confer cardiovascular benefits and favorable alterations in the lipid profile in a cohort of postmenopausal women. And a meta- analysis of 17 RCTs by Yeung et al. (2003) showed that isoflavones interventions in the forms of isolated soy protein, soy diets or soy protein capsule (ranging from 28.5 to 150 mg/d) were heterogeneous to combine and seemed to have no overall statistical and clinical benefits on serum lipids (TC, LDL-c and TG and HDL-c), and no significant effects were found among participants with normo- or hyperlipidemia and women with pre- or postmenopausal status. Weggemans et al. (2003) also found that the consumption of soy-associated isoflavones was not related to changes in LDL- or HDL-c. The inconclusive results might be due to heterogeneity, inadequate sample size and presence of potentially uncontrolled confounders (Sirtori, 2001), and these inconclusive findings lend credence to the decision of the FDA to re-evaluate the soy protein health claim issued a decade ago, but the assessment has been ongoing since December, 2007. Unfortunately, the American Heart Association also warned that ''Earlier research indicating that soy protein as compared with other proteins has clinically important favorable effects on LDL-c and other CVD risk factors has not been confirmed by many studies reported during the past 10 years" in August, 2005 (Sacks et al., 2006).

Body weight, glucose and blood pressure

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Riesco et al. (2010) reported that phyto-estrogens combined with exercise compared with exercise alone seemed to improve body composition and CVD risk profile through significantly reducing body weight, fat mass, systolic and diastolic blood pressure (BP), plasma insulin level and homeostasis model assessment in exercise-responder postmenopausal women. A meta-analysis of 11 RCTs by Liu et al. (2012) found that consumption of soy isoflavones (65-153 mg/d for 1-12 months) resulted in decrease in systolic blood pressure (SBP) of 2.5 mm Hg and diastolic blood pressure (DBP) of 1.5 mm compared to placebo. And meta-regression and subgroup analyses showed that soy isoflavones had an effect of lowering BP in hypertensive subjects, but not in normotensive subjects, which indicated that BP status may be a significant predictor of heterogeneity for the effect of soy isoflavones on BP.

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Unfortunately, mixed results were found in human clinical trials that examined the possible beneficial effects of isoflavone-rich soy products on glycemic control and insulin sensitivity. Some studies suggested that soy products significantly improved glycemic control. For example, Atteritano et al. (2007) reported that genistein (54 mg/d) with a healthy diet significantly reduced fasting glucose (FG) and insulin levels as well as insulin resistance in postmenopausal women after both 12 and 24 months of treatment. Ho et al. (2007) also found that 1-year of soy isoflavone supplementation (40 and 80 mg/d) had a favorable effect on FG in postmenopausal Chinese women. Zhang et al. (2013) suggested that Soy isoflavone supplementation in shorter duration (<6 mouth) was found to significantly reduce body weight

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and longer duration (>6 mouth) remarkably reduce blood glucose in postmenopausal women, especially, it is more effective to reduce body weight and fasting insulin level in normal weight (body mass index (BMI) <30) compared to obese (BMI >30) women. However, others observed no significant effect (Hall et al., 2006; Liu et al., 2010; Liu et al., 2011). Therefore, these results demonstrated soy isoflavone treatment had mixing effects on these CVD risk markers, and this inconsistency might be due to the forms of soy products (such as traditional soy foods, isolated soy protein, soy extracts, or purified isoflavones), study duration, the menopausal status and baseline health status of participants, intervention adherence, and degree to which dietary intake was controlled.

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Endothelial function

Phyto-estrogens may exert the protection on CVD through maintenance of intact endothelium. Atherosclerosis is a slowly progressing and multifactorial disease, in which endothelial dysfunction and damage play an initial role, There are substantial evidences to indicate that phyto-estrogens have protective and repairing effects on vascular endothelia (Colacurci et al., 2005; Gottstein et al., 2003), consequently, leading to the anti-thrombogenic and anti-atherogenic effects. It was reported that genistein produced L-arginine/NO-dependent vasodilation in the forearm vasculature of men and women with a similar potency to that of 17 β-estradiol and potentiated endothelium-dependent vasodilation (Walker et al., 2001). Cumulative evidence from 17 RCTs by Beavers et al. (2012) indicated that exposure to

isoflavones supplements (soy protein and isoflavone exposure, from 25 to 40 g/d and 33-120 mg/d, respectively, for 4-52 weeks) may beneficially influence vascular health through modestly, but significantly, improving endothelial function as measured by flow-mediated dilation (FMD). However, a meta-analysis including 9 RCT trials by Li et al. (2010) found that oral isoflavone supplementation could not improve endothelial function in postmenopausal women with high baseline FMD levels (>5.2%), although with significant improvement in women with low baseline FMD levels (<5.2%). These indicated that the baseline endothelial profile might be an important and potential factor influencing the effect on endothelial function of isoflavone supplementation. Therefore, additional high-quality rigorous studies should be performed to confirm the effect of soy isoflavone on endothelial function and explore its exact mechanisms.

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Above all, phyto-estrogens may have beneficial effects on CVD most likely through improving blood lipid metabolism, maintenance of intact endothelium, anti-atherosclerotic properties, promotion of vasorelaxation, suppression of platelet and aggregation. Although there exist some controversial results, people at risk for heart disease may want to consider adding soy protein in a healthy diet low in saturated fat and cholesterol.

3.3 Phyto-estrogens and estrogen-sensitive tumor (breast cancer)

Hormone-related cancers such as breast, endometrial, ovary, prostate, testis, and thyroid cancer share a unique mechanism of carcinogenesis, by which both endogenous and exogenous

hormones can drive cell proliferation and cell divisions, as well as increase the opportunity for random genetic errors. the incidence and mortality of hormone-related cancers vary around the world, being higher in the Western world than in Asian countries (Iwasaki et al., 2008). Especially, breast cancer is the leading cause of cancer-related deaths in women, and it becomes a serious public health concern across the world, thus, the paper mainly account for the association between phyto-estrogens and breast cancer risk.

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Development of the mammary gland and breast cancer in women depends on various factors including estrogens exposure (such as early menarche, short duration breastfeeding and low parity), which was associated with incidence of breast cancer in population-based studies (Rossouw et al., 2002). Estrogens promote breast tumorigenesis through classic mechanisms (ligand-stimulated) pathway via estrogen response element (ERE) and transcriptional regulation by ER through interaction with other DNA-binding transcriptional factors such as members of activator protein-1 (AP-1) (Paech et al., 1997), NF-κB, and specificity protein (SP-1) families (Safe, 2001), as well as through non classic (ligand-independent) pathway such as rapid activation of Src/MAP kinase (Migliaccio et al., 1996), PI3K/Akt (Castoria et al., 2001), IP3-PKC-α (Marino et al., 1998) and cAMP (Come et al., 2003). Benign mammary epithelial cells and most mammary cancer cells can express the ER (a class I nuclear receptor that exists in both α and β forms) (Mangelsdorf et al., 1995). Recent evidence suggests that the α form is most prevalent in human breast cancers and is associated with low-grade lesions and endocrine sensitivity (Knowlden et al., 2000). Moreover, ER

expression has been used as a marker of intact estrogenic signaling, probable responsiveness to antiestrogenic agents and a more favorable prognosis in breast cancer patients (Come et al., 2003). However, the effects of dietary or supplemental phyto-estrogens on estrogen signaling, mammary tumorigenesis, or exogenous estrogens/antiestrogens have not been well studied.

As phyto-estrogens bind ERs with relatively high affinity, some researchers and clinicians are concerned that high phyto-estrogens consumption might increase the risk of carcinogenesis and recurrence rate of breast cancer survivors. Whereas, others have proposed that phyto-estrogens can reduce the risk of breast cancer, citing traditionally low incidences of breast cancer in Asia as evidence. Meanwhile, the results from different *in vitro* studies, animal studies, epidemiological and clinical studies were different, which may be due to the differences in dose, form, level and duration of administered phyto-estrogens, levels of endogenous estrogen present, and genetics, life stage, and tumor type of individuals.It has proven to be one of the most challenging human health issues to address whether phyto-estrogens increase or reduce the risk of breast cancer.

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3.3.1 In vitro studies

Numberous *in vitro* studies have examined the effects of phyto-estrogens on the growth of estrogen-receptor-positive (ER+) and estrogen-receptor-negative (ER-) breast cancer cells (**Table 2**). Phyto-estrogens appear to exert biphasic effects on the proliferation of ER(+) cells, for instance, genistein can stimulate proliferation and estrogen-sensitive gene expression of ER(+) MCF-7 breast

cells at concentrations up to 10 µM (Wang and Kurzer, 1997; van et al., 2011) and potently inhibit cell proliferation at higher concentrations (>10 µM) (Hsieh et al., 1998). Genistein inhibited ER(-) cell proliferation at high concentrations (>10 µM), however, few studies showed genistein was able to stimulate the growth of ER(-) MDA-MB-231cells at low concentrations (< 10 µM) (Liu et al., 2005; Seo et al., 2006). The lignan metabolite ENL and daidzein metabolite equol has also been found to exert similar biphasic effects on the proliferation of MCF-7 cells (Mousavi and Adlercreutz,1992). Therefore, it is evident that moderate concentration is important for phyto-oestrogens in tumor cell growth response. The evidences suggest differential mechanisms of action on cell proliferation at different concentrations of phyto-oestrogens.

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At low concentrations, they appear to act as estrogen agonist resulting in the proliferation of breast cancer cells *in vitro* via a similar mechanism of action to that of oestradiol (Hsieh et al., 1998). Genistein and apigenin (<10 µM) stimulated the proliferation of ERα(+) MCF-7 and T47D cells, but did not stimulate the proliferation of an ERα(-) MDA-MB-435 cells (Seo et al., 2006); and genistein (1-10 µM) in the absence of E_2 (a situation which occurs in postmenopausal woman) remarkably counteracted the anti-tumor activity of cytostatic drugs like cisplatin in ERα (+) MCF-7 cells, but not in ERα(-) HT 29 cells (Juan et al., 2013); hence, genistein may exert an agonistic activity through interaction with the α form of the receptor. Furthermore, low concentrations of genistein could induce the expression of VEGF (which is a stimulator of angiogenesis/vascular permeability *in vivo* and acts as an autocrine growth factor for mammary

cancer cells) (Buteau-Lozano et al., 2008), proto-oncogene c-*fos* (Maggiolini et al., 2004) and proteinase inhibitor 9 (PI-9) in breast cancer cells, and block killing of breast cancer cells by immune cells (Jiang et al., 2008), the mechanisms by which are also related to ER. Moreover, low doses of phyto-estrogens could also affect the efficacy of anti-cancer drugs. For instance, a low-dose isoflavone enriched diet abrogated the growth-inhibitory effects of TAM on both human and mouse mammary tumor cells, which provided important insights into possible modulating factors that might influence the efficacy of SERMs for breast cancer prevention or treatment (Seo et al., 2006). Genistein (1-10 μM) could increase expression and activity of breast cancer-associated aromatase (which catalyzes the production of local estrogen) and negate the growth inhibitory effect of the aromatase inhibitor fadrozole at physiologically relevant concentrations in breast cancers *in vitro* (van et al., 2011). These results showed that soy-based supplements might affect the efficacy of breast cancer treatment with aromatase inhibitors.

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At higher concentrations (>10 μM), phyto-estrogens exert the antiestrogenic effects on tumor cell growth through different mechanism. Non-ER mediated actions have also been reported, including an inhibition of PTK (Manna, 2012), DNA topoisomerases I and II (essential enzymes that regulate DNA supercoiling and remove knots and tangles from the genome) (Ketron and Osheroff, 2013), ribosomal S6 kinase (RSKs) and histone H3 S10 phosphorylation, as well as attenuation of extracellular signal-regulated protein (ERK) and RSK2 pathways (Vanden

et al., 2011). Moreover, these phyto-chemicals could suppress ERα activity through stimulation of the stress-activated members of the MAPK family: c-Jun N-terminal kinase (JNK)1 and JNK2 (Collins-Burow et al., 2012). These actions are most likely mediated through transcriptional processes rather than via direct effects on ER.

Tumor growth is likely to be regulated both by cell cycle control and by apoptosis. And some experimental results showed that phyto-estrogens have inhibitory effect on breast tumor cells through activation of apoptosis and perturbations of cell cycle, and diet and supplement phyto-estrogens could be potential chemopreventive agents for ER(-) and ER(+) breast cancer. For example, Seo et al. (2011) indicated that genistein potentially caused a G_2/M arrest in breast cancer cells, the mechanism of which might be related to the reduction in cyclin B1 expression and inhibition of kinase activities of cdc2 and cdk2. Lee et al. (1999) also found that genistein (36-200 μM) inhibited $pp60^{v\text{-}src}$ activation of cyclin D1 (a gene product that has been reported to be involved in the G_1/S transition of the cell cycle) promoter activity in a dose-dependent manner, and both the JNK (c-Jun N-terminal kinase) and ERK (extracellular signal-regulated kinase) families of MAPKs have been implicated in $pp60^{src}$ signaling. Moreover, some reports also showed that phyto-estrogens could inhibit cell growth and induce apoptosis, the molecular mechanism of which might partly act by decreasing the expression of antiapoptotic protein (such as Bcl-2) and increasing the expression of proapoptotic proteins (such as Bax, caspase 3, and caspase 9) in breast cancer (Tophkhane et al., 2007), as well as

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upregulation of $p21^{WAFI}$ (an important cell cycle arrest regulatory protein) (Davis et al., 2008). These evidences indicated that phyto-estrogens act on multiple signaling pathways and ultimately affecting breast cancer cell survival and leading to repression of cell growth, whereas, rather than exclusively mediated by ERs.

Moreover, administering high doses of phyto-estrogens could improve the efficacy of anticancer therapy (Mai et al., 2007;Ferenc et al., 2010), and Some other studies have demonstrated that these estrogenic compounds also have suppressive effect on breast cancer cell invasion and metastasis (Magee et al., 2013). In addition, oxidative stress has been mechanistically linked with aging and chronic diseases, including cancer, and phyto-estrogens isoflavones have been found to have antioxidant activity, and shown to be potent inhibitors of angiogenesis and metastasis (Li et al., 2013).

Above all, antitumor actions of phyto-estrogens may involve non-oestrogen mediated mechanisms, such as inhibition of PTK; inhibition of DNA topoisomerase II; induction of apoptosis; induction of cell cycle arrest; inhibition of angiogenesis or as antioxidants. However, these findings may be difficult to relate to human studies, as the high concentration used in *in vitro* studies would rarely be achieved *in vivo*. And the *in vivo* levels (< 10 μM) would appear to be tumor-growth stimulatory; however, this is inconsistent with the apparent protective effect of the high-soy isoflavone Asian diet. (**table 3**)

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3.3.2 Animal studies

Animal studies have also shown both tumor-promoting and tumor-repressing effects of phyto-etrogens. Phyto-etrogens may stimulate existing breast tumor growth and antagonize the effects of tamoxifen (de Lemos, 2001), however, once tumors were formed, the isoflavones did not prevent tumor development but might delay tumor latency (Jin and MacDonald, 2002; Constantinou et al., 2001), and several animal studies found that the timing and duration of exposure is crucial for the effect (Lamartiniere et al., 2002).

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Fetal-perinatal exposure of phyto-estrogens and later breast cancer development

Results from fetal-perinatal exposure in animals varied, and some of the results indicated that phyto-estrogens were promising reagents for cancer chemoprevention. For example, Pei et al. (2003) found that administration of genistein (0, 0.15, 30 mg/kg/d) in the perinatal period had protective effects against NMU-induced mammary carcinoma in SD rats. And Another report also provided evidence that *in utero* and postnatal exposure to a diet rich in isoflavones but not genistein reduced the sensitivity of the mammary gland to estrogens, and indicated that isoflavones exposure *in utero* and postnatal could reduce the risk to develop breast cancer (Molzberger et al., 2012).

However, some studies showed phyto-estrogens could increase the risk of breast tumor. Foster et al. (2004) indicated that neonatal exposure to pharmacological concentrations of the dietary phyto-estrogen genistein (10 mg/kg body weight/d, subcutaneously) induced profound

morphological alterations in the mammary glands of adult rats and A recent study (Saad et al., 2011) found an abnormal perpubertal development of the rat mammary gland following exposure *in utero* and during lactation (from first gestational day to the weaning) to a mixture of genistein and the food contaminant vinclozolin (1 mg/kg/d), which was associated with significant increase in epithelial branching and proliferation, TEBs size, as well as ductal hyperplasia and periductal fibrosis in rats in post-natal day (PND) 35 or increased ER and androgen receptor (AR) expression in PND50.

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Furthermore, the biphasic effects of perinatal exposure of genistein on breast tissue development and differentiation were reported. For instance, a study (Nielsen et al., 2011) found that maternal exposure to cow's milk with high levels of isoflavones from dietary legumes (HIM, 429 ng/mL, with equol being the most abundant) during pregnancy, led to increased carcinogen-induced DNA adduct formation in the mammary gland and tend to increase mammary tumorigenesis among the offspring. In contrast, maternal intake of low levels of isoflavones from dietary legumes (LIM, 101 ng/mL, which contained more lignans than HIM), had no effect on mammary cancer risk of offspring, despite having higher estradiol and IGF-1 environment and consequently earlier puberty onset. Another study (Padilla-Banks et al., 2006) found that neonatal exposure to genistein (subcutaneous administration of 50 mg/kg/d) stunted mammary gland development characterized by less branching at 5 wk and decreased numbers of TEBs at 5 and 6 wk; and the animals, particularly those given the higher dose (5 and 50 mg/kg/d), exhibited altered ductal morphology including reduced dilated ducts, lobular alveolar development, and focal areas of ''beaded'' ducts lined with hyperplastic ductal

epithelium. Whereas, subcutaneous administration of a lower dose (0.5 mg/kg/d genistein), produced the opposite effect. These animals exhibited advanced development with increased ductal elongation, increased levels of PgR protein and ERβ mRNA, as well as decreased ERα expression, and thus a likely reduced cancer risk. These results indicated that dose and form of administered phyto-estrogens may be important factors when considering the risk of breast tumor.

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Prepubertal phyto-estrogens exposure and breast cancer growth and development

Prepubertal exposure to endogenous estradiol and phyto-estrogens, has been consistently shown to reduce breast cancer risk (Gallo et al., 2001). The incidence of chemically induced mammary tumors significantly decreased when prepubertal rats were exposed to soy extracts or the isoflavone genistein (Warri et al., 2008). Lamartiniere et al. (2002) reported that prepubertal and combined prepubertal and adult genistein treatments genistein (250 ppm diet) could inhibit the development of tumor and enhance mammary gland differentiation by regulating specific sex steroid receptors and EGF signaling pathways in SD rat mammary tumors induced by 7,12-dimethylbenz[a]anthracene (DMBA). It was concluded that route of administration and timing of exposure might determine bioavailability and biological action of genistein. Prepubertal exposure of 500 mg/kg genistein could reduce later breast cancer risk via persistent down-regulation of the expression of erbB2 (a reliable predictor of breast cancer), p-Akt, AIB1 (breast cancer 1), and PCNA and with low PTK activity in DMBA-iduced mammary tumor SD rats, which indicated the important

role of erbB2/Akt signaling in reducing mammary tumorigenesis (Peng et al., 2010). The evidences regarding prepubertal exposure to genistein are very consistent in showing a reduction in mammary cancer risk. Thus, early postnatal exposure to phyto-estrogens genistein might be considered for dietary prevention of breast cancer.

Phyto-estrogens exposure and breast cancer growth and development in adult

In adult rats, daidzein (200 ppm diet) and SPI (with normal or low levels of isoflavones) were effective inhibitors of DMBA-induced mammary tumors by reducing tumor multiplicity and increasing tumor latency (Constantinou et al., 2001). Jin et al. (2002) reported that dietary genistein (250 ppm), daidzein(250 ppm) or an isoflavone mixture (equivalent to 250 ppm genistein), significantly delayed tumor latency in the mouse mammary tumor virus (MMTV)-neu mouse, However, once tumors formed, the isoflavones did not prevent tumor development. The intraperitoneal (i.p.) administration of soy isoflavonegenistein at a dose of 10 mg/kg/d reduced tumor-induced angiogenesis in syngeneic mice implanted with B16 melanoma and F3II mammary carcinoma cells, and the antiangiogenic effects might be associated with inhibiting tumor cell migration and proteolysis (Farina et al., 2006). In a postsurgical orthotopic breast cancer model, adjuvant treatment with dietary genistein following cancer surgery can affect the growth ability of previously seeded tumor cells, potentially metastatic cells (Vantyghem et al., 2005).

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However, Cohen et al. (2000) found that isoflavone components of soy protein, or soy protein itself, had no inhibitory effect on NMU-induced rat (which were fed 20% and 10% soy protein) mammary tumorigenesis. Ueda et al. (2003) reported that genistein (at concentrations of 0, 25, or 250 ppm in soybean-free diet for 36 weeks) did not exert clear inhibitory effects on DMBA-induced mammary carcinogenesis in the promotion/progression stage in female rats under ovarian hormone-free conditions.

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Postmenopausal phyto-estrogens exposure and breast cancer growth and development

Dietary genistein was found to stimulate estrogen-dependent breast cancer in OVA animals. For instance, Ju et al. (2001) reported that dietary treatment with genistein at and above 250 ppm, which was similar to human exposure level, produced circulating blood levels of genistein sufficient to exert estrogenic effects, such as stimulating MCF-7 tumor growth and cellular proliferation, as well as inducing estrogen-responsive pS2 expression in OVA athymic mice in a dose-dependent manner similar to that seen *in vitro*. de Lemos (2001) reported that genistein and daidzein might stimulate existing breast tumor growth and antagonized the tumor-inhibiting effects of TAM. Therefore, women with estrogen-responsive breast cancer should be aware of the risks of potential tumor growth when taking soy products. Another study by Ono et al. (2012) indicated that genistein did not exert preventive effects and isoflavone components other than genistein might be preventive against rat hormone-dependent mammary carcinogenesis induced by ethyl methanesulphonate (EMS).

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Although dietary daidzein had a slight but significant stimulatory effect on MCF-7 tumor growth, dietary equol treatment (250-1000 ppm, for 37 weeks) did not stimulate MCF-7 tumor growth, proliferation and pS2 expression among any treatment groups in OVA athymic mice (Ju et al., 2006). These results suggested that pharmacokinetic and/or metabolic factors of daidzein and equol might attenuate their estrogenic effects *in vivo*.

However, some studies reported no stimulatory but inhibitory effect on tumor growth of lignans. Chen et al. (2004) found that supplementation of 10% dietary flaxseed (FS) in the animal diet, which was equivalent to human intake of 25 to 50 g/d of FS, inhibited the growth of human ER(+) breast cancer MCF-7 and enhanced the tumor-inhibitory effect of TAM in nude mice in the presence of low (35 pMin OVA mice, and within the range of plasma E_2 levels in postmenopausal women) or high circulating E_2 (0.3-0.4 nM in OVA mice, which is also within the range of physiological E_2 level in premenopausal women) levels. A study by Lindahl et al. (2011) found that TAM, dietary ENL (100 ppm) and flaxseed (10% ground flaxseed), but not genistein (100ppm), reduced tumor growth and tumor angiogenesis, as well as decreased extracellular stroma-derived IL-1β and increased extracellular stroma- and cancer cell--derived IL-1Rα in OVA athymic nude mice implanted with estrogen-dependent MCF-7 cells. These results implicated profound differences between various phytoestrogens on breast cancer growth, and suggested that ENL might prove to be beneficial in breast cancer prevention strategies, even in combination with genistein, by

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downregulating E_2-stimulated angiogenic factors in tumor cells, whereas caution regarding genistein may be warranted. (**table 4**)

Above all, whether or not phyto-estrogens have beneficial or harmful effect, may be due to the timing, dose and form of exposure in animal models. For example, prepubertal and adult phyto-estrogens exposure have inhibitory effects on breast cancer growth and development; and postmenopausal (physiological dose) isoflavones exposure increase the risk of breast cancer. However, the results of fetal-perinatal exposure are variable.

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3.3.3 Epidemiological studies

High intake of soy foods has been proposed to contribute to the low breast cancer risk in Asian countries, and this risk increases in Asian women after emigration to the United States. Several investigators have raised the hypothesis that this lower incidence of breast cancer in Asian women may be related to their traditional soy-riched diet (Goodman et al., 2009). A relatively large number of epidemiological studies reported the association between phyto-estrogens consumption and breast cancer risk, however, results of this association are highly variable, and the variability may be due to the differences of sample size, region, population and methodology of different studies. Moreover, i*n vitro* and animal studies suggest that they can be estrogenic and potentially risk enhancing, is the consumption of a phyto-estrogens-rich diet really associated with a reduction of breast cancer risk in women? Therefore, rigorous evaluation of epidemiological results is necessary

before appropriate recommendations can be made, especially for women at high risk of breast cancer or those who have survived the disease. Retrospective case-control studies, prospective studies and clinical studies are three common research methods for epidemiological studies, from this order, the intensity of verification testing is stronger and stronger, and the results are more and more reliable. Thus, epidemiological studies are divided into the three parts.

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3.3.3. 1 Retrospective case-control studies

Limited evidences from epidemiological studies suggest childhood and adolescent dietary phyto-estrogens intake may be associated with reduced risk of developing breast cancer (Table 5). For example, Shu et al. (2001) found adolescent soy food intake was inversely associated with breast cancer risk, with ORs of 1.0 (reference), 0.75 (95% CI, 0.60--0.93), 0.69 (95% CI, 0.55-- 0.87), 0.69 (95% CI, 0.55-- 0.86), and 0.51 (95% CI, 0.40--0.65), respectively, for the lowest to highest quintiles of total soy food intake in Chinese women aged less than 45 years. Wu et al. (2002) conducted a population-based case-control study (501/594) of breast cancer among Asian-Americans aged 20--74 years, and suggested that soy food intake, particularly during adolescent, might have a lasting protective effect on breast cancer risk. A recent population-based case--control study (3024/3420) of breast cancer on Canadian women aged 20--74 years, and found that adolescent dietary phyto-estrogens intake was significantly associated with a decreased risk of adult breast cancer (Thanos et al., 2006). These results

potentially produce transformative knowledge to inform breast cancer prevention strategies through dietary intervention during childhood and adolescence, rather than later in life, as it is perhaps less effective from current practice. (**table 5**)

For phyto-estrogens exposure in adults, many case-control studies revealed that high levels of lignans and isoflavones were frequently associated with low risk of breast cancer. For example, a meta-analysis of 18 epidemiological studies published from 1978 to 2004 that examined soy exposure and breast cancer risk by Trock et al. (2006), concluded that soy intake may be associated with a small reduction in breast cancer risk; moreover, the inverse association was somewhat stronger in premenopausal women (OR = 0.70, 95% CI:0.58-0.85) than in postmenopausal women (OR = 0.77, 95% CI:0.60-0.98) in the 10 studies that stratified by menopausal status; however, six did not support an association in the 8 studies that did not provide menopause specific results. And the inconclusive result might be due to potential exposure misclassification, confounding, and lack of a dose response. Bouche et al. (2013) found that isoflavone supplements were associated with decreased postmenopausal breast cancer risk (OR<1). Cotterchio et al. (2008) also reported that the high consumption of lignans and isoflavones was related to a low breast cancer risk, especially in premenopausal women or Asians.

However, some reports did not show the inverse association. For example, a population-based case-control study on breast cancers with 1,326 cases and 1,657 controls of non-Asian US women aged 35--79 years found that intake of all seven phytoestrogenic compounds was not associated

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with breast cancer risk (OR = 1.0; 95% CI, 0.80-1.3; for the highest vs. lowest quartile), and results were similar for pre- and postmenopausal women (Horn-Ross et al., 2001). And Cotterchio et al. (2008) found no statistically significant association between breast cancer risk and isoflavones or lignans intakes, although total phyto-estrogens intake was associated with a reduction in breast cancer risk among overweight women in premenopausal women (MVOR = 0.51; 95% CI: 0.30-0.87; for highest vs. lowest quintiles). Although soy food intake in the amount consumed in Asian populations may have protective effects against breast cancer. Thus, the results of phyto-estrogens exposure in adults of retrospective case-control studies are inconclusive. (**table 6**)

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3.3.3. 2 Prospective studies

Prospective studies have been conducted in Japanese, Chinese and Western women. In opposition to case-control studies, they generally failed to find a correlation between consumption of phyto-estrogens and breast cancer risk. For example, Keinan-Boker et al. (2004) found that a high intake of isoflavones or mammalian lignans was not significantly related to breast cancer risk in Western populations after the median follow-up of 5.2 years. Ward et al. (2010) also found that phyto-estrogens intake was not associated with breast cancer among European women. And a Japan prospective study by Nishio (2007) also reported that consumption of soy food had no protective effects against breast cancer, and hazard ratios (HRs, 95% CI) were 1.14 (0.74-1.77), 0.77 (0.47-1.27) and 1.01 (0.65-1.56) for the highest category of tofu, boiled beans, and miso soup consumption. Travi

et al. (2008) found no evidence for a strong association between dietary isoflavone intake and risk for breast cancer in a population of British women with heterogeneous diets.

However, breast cancer risk was found to be reduced for at least one study. Mason reported that flaxseed (FS), rich in the phytoestrogen lignans and alpha-linolenic acid-rich oil were effective in the risk reduction and treatment of breast cancer and safe for consumption by breast cancer patients throughout in vitro, animal, observational, and clinical studies (Mason and Thompson, 2013). Verheus et al. (2007) found that high genistein circulation levels were associated with reduced breast cancer risk in the Dutch population, although no effects of lignans on breast cancer risk were observed in a prospective nested case-control study within the cohort. Iwasaki et al. (2008) also reported an inverse association between plasma genistein and the risk of breast cancer in Japan (OR = 0.34; 95% CI, 0.16 ~0.74; for highest versus lowest quartile). Shu et al. (2009) suggested that in breast cancer survivors, soy food consumption was significantly associated with decreased risk of death and recurrence in ER(+)/ER(-) breast cancer and users/non-users of TAM. These results might provide the necessary epidemiologic evidence that clinicians no longer need to advise against soy consumption for women with a diagnosis of breast cancer.

In conclusion, the present prospective studies have failed to corroborate such a significant reduced risk of breast cancer incidence in Western population, however, we could find their beneficial effect in Asian populations. Moreover, some studies show a decreased risk of death and

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recurrence in breast cancer survivors. Thus, further studies are warranted to confirm the inverse association of soy consumption with risk of breast cancer recurrence. (**table 7**)

3.3.3. 3 clinical studies

Relatively few human intervention studies explore the possible link between phyto-estrogens and breast cancer, possibly due to the methodological difficulty with ethics, the sample size, as well as dose, form and duration of treatment. Generally, sample size was small, study duration was short, and subjects (including healthy women, breast cancer patients or a combination of breast cancer patients and women with benign breast disease) varied in different studies.

Human dietary intervention studies have generally produced negative results. Maskarinec et al. (2003) reported that isoflavone supplement (100 mg/d) in premenopausal women over 12 months did not decrease mammographic density (a biomarker of increased risk) in a double-blind randomized trial. A 2-year soy intervention (quivalent to 50 mg/d of isoflavones) in premenopausal women by Maskarinec et al. (2004) also had no significant effects in mammographic densities by intervention status. However, women who ate more soy during their lives had significant higher breast densities than women who ate little soy in Caucasians. Results also indicated that lower soy intake during early life and higher soy intake during adulthood may predict a greater reduction in the density, thus it appears that soy consumption throughout life might have some effect on breast density. Atkinson et al. (2004) reported that 1 year dietary supplement containing red clover

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derived isoflavones (26 mg biochanin A, 16 mg formononetin, 1 mg genistein and 0.5 mg daidzein) also failed to alter mammographic breast density. Even equol producer status did not modify the mammographic density (Verheus et al., 2008). Khan et al. (2012) reported that a 6-month intervention of soy isoflavones in healthy, high-risk adult Western women did not reduce breast epithelial proliferation, and had no treatment effect on cytologic atypia or nipple aspirate fluid (NAF) parameters. All these findings offer reassurance that isoflavones do not act like hormone replacement medication on breast density.

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However, some human studies suggested that dietary soy protein might have estrogen agonist effects on the breast. For instance, Petrakis et al. (1996) found that consuming soy protein isolate (containing 38 mg of genistein) had a estrogenic stimulus effect on the premenopausal female breast by increasing hyperplastic epithelial cells, secretion of breast fluid and the levels of plasma estradiol. McMichael-Philips et al. (1998) found that short-term soy treatment (containing 45 mg isoflavones) of premenopausal human breast tissue *in vivo* increased the proliferation rate of breast lobular epithelium significantly and up-regulated PR expression when accounted for both the day of menstrual cycle and the age of patient. Whereas, one study reported that the total intake of flaxseed reduced c-erbB2 score ($r = -0.373$; $P = 0.036$) and increased apoptotic index ($r = 0.495$; $P < 0.004$), which indicated dietary flaxseed has the potential to reduce tumor growth in patients with breast cancer (Thompson et al., 2005). **(table 8)**

Above all, it remains unclear whether or not phyto-estrogens are helpful or harmful to breast cancer. Results of the relationship between phyto-estrogens and breast cancer from *in vivo*, animal, epidemiological and human intervention studies have been frustratingly incongruous over the past two decades, the conflictive evidence might be due to timing of exposure; dose and form of phyto-estrogens; digestion, absorption and individual metabolism; hormonal status; consumption of prescribed and non-prescribed therapies; other foods and previous processing; previous and current health condition; and individual genetics. Thus, a clear dietary guidelines for phyto-estrogens is urgently needed. Given the evidence that soy foods could reduce the risk of breast cancer in some animal and human epidemiological studies, women without serious risk factors for breast cancer or a family history of breast cancer could likely accept phyto-estrogens diet without significant concern.

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Conclusion

Phyto-estrogens which share structural similarities with 17-β-estradiol are very intriguing because they have two-sided effects on human health, and the issue of whether or not phyto-estrogens have beneficial or harmful effects on human health remains unknown. However, generally, phyto-estrogens are considered to have beneficial effect on menopausal symptoms, osteoporosis and cardiovascular disease, although the beneficial effects were frequently overstated. However, phyto-estrogens are also considered endocrine disruptors, and evidence of the relationship between phyto-estrogens and breast cancer risk is frustratingly incongruous. Some studies suggest

that they might increase the breast cancer risk, but the potentially adverse effects of phyto-estrogens are likely under appreciated. Moreover, a clear consensus-based set of dietary guidelines for clinical use on breast cancer survivors and also in healthy women should be built.

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Table 1 Phyto-estrogens and urinary metabolites

Phyto-estrogens Class	Phyto-estrogens or Metabolite
Isoflavones	Daidzein
	O-Desmethylangolensin
	Equol
	Genistein
Lignans	Enterolactone
	Enterodiol

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Table 2 Effect of phyto-estrogens on breast cell proliferation in vitro

Reference	Phyto-estrogens tested	Concentration (μM)	Cell line	Rusults
(Wang et al., 1997)	Coumestrol, genistein, biochanin A, and enterolactone	0.1-10	MCF-7	↑
		10-100	MDA-MB-231	↓
		10-100	MCF-7	↓
(Hsieh et al.,1998)	Genistein	0.01-10	MCF-7	↑
(Le Bail et al.,1998)	Genistein	10, 50	MCF-7	↓
(Mousavi et al.,1992)	Enterolactone	0.5--2	MCF-7	↑
		>10		↓
(Schmitt et al.,2001)	Daidzein, Equol and O-DMA	0.010--10	MCF-7	↑
(Liu et al.,2005)	Genistein	50	MCF-7, MDA-MB-231	↓
(Seo et al.,2006)	Genistein	0.1-10	MCF-7, T47D	↑
		>10	MCF-7, T47D	↓
		0.1-10	MDA-MB-231	No effect
(Van Duursen	Genistein	10-Jan	MCF-7	↑

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et al.,2011)				
(Juan et al.,2013)	Genistein	10-Jan	MCF-7	↑

Abbreviations: ↑, stimulated proliferation; ↓, promote proliferation

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Table 3 Effects of phyto-eatrogens on cancer risk *in vitro* studies

Reference	Phyto-estrogens tested	Concentration	Cell line	Results and mechanisms
(Seo et al., 2006)	Genistein, apigenin	<10μM	MCF-7, T47D cells, MDA-MB-435	Stimulated ERα(+) cell proliferate, exert agonistic activity through interaction with the receptor of α form
(Lucki and Sewer, 2011)	Genistein	20-200nM	MCF-7	Stimulated cell proliferation and cyclin B_2 expression by activating a GPR_{30}-dependent pathway that requires the activation of c-Src and ERK1/2.
(Naragoni et al.,2009)	Genistein	100nM	MCF-7	Significantly increase in GNB1 mRNA expression levels

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(Valaciiovicova et al., 2004)	Genistein, daidzein	>10μM	MDA-MB-231	inhibited cell adhesion and migration via inhibiting NF-κB and AP-1 and suppressing the secretion of uPA
(Mai et al., 2007)	Genistein	25μM	BT-474	synergistically inhibited cell growth and induced cell apoptosis via downregulation of expression of surviving, EGFR, HER2 and ERα
(Sakamoto et al., 2010)	Genistein, resveratrol and glycitein	100μM	MCF-7	Significantly inhibited the cells growth activity via increasing apoptosis and reducing the Bcl-2/Bax ratio

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(Davis et al., 2008)	Genistein and other synthetic isoflavones	10,25,50µM	MCF-7,MDA-MB-231	Induced cell apoptosis via activation of apoptotic mediated mechanisms
(Ferenc et al., 2010)	Genistein	50µM	MCF-7,MDA-MB-231	reduced cell proliferation; induced cell apoptosis via alterations in Bcl-2, Bax and Akt protein levels and cell cycle perturbations.
(Pavese et al., 2010)	Genistein	1-10µM	prostate and breast cancer cell	inhibited the prometastatic processes of tumor cell detachment, migration, and invasion via FAK, TGF-β signaling pathway and MMPs

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(Seo et al., 2011)	genistein, apigenin, quercetin	100μM	MCF-10A,	Induced the apoptotic cell death, as well as increasing in the sub-G_0/G_1 apoptotic fractions and accumulation of cell population in the G_2/M phase
			MDA-MB-231	
(Engel et al., 2012)	Genistein	>10 μM	MCF-7, MCF-12A	Decreased tumor progression signaling via sphingolipids by decreasing the expression of Sphk, increasing the S1P lyase expression in MCF-7
	root flax extract	>1 μg/ml		
(Jawaid et al., 2010)	Genistein(40-60d)	10^{-8}M	MCF-7	reduced expression of acetylated H3 protein, increased expression of pro-caspase 9 and inhibited the mitogenic response to E2 and EGF.

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(Magee et al., 2013)	Daidzein and equol	50μM.	MDA-MB-231	inhibit the invasion of human breast cancer cells in part though the down-regulation of MMP-2 expression

Abbreviations: GPR_{30}, a G-protein-coupled cell surface receptor; ERK1/2, xtracellular signal regulated kinase 1/2; GNB1, Guanine Nucleotide-Binding Protein, β-1 Subunit;NF-kB, nuclear factor-kappa beta; AP-1, activator protein-1; uPA, urokinase-type plasminogen activator; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor 2; Bcl-2/Bax,;Akt,; FAK, focal adhesion kinase; TGF-β, transforming growth factor β; MMPs, matrix metalloproteinases; Sphk, sphingosine-1-phosphate kinase; S1P, sphingosine-1-phosphate; H3 protein, histone protein 3; EGF, epidermal growth factor; MMP-2, matrix metalloproteinase-2

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Table 4 Effects of phyto-estrogens on cancer risk in animal studies

Reference	PEs	Exposure (plasma level)	Animal model	Treatment length	Rusults and mechanisms
(Foster et al., 2004)	Genistein	10mg/kg BW, subcutaneously	SD rats following treatment with an organochlorine mixture *in Utero* Genistein	GD 9-16	Induced profound morphological alterations in the mammary glands
		4-500mg/kg, gavage	SD rats following treatment with an organochlorine mixture *in*	On postnatal days 2-8	Produced morphological changes

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			Neonatal Genistein		
(Saad et al., 2011)	genistein plus vinclozolin	1 mg/kg/d, gavage	Wistar Han rats following treatment with genistein and vinclozolin *in utero*	GD1-PND21	abnormal prepubertal development of the rat mammary gland
(Ueda et al., 2003)	Genistein	0, 25, 250ppm diets	DMBA-induced mammary cancers in rats	36 weeks	No protective effect
Jin and MacDonald, 2002)	Genistein Daidzein	250ppm diet	MMTV-neu mouse	28 weeks	Delayed tumor latency, but isoflavones did not prevent tumor development

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(Vantyghem et al., 2005)	Genistein	750ppm diet	Postsurgical orthotopic breast cancer nude mice model	5 weeks	Affect the growth ability of previously seeded tumor cells, potentially metastatic cells
(Juet al., 2006)	Equol	250-1000ppm diet (2.10--3.21μM)	OVA athymic nude mice implanted with MCF-7 cells	37 weeks	Did not stimulate MCF-7 tumor growth
(Lindahl et al., 2011)	ENL, flaxseed	100ppm ENL and 10% ground flaxseed diet	OVA athymic nude mice implanted with MCF-7 cells	14 days	Reduced tumor growth, angiogenesis, and extracellular stroma-derived IL-1β;

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					increased extracellular stroma- and cancer cell--derived IL-1Rα
(Saarinen et al., 2010)	ENL and ENL+genistein	100ppm diet (~0.5μM)	OVA athymic nude mice implanted with MCF-7 cells	18 days	inhibited E_2-induced cancer growth and angiogenesis, decreased both stroma- and cancer cell-derived VEGF
(Zhang et al., 2012)	soy isoflavone	40g Soybean meal	Five-week-old mouse	tumour was 1.5	promoting the occurrence and

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			mammary tumour virus (MMTV)-erbB2 female transgenic mice	cm^2 or the mouse was 60 weeks	development of breast cancer at low oestrogen levels; inhibiting breast cancer growth at high oestrogen levels
(Watson et al., **2015)**	soy diet	40 g of soy/kg body weight/day	MTB-IGFIR transgenic mice was developed mammary tumors	entire lives	high levels of soy protein promote mammary tumor development and decrease tumor latency

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Abbreviations: BW, body weight; MMTV-neu mouse, mouse mammary tumor virus-neu mouse; DMBA (7,12-Dimethylbenz[a]anthracene); PND, post-natal day; GD, gestational day; ENL, mammalian lignan enterolactone; VEGF, vascular endothelial growth factor-α.

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Table 5 Adolescent dietary phyto-estrogen intake and breast cancer risk in women

Reference	Design	Population	OR(95%CI)	Adolescent or childhood exposure differences	Adjustment factors
(Shu et al., 2001)	Population-based case-control (296 /359)	Chinese(≤45 y of age)	0.51(0.40-0.65)	Soy intake (lowest vs. highest quintile)	Menstrual and reproductive history, hormone use, dietary habits, prior disease history, tobacco and alcohol use, weight, and family history of cancer
(Wu et al., 2002)	Population-based case-control (501 /594)	Asian-Americans	0.51(0.31-0.84)	Tofu (≥4 times/week vs. less than monthly)	Age, specific Asian ethnicity, education, migration history and

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					menstrual and reproductive factors
(Thanos et al.,2006)	Population-based case-control (3024/3420)	Canadians(25-74y of age)	0.81(0.71,0.94)	Isoflavone intake	Age
			0.74(0.64, 0.85)	Lignan intake	
			0.71 (0.62, 0.82)	Total PE(4^{th} vs. 1^{st} quartile)	
(Korde et al., 2009)	Population-based case-control (597/966)	Asian-Americans(20-55y of age)	0.42(0.20,0.90)	Childhood soy intake	parity/age at first live birth, menopausal status at diagnosis, age at menarche, family history of breast cancer, and personal history of benign breast
			0.80(0.59,1.08)	Adolescent soy intake (highest vs. lowest tertile)	

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					disease
(Lee et al., 2009)	Prospective cohort study (73223, 305 breast cancer cases)	Chinese (premenopausal)	0.57(0.34-0.97)	Adolescent soy intake (highest vs. lowest quintiles)	age, education, physical activity, age at first live birth, BMI, season of recruitment, family history of breast cancer, and total energy intake
(Anderson et al., 2013)	Population-based case-control (2438/3370)	Americans	0.79(0.65-0.96)	Adolescent soy intake (highest vs. lowest tertile)	Age

Abbreviations: OR, odds ratio; CI, confidence interval; BMI, body mass index.

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Table 6 Case-control studies of phyto-estrogens and breast cancer risk

Reference	Design	Population	OR or RR(95%CI)	Exposure differences	Adjustment factors
(Wu et al., 1996)	Population-based case-control	Premenopausal	0.84(0.79-0.99)	OR per 1 times/week	Age, study area, ethnicity and migration status
		Postmenopausal	0.86(0.63-1.03)		
(Yuan et al., 1995)	Population-based case-control	Shanghai(534/534)	0.9(0.6-1.3)	Soy intake (95^{th} vs. 5^{th} percentile)	Age, age of menarche, parity,age at first use of oral contraceptives, benign breast disease, energy and education, etc.
		Tianjin(300/300)	1.4(0.7-3.0)		
(Horn-Ross et al., 2001)	Population-based case-control	non-Asian US women(1326/1657)	1.0 (0.80-1.3)	PEs intake (4^{th} vs. 1^{st} quartile)	age; race; age at menarche; parity; lactation; history of benign breast disease; family history of breast cancer; BMI, and HRT use etc.

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(Pietinen et al., 2001)	Population-based case-control (Finland)	All women	0.38(0.18-0.77)	ENL (highest vs. lowest quintiles)	Age at menarche, age at first full-term pregnancy, use of oral contraceptives, use of HRT, first-degree family history of breast cancer, smoking habits,etc.
		Premenopausal	0.42(0.10-1.77)		
		Postmenopausal	0.50(0.19-1.28)		
(Shu et al., 2001)	Population-based case-control (Shanghai)	Chinese women (1459/1556)	0.46(0.28-0.75)	Soyfood intake for the highest decile	Age, education, first degree family history of breast cancer, history of breast fibroadenoma,waist-to-hip ratio,menopausal status, age at menopause, total energy
(Linseisen et al., 2004)	Population-based case-control (German)	Premenopausal women (≤50y of age)	0.62(0.40-0.95)	DAI intake	history of breast cancer, number of births, duration of breast-feeding, energy intake, BMI, alcohol

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			0.47(0.29-0.74)	GEN intake (4th vs. 1st quartile)	consumption.
(Cotterchioet al., 2008)	Population-based case-control (Canada)	Premenopausal ((BMI>25)	0.51(0.30-0.87)	Lignan intake (highest vs. lowest quintiles)	Age, family history breast cancer, duration of HRT, breast disease, dietary fiber intake, age at first live birth; stratified by BMI
(Boucher et al., 2013)	Population-based case-control	Canada (3101/3471)	0.23(0.06-0.93)	High content supplements (duration >5y vs. <1y)	Age, and stratified by joint hormone receptor status

Abbreviations: ENL; enterolactone; HRT, hormone replacement therapy; BMI, body mass index, PEs, phyto-estrogens; GEN, genistein; DAI, daidzein;

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Table 7 Prospective studies of phyto-estrogens and breast cancer risk

Reference	Population	Cases (Total)	Design	Endpoint	Mean follow-up (years)	OR or RR(95%CI)	Exposure differences	Adjustment factors
(Nishio et al., 2007)	Japanese (49-70y)	145 (30454)	Cohort	Incidence	7.6	1.14(0.74-1.77)	Tofu intake (Almost daily vs. <3 times/wk)	Age, family history of breast cancer,use of exogenous female hormone, smoking, consumption of vegetables, BMI, and energy
(Travis et al., 2008)	British (20-90y)	585 (37643)	Cohort	Cohort	7.4	1.08(0.85-1.38)	ISO intake (10~20 vs. <10mg/d)	Age, BMI, age, menopausal status, HRT use, alcohol consumption and daily energy intake
						1.17 (0.79-1.71)	ISO intake (≥20 vs. <10mg/d)	

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(Iwasaki et al., 2008)	Japanese (40-69y)	144 (24226)	NCC	Incidence	10.6	0.34(0.16-0.74)	Plasma GEN	Age at menarche, menopausal status at baseline, age at menopause, BMI, and alcohol consumption
						0.71(0.35-1.44)	Plasma DAI (4th vs. 1st quartile)	
(Ward et al., 2010)	European	244/941	NCC	Incidence	9	1.10(0.96-1.25)	Total ISO intake	Age, weight, family history of breast cancer, oral contraceptive use, HRT use, surgical removal of ovaries, average daily intake of fat and energy.
						1.04(0.90-1.19)	GEN intake	
						1.03(0.89-1.18)	DAI intake	
						0.99(0.84-1.17)	ENL (µg/d)	

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(Caan et al., 2011)	US and Chinese women	271 (3088)	Cohort	Mortality	7.3	0.46(0.2-1.05)	ISO intake (≥16.33 vs. <0.07 mg/d)	Stage, grade, ER/PR status, menopausal status, chemotherapy treatment, radiation, age, education, race, soy supplements intervention group,
(Goodman et al., 2009)	US	251/462	NCC	Incidence	1.57	0.88(0.78-0.99)	Urinary GEN	Crude OR. alcohol use, parity, and family history of breast cancer
						0,80(0.65-0.99)	Urinary ISO (25^{th} vs. 75^{th} percentile)	

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(Guha et al., 2009)	US	282 (1954)	Cohort	Recurrence	6.31	0.95(0.52-1.75)	GEN intake (≥13 vs. 0 mg/d)	Soy supplement use, BMI, menopausal status, tobacco pack-years, tumor stage, ER status, age, race
(Lowcock EC et al., 2013)	Canadian	2990(6369)	NNC	Incidence	2	0.82(= 0.69–0.97)	1/4 cup serving of flaxseed or 1 slice of flax bread	ages,last menstrual period; parity; hormone replacement therapy;benign Breast disease; BMI; alcohol intake

Abbreviations: ENL; enterolactone; HRT, hormone replacement therapy; BMI, body mass index, GEN, genistein; ISO, isoflavones; DAI, daidzein; CI, confidence interval; ER, estrogen receptor; NCC, nested case-control; OR, odds ratio; RR, relative risk; PR, progesterone receptor;

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Table 8 Human intervention and clinical studies of phyto-estrogens and breast cancer risk

Reference	PEs	Exposure	Subjects	Treatment length	Results
(Petrakis et al., 1996)	GEN	38 mg/d	24 normal (14 pre- and 10 post-menopausal) women	6 months	Cytological detection of epithelial hyperplasia in 7 of 24 women (29.2%)
(McMichael-Phillips et al., 1998)	ISO	45 mg/d	48 premenopausal women (9 malignant breast, others are with benign breast conditions), 19 consumed ISO	14 days	Increased the proliferation rate of breast lobular epithelium and PR expression

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(Thompson et al., 2005)	Flaxseed -containing muffin	25g/d	flaxseed-containing muffin (n = 19, postmenopausal breast cancer)	32d	Reduced c-erbB2 score and increased apoptotic index
(Cheng et al., 2007)	ISO	60mg/d	60 healthy postmenopausal women	3 months	Did not affect expression levels of steroid receptors; estrogen receptors α, β, and βcx; progesterone receptors A and B; or the proliferation marker Ki67
(Verheus et al., 2008)	Soy ISO	99 mg/d	202 Dutch postmenopausal	1 year	Did not significantly

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			women ages 60~75 years were randomized		differ the mammographic density
(Khan et al., 2012)	Soy ISO	150mg GEN +74mg DAI +11mg GLY	ISO intervention (n = 49, healthy, high risk women aged 25~55) or placebo (n = 49)	6 months	Did not reduce breast epithelial proliferation
(Chen and Liu, 2014)	Soy ISO	70 mg/d	75 menopausal women aged 50-70 years with estrogen reduction symptoms	1 year	Did not significantly differ in the TG,TC,LDL and Bone densities between control group and study group
(Clifton-Bligh et	isoflavone	50 mg/d	women more than	1 year	no beneficial

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al.,2015)	from red clover		10 years after menopause		effect on bone density,12% fall in serum LDL

Abbreviations: GEN, genistein; ISO, isoflavones; DAI, daidzein; GLY, glycitein; PR, progesterone receptor;

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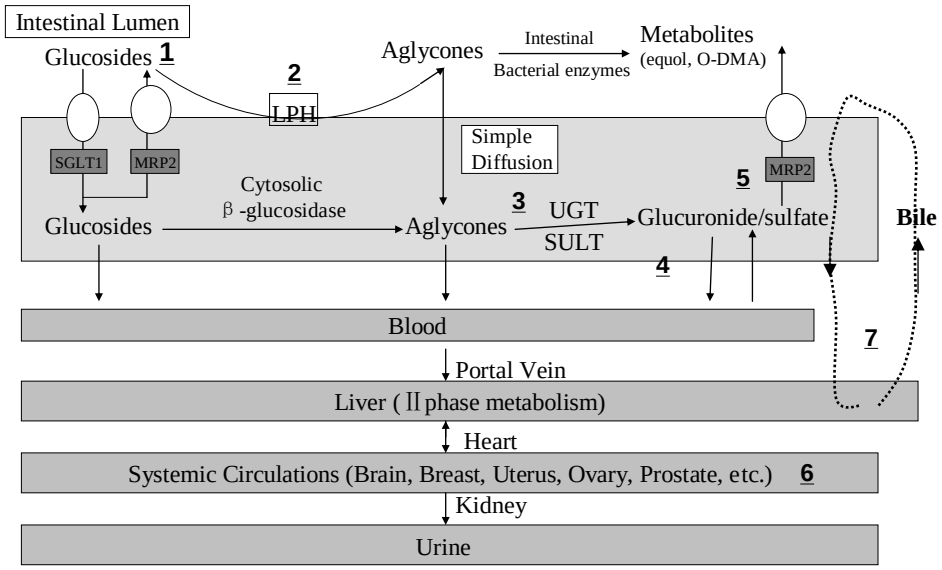

Fig. 1. Proposed pathways for small intestinal absorption and metabolism of isoflavone glucosides and their distribution in the body. LPH, lactase phloridzin hydrolase; SGLT1, sodium-dependent glucose transporter 1; MRP-2, multidrug resistance-associated protein-2. UGT, UDP-glucuronosyltransferase; SULT, phenol sulfotransferase. **1**, Glucosides are directly absorbed via SGLT-1followed by cytosolic β-glucosidase hydrolysis or excretion into the lumen via MRP-2; **2**, Glucosides are hydrolyzed by luminal LPH followed by the absorption of resulting aglycones via lipophilicity-dependent simple diffusion. **3**, Aglycone in the mucosa is converted into its conjugated metabolites by UGT and/or SULT. **4,5,** Conjugated metabolites are transported into the circulatory systems (4) or are excreted into the lumen (5). **6,** The metabolites are distributed in many tissues, such as brain, breast, uterus, ovary, prostate, etc.

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7, Enterohepatic recirculation: the metabolites recycle back to the intestine tract through bile excretion and reabsorbed by intestinal.

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