Isoflavones, equol producing status, and atherosclerosis in Japanese men in Japan

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Coronary heart disease (CHD) is the leading cause of morbidity and mortality worldwide. Soy is a potential nutritional source for preventing CHD and is a standard part of the traditional Asian diet. The main components of soy that may exert cardioprotective effects are soy isoflavones (ISFs). The predominant ISFs, daidzein, and genistein are structurally similar to estradiol and mimic some effects of estrogen. Estradiol exerts its biological action by binding both estrogen receptor α (ERα) expressed in reproductive, central nervous, cardiovascular and other systems and estrogen receptor β (ERβ) expressed in cardiovascular, central nervous and other systems. ISFs, however, preferentially bind to ERβ. ISFs may reduce CHD risk by reducing inflammation and oxidation; the latter may prevent the oxidative damage to low-density lipoprotein (LDL) that contributes to atherogenesis.

Although there are clear cardiovascular benefits of ISFs in preclinical studies, evidence in humans is conflicting. Furthermore, ISFs have very small or no effects on traditional CVD risk factors. A growing hypothesis is that the ability of humans to metabolize daidzein to equol may contribute to the cardioprotective effects of ISFs. Cell culture and preclinical studies show that equol has a greater affinity for ERβ than its precursor daidzein, a longer half-life, greater bioavailability than daidzein and genistein, and more potent antioxidant activity than any other ISFs. Therefore, equol may be more cardioprotective than ISFs.

The mechanistic model of action of equol on atherosclerosis is not completely understood. Investigation of the effects of equol has primarily been conducted in the in vitro assays and

preclinical studies and lacks a sturdy conclusion. In addition, very few studies have explored the association between equol-producing status and atherosclerosis in humans. In this dissertation, I first conducted a systematic review summarizing the current knowledge about the mechanisms underlying the potential cardioprotective effect of equol on inflammation, oxidation, and endothelial function. I then performed two cross-sectional analyses to delineate the link between equol producing status and aortic calcification in Japanese, a population being widely acknowledged to have a high prevalence of equol-producers.

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Preface

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1.0 Introduction on soy isoflavones (ISFs) and equol

1.1 ISFs

ISFs are a class of phytoestrogens that are highly present in soy. The major types of ISFs in soy are daidzein and genistein which are both structurally similar to estradiol (Figure 1) [1]. Estradiol exerts its biological action by binding both estrogen receptor α (ERα) expressed in the reproductive, central nervous, cardiovascular and other systems [2] and estrogen receptor β (ERβ) expressed in the cardiovascular, central nervous and other systems. ISFs, however, preferentially bind to ERβ [3]. The dietary intake of ISFs is 25-50 mg/day in northeast Asia in contrast to <2 mg/day in the US and other Western countries [4].

The cardioprotective effects of soy and ISFs have been studied for decades. In 1999, the US Food and Drug Administration approved a food-labeling health claim for soy protein (25 g/day) for prevention of coronary heart disease (CHD), based on the evidence acquired from a meta-analysis of 38 randomized controlled trials (RCT): 47 g/day of soy protein intake was associated with 23 mg/dL decrease in total cholesterol and 22 mg/dL decrease in low-density-lipoprotein cholesterol (LDL-C) [5]. However, soy protein was later shown with small clinical significances in preventing CHD: a reduction of LDL-C by 3-5% was achieved by treating participants with 50 g/day of soy protein [6]. RCTs have shown that ISFs, independent of soy protein, reduce the progression of coronary and carotid atherosclerosis in both male and female monkeys [7-9].

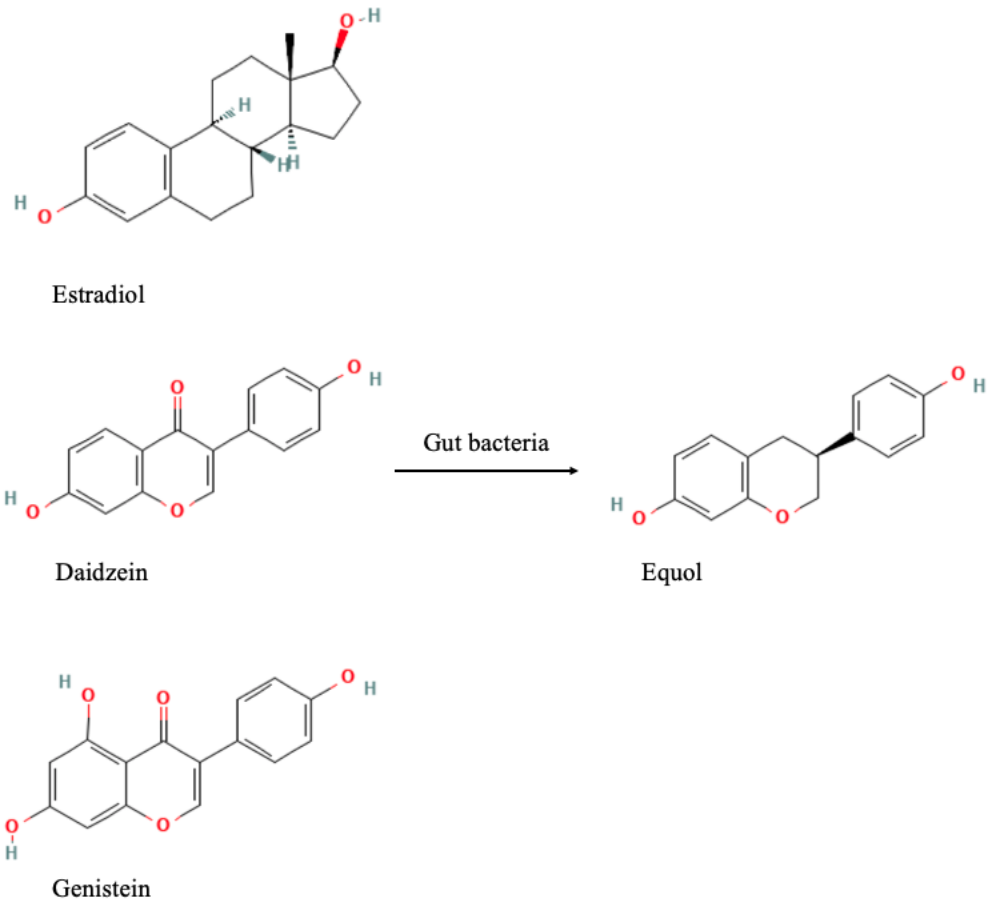


Figure 1 Structures of estradiol, daidzein, genistein, and equol

Structures of genistein, daidzein, and equol [10-12]. Genistein and daidzein are two major ISFs and comprise >95% of dietary sources. Equol is a metabolite of daidzein, bio-transformed by the gut bacteria.

ISFs possess antioxidant [13-20] and anti-inflammatory properties [21-24], suggested by the studies showing that ISFs reduced the levels of oxidative and inflammatory markers that play essential roles in the pathogenesis of atherosclerosis. In addition, ISFs improve the vascular endothelial function [25-27]. Some of the above effects are achieved through binding to the ER [28].

1.2 Equol

However, researchers think that only individuals who can produce equol by the gut microbiome after consuming ISFs can benefit from ISFs. Equol exists in two mirror-image forms known as enantiomers, S-equol and R-equol; only S-equol is secreted by the gut in humans and animals after the consumption of daidzein [29]. S-equol (referred to as "equol" throughout this dissertation) has the following characteristics that make it superior to ISFs: 1) Equol does not undergo phase II biotransformation, unlike ISFs, and thus possesses higher bioavailability than ISFs [30]. 2) Equol has higher anti-oxidant properties than ISFs [31-35], its anti-oxidant properties are even greater than vitamins C and E in *in vitro* studies [36]. 3) Equol has a slower clearance rate than ISFs [31-35]. 4) The affinity of equol to ERβ is much higher than daidzein and similar to genistein [37]. 5) Equol has a lower ability to bind to serum proteins (*i.e.*, albumin, sex-hormone-binding globulin and alpha-fetoprotein) and thus have greater availability for receptor occupancy than endogenous estrogens and ISFs. It is reported <5% of estradiol and 18.7% of daidzein are present in the free form in contrast to 49.7% of equol that circulates in the free form [38].

There is a variation in the equol-producing capacity across populations. Equol-producing status can be determined based on the amount of equol that is isolated from the urine or plasma [39]. There has been no widely used definitions of equol-producers [39]. Equol-producing bacteria in the intestine are anaerobes, rod-shaped, and gram-positive [40, 41]. We are able to generate equol *in vitro* by using bacteria isolated from the feces of animals and humans [42]. Individual who can produce equol is classified as an "equol-producer".

Several observational studies showed that soy intake increased the capacity. Equol excretion was significantly increased following weeks of ingestion of soy products among healthy people [43]. The rate of equol-producers was lifted to 50-60% after a couple of weeks of

soy intake while it was 13-27% under the usual diet [44, 45]. Two out of ten healthy non-producers became equol-producers after the 3-month ISFs intervention [46]. On the contrary, equol producing status were unchanged following a high dose of soy intake in the RCTs in healthy people [47].

Plant-based diet may also influence the equol-producing capacity [48]. Setchell *et al.* reported that the rate of equol-producers in the vegetarians in the U.S. was 59%, similar to the rate in Japanese adults consuming soy, and much higher than that of non-vegetarian American adults (25%) [49]. Peeters *et al.* also showed that vegetarian than non-vegetarian was more likely to be equol-producers [50]. Studies also showed that subjects with lower intake of total fat [51-53] and alcohol [54], or higher intake of dietary fiber [51-53], polyunsaturated fatty acids [54] and probiotic supplementation [55] were more likely to be equol-producers and had increased equol excretion.

The rate of equol-producers depends on the definition of the equol-producers [39]. Sekikawa *et al.* recently published a review that summarized all studies that had ever reported the rate of the equol-producers [39]. The cut-off points used to define the equol-producing status based on serum and urinal equol level varied across studies – some studies used the limit of the detection while others used a value that can distinguish one part of participants from the other. Correspondingly, equol-producers could be participants whose equol in the urine >0.27 ng/mL or >0.4 μM/day; or whose equol in the serum >2.1 nM/L or >0.5 ng/mL [39]. In Japanese populations, the rate of equol-producers ranged from 20 to 77%; in Korean, the number was 24 to 75%; in Chinese, 13 to 60%; and in the Americans, 14 to 69% [39]. Previous studies also showed that the rate of equol-producers was 26-32% in Westerners and up to 75% in Asians. However, it should be noted that in some of the Western studies the soy products were provided to the participants

before the equol was quantified [56, 57] while in some Asian studies the participants were not treated with soy and therefore the rate of equol-producers could be underestimated in Asian individuals [40, 58-61].

Given the fact that people living in Western countries seldom eat soy, Setchell defined equol-producers as below: following a 3-day period of ISF intake, people with the log 10-transformed 24-hour urinary equol to daidzein ratio >-1.75 were classified as the equol-producer [49]. This classification provided a clearer distinction of equol producing status than the absolute serum or urinary equol concentrations because it was independent of ISFs intake and minimizes inter-individual variation in ISFs pharmacokinetics in a population with low soy consumption.

1.3 Biological effects of ISFs

Both clinical and preclinical studies showed that ISFs exert antioxidant and anti-inflammatory effects and improve endothelial function. Evidence has been consistent in animals but not in humans. Main biological effects are summarized in Figure 2.

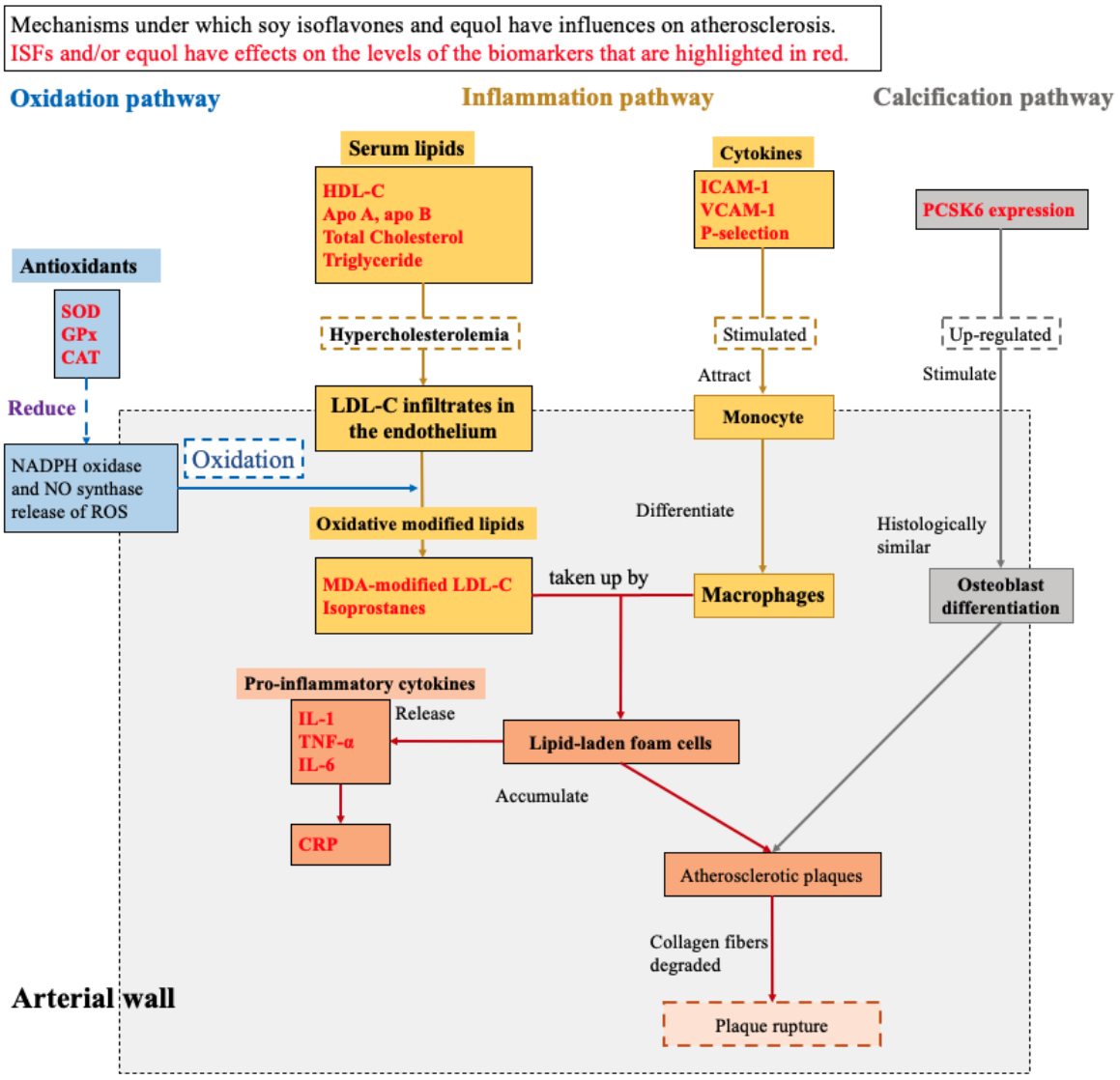


Figure 2 Antioxidative, anti-inflammatory, and vasodilatory effects of ISFs in the atherogenic process

ISFs: soy isoflavones, CAT: catalase, Apo: Apolipoprotein, GPx: glutathione peroxidase, HDL-C: high-density lipoprotein, cholesterol, ICAM: intercellular cell adhesion molecule 1, IL: interleukin, LDL-C: low-density lipoprotein cholesterol, TNF-α: tumor necrosis factor alpha, NAPDH: nicotinamide adenine dinucleotide phosphate, NO: nitric oxide, ROS: reactive oxygen species, SOD: superoxide dismutase, VCAM-1: vascular cell adhesion molecule 1, PCSK6: proprotein convertase subtilisin/kexin type 6.

1.3.1 Antioxidative effects

Atherosclerosis progressively narrows the arteries that reduces the flow of blood and leads to plaque formation [62]. The early stages of atherogenesis characterizes by molecular changes induced by cytokines and reactive oxidant species (ROS), involving endothelial cells, LDL-C, and macrophages [63, 64]. ROS oxidize LDLs, giving formation to oxidized LDL (oxLDL). OxLDL activates the endothelium by inducing the production of adhesion molecules, which recruit monocytes and T-cells. Monocytes differentiate into foam cells and, along with T-cells, release pro-inflammatory cytokines and contributes to the formation of an atherosclerotic plaque [62].

Both preclinical studies and human studies have provided solid evidence on the association between pro-oxidant agents and atherosclerosis. In preclinical studies, nicotinamide adenine dinucleotide phosphate oxidase and myeloperoxidase (MPO) serve as an enzymatic source of oxidant species to generate oxLDL in the vulnerable plaques [65]. Moreover, the increase in the NADPH oxidase activity is associated with a higher risk of atherosclerosis. In human studies, higher levels of F2-isoprostanes, oxLDL and MPO were associated with increased risks of CHD [66]. Moreover, an up-regulation of genes of ROS-producing enzymes boosted the development of CHD [67]. An over-expression of NADPH oxidase was associated with accelerated atherosclerotic lesion progression [68]. In contrast, a higher intake of dietary antioxidants (such as β-carotene, α-tocopherol, and ascorbic acid) was associated with lower risks of major adverse cardiovascular events [69, 70]. Moreover, individuals with MPO deficiency have presented a reduced rate of CHD [71].

ISFs reduce levels of antioxidant biomarkers in both animal and human studies. However, the evidence has been inconsistent in humans. Preclinical studies have shown that ISFs enhance the overall antioxidant capacity of plasma: ISFs significantly increase the activities of antioxidants

such as superoxide (SOD) and glutathione peroxidase (GPx), and elevate the concentration of antioxidant enzymes such as catalase [14-16]. ISFs also reduce the concentration of oxidation products such as malondialdehyde (MDA) [14-16]. Some studies showed that only high pharmacological levels of ISFs have antioxidant effects [15].

However, the antioxidative effect of ISFs has been inconsistently shown in humans. A couple of RCTs are summarized as follows: on the one hand, an RCT of 182 postmenopausal women aged 47-60 showed that 100mg/day ISFs for 1-year reduced MDA concentration [72]. Similarly, another crossover study of 24 participants showed that ISFs reduced plasma 8-epi-prostaglandin F2 alpha and increased the resistance of LDL to oxidation [18]. On the other hand, one RCT that treated 42 participants with 50mg/1000 kcal of ISFs for 42 days reported that ISFs did not reduce urinary F2-isoprostanes [73]. Another RCT treated 43 women with 75mg/day of ISFs for 3 months reported that ISFs did not improve the lipid peroxidation and the level of catalase, SOD, or GPx [74]. Moreover, a trial that treated 32 postmenopausal women with 100 mg/day of ISFs for 10 weeks showed that there were no differences in the changes between groups for SOD, and total antioxidant capacity. Finally, a trial that treated 82 women with > 40 mg/day of ISFs for 6 months did not support the hypothesis that ISFs reduce the level of isoprostane [75]. These null results may be partially due to the short intervention periods of these trials and the heterogeneity of the participants across studies.

Overall, ISFs may have an antioxidant effect in humans. Given inconsistent results from RCTs, at least part of the cardioprotective mechanism of ISFs might be due to some other reasons apart from the antioxidant pathway.

1.3.2 Anti-inflammatory effects

Inflammation participates in atherosclerosis from its inception to development [76]. The accumulation of oxLDL triggers the activation of proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and nuclear factor-κB, which initiate the expression of vascular cell adhesion molecule-1 (VCAM-1) produced by endothelial cells (ECs). VCAM-1 thus allows the attachment of leukocytes, monocytes, and T lymphocytes to the arterial wall [77]. Once adhered to the arterial endothelium, monocytes penetrate the endothelial lining and enter the intima of the vessel wall by diapedesis between ECs. Within the intima, monocytes mature into macrophages, engulf modified lipoproteins, and become foam cells.

Despite the abundance of laboratory and observational study evidence indicating that the presence of an oxidative imbalance is associated with an increased risk of CVDs, the results were not supported by large-scale primary and secondary prevention RCTs of CVD by supplementation of antioxidants. In particular, many of these trials reported little or no observed risk reduction in antioxidants among participants of a variety of characteristics [78-84].

ISFs reduce the levels of inflammatory biomarkers in animal and human studies although evidence is not consistent in humans. Observational studies in humans showed that ISFs were inversely associated with circulating levels of CRP [21], IL-6, TNFα, and soluble TNF receptors 1 and 2 which are essential for TNFα-signaling [22]. On the contrary, a meta-analysis of RCTs showed that ISFs had no effect on the levels of TNFα [23], IL-6 [23] and CRP (Figure 3) [24]. In a meta-analysis (6 RCTs, 438 participants), the mean difference for the change of plasma concentration of TNFα between ISFs and control group was 0.087 (95%CI: -0.051, 0.224), and the mean difference for IL-6 was 0.017 (95%CI: -0.001, 0.036) [23]. However, the small number of studies (6 RCTs), the small sample size and the short duration of intervention across studies

limited the interpretation of the pooled results. In another meta-analysis (36 RCTs), the effect of ISFs on CRP was -0.19 (95%CI: -0.49, 0.09) [24]. The results from recent RCTs add to the controversy of this issue. ISFs significantly reduce serum VCAM-1 and ICAM-1 in one RCT with peritoneal dialysis participants [85] but ISFs did not reduce serum VCAM-1 in another RCT with postmenopausal women who were equol-producers [86].

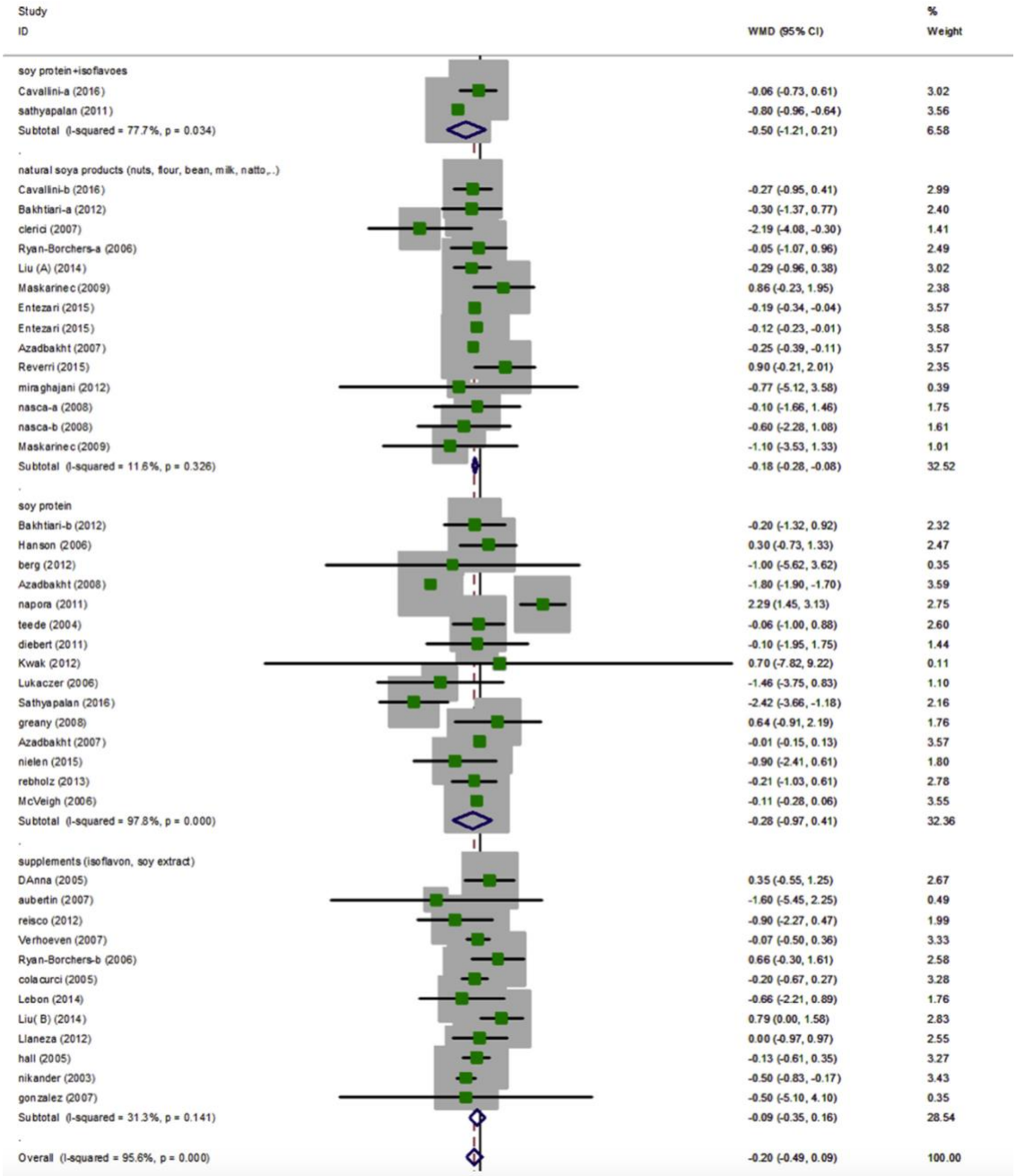


Figure 3 Effect of ISFs (and soy protein) on CRP

Reprinted from Meta-Analysis Clin Nutr. Mahdieh Khodarahmi. A systematic review and meta-analysis of the effects of soy on serum hs-CRP. Meta-Analysis Clin Nutr. 2019 Jun;38(3):996-1011.[24].

In conclusion, RCTs have not provided strong evidence regarding the beneficial effect of ISFs on inflammation which may be partly explained by the short intervention duration of RCTs and the heterogeneity of the participants. However, the observational studies supported that ISFs reduced the levels of inflammatory markers.

1.3.3 The effects on endothelial function

All of the above oxidative and inflammatory processes cause the damage of vascular endothelium, leading finally to the formation of arteriosclerotic plaque. Meanwhile, the rise of VCAM-1, platelet-selectin and others cytokines are the biomarkers of endothelial dysfunction [87].

In RCTs in humans, the effect of ISFs on endothelial function has been inconsistent. Flow-mediated vasodilation (FMD) of the peripheral conduit arteries is one of the most widely used tests of endothelial function. FMD measures the endothelial vasomotor response during reactive hyperemia. In a meta-analysis of 17 RCTs of ISFs, the overall mean absolute change in FMD for ISFs-containing soy product interventions was 1.15% (95% CI: -0.52, 2.75) [88]. This meta-analysis included both men and women with a mean age of about 60. Seven of the 17 studies found statistically significant changes in FMD as compared to controls. The inconsistency of the results may be due to the heterogeneity in the intervention duration (ranging from 6 to 52 weeks) and the characteristics of the participants (health status varies, including dyslipidemia, ischemia, and healthy). It is also likely that three studies reporting no effect were underpowered ($n<20$ participants per treatment group), as defined in a recent report of the International Brachial Artery Reactivity Task Force [89]. Another meta-analysis of nine RCTs with post-menopausal women

reported that ISFs supplementation even exerts harmful effects on endothelial function. The overall effect on FMD was a weighted mean difference 1.75% (95% CI: 0.83, 2.67) [90].

ISFs may improve vasodilation. *In vitro* studies showed that ISFs can inhibit vasoconstrictive responses to endothelin-1, histamine, serotonin, angiotensin II, and noradrenaline [25-27]. These functions are exerted through binding to the ERβ. In addition, genistein serves as the tyrosine kinase inhibitor and reduces intracellular calcium activity in arteries stimulated with noradrenaline, which antagonizes contractions [91]. ISFs, because of their vasodilatory effect by binding to ER and the effect for inhibiting the oxidized-LDL by stimulating the sirtuin-1 pathway [92], have a higher efficacy of the prevention of endothelial dysfunction than the ovarian steroid 17β-estradiol [25].

1.4 Biological effects of equol

There is a paucity of human studies on the effect of equol. We extract evidence of the anti-oxidative, vasodilative, anti-inflammatory, and anti-calcification effects of equol mainly from the cell line and animal studies. The justification of a superior effect of equol than ISFs is also summarized.

1.4.1 Anti-oxidant effect

Cell culture studies show that equol is a potent antioxidant in numerous models (Table 1). Equol protected macrophages from oxidative stress induced by L-lactate dehydrogenase or oxLDL by reducing lipid peroxidation product MDA, enhancing antioxidant glutathione, and increasing

the activities of the antioxidant enzyme SOD [93, 94]. Equol caused reduced toxic action of ROS produced by neutrophils, achieved through decreasing the phosphorylation of proteins regulating NADPH oxidase [95]. Phagocytic cells, after being incubated with equol for 1 hour, showed a significant reduction in the intracellular production of superoxide anion and hydrogen peroxide [96]. In the intestinal epithelial cells with oxidative stress induced by hydrogen peroxide, equol promoted the expression of antioxidant genes, increased the activities of SOD, and increased the abundance of nuclear factor erythroid 2 (Nrf2) transcripts [97]. Finally, equol decreased the ratio of reduced/oxidized glutathione in primary cortical neuron cells [98]. Although some antioxidant effects are independent of ERβ [99], these effects are generally considered to be mediated through ERβ.

Table 1 Antioxidant effects of equol

#	Authors	Type	Findings	Has effect
1	Lin [97]	In vitro	Equol was shown to protect chicken intestinal epithelial cells from oxidative damage by promoting the expression of antioxidant genes, increasing the activities of antioxidant enzymes, and enhancing antioxidant capacity. Equol significantly enhanced total SOD activity and the Nrf2 transcript.	Yes
2	Pereboom [96]	In vitro	Equol decreased the intracellular production of the superoxide anion and hydrogen peroxide content of phagocytic cells.	Yes
3	Hwang [100]	In vitro	Equol and ascorbic acid interacted synergistically in preventing LDL oxidation. All phases of LDL oxidation were affected by these compounds, which is atypical of the behavior of antioxidants that are consumed during the early phases. Equol was more potent than daidzein and genistein because of its absence of a carbonyl group, C2-C3 double bond and flanking hydroxyl groups in the pyran ring.	Yes

#	Authors	Type	Findings	Has effect
4	Pažourekov á [95]	In vitro	Upon activation by ROS, neutrophils treated by equol produced less p40 phox (a component of NADPH oxidase, responsible for the assembly of functional oxidase in intracellular membranes) both extra- and intracellularly to the control.	Yes
5	Choi [101]	In vitro	Equol pretreatment significantly decreased levels of oxidative stress biomarkers such as thiobarbituric acid-reactive substances, carbonyl content and serum 8-hydroxy-2-deoxyguanosine. Moreover, equol increased the activity of CAT, superoxide dismutase, GPx and glutathione reductase. In addition, equol possessed anticancer activity through acting as an antioxidant therefore reduced apoptosis.	Yes
6	Wei [102]	In vitro	Low doses of equol could prevent skeletal muscle cell damage induced by hydrogen peroxide. Equol increased cell viability, the concentration of MDA content, and LDH activity.	Yes
7	Kamiyama [103]	In vitro	Equol might contribute to a reduced level of oxLDL-stimulated apoptosis linked to the reduced generation of intracellular ROS in human umbilical vein endothelial cells.	Yes
8	Sierens [104]	In vitro	Equol was able to function as an antioxidant, scavenging potentially harmful free radicals. Equol protected against oxidative-induced DNA damage. Pretreatment of a physiological range of equol offered protection against the hydrogen peroxide-mediated DNA damage in human lymphocytes cells. This protection was greater than that offered by the addition of antioxidant vitamins ascorbic acid and alpha-tocopherol, or the compounds 17β-estradiol and tamoxifen, which have similar structures to ISFs and are known to have moderate antioxidant activity.	Yes
9	Rüfer [105]	In vitro	Equol exhibited higher antioxidant activity than daidzein and about the same antioxidant capacity as the oxidative metabolites of daidzein and genistein despite the lack of the 2,3-double bond with the 4-oxo group and a 5,7-dihydroxyl structure. The antioxidative effect was tested by an ORAC assay which determined the ability of compounds to scavenge peroxyl radicals.	Yes
10	Hwang [34]	In vitro	Equol inhibited LDL oxidation in vitro and LDL oxidative modification by monocyte/macrophages. The antioxidant effect of equol was found to be mediated by inhibition of superoxide radical production and manifested through enhanced levels of free NO. Equol	Yes

#	Authors	Type	Findings	Has effect
			had a greater antioxidant activity than genistein and daidzein.	
11	Sierens [106]	In vitro	Pretreatment with equol significantly protected sperm DNA against oxidative damage. Compared with ascorbic acid and alpha-tocopherol, being added at physiological concentrations, genistein was the most potent antioxidant, followed by equol, ascorbic acid, and alpha-tocopherol. Equol might have a role to play in antioxidant protection against male infertility.	Yes
12	Arora [107]	In vitro	Compared to genistein and daidzein with their glycosylated and methoxylated derivatives, equol and its 4-hydroxy and 5-hydroxy derivatives were more potent antioxidants, suggesting that the absence of the 2, 3-double bond and the 4-oxo group on the ISF nucleus enhanced antioxidant activity.	Yes
13	Turner [108]	In vitro	Equol inhibited the oxidation of LDL 2.65-fold more than its parent compound daidzein.	Yes
14	Choi [98]	In vitro	Equol acted as an antioxidant in the brain of rats. The ratio of GSH/GSSG in primary cortical neuron cells exposed equol for 24 and 72 hours significantly decreased in a time- and dose-dependent manner. Moreover, equol treatment was significantly increased the LDH release in a time-and dose-dependent manner.	Yes
15	Gou [94]	In vitro	Equol protected chicken macrophages from oxidative stress induced by lipopolysaccharide through reducing lipid peroxidation products such as MDA and enhancing contents of antioxidants such as glutathione, and activities of relevant antioxidase enzymes such as total SOD; effects were also seen in gene expression related to the immune response and increased contents of cytokines.	Yes
16	Liu [109]	In vitro	Equol elevated brain antioxidant activity by increasing SOD, CAT and GPx levels. MDA levels and AChE activity were decreased in hypertensive and vascular dementia rats. Equol further improved the long- and short-term memory of the rats.	Yes
17	Vedavanam [110]	In vivo	The order of the half-maximal inhibitory concentration values, the indication of the potency of inhibiting glucose-induced LDL lipid peroxidation observed for the compounds was equol > genistein > daidzein.	Yes

#	Authors	Type	Findings	Has effect
18	Choi [93]	In vivo	Equol might act as an antioxidant through an inhibition of oxidative stress and stimulation of CAT and SOD, but could also cause pro-oxidant effects, such as reduction of the GSH/GSSG ratio, depending on the treatment period. Study in mice showed that equol administration significantly inhibited biomarkers of oxidative stress (thiobarbituric acid-reactive substances value, carbonyl content, and serum 8-hydroxydeoxyguanosine). Moreover, CAT and total SOD activities and their transcripts were significantly increased by equol. Although equol increased the glutathione peroxidase activity in mice treated with equol for 1-week, long-term administration of equol (7 weeks) caused a decrease in the ratio of GSH/GSSG and the activities of GPx and glutathione reductase .	Yes
19	Ma [111]	In vivo	A study in male and ovariectomized female rats with transient middle cerebral artery occlusion revealed that pretreatment of equol significantly reduced infarct size in both sexes. This neuroprotection was accompanied by a decrease in NADPH oxidase activity and superoxide levels in the brain. In addition, equol reduced plasma thiobarbituric acid reactive substances and neurological deficits up to 7 days after injury.	Yes
20	Horiuchi [112]	In vitro	The study demonstrated that equol had suppressive effects against oxidative stress in pancreatic β-cells in a dose-dependent manner and presumably through activating PKA signaling.	Yes
21	Jackman [31]	In vivo	Equol exerted weak antioxidant effects in cerebral arteries, whereas effects of daidzein were insignificant. Antioxidant activity was assessed as the reduction of NADPH-induced superoxide levels.	Yes
22	Widyarini [113]	In vivo	In addition to the activation of estrogenic signaling pathways for photoprotection, equol also provided UV-protective antioxidant effects that depend partially on HO-1 induction. Equol dose-dependently inhibited the oxidative stress measured as UVA-induced lipid peroxidation on mouse skin. A component of equol lipid protection capacity is attributed to endogenous cutaneous antioxidant enzymes including the inducible stress protein HO-1.	Yes
23	Nhan [114]	Human	Urinary equol was not associated with the secretion of urinary F2 isoprostane,a measure of cellular lipid peroxidation, after ISF treatment in postmenopausal women. But the observations on the effect of equol were	No

#	Authors	Type	Findings	Has effect
			limited because only 2 of the 8 subjects were equol producers, one of whom experienced a large increase in the biomarker excretion, whereas the other experienced small decreases.	
24	Hidayat [86]	Human	The level of MDA, an oxidative stress marker, was lower in equol producers than non-producers. This RCT was conducted with 190 postmenopausal women aged 47-60 and who received 100 mg ISFs for 6 months. The random allocation of ISFs intervention was carried out separately by equol producing status.	Yes
25	Richardson [99]	In vitro	Equol might have a beneficial effect in delaying the onset and decreasing the severity of symptoms in Friedreich's ataxia patients by an antioxidant mechanism such as reducing ROS-induced modification of proteins and lipids and impaired mitochondrial function. These effects were independent of ERβ.	Yes

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; MDA, malondialdehyde; AChE, acetylcholinesterase; PKA, protein kinase A; Nrf2, nuclear factor erythroid 2–related factor 2; NADPH, nicotinamide adenine dinucleotide phosphate; LDH, L-lactate dehydrogenase; ORAC, oxygen radical absorbance capacity; GSH/GSSG, reduced/oxidized glutathione; DNA, deoxyribonucleic acid; HO-1, heme oxygenase-1; NQO1, NADPH-quinone oxidoreductase 1; UV, ultraviolet.

Animal studies also demonstrated the antioxidant properties of equol. Two mice studies demonstrated that equol improved vascular health in the brain [31, 109]. It was suggested that equol elevated the activities of SOD, catalase, acetylcholinesterase, and glutathione peroxidase, and decreased MDA levels in mice that had deoxycorticosterone acetate salt-induced hypertension and associated vascular dementia [109]. Equol also reduced NADPH-induced superoxide production in the cerebral arteries of normotensive rats and hypertensive rats that were induced by angiotensin II [31]. However, the antioxidant effect was not observed in daidzein [31]. One study showed that equol suppressed tumor formation in rats, presumably through decreasing the concentrations of thiobarbituric acid-reactive substances and 8-hydroxy-2-deoxyguanosine, and increasing the activity of catalase, SOD, glutathione peroxidase [101].

One RCT in humans demonstrated that equol-producing individuals could receive the antioxidant benefits from ISFs but non-producers could not. An RCT of 190 post-menopausal women found that after 6 months of supplementation with ISFs, blood MDA concentrations were significantly lower in the equol producers compared with non-producers in the ISFs group [86].

At the normal physiological concentrations, equol is a more potent antioxidant compared to ISFs probably due to the absence of 2,3-double bond, the 4-oxo group, and a 5,7-dihydroxyl [100, 105-107, 110]. The antioxidant property of equol is even more potent than ascorbic acid, alpha-tocopherol, and 17β-estradiol. Comparative studies found that the potency of inhibiting LDL lipid peroxidation was in such order: equol > genistein > daidzein [100, 110].

1.4.2 Anti-inflammatory effect

Equol inhibits the overproduction of inflammatory biomarkers expressed by macrophages, microglia cells, and adipose tissues. In addition, equol ameliorates inflammatory processes important to the pathogenesis of rheumatoid arthritis, metabolic syndrome and intracranial aneurysm (Table 2).

Table 2 Anti-inflammatory effect of equol

#	Authors	Type	Findings	Has effect
1	Blay [115]	In vitro	Equol (10 μM) significantly inhibited the overproduction of NO and PGE2 induced by LPS plus INF-γ when a pre-treatment was performed or when administered during activation. Moreover, equol regulated gene transcription of cytokines and inflammatory markers. Genistein (20 μM) exerted similar anti-inflammatory effects, but daidzein did not.	Yes

#	Authors	Type	Findings	Has effect
2	Johnson [116]	In vitro	Equol exhibited protective effects against pro-inflammatory cytokines (IL-6 and TNF-α) and NO production in murine microglia cells. Equol also showed greater permeability through artificial gut and blood-brain barriers compared to daidzein.	Yes
3	Obiorah [117]	In vitro	Equol and ISFs induced endoplasmic reticulum stress and inflammatory response stress-related genes in a comparable manner to estrogens. Equol and ISFs induced proliferation of estrogenized breast cancer cells (simulating a perimenopausal state) but induced apoptosis of estrogen-deprived cells (simulating a postmenopausal state).	Yes
4	Nagarajan [118]	In vitro	In an in vitro LPS-induced inflammation model, equol dose-dependently inhibited LPS-induced MCP-1 secretion by macrophages.	Yes
5	Subedi [119]	In vitro	In microglial cells, equol inhibited TLR4 activation, MAPK activation, NF-kB-mediated transcription of inflammatory mediators, production of NO, release of PGE-2, secretion of TNF-α and IL-6, in LPS-activated murine microglia cells.	Yes
6	Moriyama [120]	In vitro	Equol attenuated LPS-induced NO production with a concomitant decrease in expression of iNOS. Equol did not affect LPS-induced increase in intracellular ROS production. Increased NO production is a well-known inflammatory change in astrocytes stimulated by LPS. Attenuation of NO production by equol may mitigate LPS-induced neuroinflammation in astrocytes.	Yes
7	Lin [121]	In vivo	Equol-administered collagen-induced arthritis mice had lower severity of arthritis symptoms. Equol administration suppressed the expression of IL-6 and its receptor in the inflamed area of collagen-induced arthritis mice.	Yes
8	Yokosuka [122]	In vivo	In ovariectomized mice induced to have intracranial aneurysms, equol protected against aneurysm formation; the disruption of the intestinal microbial conversion of daidzein to equol abolished daidzein's protective effect against aneurysm formation. Moreover, mice treated with equol had lower inflammatory cytokines in their cerebral arteries.	Yes
9	van der Velpen [123]	Human	In the adipose tissue of postmenopausal women, expression of inflammation-related genes was upregulated in equol producers but downregulated in non-producers.	Yes
10	Törmälä [124]	Human	ISFs caused a decrease in the VCAM-1 and platelet-selectin. The fall in platelet-selectin was more marked in	Yes

#	Authors	Type	Findings	Has effect
			equol producers. No changes appeared in SHBG, CRP or ICAM-1.	
11	Reverri [125]	Human	Consuming soy improved arterial stiffness as was assessed by the augmentation index, but did not improve the inflammatory biomarkers (CRP, TNF-α, IL-6, IL-18, IL-10). The addition of equol producing status as a covariate did not significantly change these results.	No
12	Nicastro [21]	Human	Equol, while not associated with a decrease in CRP level, was associated with decreased geometric mean WBC counts comparing the highest quartile to the lowest.	Yes
13	Greany [126]	Human	An RCT of 34 postmenopausal women on 44 mg/day of ISFs showed that ISFs did not influence the concentrations of Hcy, CRP, sE-selectin, sVCAM-1, and sICAM-1. Equol producing status did not modify the associations.	No
14	Mangano [127]	Human	In women who received the ISFs intervention, there was no significant differences in percent change in serum inflammatory markers between equol producers and non-producers.	No

Abbreviations: NO, nitric oxide; PGE2, prostaglandin E_2; LPS, lipopolysaccharide; INF-γ, interferon gamma; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractant protein-1; MetS, metabolic syndrome; CRP, C-reactive protein; sICAM, soluble intercellular adhesion molecule; VCAM-1, vascular cell adhesion molecule 1; SHBG, sex hormone binding globulin; ICAM-1, intercellular adhesion molecule 1; IL-8, interleukin-8; IL-10, interleukin-10; WBC, white blood cell; Hcy, homocysteine; sE-selectin, soluble endothelial leukocyte adhesion molecule-1; TLR4, toll-like receptor 4; MAPK, mitogen-activated protein kinase; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; RCT: randomized controlled trial.

In vitro studies have consistently shown that equol is anti-inflammatory. First, in macrophages, equol down-regulated the expression of genes related to the production of various kinds of cytokines and inflammatory biomarkers [115], and dose-dependently reduced the secretion of inflammatory biomarkers such as prostaglandin E2 [115] and MCP-1 [118], whereas daidzein did not have such effect [115]. In microglia cells, equol reduced the release of interleukin-6 (IL-6) [116, 119] and TNF-α [116, 119]. These effects may be mediated through the inhibition of Toll-like receptor 4, mitogen-activated protein kinase (MAPK), or NF-κB [119]. In astrocytes, equol attenuated nitric oxide (NO) production and concomitantly decreased the expression of inducible NO synthase [120].

Several in vivo studies in mice reported the effect of equol on inflammatory diseases and inflammation. Equol administration in a rheumatoid arthritis mouse model improved arthritis-induced bone mineral density and suppressed the expression of IL-6 and its receptor in inflamed areas [121]. In ovariectomized mice induced to have intracranial aneurysms, daidzein with equol supplementations protected against aneurysm formation, whereas the disruption of the intestinal microbial conversion of daidzein to equol abolished daidzein's protective effect against aneurysm formation, indicating that equol alone had a protective effect [122]. The same study also suggested that mice treated with equol had lower inflammatory cytokines in their cerebral arteries [122]. All of the above effects were exerted through the activation of ERβ.

In some human RCTs of ISFs, the anti-inflammatory effects of ISFs were more pronounced in equol producers than non-producers, while in other RCTs, the anti-inflammatory effects of ISFs were only observed in equol producers. One RCT of ISFs among 110 postmenopausal women on tibolone showed that equol producers had a larger reduction in platelet-selectin levels than non-producers after being treated with ISFs [128]. Platelet-selectin is a cell adhesion molecule on the surface of activated ECs [129]. In the peripheral blood mononuclear cells in 30 equol-producing postmenopausal women, ISFs intervention down-regulated clusters of genes that were involved in inflammation, oxidative phosphorylation, and cell cycle regulation, as was suggested by the analysis in the whole-genome gene expression profiles [130]. Further analysis in the same group of participants later reported that ISF supplements upregulated the expression of anti-inflammatory genes in the adipose tissue of equol producers but down-regulated the expression in non-producers [123].

1.4.3 Improvement of endothelial function

It has been observed in several categories of vessels (umbilical vein, aorta, pulmonary artery, and cerebral basilar artery) that equol stimulated endothelial redox signaling and increased NO production in the ECs [35, 131, 132] (Table 3). Studies indicated that equol, by binding to ERβ, could rapidly stimulate phosphorylation of extracellular signal-regulated protein kinase 1/2 and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), leading to the activation of endothelial NO synthase (eNOS) [131, 133]. NO can react with superoxide anions to form peroxynitrite. NO and peroxynitrite then, in turn, enhance the nuclear accumulation of Nrf2, which binds to an antioxidant response element in target genes to enhance the transcription of the phase II antioxidant defense enzymes, such as superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase, and heme oxygenase-1 [134]. A second pathway by which equol improved endothelial function was through transactivation of the epidermal growth factor receptor kinase, resulting in a reorganized F-actin cytoskeleton [135]. A third pathway in which equol was involved was through the direct upregulation of eNOS, resulting in reduced oxidative stress in ECs [35, 136]. The target gene eNOS contains an estrogen-response element. Thus, it can be reasoned that the binding of equol to ERβ may be responsible for enhanced eNOS expression. In a fourth pathway, equol could increase the expression of phospho-p38 MAPK and B-cell lymphoma-2 to reduce intracellular ROS production in ECs [137]. Furthermore, equol directly inhibited apoptosis of ECs [138], presumably through stimulating the thymidine incorporation which is important for the deoxyribonucleic acid (DNA) synthesis of ECs [138]. This stimulatory effect on cell growth was not observed for daidzein or genistein [138].

Table 3 Equol improves endothelial function

#	Authors	Type	Findings	Has effect
1	Joy [133]	In vitro	Nutritionally relevant plasma concentrations of equol rapidly stimulated phosphorylation of ERK1/2 and PI3K/Akt, leading to the activation of NOS and increased NO production at resting cytosolic Ca^{2+} levels.	Yes
2	Rowlands [135]	In vitro	Equol-stimulated mitochondrial ROS modulated endothelial redox signaling and NO release through transactivation of epidermal growth factor receptor kinase and reorganization of the F-actin cytoskeleton.	Yes
3	Cheng [35]	In vitro	Equol prevented oxidative damage to vascular function in pulmonary cells via downregulating eNOS and oxidative stress.	Yes
4	Zhang [131]	In vitro	In HUVEC, equol increased Nrf2 mRNA as well as mRNA of gene products of HO-1 and NQO1. Pretreatment of cells with specific endoplasmic reticulum inhibitors or PI3K/Akt increased Nrf2, HO-1, and NQO1 protein.	Yes
5	Chung [137]	In vitro	Equol had a significant antioxidant effect on the bAECs that were exposed to hydrogen peroxide. Equol pretreatment effectively inhibited the hydrogen peroxide-induced cell death by the reduction of intracellular ROS production, probably through increasing phospho-p38 MAPK.	Yes
6	Zhang [139]	In vitro	The improvement of atherosclerosis by equol through attenuation of endoplasmic reticulum stress is mediated by activating the Nrf2 signaling pathway. Equol treatment inhibited cell apoptosis and attenuated upregulation of endoplasmic reticulum stress markers in HUVECs. In an oxidative stress environment, equol treatment dose-dependently activated the Nrf2 signaling pathway.	Yes
7	Somjen [138]	In vitro	Equol, but not daidzein and genistein, had a monophasic stimulatory effect on thymidine incorporation, which boosts DNA synthesis. In human endothelial cells, equol, daidzein, and genistein stimulated DNA synthesis in a dose-dependent manner. The administration of equol, daidzein, and genistein to immature and ovariectomized female rats resulted in increased creatine phosphokinase in the aorta and in the left ventricle of the heart.	Yes

#	Authors	Type	Findings	Has effect
8	Kim [140]	In vitro	Equol had a vasodilatory effect on human uterine arteries vascular smooth muscle, which was mediated through antagonistic action for receptor-dependent Ca^{2+} channel.	Yes
9	Johnson [116]	In vitro	Equol exhibited protective effects against NO production in murine microglial cells. Equol also showed greater permeability through artificial gut and blood-brain barriers compared to daidzein.	Yes
10	Chin-Dusting [25]	In vivo	Equol had a dose-dependent inhibition of the contractile responses to noradrenaline in rat isolated aortic rings. Equol independently increased the release of a vasoconstrictor prostanoid, such as thromboxane.	Yes
11	Jackman [31]	In vivo	In normotensive rats, equol displayed vasorelaxant activity similar to daidzein. The relaxant effect of equol was independent of intact endothelium, NOS activity, K^+ channels and gender. In the basilar artery, where superoxide levels are higher, equol exerted weak antioxidant effects, whereas effects of daidzein were insignificant. During hypertension, equol-induced vasorelaxation was preserved, whereas relaxant responses to daidzein were impaired.	Yes
12	Matsumoto [141]	In vivo	Contractions induced by a selective 5-HT receptor agonist increased with insulin treatment, but less so with equol + insulin. In the endothelium-denuded preparations, 5-HT-induced contractions were augmented with insulin treatment but less so by equol + insulin treatment. These differences in 5-HT-induced contractions were eliminated by a large-conductance Ca^{2+}-activated K^+ channel inhibitor.	Yes
13	Yu [132]	In vivo	Equol significantly increased regional cerebral blood flow in rats and produced an endothelium-independent relaxation in rat cerebral basilar arteries. Selective Ca^{2+}-activated K^+ channel blockers significantly inhibited equol-induced vasodilation in cerebral arteries.	Yes
14	Ohkura [136]	In vivo	Ovariectomized rats were assigned to 1) ISF-deficient but equol-sufficient group, 2) ISFs-deficient and equol-deficient group. In the thoracic artery, endothelium-dependent relaxation, cyclic guanosine monophosphate levels in the tissue, and eNOS synthase expression and phosphorylation were significantly higher in the first group compared to the second group.	Yes

#	Authors	Type	Findings	Has effect
15	Törmälä [128]	Human	Before ISF intervention, women with a 4-fold elevation in equol levels had a lower endothelial function index compared to women without this capacity. Soy supplementation had no effect on arterial stiffness or endothelial function in either group.	Yes
16	Kreijkamp-Kaspers [142]	Human	This RCT did not support the hypothesis that ISFs have beneficial effects on endothelial function in older postmenopausal women. However, in the soy-only group, systolic and diastolic blood pressure decreased and endothelial function improved in the equol producers, whereas blood pressure increased and endothelial function deteriorated in the non-producers.	Yes
17	Hidayat [86]	Human	ISFs did not improve endothelial functions in both equol producer and non-producers. The VCAM-1 and NO did not differ by equol-producing status.	No
18	Clerici [143]	Human	After ISFs treatment, brachial artery flow-mediated vasodilatation was improved more obviously in equol producers.	Yes

Abbreviations: ERK1/2, extracellular signal-regulated protein kinases 1 and 2; PI3K/Akt, protein kinase 1/2 and phosphatidylinositol 3-kinase/protein kinase B; NOS, nitric oxide synthase; NO, nitric oxide; ROS, reactive oxygen species; HUVEC, human umbilical vein endothelial cell; eNOS, endothelial nitric oxide synthase; HO-1, heme oxygenase-1; NQO1, NADPH-quinone oxidoreductase 1; Nrf2, nuclear factor-erythroid 2-related factor 2; bAECs, bovine aortic endothelial cell; MAPK, mitogen-activated protein kinase; HUVECs, human umbilical vein endothelial cells; cfPWV, carotid-femoral pulse wave velocity; VCAM-1, vascular cell adhesion molecule-1; 5-HT, 5-hydroxytryptamine; Ca, calcium; K, potassium.

Independent of an effect on the intact endothelium and NOS activity, equol exhibited vasodilator activity in vascular smooth muscle cells of various of arteries which may be induced by antagonistic of the Ca2+-activated K+ channel [31, 132, 140, 141]. This vasodilatory effect was not observed with daidzein [31].

Equol-producing status in humans may be critical in unlocking the endothelial benefits of ISFs. In a long-term RCT of ISFs among 202 older postmenopausal women, endothelial function was significantly improved in equol producers whereas it deteriorated in non-producers, when both groups were treated with ISFs [142]. Secondary analysis of RCT of ISFs with 110 women [128] reported significantly improved endothelial function in equol producers as compared to controls

whereas ISFs themselves had no significant effects on endothelial function. Another RCT of ISFs in 62 adults with hypercholesterolemia observed the vasodilatory effect of ISFs on brachial artery, and determined that improvement was larger in equol producers [143].

1.4.4 Anti-calcification effect of equol

It has been suggested that equol stimulates osteoblast differentiation by upregulating the expression of proprotein convertase subtilisin/kexin type 6 (PCSK6) by binding to the equol receptor β [144]. The process of osteoblast differentiation is histologically similar to the process of calcium deposition in the arterial wall [145].

1.5 ISFs, equol, and cardiovascular diseases (CVDs)

Soy was suggested as reducing the likelihood of a CHD event in 1998. One study was conducted on Chinese adults who were 60+ years old and it had 21,494 deceased cases and 10,968 living controls. Soy consumption was inversely associated with ischemic heart disease. The odds ratio for CHD mortality in participants whose soy intake was ≥ 4 times/week was 0.61 (95%CI: 0.42, 0.88) for men and 0.60 (95%CI: 0.42, 0.87) for women compared to those whose soy intake was < 1 time/month [146].

Starting in 2001, studies have specifically focused on ISFs. The WHO CARDIAC Study presented ecological evidence that urinary ISFs were significantly inversely associated with CHD mortality in a total of 61 populations from 25 countries [147, 148]. In the same year, a series of preclinical evidence supported that ISFs possess anti-atherogenic properties independent of soy

protein. An RCT with three arms: soy protein only, soy protein plus ISFs, and soy protein plus estrogen was conducted among post-menopausal female monkeys for a 36-month intervention. The result showed that the soy protein plus ISFs group had significantly slower progression of where atherosclerosis as compared to the soy protein only group. Another RCT with three arms: casein/lactalbumin, soy protein only, and soy protein plus ISFs in male monkeys for 14-months intervention showed that compared to casein/lactalbumin group, the soy protein only group and the soy protein plus ISFs group had 50% and 90% less coronary atherosclerosis, respectively [9].

In 2003, the Shanghai Women's Health Study in China was the first prospective cohort study that reported a significant inverse relationship between soy protein intake and CHD [149]. After a mean follow-up of 2.5 years of 64,915 women aged 40-70 years at baseline, 62 incident cases of CHD (43 non-fatal myocardial infarction (MI) and 19 CHD deaths) occurred. A multivariable-adjusted hazard ratio (HR) in the highest tertile of soy protein intake compared to the lowest was 0.25 (95%CI: 0.10, 0.63) for CHD and 0.14 (95%CI: 0.04, 0.48) for non-fatal MI. However, the HRs could potentially be overestimated because of self-reported hypertension and the absence of measurement of serum cholesterol.

In 2007, the Japan Public Health Center-Based Study Cohort I was the first prospective cohort study that reported a significant inverse association of ISFs intake with CHD [150]. After 12.5 years of follow-up of 40,462 men and women aged 40-59 years at baseline, 308 incident cases of MI occurred. A multivariable-adjusted HR in the highest quintile of ISFs intake compared to the lowest was 0.37 (95%CI: 0.14, 0.98) for women. The mean dietary intake of ISFs in the highest and lowest quintile was 45.2 mg/day and 10.6 mg/day, respectively. Again, the HRs could potentially be overestimated because hypertension and lipid medication used for statistical adjustment was self-reported and the blood levels of lipids were not assessed. On the contrary, in

2005, no association of ISFs with CHD was found in the European Prospective study Into Cancer and Nutrition Dutch cohort during a median follow-up of 75 months [151]. The null result is likely to be due to very low dietary intake of ISFs in this population which was less than 1 mg/day.

In 2012, equol was first suggested to be inversely associated with the incident CHD [152]. This was a case-control study nested within the Shanghai Women's Health Study and Shanghai Men's Health Study. There were 536 and 559 incident CHD cases for women (mean follow-up time of 10 years) and men (mean follow-up time of 5 years), respectively. Total urinary ISFs excretion was not associated with the development of CHD in either women or men. Among individual ISFs, equol excretion was significantly and inversely associated with CHD in women. The fully adjusted ORs (95% CIs) for CHD across increasing quartiles of equol levels in women were 1 (reference), 0.61 (0.32, 1.15), 0.51 (0.26, 0.98) and 0.46 (0.24, 0.89) (P = 0.02 for trend). In men, none of the individual ISFs showed significant associations with CHD.

Up to now, there have been no additional observational studies or RCTs examining equol and CVDs.

1.6 ISFs, equol and CVD risk factors

The effect of ISFs on CHD risk factors could be modified by equol producing status (Table 4). We showed a summary of the RCTs that have found statistically significant improvements in the risk factors for CHD after the ISFs intervention compared with placebo, more importantly, equol producer status further improved risk factors for CHD (including LDL-C, TG, SBP, DBP, FMD, soluble intercellular adhesion molecule-1, platelet-selectin and CRP). We also showed the RCTs that found no association between ISFs and risk factors for CHD compared with placebo,

yet equol producer status significantly improved risk factors for CHD (including total cholesterol, LDL-C, TG, apoA, apoB, blood pressure, mean arterial pressure, carotid to femoral pulse wave velocity).

Table 4 RCTs of ISFs that showed the modification effect of equol producing status

First author, year	Parameters of risk factors		Effect of ISFs intervention on the parameters		Differential effect by equol producing status	
Acharjee et al., 2015	Inflammatory	C-reactive protein (CRP)	11.8% (p=0.04) and 30% (p=0.01) reduction in CRP compared to placebo in women with and without metabolic syndrome (MetS), respectively.	+	21.4% (p=0.01) and 30% (p=0.04) reduction in CRP compared to placebo in equol-producers with and without MetS, respectively. Regarding non-producers, there were no significant effects.	+
		Intercellular adhesion molecule-1 (ICAM-1)	5.2% (p=0.04) reduction in ICAM-1 compared with placebo in women with MetS.	+	7.3% (p=0.03) reduction in ICAM-1 in equol-producers with MetS compared with placebo. Regarding non-producers, there were no significant effects.	+
Pusparini & Hidayat, 2015	Inflammatory	Malondialdehyde (MDA)	Reduction in MDA (p=0.021)	+	Equol-producers had a greater decline in MDA than non-producers	+
Törmälä et al., 2018	Inflammatory	Platelet selectin (P-selectin)	P-selectin decreased by 10.3 % (p=0.002) compared with placebo	+	equol-producers had a greater decline (13.5 %; p=0.007) than non-producers (7.7 %; non-significant)	+

First author, year	Parameters of risk factors		Effect of ISFs intervention on the parameters		Differential effect by equol producing status	
Clerici et al., 2007	Inflammatory	CRP	Reduction in CRP (2.2 (SD 0.9) mg/l, p=0.03) compared with placebo	+	CRP decreased 0.9 (SD 0.5) mg/l more in equol-producers than non-producers (p=0.025)	+
Clerici et al., 2007	Endothelial function	Flow-mediated dilation (FMD)	Increase in FMD (2 (SD 0.8) %; p=0.012) compared with placebo	+	Increase in FMD in equol-producers (p=0.03), unlike in non-producers.	+
Acharjee et al., 2015	Lipid	Triglyceride	17.8% reduction in triglyceride in women with MetS (p=0.04) compared with placebo	+	Reduction in triglyceride in equol-producers with MetS (22.9 %, p=0.02) compared with placebo. There were non-significant effects on non-producers with or without MetS in triglyceride	+
Clerici et al., 2007	Lipid	Low-density lipoprotein - cholesterol (LDL-C)	8.6% reduction in LDL-C (p=0.002) compared with placebo	+	LDL-C reduced 15 (SD 7) mg/dl more in equol-producers than in non-producers (p=0.042) after the intervention.	+
Hall et al., 2006	Lipid	% small density LDL-C (% sdLDL-C)	The ISFs intervention was associated with a greater reduction of % sdLDL-C compared with placebo (24.14 (SD 14.26) and 22.22 (SD	+	The interaction between equol producing status and intervention was significant (p< 0.05)	+

First author, year	Parameters of risk factors		Effect of ISFs intervention on the parameters		Differential effect by equol producing status	
			11.87), respectively; p=0.044)			
Mangano et al., 2013	Lipid	Total cholesterol (TC) : high-density lipoprotein - cholesterol (HDL-C),	The intervention had a non-significant effect on TC:HDL-C compared with placebo	0	Equol-producers had lower TC:HDL compared with non-producers (p=0.018) after the intervention	+
		LDL-C:HDL-C	The soy intervention had a non-significant effect on LDL-C:HDL-C compared with placebo	0	Equol-producers had lower LDL-C:HDL-C compared with non-producers (p=0.043) after the intervention	+
Wong et al., 2012	Lipid	HDL-C	The soy intervention had a non-significant effect on HDL-C compared with placebo	0	HDL-C reduced in non-producers but not in equol-producers (−0.07 (SD 0.02) and 0.0 (SD 0.03) mmol/l, respectively; p=0.036)	+
		Apolipoprotein A-1 (ApoA-I)	The soy intervention had a non-significant effect on apoA-I compared with placebo	0	Apo A-I reduced in non-producers but not in equol-producers (−0.08 (SD 0.02) and −0.02 (SD 0.02) g/l, respectively; p=0.010)	+
Pipe et al. 2009	Lipid	TC	The ISFs intervention had a non-significant effect on the risk factors	0	There was an interaction between equol producing status and TC (p=0.05).	+

First author, year	Parameters of risk factors		Effect of ISFs intervention on the parameters		Differential effect by equol producing status	
			compared with placebo			
		Apolipoprotein B (apoB)	The ISFs intervention had a non-significant effect on the risk factors compared with placebo	0	There was an interaction between equol producing status and apoB (p=0.04).	+
Hall et al., 2006	Lipid	Lipoprotein (a)	The ISFs intervention had a non-significant effect	0	There was an interaction between equol producing status and treatment (p< 0.05)	+
McVeigh et al., 2006	Lipid	LDL-C	The soy intervention had a non-significant effect on LDL-C compared with placebo	0	Equol producing status is associated with a significant decrease on the low-ISFs diet (p=0.035) and high-ISFs diet (p=0.041) compared with placebo	+
Meyer et al., 2004	Lipid	TC	The soy intervention had a non-significant effect compared with placebo	0	8.5% significant reduction in equol-producers (p<0.001) but no significant reduction is observed in non-producers.	+
		LDL-C	The soy intervention had a non-significant effect compared with placebo	0	10% significant reduction in equol-producers (p<0.001) but no significant reduction is observed in non-producers.	+
		LDL-C:HDL-C	The soy intervention had a non-significant effect	0	13.5% significant reduction in equol-producers (p<0.001) but no significant reduction is observed in non-producers.	+

First author, year	Parameters of risk factors		Effect of ISFs intervention on the parameters		Differential effect by equol producing status	
			compared with placebo			
		Triglyceride	The soy intervention had a non-significant effect compared with placebo	0	21% significant reduction in equol-producers ($p<0.001$) but no significant reduction is observed in non-producers.	+
		Lipoprotein (a)	The soy intervention had a non-significant effect compared with placebo	0	11% significant reduction in equol-producers ($p<0.001$) but no significant reduction is observed in non-producers.	+
Acharjee et al., 2015	Blood pressure	Diastolic blood pressure (DBP)	5.4% ($p=0.03$) and 3.4% ($p=0.0008$) reduction in women with and without MetS, respectively	+	Equol-producers with and without MetS had reduced DBP (7.7 %, $p=0.02$ and 3.3 %, $p=0.02$, respectively) compared with placebo. There were non-significanteffects on non-producers with or without MetS in DBP	+
Curtis et al., 2013	Blood pressure	Blood pressure	The intervention had a non-significant effect compared with placebo.	0	Equol-producers compared with non-producers had reduced BP ($p=0.01$), DBP (EP: −2.24 (se 1.31) mmHg; non-producers: 1.00 (se 0.89) mmHg; $p<$ 0.01), MAP (EP: −1.24 (se 1.30) mmHg; non-producers: 1.90 (se 1.08) mm Hg; $p=0.01$) .	+

First author, year	Parameters of risk factors		Effect of ISFs intervention on the parameters		Differential effect by equol producing status	
		DBP	The flavonoid intervention had a non-significant greater reduction compared with placebo (p=0.06)	0	Inverse correlation (P=0.08) between DBP and equol concentration in equol-producers but no association in non-producers	+
		MAP (mean arterial pressure)	The flavonoid intervention had a non-significant greater reduction compared with placebo (p=0.06)	0	Inverse correlation (P<0.1) between MAP and equol concentration in equol-producers but no association in non-producers	+
Welty et al., 2007	Blood pressure	Systolic blood pressure (SBP)	Reduction in SBP in hypertensive women (9.9 %, p=0.003) and normotensive women (5.2 %, p< 0.001) compared with the placebo	+	The percentage reduction in SBP was positively correlated with the level of equol in the intervention group (p=0.02)	+

SD: standrad deviation, ISFs: soy isoflavones.

+ in column 5: beneficial effect of ISFs.

+ in column 7: beneficial effect of equol-producing status on risk factors after ISFs (or soy) interventions.

This table is partially built upon Rahel L Birru, et al. The impact of equol-producing status in modifying the effect of soya isoflavones on risk factors for CHD: a systematic review of randomised controlled trials. J Nutr Sci. 2016 Jul 19;5:e30. [153].

1.6.1 ISFs, equol, and lipids

A series of meta-analyses have reported the beneficial effect of ISFs on lipids (Table 5). In 2007, Taku *et al*. conducted a meta-analysis of 11 RCTs (471 participants) and showed that soy protein that enriched with ISFs (on average 102 mg) significantly decreased serum total cholesterol (3.9 mg/dL, 1.77%) and LDL-C (5.0 mg/dL, 3.58%) compared with the same amounts of ISFs-depleted soy protein over 1-3 months [154]. The above effects were especially apparent in hypercholesterolemic subjects. These results suggest that ISFs may have synergistic effects on lipids when provided concurrently with soy protein. In 2011, Yang *et al.* conducted a meta-analysis of 8 RCTs (183 participants with type 2 diabetes) of soy products on lipids [155]. This study concluded that the consumption of soy products significantly reduced total cholesterol by 16.2, LDL-C by 11.6, and triglycerides by 19.5 mg/dL, as well as significantly increased HDL-C by 1.9 mg/dL, compared to the placebo groups. Similarly, Tokede *et al*. also conducted a meta-analysis of 35 RCTs (1,687 participants) of soy products on lipids [156], showing significant reductions in LDL-C by 4%, TG by 3% and total cholesterol by 2% and a significant increase in HDL-C by 3%. The meta-regression analysis showed that baseline levels of total cholesterol, LDL-C and triglycerides were significant determinants of reduction in lipid levels.

Table 5 The effect of ISFs on lipids

Author, year	# of RCTs, participants	Comparison	Effect
Taku, 2007	11, general people	Soy protein enriching ISFs (102 mg) vs. soy protein depleting ISFs	Significantly decreases total cholesterol by 3.9 mg/dL, and LDL-C by 5.0 mg/dL
Yang, 2011	8, diabetic patients	Soy products containing ISFs vs. placebo	Significantly decreases total cholesterol by 16.2 mg/dL, LDL-C by 11.6 mg/dL, and triglycerides by 19.5 mg/dL
Tokede, 2015	35, hyperlipidemia patients	Soy products containing ISFs vs. placebo	Significantly decreases total cholesterol decreases by 2%, LDL-C by 4%, and triglycerides by 3%
Taku, 2008	23, menopausal women	70 mg ISFs vs. placebo	Total cholesterol and LDL-C were not significantly decreased (0.38 mg/dL and 1.2 mg/dL, respectively)
Balk, 2005	178, general people	A median of 80 mg ISFs vs. placebo	Total cholesteroal and LDL-C were not significantly decreased (data not shown)

Notably, although ISFs may have additive and independent effects on lowering LDL-C when offered concurrently with soy protein, the extracted form of ISFs alone may not have these effects [154, 157, 158]. However, in another meta-analysis, there was no effect of ISFs on triglycerides or LDL-C over 1-3 months [157]. The lack of any beneficial effect of extracted ISFs on LDL-C, HDL-C, and triglycerides was consistent with another meta-analysis of 178 RCTs with at least 4 weeks' duration [158]. In the meta-analysis by Balk et al., the dose of ISFs extracts was not associated with any lipid; however, soy products appear to exert a small benefit on LDL and triglycerides. These effects of soy products may be of small clinical effect in individuals yet possibly have a larger population-wise effect. The inconsistency of the results among Taku *et al.* (2007), Taku *et al.* (2008), and Balk *et al.* (2005) may be due to a large amount of heterogeneity across studies that were included in the meta-analyses, as well as poor reporting or inadequate study design of a large proportion of studies. For example, Taku *et al.* (2007) included more trials evaluating the effects of soy protein using larger amounts of ISFs in hypercholesterolemic subjects and used different approaches to determine the combined treatment effects. Nevertheless, additional well-designed and especially large-scale RCTs are still needed to clarify this issue

because the previous evidence summarized above was based on trials with relatively small sample sizes and short duration.

Equol has been available as dietary supplement and has been used as treatment in one RCT. In this RCT of equol supplements (10 mg equol were given daily for 12 weeks) in 54 Japanese overweight or obese people, equol led to a significant 0.2 mmol/L decrease in serum LDL-C level as compared with placebo group [41].

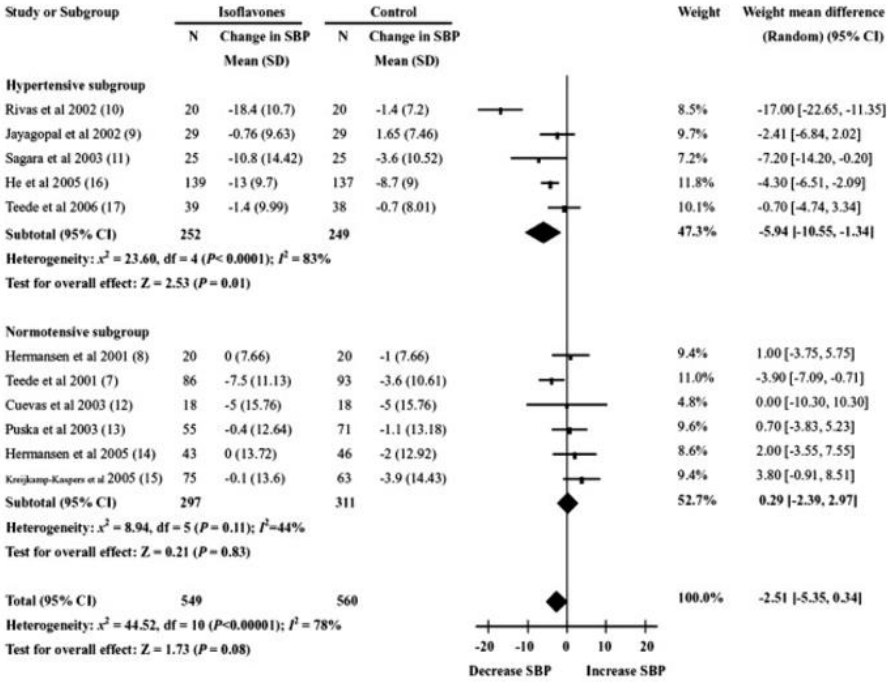
Equol-producing status may modify the effect of ISFs on lipids (Table 4). In the secondary analyses of RCTs of ISFs that have already observed the favorable effect of ISFs on lipids, equol-producers had significantly improved LDL-C, HDL-C, total cholesterol and triglycerides than non-producers [143, 159, 160]. In addition, in RCTs of ISFs that did not observe the favorable effect of ISFs, equol-producers also had significantly improved lipids [127, 161-164]. On the other hand, equol-producers did not modify the effect of ISFs on lipids in some other RCTs [143, 162, 163, 165-170]. Finally, a cross-sectional study suggested that the benefits of ISFs on lipids may be more apparent among equol-producers only [59]. Equol-producers with higher daily ISFs intakes (5.4 mg/d) tended to have higher HDL-C ($P = 0.055$) than did those with lower ISFs intakes (1.5 mg/d) while no association was observed in non-producers [59].

In addition, RCTs have shown that the effects of ISFs on adipose tissue gene expression were influenced by equol-producing status [123]. ISFs resulted in a caloric restriction-like gene expression profile for both producer status and pointed toward a potential beneficial effect, whereas ISFs induced anti-inflammatory gene expression in equol-producers only [123].

1.6.2 ISFs, equol, and blood pressure

A series of studies have reported the beneficial effect of ISFs on blood pressure (BP) (Figure 4). He *et al*. in the meta-analysis found that ISFs reduced SBP by 2-6 mmHg - especially in hypertensive subjects - which corresponds to the effect of decreasing salt intake by 2-4 g/day [171]. In 2010, Taku *et al.* conducted a meta-analysis of 14 RCTs (789 participants) of ISFs on BP [172], concluding that the consumption of ISFs significantly decreased SBP by 1.9 mmHg compared to the placebo group. In 2012, Liu *et al.* conducted a meta-analysis of 11 RCTs (1,109 participants) of ISFs on BP [173], suggesting that ISFs had an effect of lowering BP only in hypertensive subjects but not in normotensive subjects. In the overall sample, the ISFs-treated group did not have significantly reduced systolic BP (-2.5 mmHg, 95% CI: -5.4, 0.3 mmHg) or reduced diastolic BP (-1.5 mmHg, 95% CI: -3.1, 0.2 mmHg) compared to the placebo group. Subgroup analyses in hypertensive group showed the significant results: SBP: -5.9 (95% CI: -10.6, -1.3) mmHg; DBP: -3.4 (95% CI: -6.5, -0.19) mmHg. Yet, it should be noticed that the subgroup analysis included only five trials. In 2017, Kou *et al.* conducted a meta-analysis of 12 RCTs (1,551 participants) of ISFs on BP among postmenopausal women [174]. Compared to the placebo group, the soy protein-treated group had significantly reduced systolic BP and diastolic BP by 3.03 mmHg and 0.71 mmHg, respectively.

(A) Systolic blood pressure



(B) Diastolic blood pressure

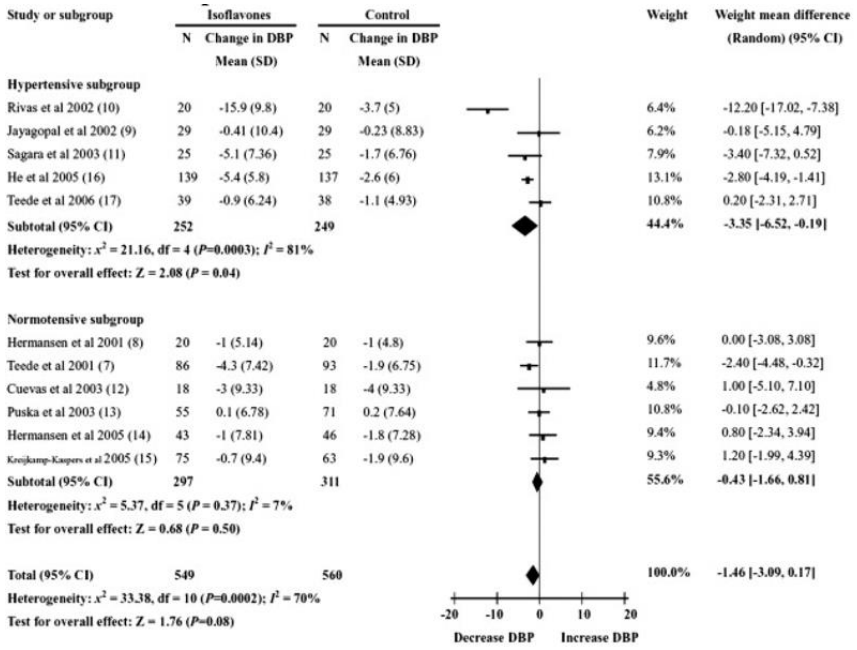


Figure 4 The effect of ISFs on blood pressure

Reprinted from Nutrition, metabolism, and cardiovascular diseases: NMCD. 2012;22, Liu XX, Li SH, Chen JZ, et al. Effect of soy isoflavones on blood pressure: a meta-analysis of randomized controlled trials. Nutrition, metabolism, and cardiovascular diseases: NMCD. 2012;22(6):463-470. Copyright © (2012), with permission from Elsevier.

Note that equol-producing status may modify the effect of ISFs on BP (Table 4). In the secondary analyses of RCTs of ISFs on BP, equol-producers significantly improved systolic and diastolic BP in RCTs that have observed the favorable effect of ISFs [143, 159, 175] and others that have not observed the favorable effect of ISFs, though equol-producers did not modify BP in some other RCTs [125, 159, 164, 175]. In summary, RCTs of equol treatment on BP are awaited to justify the effect of equol producing status on the BP.

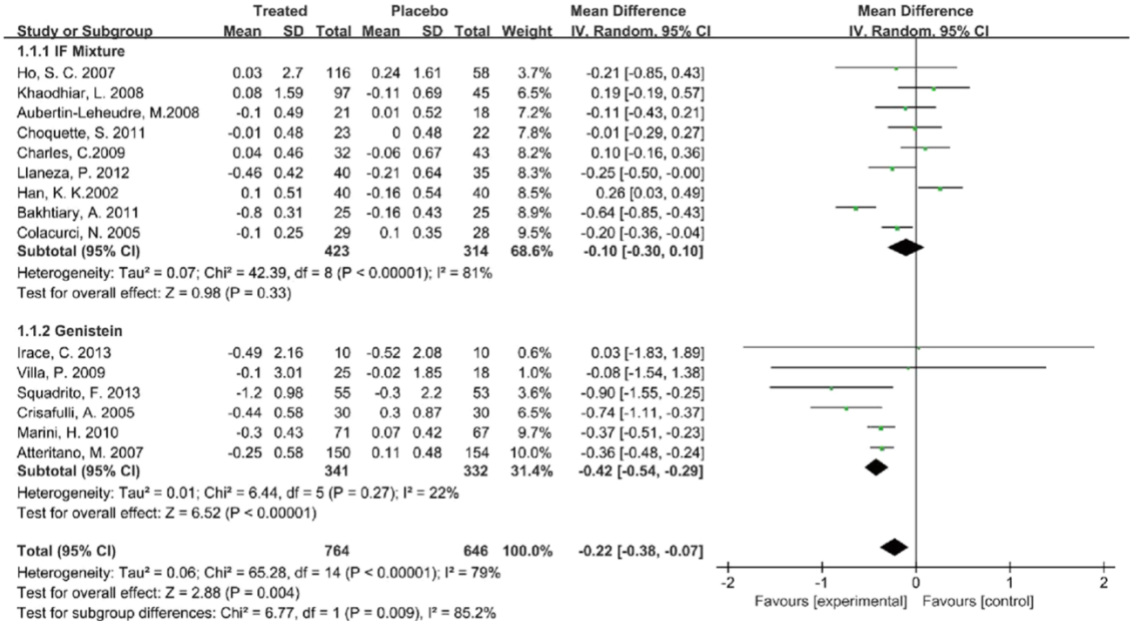
1.6.3 ISFs, equol, and blood glucose

ISFs have been suggested to improve glucose metabolism in observational studies and RCTs. Systematic reviews of observational studies showed that ISFs reduced the risk of type 2 diabetes (T2D). In 2016, Ding *et al*. in a pooled analysis of three large US cohorts (the Nurse's Health Study, the Nurse's Health Study II, and the Health Professionals Follow-up Study) reported that ISFs were inversely associated with incident T2D (the multivariable-adjusted HR in the highest compared to the lowest consumption of ISFs was 0.89 (95%CI: 0.83, 0.96) [176]. Soy food intake, however, was not associated with T2D. The result might be debated because ISFs intake is very low in the US and the effect of ISFs on health outcomes in Westerners is weak. In 2018, Li et al. in a meta-analysis of 8 prospective and 2 cross-sectional studies reported that soy intake was associated with a 23% (95% CI: 0.66, 0.91) lower risk of T2D [177]. Moreover, of those studies that reported on soy constituent, ISFs intake was associated with a 12% lower risk of T2D (95% CI: 0.80, 0.97) [177]. This study further showed that significant inverse associations between soy consumption and T2D were significant in women but not men and in Asians but not in non-Asians.

Systematic reviews of RCTs showed that ISFs reduced fasting blood glucose and improved insulin resistance. Fang *et al.* in a meta-analysis of 17 RCTs (1,529 menopausal women) concluded that ISFs group had 8.6 mg/dL lower fasting blood glucose, 16.6 mg/dL lower insulin, as well as 20 Mass Units lower homeostasis model assessment insulin resistance (HOMA-IR) than compared to placebo (

Figure 5) [178]. Ricci *et al.* in a meta-analysis of 10 RCTs (794 peri- and post-menopausal non-Asian women) and reported that the ISFs group had significantly lower insulin (-42 mIU/L) and HOMA-IR (-0.39) but non-significantly lower blood glucose [179]. Subgroup analysis showed that genistein alone significantly reduced fasting blood glucose by -7.15 mg/dL. Overall, ISFs reduced fasting blood glucose and improved insulin resistance.

(A) Glucose



(B) Insulin

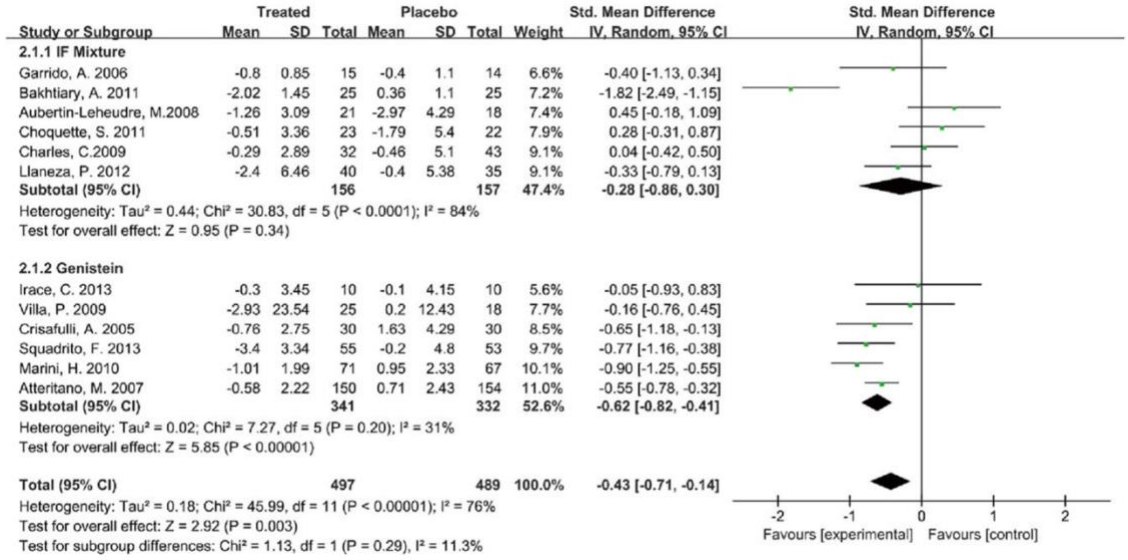


Figure 5 The effect of ISFs on glucose and insulin

Reprint from Mol Nutr Food Res. Ke Fang. Soy isoflavones and glucose metabolism in menopausal women: A systematic review and meta-analysis of randomized controlled trials. Mol Nutr Food Res. 2016 Jul;60(7):1602-14.

2.0 Coronary artery calcification (CAC) and aortic calcification (AC)

CAC has been widely accepted as an biomarker of atherosclerosis [180, 181]. It has also been suggested that CAC and AC provide incremental prognostic value beyond cardiovascular risk models based on traditional cardiovascular risk factors (e.g., Framingham Risk Score) [182]. A recent study suggested that aortic calcification may be another predictor of atherosclerosis [183].

2.1 CAC

CAC is a highly specific biomarker of coronary atherosclerosis [145, 184]. Based on the clinical and population-based studies in both Western [185-187]and East Asian populations[188], CAC is a consistent and reproducible measurement of assessing risk for major cardiovascular outcomes.

2.1.1 Histology of vascular calcification

Ectopic bone production is the basis for calcification [189]. Study has found that matrix gla protein, an inhibitor of bone morphogenetic protein, is highly expressed in calcified human arteries [190]. Moreover, CHD risk factors are critical to the development of CAC. Modified low-density lipoprotein and oxidized phospholipids are pro-osteogenic mediators [191]. The apolipoproteins and oxidized phospholipids in the artery wall propagate inflammation and stimulate the vascular calcification [192]. Several factors associated with oxidative stress are

implicated in calcification [191]. Glucose promotes vascular cell calcification [193], yet insulin inhibits it [194]. Adipose-derived factors affect calcification, with leptin promoting and adiponectin inhibiting vascular calcification [195].

2.1.2 Predictive value of CAC presence and CAC progression

CAC increased the risk-predictive value. CAC has incremental value over traditional risk factors [196-198]. Multi-Ethnic Study of Atherosclerosis (MESA) of >6000 adults aged from 45 to 84 years showed that CAC predicted cardiovascular events beyond traditional risk factors, with similar strength in white, African American, Hispanic, and Asian [197]. The Rotterdam Study of 8000 German adults at least 55 years old found similar results compared to that of MESA, despite age and ethnicity differences between cohorts [198]. Moreover, CAC further risk-stratified subjects deemed at intermediate risk by the Framingham risk score[199].

CAC is commonly presented in people of low risk of CVD which adds to their value in the prediction of CVD. A meta-analysis in middle-aged to elderly low-risk women found that prevalent CAC was associated with an increased risk for CVD and improved the prognostic accuracy compared with traditional risk factors [200].

2.2 Aortic calcification (AC)

2.2.1 Histology of AC

AC occurs in the intimal and in the medial layer of the arterial wall [201]. AC seems to be an independent process from atherosclerosis. It is considered a metabolic disease that starts around the internal elastic lamina and expands into the medial layer. Medial calcification in aorta is related to aging, diabetes, chronic kidney disease and several rare monogenetic diseases [202].

2.2.2 Prevalence of AC

Atherosclerotic plaques already develop in childhood and their extent increases with age. Compared to CAC, AC develops at a relatively younger age. The Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study showed that atherosclerotic lesions were presented in all of the abdominal aortas of deceased Japanese aged 15-19 years, whereas in only half of the coronary arteries [203].

Men have higher AC prevalence than women. In participants under 45 years in the FHS (3285 participants), abdominal AC was present in 22% of male and 16% of female. In participants without cardiovascular risk factors and under 45 years in FHS, abdominal AC was present in 16% of males and 8% of females. The prevalence increased to 100% in both males and females over 75 years old [204].

2.2.3 Risk factors of AC

Prevalent abdominal AC and AC progression are independently associated with LDL-C, SBP, DM, BMI, pulse pressure, and baseline abdominal AC [205-208]. Risk factors appear to differ between males and females. In a Korean study, smoking was associated with abdominal AC in males, whereas DM and hypertension were associated with abdominal AC in females[209].

2.2.4 AC and CVDs

Epidemiological studies in several populations showed that prevalent and progression of AC are associated with cardiovascular mortality [210], incident CHD [211], MI [212] and stroke[213]. Some studies showed that the above associations are independent of CAC [183, 211, 214, 215]. Other studies suggested that abdominal AC was a stronger predictor for CVD mortality or all-cause mortality than CAC [205, 210, 215]. AC also has high specificity for detection of severe coronary atherosclerosis [216]. A meta-analysis of 10 longitudinal studies showed that abdominal AC presence was associated with an increased relative risk of coronary events (RR 1.81, 95% CI 1.54–2.14), cerebrovascular events (RR 1.37, 95% CI 1.22–3.34), all cardiovascular events (RR 1.64, 95% CI 1.24–2.17) and cardiovascular death (RR 1.72 95% CI 1.03–2.86) (

Figure 6) [183]. A retrospective study among 829 asymptomatic patients (mean age, 57.9 years; 451 women, 378 men) who underwent non-enhanced CT colonography screening found that abdominal AC was a strong predictor of cardiovascular events independent of Framingham

risk score. The area under the receiver operating characteristic curve was higher for abdominal AC than Framingham risk score at all evaluated time points [217].

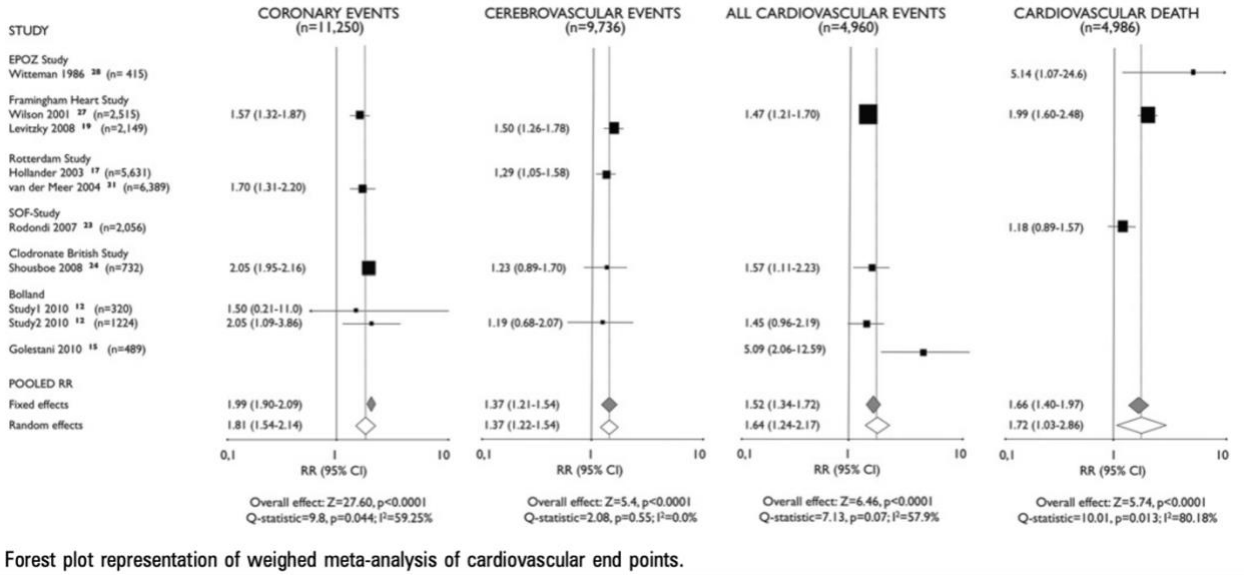


Figure 6 The association between AC and CVD

Reprinted from Heart. Frederico Bastos Gonçalves. Calcification of the abdominal aorta as an independent predictor of cardiovascular events: a meta-analysis. Heart. 2012 Jul;98(13):988-94.

3.0 ISFs, equol, and atherosclerosis

Preclinical studies in monkeys demonstrated that ISFs reduced the progression of atherosclerosis [8, 9]. Because all monkeys are equol-producers, this beneficial effect may be attributed to equol. Next, we introduce the effect of equol on atherosclerosis. In general, in humans, observational studies and RCTs in eastern Asia showed that ISFs were significantly inversely associated with atherosclerosis but only among equol-producers [59, 218] whereas the RCTs in the US did not show any beneficial effect of ISFs among both the equol-producers and non-producers [219]. Observational studies reported that equol producing status but not ISFs was related to atherosclerosis [220].

3.1 ISFs, equol, and CAC

Ahuja et al. conducted a cross-sectional study with 272 middle-aged men in Japan in the ERA JUMP study and found that equol-producers compared to non-producers had a significantly lower prevalence of CAC. The odds ratio for the presence of CAC in equol-producers compared to non-producers was 0.10 (95% CI: 0.01, 0.90) [220]. Additionally, baseline blood levels of dietary sources of ISFs (daidzein and genistein) were not significantly associated with baseline CAC. These results suggested that equol-producing status is a key factor for anti-atherogenic properties of ISFs, though these findings need to be confirmed in larger studies.

3.2 ISFs, equol, and other atherosclerotic markers

3.2.1 ISFs, equol, and cIMT

The carotid intima-media thickness (cIMT) is considered to be a surrogate endpoint of cardiovascular outcomes [221] and is a different measure of the pathogenesis of atherosclerosis compared to vascular calcification [222] while CAC is a more direct gauge of the volume of coronary artery plaque [184]. cIMT is associated with several cardiovascular risk factors and incident CVDs [223-225].

Observational studies and RCTs in eastern Asia have showed that soy or ISFs were significantly inversely associated with cIMT among equol-producers [59, 218]whereas the RCTs in the US did not show any beneficial effect of ISFs even among the equol-producers [219]. However, it should be noted that the intervention duration of all these RCTs were relatively short and the proportion of the equol-producers were low in the U.S. which contributed to the null results. A recent observational study with 8.8-year follow-up in 2,572 subjects found that serum ISFs and equol were bot associated with reduced cIMT progression, mediated by sex-hormone-binding globulin and SBP [226].

Studies in China have shown the beneficial effect of soy and ISFs on cIMT. Cai et al. conducted a cross-sectional study with 572 subjects and reported that equol-producers had a significantly lower cIMT (-4.9, 95% CI: -9.7, -0.3) than non-producers following their usual diet [59]. Among equol-producers, those with higher ISFs intake (5.4 mg/d, measured by food frequency questionnaire) had significantly lower cIMT than those with lower ISFs intake (1.5 mg/d). Liu *et al*. in a 6-month, three-arm RCT with 270 prehypertensive postmenopausal women who were equol-producers reported that whole soy (40 g), but not purified daidzein (63 mg),

significantly reduced LDL-C (7.95%, 95% CI: -15.09, -0.81) and hs-CRP (0.164, 95% CI: -0.309, -0.019) [218]. The null effect of daidzein may be due to the short follow-up of the trial as 1 year or less could be too short to detect changes in cIMT [227].

Conversely, studies in the U.S. did not show any effect of ISFs on cIMT. The Women's Isoflavone Soy Health (WISH) trial with 350 healthy postmenopausal women aged 45-92 years reported that subjects in the treatment group (25 g soy protein containing 91 mg ISFs) had non-significantly slower cIMT progression (16%, p=0.36) as compared to the placebo group over a 2.7-year intervention [219]. Subgroup analysis showed that among women within 5 years after menopause, the ISFs group had a marginally significantly slower progression of cIMT compared to the placebo group: the mean annual cIMT progression was 2.16 μm (95% CI: -1.10, - 5.43) and 6.79 μm (95% CI:3.56-10.00), respectively (p=0.05). The positive result in the subgroup analysis supported the timing-related hypothesis that CVD risk is reduced in young postmenopausal women who initiate hormone therapy in close proximity to menopause versus a null CVD effect in women who initiate hormone therapy when distant from menopause [228, 229]. Additionally, another post-hoc sub-group analysis showed that equol-producers did not have a significantly lower progression of cIMT as compared to non-producers, which is likely due to a small number of equol producers (n=39). Curtis *et al*. conducted a RCT with 93 postmenopausal diabetic women and reported that subjects in the intervention group who were treated with flavanol (850 mg) plus ISFs (100 mg) for 1 year did not have significantly different cIMT progression than those in the placebo group. Their subgroup analysis showed that in the intervention group, equol-producers (n=17) compared to non-producers (n=30) had significantly larger reductions in the arterial stiffness and BP but not cIMT. The interpretation of these RCTs was very limited because the secondary analyses in these RCTs may lack the statistical power to detect a significant result with

very low proportions of equol-producers, and because “equol-producers” were not randomized across ISF arms, they may be subjected to selection bias and unmeasured confounding bias [230].

4.0 Aims

Aim 1: To determine the cross-sectional association between equol producing status and aortic calcification in Japanese population.

Paper 1: Hypothesis: In 302 middle-aged Japanese men in "The EBCT and Risk Factor Assessment among Japanese and U.S. Men in the Post World War II Birth Cohort" (ERA JUMP) study, equol producing status is significantly, cross-sectionally associated with aortic calcification.

Paper 2: Hypothesis: In 979 middle-aged to elderly Japanese men in "Shiga Epidemiological Study of Subclinical Atherosclerosis (SESSA)" study, equol producing status is significantly, cross-sectionally associated with aortic calcification.

Aim 2: To systematically review potential cardioprotective effects of equol from basic research through human studies.

The above aims are summarized in Figure 7.

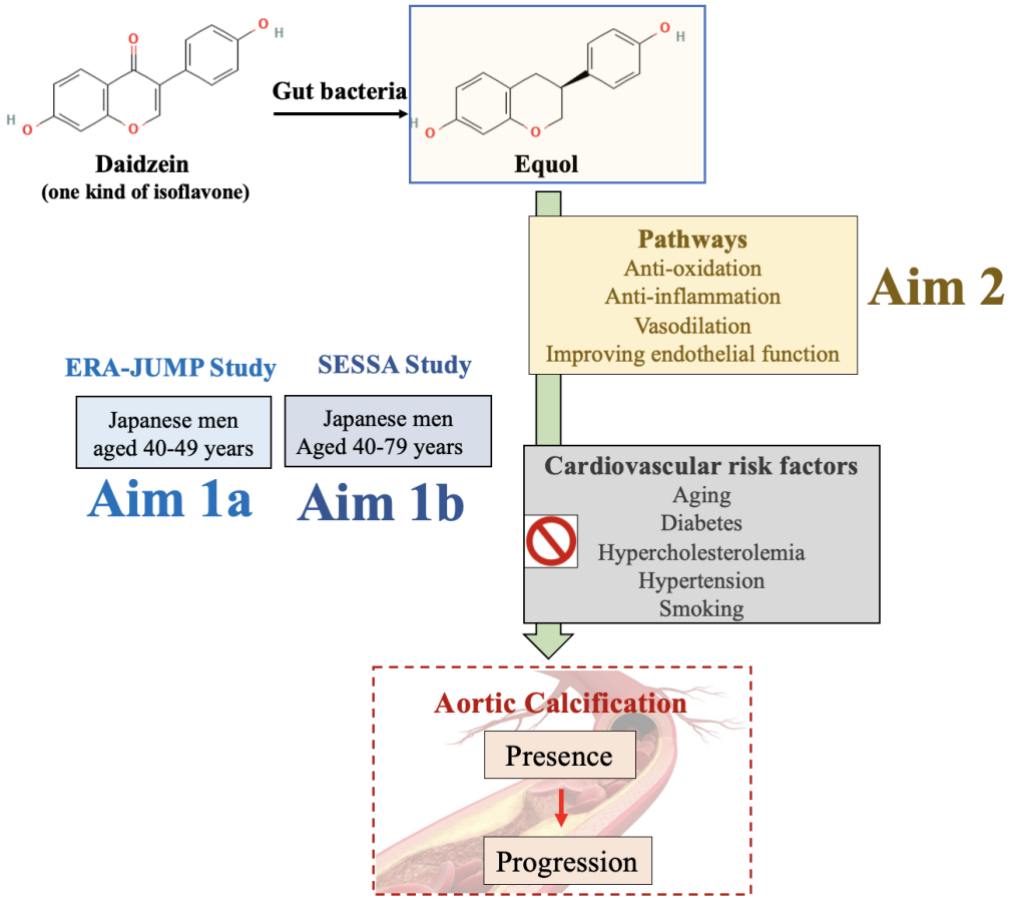


Figure 7 Aims in the dissertation

SESSA: Shiga Epidemiological Study of Subclinical Atherosclerosis; ERA-JUMP: The EBCT and Risk Factor Assessment among Japanese and U.S. Men in the Post World War II Birth Cohort.

5.0 Paper 1 – Association of equol producing status with aortic calcification in middle-aged Japanese men: The ERA JUMP Study

5.1 Summary

Background: Equol, an isoflavone (ISF)-derived metabolite by the gut microbiome in certain individuals termed as equol-producers, might be the key anti-atherogenic component of ISFs. Our objective was to determine the association between equol-producing status and aortic atherosclerosis assessed as aortic calcification (AC).

Methods: This population-based study of 302 Japanese men aged 40–49, free of cardiovascular disease, examined serum levels of equol and ISFs, AC in the entire aorta by electron-beam computed tomography with Agatston method, and cardiovascular risk factors. We defined equol-producers as individuals with serum levels of equol ≥20 nM and prevalent AC as an AC score ≥10. We analyzed the association between equol-producing status and AC using Tobit and logistic regressions. We performed age-stratified analyses since age was a significant effect-modifier.

Results: The 60th to 90th percentile AC scores were 4 to 243 in equol-producers and 15 to 444 in non-producers, respectively. Overall, equol-producers (41% of the sample) had lower AC scores (-209, [95% confidence interval (CI): -455, 36]) and odds of AC (odds ratio (OR): 0.7 [95% CI: 0.4, 1.3]), although not statistically significant, compared to non-producers after controlling for cardiovascular risk factors. Among men aged 46-49, equol-producers had significantly lower AC scores (-428 [95% CI: -827, -29]). Furthermore, there were null associations between serum levels of ISFs and both AC score and the odds of AC.

Conclusion: In middle-aged Japanese men, equol-producers had a non-significantly lower burden of aortic atherosclerosis than non-producers whereas ISFs had a null association. Studies with larger sample sizes in both sexes are warranted.

5.2 Introduction

Soy isoflavones (ISFs) are non-steroidal phytoestrogens regularly consumed in East Asian countries whereas their intake in the US is very limited (an average of 25-50 mg/day in East Asia vs. <2 mg/day in the US [4]). As compared to estrogen which preferably binds to estrogen receptor α that are abundant in reproductive tissues, ISFs show a greater affinity toward estrogen receptors β (ER β) which are expressed in many systems including the vascular system [42]. Several studies in East Asian countries reported that dietary intake of ISFs is significantly and inversely associated with incident coronary heart disease (CHD) [150, 152]. However, a randomized clinical trial in the US among 350 postmenopausal women showed that ISFs intervention for 2.7 years had a null treatment effect on atherosclerosis, overall [219]. This discrepancy may be due to the higher capacity of producing equol after consuming ISFs among people living in the East Asian countries than in the US; such individuals are referred to as "equol-producers". Equol is a metabolite of an ISF daidzein by the gut bacteria [33]. Compared to ISFs, equol is more biologically active, a more potent antioxidant, and has a similar or greater affinity to ERβ and thus may have a higher anti-atherogenic effect [31, 33]. In fact, a nested case-control study within a prospective cohort study in China demonstrated that urinary equol but not ISFs or their other metabolites is significantly associated with incident CHD [152]. Interestingly, 50 to 70% of the population residing in East Asian countries are equol-producers in contrast to 20 to 30% in Western countries. This is due to the differences in microbiome but not genetics and suggests that equol may be the key anti-atherogenic component of ISFs.

Aortic calcification (AC) and coronary artery calcification (CAC) are biomarkers of atherosclerosis. While CAC is a powerful predictor of future cardiovascular events and significantly improves the classification of cardiovascular risk status [231], several studies have

suggested that both presence and progression of AC significantly predict future cardiovascular events independent of CAC [215, 232]. To date, no study has examined the association between equol producing status and AC.

This study aimed to assess the association between equol producing status and AC in a sample of middle-aged Japanese men from the ERA JUMP (Electron-Beam Tomography, Risk Factor Assessment Among Japanese and U.S. Men in the Post-World War II Birth Cohort) study. The ERA JUMP Study reported that Japanese men in Japan had a significantly lower odds of AC presence compared to White and Japanese Americans (32%, 49%, and 43%, respectively) despite similar or greater exposure to traditional cardiovascular risk factors in Japanese in Japan, including hypertension, diabetes, hypercholesterolemia and especially smoking [233]. The Pathobiological Determinants of Atherosclerosis in Youth study has reported that smoking is a stronger determinant of atherosclerosis in the aorta than in the coronary artery [234]. Therefore, significantly lower AC in Japanese in Japan may suggest some protective factors in this population. In this study, we hypothesize that equol-producers had a significantly lower degree of AC than non-producers in middle-aged men in Japan.

5.3 Material and methods

5.3.1 Study population

ERA JUMP was a population-based study of 926 men aged 40 to 49 that was established to compare the levels of subclinical atherosclerosis and explore their risk factors in Japanese in Japan, White and Japanese Americans. This age group was selected because exposure to traditional

risk factors was similar or worse in Japanese men in Japan than American men [233]. From 2002 to 2007, 313 Japanese men from Kusatsu, Shiga, Japan; 310 white men from Allegheny County, Pennsylvania; and 303 Japanese American men from a representative sample of offspring of fathers who participated in the Honolulu Heart Program, Honolulu, Hawaii were randomly selected. All participants were free of cardiovascular disease (CVD) or other severe diseases [235]. We originally intended to study the association between equol-producing status and AC in the three populations. However, sub-sample of White (n=57) and Japanese Americans (n=60) showed extremely low levels of ISFs and almost zero equol-producers (data not shown) which prohibited us to analyze the data in these two populations. Therefore, this present study is limited to the sample of Japanese in Japan. Of this sample, we excluded participants with missing AC scores (n=3) or blood levels of equol (n=8), resulting in a total sample size of 302. All participants provided informed consent. The study was approved by the Institutional Review Boards of the Shiga University of Medical Science, Otsu, Japan, and the University of Pittsburgh, Pittsburgh, USA.

5.3.2 Measurement of covariates

Physical examination, a questionnaire for lifestyle habits, and laboratory assessment were conducted on all participants [235]. Bodyweight and height were measured while the participant was wearing light clothing barefoot. Body mass index (BMI) was calculated as weight (kg) divided by the square of the height (m^2). Blood pressure (BP) was measured after the participant emptied his bladder and sat quietly for 5 minutes. The BP was the average of the two measurements on the right arm with an automated sphygmomanometer (BP-8800, Colin Medical Technology, Komaki, Japan) using an appropriately sized cuff. Hypertension was defined as systolic blood pressure

(SBP) greater than or equal to 140 mmHg, diastolic blood pressure greater than or equal to 90 mmHg, or use of anti-hypertensive medications. The frequency of soy product intake in the past year was assessed through the food frequency questionnaire (FFQ). Alcohol intake was measured as grams of daily ethanol intake. Pack-years of smoking were calculated as smoking years multiplied by the number of cigarettes per day divided by 20. The use of medication (antihypertensive, anti-diabetic, and lipid-lowering) was reported as "yes" or "no". Blood samples were collected in the morning after a 12-hour fast. All the blood samples were stored at -80 degrees Celsius and shipped on dry ice to the Heinz Nutrition Laboratory, University of Pittsburgh (glucose, lipid panel, daidzein, genistein, and equol), and University of Vermont (C-reactive protein (CRP)). Serum glucose was determined using a hexokinase glucose-6- phosphate-dehydrogenase enzymatic assay. Type 2 diabetes was defined as a fasting serum glucose level greater than or equal to 126 mg/dL or use of anti-diabetic medications. Serum low-density lipoprotein cholesterol (LDL-C) was determined using standardized protocols from the Center for Disease Control and Prevention [235] and estimated by the Friedewald equation. When triglyceride level exceeded 400mg/dL, LDL-C was measured directly using an automated spectrophotometric assay [LDL Direct Liquid Select (Equal Diagnostics, Exton US)]. Hypercholesterolemia was defined as a fasting serum LDL-C level greater than or equal to 140mg/dL or the use of lipid-lowering medication.

5.3.3 Measurement of serum ISFs and equol

Serum daidzein, genistein, and equol concentrations were measured using a modified method of Pumford et. al. [236]. Daidzein-d4, genistein-d4 (Cambridge Isotope Laboratories), and equol-d4 (Medical Isotopes Inc.) was added and the samples were incubated with beta-

glucuronidase. The samples were extracted with diethyl ether, dried under N2, and silylated. The samples were analyzed by gas chromatography-mass spectrometry in the selected ion monitoring mode. Ions monitored (m/z) were: 425/482, 234/470, and 555 for daidzein, equol, and genistein, respectively; 428/485, 236/474, and 559 for daidzeind4, equol-d4, and genistein-d4, respectively. The coefficient of variation was 10, 15, and 5 percent for daidzein, genistein, and equol, respectively.

5.3.4 Measurement of AC

For AC measurements, an electron-beam computed tomographic (EBCT) scanner (GE Medical Systems, South San Francisco, California) was used. All images were saved to optical discs and shipped to the Cardiovascular Institute, University of Pittsburgh Medical Center, and were read by one trained reader blinded to the participants' characteristics. For AC evaluation, 6-mm-thick transverse contiguous images were obtained from the level of the aortic arch to the iliac bifurcation using 300-ms exposure time. Calcium was considered to present in the aorta when at least three contiguous pixels of 130 Hounsfield Units were present on a 30-cm matrix. AC was quantified by the Agatston method [237]. The within-reader intra-class correlation was 0.98.

5.3.5 Statistical analysis

Characteristics of participants were expressed as medians (interquartile range) for continuous variables and as percentages for categorical variables by equol producing status. No continuous variables except for BMI conformed to the normal distributions. The difference in characteristics between equol-producers and non-producers was tested by the Kruskal-Wallis test

when the variable was continuous and by the Chi-square test when the variable was categorical. Equol-producers were defined as the blood level of equol of ≥20 nM. We defined the AC presence as AC scores of ≥10 units because scores <10 was considered artifact [220]. A cut-off point of 200 was used to distinguish "moderate" from "more advanced" AC.

To examine the association of equol producing status with the AC score, Tobit conditional regression was used because it is suited to the right-skewed AC score distribution with many zeros [238]. The Tobit conditional regression thus provides an estimate with the combination of the following two regressions: i) logistic regression of the odds of AC ≥10, and ii) linear regression of log-transformed AC when AC ≥10. We also performed logistic regression to examine the association of equol producing status with prevalent AC (AC ≥10 vs. AC<10). Models were performed in the following orders: i) crude, ii) adjusted for age and the traditional CVD risk factors (diabetes, pack-year of smoking, hypercholesterolemia, and hypertension), iii) further adjusted for other confounders (BMI, CRP, and alcohol consumption), iv) further adjusted for daidzein or genistein. A p-value <0.05 was considered statistically significant.

We assessed the modification impact of all risk factors (age, diabetes, BMI, pack-year of smoking, LDL-C, SBP, and CRP) by including an interaction term between each of them and the equol producing status in the fully adjusted model. The p-value for the modification effect of age was 0.09. Thus, we performed age-stratified analyses by using the median value of age (i.e., 45) as the cut-off point (40-45 vs. 46-49 years).

The correlations between levels of equol, daidzein, and genistein were assessed by Spearman correlation coefficients due to their skewed distributions. The association of the log-transformed ISFs (the sum of daidzein and genistein) with the AC score and AC presence were also assessed with linear regressions and logistic regression, respectively.

To illustrate the predicted probabilities of having more advanced AC (≥200) or moderate AC (≥10 and <200) in equol-producers and non-producers, a figure was depicted using proportional odds models with all risk factors controlled (Figure 8). All analyses were performed using R Statistical Software Version 4.0.2 (Foundation for Statistical Computing, Vienna, Austria). The plot was drawn by using the "effects v4.2-0" package in R.

5.4 Results

Among the 302 participants, 41% (n=125) were equol-producers. The characteristics of equol-producers and non-producers (n=177) are described in Table 6. There was no significant difference between equol-producers and non-producers in the frequency of pack-year of smoking, consumption of soy products, hypertension, or hypercholesterolemia. Equol-producers compared to non-producers had significantly lower diabetes prevalence and AC scores at 70th percentile and above.

Table 6 Characteristics of Japanese men in ERA-JUMP

	Equol-producers		**Non-producers**	
	n=125		n=177	
Age, years	46	43-48	45	43-47
Body mass index, kg/m^2	24	22-25	23	21-25
Diabetes, %	**2**		**9 ***	
Hypertension, %	22		30	
Hypercholesterolemia, %	45		42	
C-reactive protein, mg/dL	0.3	0.2-0.7	0.3	0.2-0.7
Pack-year of smoking	18	3-29	19	4-29
Alcohol consumption, g/day	18	2-42	14	3-43
Soy product consumption frequency, %				
Daily	9		4	
2-5 times/week	39		42	

	0-4 times/month	52		54	
Equol, nM		**55**	**25-138**	**0.1 ***	**0.1-5 ***
Isoflavones, nM		484	200-1227	472	182-1028
Daidzein, nM		92	34-231	82	36-199
Genistein, nM		394	171-923	357	156-784
AC score ≥10, %		29		34	
AC score					
	50th percentile	0		0	
	60th percentile	0		0	
	70th percentile	**4**		**15 ***	
	80th percentile	**66**		**95 ***	
	90th percentile	**243**		**444 ***	
	100th percentile	**1224**		**4139 ***	

AC: aortic calcification, IQR: interquartile ranges.
Median values and IQR are shown for continuous variable. Median values are show for AC score across percentiles.
* indicates the difference by equol producer status is significant at 0.05 level. The differences were tested by Kruskal–Wallis test (continuous variables) or chi-square test (categorical variables)
Definition: equol-producers: blood level of equol ≥20 nM; diabetes: fasting serum glucose ≥126 mg/dL or taking anti-diabetes medication; hypertension: systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or taking anti-hypertension medication; hypercholesterolemia: low-density lipoprotein-cholesterol ≥140 mg/dL or taking lipid medication

When stratified by age, participants aged 46-49 years (n=163) compared to 40-45 years (n=139) had significantly higher prevalent hypertension, pack-year of smoking, genistein levels, AC scores, and the odds of prevalent AC (Table 7). Only among participants aged 40-45 years, equol-producers had significantly lower prevalent diabetes and levels of daidzein and genistein than non-producers. The frequency of consuming soy products was not significantly different by equol-producing status in either age group.

Table 7 Characteristics of Japanese men by age groups and equol producing status

	Age 40-45 years			**Age 46-49 years**		
	Overall	Equol-producer	Non-producer	Overall	Equol-producer	Non-producer
Age, years	42	42	42	47	47	47

Body mass index, kg/m^2		24	24	24	23	24	23
Diabetes, %		6	**0 §**	**9**	7	4	9
Hypertension, %		18 *	13	20	33	28	37
Hypercholesterolemia, %		39	42	38	46	46	46
C-reactive protein, mg/dL		0.3	0.3	0.7	0.3	0.3	0.3
Pack-year of smoking		14 *	11	15	22	21	23
Alcohol consumption, g/day		14	14	26	20	20	16
Soy product consumption frequency, %							
	Daily	6	7	3	6	10	3
	2-5 times/week	37	33	44	43	43	44
	0-4 times/month	57	60	53	51	48	53
Equol, nM		7.0	**39 §**	**0.1**	12	**70 §**	**0.1**
Equol-producers, %		38	/	/	44	/	/
Isoflavones, nM							
Daidzein, nM		75	**71 §**	**154**	95	98	90
Genistein, nM		**294 ***	**252 §**	**525**	**405**	484	384
AC score							
	50th percentile	0	0	0	0	0	0
	60th percentile	**0 ***	0	0	**5**	**1.2 §**	**14**
	70th percentile	**0 ***	0	0	**49**	30	63
	80th percentile	**11 ***	**5 §**	**12**	**126**	99	199
	90th percentile	**113 ***	122	90	**497**	358	645
	100th percentile	**947 ***	629	957	**4139**	**1224** §	**4139**

AC: aortic calcification.

Median values are shown for continuous variable.

Definition: equol-producers: blood level of equol ≥20 nM; diabetes: fasting serum glucose ≥126 mg/dL or taking anti-diabetes medication; hypertension: systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or taking anti-hypertension medication; hypercholesterolemia: low-density lipoprotein-cholesterol ≥190 mg/dL or taking lipid medication.

* indicates the difference by age categories was significant at 0.05 level. The difference was tested by Chi-square test (categorical variable) or Kruskal–Wallis test (continuous variable). § indicates the difference by equol-producing status within each age category was significant at 0.05 level.

Serum levels of daidzein and genistein were significantly and highly correlated (Spearmen correlation coefficient was 0.94). Serum level of equol was significantly correlated with those of daidzein and genistein but only in equol-producers (Spearmen correlation coefficients were 0.28 and 0.21, respectively). Daidzein was detected in all participants.

Table 8 describes the association of equol producing status with AC. Equol-producers compared to non-producers had lower AC scores (-209, 95% CI: -455, 36, Model 2) and lower odds of AC presence (0.7, 95% CI: 0.4, 1.3, Model 2), although not statistically significant, after controlling for all risk factors. Further adjustment for serum levels of daidzein or genistein did not materially change the results (data not shown). When the above association was examined by age sub-group, equol-producers had significantly lower AC scores in participants aged 46-49 years (-428, 95% CI: -827, -29, p-value = 0.03, Model 2) but not in 40-45 years (8, 95% CI: -194, 210, Model 2).

Table 8 Mean AC score and odds of prevalent AC in equol producers (vs. non-producers)

	Overall Sample	Age 40-45 years	Age 46-49 years
	n=302	n=163	n=139
Coefficients (95% CI)			
Crude	-174 (-441,93)	22 (-195, 239)	**-440 (-852, -27) ***
Model 1	-193 (-451, 51)	5 (-195, 205)	-370 (-763, 23)
Model 2	-209 (-455, 36)	8 (-194, 210)	**-428 (-827, -29) ***
ORs (95% CI)			
Crude	0.8 (0.5, 1.3)	1.0 (0.4, 2.0)	0.6 (0.3, 1.1)
Model 1	0.8 (0.4, 1.3)	0.9 (0.4, 2.0)	0.6 (0.3, 1.4)
Model 2	0.7 (0.4, 1.3)	0.5 (0.2, 1.2)	0.5 (0.2, 1.2)

CI: confidence interval, ORs: odds ratios, AC: aortic calcification
For overall sample: Model 1, adjusted for age, pack-year of smoking, hypercholesterolemia, diabetes, and hypertension; Model 2, Model 1 + body mass index, alcohol intake, and C-reactive protein
* indicates that we reject the null hypothesis at a 0.05 significance level

Figure 8 shows the probability that more advanced AC (≥200) increased with age in both equol-producers and non-producers with the former group having a slightly flatter trend, although

the difference was not statistically significant. The same pattern of change was also shown for moderate AC (≥10 and <200).

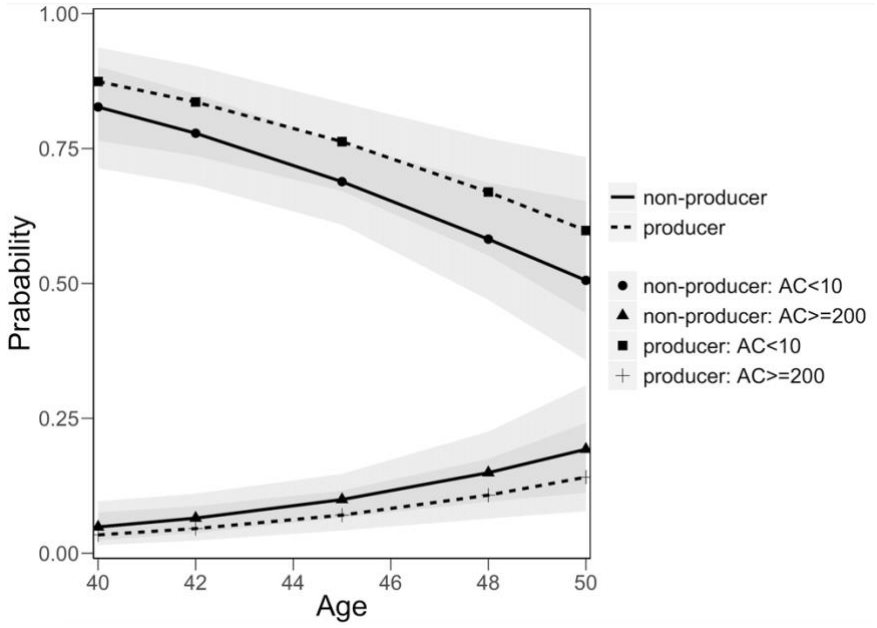


Figure 8 The predicted probability (95%CI) of "no AC" and "more advanced AC" as function of age by equol-producing status

The probabilities (95% CIs) of being in each AC category was estimated at age 40, 42, 45, 48, and 50 years by using multivariate proportional odds models. The differences by equol producing status were not significant at 0.05 level.

The serum level of ISFs was not associated with AC score or AC presence (Table 9). In the overall sample, a 1 unit increase in log-transformed ISFs had 39 (95% CI: -187, 264, Model 2) higher AC scores. The OR for AC presence was 1.0 (95%CI: 0.6, 1.8, Model 2). The associations were also non-significant in participants aged 40-45 and 46-69. When the associations for daidzein or genistein were analyzed separately, similar null associations were observed (data not shown).

Table 9 Mean AC score, odds of prevalent AC with 1 unit increase in log-transformed ISFs

	Overall Sample	Age 40-45 years	Age 46-49 years
	n=302	n=163	n=139
Coefficients (95% CI)			
Crude	69 (-182, 320)	-65 (-261, 131)	141 (-271, 554)
Model 1	30 (-198, 257)	-71 (-248, 106)	243 (-153, 641)
Model 2	39 (-187, 264)	-75 (-254, 104)	284 (-119, 686)
ORs (95% CI)			
Crude	1.1 (0.7, 1.8)	0.7 (0.4, 1.5)	1.4 (0.7, 2.8)
Model 1	1.0 (0.6, 1.8)	0.7 (0.3, 1.4)	1.8 (0.8, 4.3)
Model 2	1.0 (0.6, 1.8)	0.7 (0.3, 1.4)	1.9 (0.8, 4.6)

CI: confidence interval, AC: aortic calcification, ISFs: soy isoflavones (daidzein + genistein), ORs: odds ratios.
For overall sample: Model 1, adjusted for age, pack-year of smoking, hypercholesterolemia, diabetes, and hypertension; Model 2, Model 1 + body mass index, alcohol intake, and C-reactive protein.
For age-specific analyses, age was dropped from models

5.5 Discussion

This population-based cross-sectional study among Japanese men aged 40-49 years showed that equol-producers tended to have lower AC scores, lower odds of prevalent AC, and lower odds of being in a higher AC category than equol-non-producers. The difference in AC scores between equol-producers and non-producers reached statistical significance in participants aged 46-49 years. Meanwhile, serum levels of ISFs in overall or age-stratified analyses showed null associations with AC suggesting that equol may be the key anti-atherogenic factor of ISFs.

Several previous observational studies in humans and RCTs in monkeys support the equol hypothesis. A nested case-control study of CHD in 1,130 Chinese women found a significant inverse association of incident CHD with equol (OR: 0.46, 95% CI: 0.24, 0.89, comparing the lowest to highest quartile) but not with ISFs and their other metabolites [152]. We reported that that equol-producing status, but not serum levels of ISFs, was significantly and inversely associated with prevalent CAC (OR: 0.1, 95%CI: 0.01, 0.9) in Japanese men in ERA JUMP [220]. RCTs in monkeys showed that supplementation of ISFs significantly reduced the progression of atherosclerosis than placebo [9]. Given that all monkeys are equol-producers, equol may contribute to the anti-atherogenic property of ISFs in these RCTs. Although secondary analyses of three RCTs of ISFs in Western countries reported no significant association of equol producing status with the progression of atherosclerosis [219], the negative results are very likely to be a lack of statistical power due to low rates of equol-producers in Westerners [219], as well as short duration of intervention.

We observed that equol-producers had significantly lower AC scores only among participants aged 45-49 years but not in younger age group. Although the reasons for this are unknown, this may be partly due to higher AC scores with a larger variation in older than younger

age groups. On the other hand, we observed null associations between ISFs and AC in overall and age-stratified analyses. Previous observational studies of the association of dietary intake of ISFs and CHD in Asian populations showed inconsistent results. Dietary intake of ISFs was significantly inversely associated with CHD in 40,462 Japanese in Japan [150] whereas it was not in 63,257 Chinese in Singapore [239]. Much higher dietary intake of ISFs in Japanese than in Singapore Chinese may in part account for this different result. Unfortunately, equol or equol producing status was not reported in these studies.

The serum levels of daidzein and genistein were highly correlated. This observation is consistent with the results of a previous study in Japan [240]. This is expected because soy foods in the Japanese diet contain both daidzein and genistein to some extent [241]. We observed equol producers had a significantly lower rate of diabetes than non-producers. A recent meta-analysis of 15 prospective cohort studies showed that dietary intake of ISFs is inversely associated with the risk of diabetes [242]. This may raise a possibility that the observed inverse association of equol producing status with AC is mediated through diabetes. However, neither adjusting for diabetic status nor excluding diabetic participants materially changed the results.

The significant difference in AC scores by 428 units between equol-producers and non-producers in the higher age group may suggest a substantial improvement in CVD risks. The Multi-Ethnic Study of Atherosclerosis (MESA) reported that the hazard ratios (HRs) for non-fatal and fatal CVD events for abdominal AC at 242 to 1,437 units compared to 0 to 241 units were 1.87 ($p=0.07$) and 3.77 ($p=0.002$), respectively [215]. MESA also reported that the presence of thoracic AC at baseline was significantly associated with incident CHD during a mean follow-up of 4.5 years. The association remained significant even after adjusting for traditional CVD risk factors and CAC in women but not in men [232]. The Framingham Heart Study (Offspring and Third

Generation cohorts) showed that the HRs (95% CI) for major CHD and major CVD incidence for each standard deviation increase in the log-transformed abdominal AC was 1.95 (1.27, 3.00) and 1.50 (1.11, 2.05), respectively [210].

Age-adjusted CHD mortality in Japan was less than a third of that in the US despite a lifetime exposure to traditional risk factors similar or worse in Japanese [243]. This low CHD mortality in Japan was unlikely to be due to genetic susceptibility because migrant studies of Japanese to the US documented a dramatic rise in CHD rates [243]. The finding from ERA JUMP are consistent with these observations: the level of subclinical atherosclerosis was significantly lower in Japanese in Japan than White and Japanese Americans despite similar levels of CVD risk factors [235]. This finding led us to investigate the difference in environmental factors, especially diet. The Japanese diet is characterized by a very high dietary intake of long-chain n-3 fatty acids and ISFs. We reported that the difference in blood levels of long-chain n-3 fatty acids partly accounted for the difference in levels of subclinical atherosclerosis between Japanese and Americans [235, 244]. The current study suggests that high dietary intake of ISFs may not be sufficient but proper processing of ISF to equol may be crucial.

Although this study examined the association of equol with atherosclerosis, other vascular effects of equol have been reported. A cross-over RCT of equol for 12 weeks among 54 overweight or obese subjects in Japan reported a significant improved cardio-ankle vascular index, a biomarker of arterial stiffness [41]. Three small RCTs of equol in the US and Japan reported significant reduction in vasomotor symptoms and hot flashes in peri- and postmenopausal women [245-247]. We recently reported that among 91 cognitively normal older adults in Japan, equol-producers had significantly lower white matter lesion volume (WML) in the brain, a biomarker of cerebral small vessel disease examined by structural brain magnetic resonance, compared to non-

producers. Equol-producing status was determined 6-9 years before the imaging study. As with the current study, however, serum levels of ISFs did not relate to WML [240].

The association between equol producing status and AC remained similar after adjusting for traditional risk factors, suggesting that equol may exert its effect through pathways other than the traditional risk factors. These pathways include antioxidation, anti-inflammation, vasorelaxation and anti-calcification properties of equol [42]. Preclinical studies showed that equol exerts many of these effects through ERβ [37].

Our study has several limitations. First, we cannot establish causality due to the cross-sectional study design. However, equol producing status is reported to be stable over years and it is unlikely that the presence of AC affected equol producing status. Second, because no optimal cut-off points for equol-producers and prevalent AC have been established, we chose cut-off points that are consistent with previous studies [60]. Nevertheless, sensitivity analyses using different cut-off points for equol producing status and prevalent AC did not materially change the results (data not shown). Third, all the participants were men. While sex may modify the association between equol and AC, further studies in both sexes are needed. Fourth, our findings may not be attributed purely to equol but phenotypes related to equol-producing status. Finally, our sample size was relatively small.

Our study has several strengths. First, this was a community-based study that utilized a random sampling method. Second, daidzein was detected in all the participants and thus misclassification of equol-producing status was unlikely. Third, we collected blood equol as well as daidzein and genistein to delineate the association between not only equol but also ISFs with AC.

In conclusion, we found that equol-producers had non-significantly lower AC compared to non-producers whereas ISFs had a null association with AC in middle-aged Japanese men. Research on the association of equol producing status and atherosclerosis is almost impossible in Western countries because of the very limited dietary intake of ISFs and the low rate of equol-producers in Western countries. Therefore, additional studies in East Asian countries with larger sample sizes and a wider age range in both sexes are warranted.

6.0 Paper 2 – Association of equol producing status with aortic calcification in Japanese men aged 40-79 years: The SESSA Study

6.1 Summary

Background: Equol is an isoflavone (ISF)-derived metabolite by the gut microbiome in certain individuals termed as equol-producers (EP). Equol might be the key anti-atherogenic component of ISFs. Our objective was to determine the association between equol-producing status and aortic atherosclerosis assessed as aortic calcification (AC).

Methods: In a population-based study of 979 Japanese men aged 40–79 without cardiovascular (CVD) or chronic kidney disease, we measured the urinary levels of equol and ISFs, AC and other factors. AC in the entire aorta was assessed by electron-beam or multi-detector-row computed tomography and was reported in the Agatston unit. Subjects with log10(urinary equol to daidzein concentration) > -1.5 were classified as EP. Using the median value among EP, EP was classified as low- and high-equol. The presence of AC was defined as AC>0. AC scores were further categorized into 0, 1-99, 100-299, 300-399, ≥1000 categories. We analyzed the association between equol producing status as well as equol categories (low-equol vs. non-EP, high-equol vs. non-EP) and AC presence by the logistic regressions. We explored the association between equol-producing status and AC categories by the multinomial logistic regressions. We further explored the association between equol producing status and AC>0 in strata of CVD risk factors. Finally, we examined the associations between ISFs and AC>0, AC≥300, and AC≥1000. CVD risk factors were adjusted in all models.

Results: EP (50% of the sample) had significantly lower odds of AC>0 (odds ratio (OR): 0.62, 95% confidence interval (CI): 0.39, 0.98) compared to non-EP, and this association was independent of CVD risk factors and ISFs. For the dose-response association of equol, compared to non-EP, subjects with low-equol had 0.47 times (95%CI: 0.28, 0.80) the odds of AC>0 and that number for high-equol was 0.82 (95%CI: 0.46, 1.44). Moreover, the odds of being in the AC category of 1 to 99 compared to no AC decreased by 47% (95%CI: 0.32, 0.86) if a non-EP was an EP. Furthermore, EP had significantly lower odds of AC>0 than non-EP in subjects who was not obese, had hypertension, aged 50-59 years, consumed ethanol ≥69 g/day, or had pack-year of smoking ≥40. ISFs were not significantly associated with AC>0.

Conclusion: In Japanese men aged 40-79 years, EP had a significantly lower burden of the presence of aortic atherosclerosis than non-EP. The association was pronounced in heavy drinkers, heavy smokers or individuals with hypertension.

6.2 Introduction

Soy isoflavones (ISFs) are non-steroidal phytoestrogens regularly consumed in East Asian countries whereas their intake in the US is very limited (an average of 25-50 mg/day in East Asia vs. <2 mg/day in the US [4]). As compared to estrogen which preferably binds to estrogen receptor α that are abundant in reproductive tissues, ISFs show a greater affinity toward estrogen receptors β (ER β) which are expressed in many systems including the vascular system [42]. Several studies in East Asian countries reported that dietary intake of ISFs is significantly and inversely associated with incident coronary heart disease (CHD) [152]. However, a randomized clinical trial in the US among 350 postmenopausal women showed that ISFs intervention for 2.7 years had a null treatment effect on atherosclerosis, overall [219]. This discrepancy may be due to the higher capacity of producing equol after consuming ISFs among people living in the East Asian countries than in the US; such individuals are referred to as "EP". Equol is a metabolite of an ISF daidzein by the gut bacteria [3]. Compared to ISFs, equol is more biologically active, a more potent antioxidant, and has a similar or greater affinity to ERβ and thus may have a higher anti-atherogenic effect [31]. In fact, a nested case-control study within a prospective cohort study in China demonstrated that urinary equol but not ISFs or their other metabolites is significantly associated with incident CHD [152]. Interestingly, 50 to 70% of the population residing in East Asian countries are equol-producers in contrast to 20 to 30% in Western countries. This is due to the differences in microbiome but not genetics [33] and suggests that equol may be the key anti-atherogenic component of ISFs.

Aortic calcification (AC) and coronary artery calcification (CAC) are biomarkers of atherosclerosis. While CAC is a powerful predictor of future cardiovascular events and significantly improves the classification of cardiovascular risk status [231], several studies have

suggested that both presence and progression of AC significantly predict future cardiovascular events independent of CAC [215, 232]. To date, no study has examined the association between equol producing status and AC.

This study aimed to assess the association between equol producing status and AC in a population-based Japanese men aged 40-79 years old from the Shiga Epidemiological Study of Subclinical Atherosclerosis (SESSA) study. We hypothesize that EP had a significantly lower burden of AC than non-EP.

6.3 Material and methods

6.3.1 Study population and measurements

The SESSA is an ongoing prospective, population-based study of a random sample from a general Japanese population, as described elsewhere [248]. Participants eligible for the present study were 1,094 men aged 40 through 79 years (mean 63.8; standard deviation [SD], 9.8 years) enrolled at baseline (May 2006–March 2008). A total of 979 participants were analyzed in the present study. We excluded 2 participants missing urinary equol, 7 missing AC score, 62 had myocadiac infarction or stroke, 18 whose estimated glomerular filtration rate (eGFR) <45, 25 missing daily ethanol amount or smoking status or cigarette numbers. The present study was approved by the Institutional Review Board of Shiga University of Medical Science (Otsu, Japan) and the University of Pittsburgh (Pittsburgh, U.S.), and all participants provided written informed consent.

A self-administered questionnaire was used to obtain information on demography, alcohol drinking, smoking habits, physical activity, medication use, medical history, and other lifestyle factors. After the participants completed the questionnaires, trained nurses confirmed answers to the questionnaire with the participants. Based on the questionnaire, the frequency of alcohol consumption during a typical week or month and the total alcohol intake on each occasion were determined and used to calculate the alcohol intake per week [249]. Specifically, weekly intake of alcohol was assessed in units of "go" (a traditional Japanese unit of volume corresponding to 23 g of ethanol, that is, 7 go, 14 go, and 21 go correspond to 161 g, 322 g, and 483 g of ethanol, respectively), and was converted to grams of ethanol per week. One go is equivalent to 180 mL of Sake (Japanese rice wine) and corresponds to one bottle (500 mL) of beer, two single shots (75 mL) of whisky, or two glasses (180 mL) of wine. Smoking status was categorized into three groups: current, former, and never smokers. Participants who smoked in the last 30 days were defined as current smokers, whereas participants who had never smoked before were defined as never smokers, and smokers were queried for the average number of cigarettes smoked each day. Packyear of smoking was calculated, which is a measure of how much a person has smoked over a period of time.

Body weight and height were measured while the participants were wearing light clothing without shoes. BMI was calculated as weight (kg) divided by height squared (m2). Obesity was defined as BMI≥25 kg/m2, a definition tailored for the Asian populations [250]. Blood pressure was measured twice consecutively in the right arm of the seated participant, after sitting quietly for 5 min, using an automated sphygmomanometer (BP-8800; Omron Health Care Co. Ltd, Tokyo, Japan). The mean of these two measurements was used for analyses. Hypertension was defined as

the use of antihypertensive medication, systolic blood pressure (SBP) ≥ 140 mmHg, or diastolic blood pressure ≥ 90 mmHg.

Blood specimens were obtained early in the clinic visit, after fasting for at least at 12 h, and used for laboratory testing. Serum lipid concentrations were determined at a single laboratory (Shiga Laboratory; MEDIC, Shiga, Japan) that had been certified for standardized lipid measurements according to the protocols of the United States Centers for Disease Control and Prevention/Cholesterol Reference Method Laboratory Network. Total cholesterol and triglycerides were measured using enzymatic assays, and high-density lipoprotein cholesterol (HDL-C) was determined using a direct method. Hypercholesterolemia was defined as non-HDL-C ≥170 mg/dL or the use of dyslipidemia medication. Non-HDL-C was the difference between total cholesterol (mg/dL) and HDL-C (mg/dL) and was a biomarker of dyslipidemia [251]. Plasma glucose levels were determined from NaF-treated plasma using a hexokinase glucose-6 phosphate-dehydrogenase enzymatic assay. Glycated hemoglobin A1C (HbA1c) was measured using latex agglutination immunoassays according to the protocol by the Japanese Diabetes Society and converted to the National Glycohemoglobin Standardization Program value. Diabetes was defined as either fasting glucose ≥126 mg/dL, HbA1c ≥6.5%, or the use of medication [252]. C-reactive protein (CRP) levels were measured by nephelometry using a BN II Analyzer [253]. Serum creatinine levels were measured using an enzymatic method (Espa CRE-liquid II; NIPRO, Osaka, Japan) [254]. We used the CKD Epidemiology Collaboration (CKD-EPI) equation modified for the Japanese in the main analysis [255], which was also used in previous SESSA study [254]. We chose the CKD-EPI equation over the Modification of Diet in Renal Disease (MDRD)-based equations because of its superior accuracy. Participants with eGFR <45 ml/min/1.73m2 were categorized as low eGFR and may not produce accurate urinary equol.

6.3.2 Measurement of serum ISFs and equol

Serum daidzein, genistein, and equol concentrations were measured using a modified method of Pumford et. al. [236]. Daidzein-d4, genistein-d4 (Cambridge Isotope Laboratories), and equol-d4 (Medical Isotopes Inc.) was added and the samples were incubated with beta-glucuronidase. The samples were extracted with diethyl ether, dried under N2, and silylated. The samples were analyzed by gas chromatography-mass spectrometry in the selected ion monitoring mode. Ions monitored (m/z) were: 425/482, 234/470, and 555 for daidzein, equol, and genistein, respectively; 428/485, 236/474, and 559 for daidzeind4, equol-d4, and genistein-d4, respectively. The coefficient of variation was 10, 15, and 5 percent for daidzein, genistein, and equol, respectively.

6.3.3 Measurement of AC

AC measurements have been described in detail elsewhere [256]. In brief, AC was measured using electron-beam computed tomographic (EBCT) with the C-150 scanner (Imatron, South San Francisco, CA, US) or 16-channel multi-detector-row computed tomography (MDCT) with the Aquilon scanner (Toshiba, Tokyo, Japan). Images from the aortic bulb to the abdominal aortic bifurcation with slice thickness of 7.0 mm were considered with a scan time of 100 ms (EBCT) or 320 ms (MDCT). AccuImage software (AccuImage Diagnostic, San Francisco, California) was used to quantify AC scores. AC was considered to be present with three contiguous pixels $\geq$ 130 Hounsfield Units. AC scores were evaluated according to the Agatston method [237]. All CT images were evaluated by one physician who was trained and blinded to the information

of participants. The definitions of AC by EBCT and MDCT were considered to be equivalent [256].

6.3.4 Statistical analysis

Characteristics of participants were expressed as medians (interquartile range) for continuous variables and as percentages for categorical variables by equol producing status. The difference in characteristics between equol-producers and non-producers was tested by the Kruskal-Wallis test when the variable was continuous and by the Chi-square test when the variable was categorical. Similar to that was proposed by Setchell et al. [49], equol-producers in our study were defined as log10-transformed urinary equol to daidzein ratio of −1.5. The log10-transformed urinary equol to daidzein ratio provided a clearer distinction of equol-producer status than the absolute serum or urinary equol concentrations because it is independent of ISFs intake and minimizes interindividual variation in ISFs pharmacokinetics or differences in analytical methodologies [49]. A threshold value for the log10-transformed urinary equol to daidzein ratio of −1.5 provided a demarcation to define equol-producer status. AC presence was defined as AC score>0 [257, 258]. We also categorized AC score into 0, 1-9, 10-99, 100-299, 300-999, ≥ 1000 categories.

We used logistic regression to examine the association of equol producing status with the AC presence (AC>0), and multinomial logistic regression to examine the association of equol producing status with AC categories (0, 1-99, 100-299, 300-999, ≥ 1000). Models were performed in the following orders: 1, adjusted for CT and age. Age is a strong indicator of AC in this study (OR of AC>0 for a 1-year increase in age was 1.18 (95%CI: 1.14, 1.21), and AC value was directly determined by the type of CT that was used, 2, further adjusted for the traditional CVD risk factors

(diabetes, hypercholesterolemia, and hypertension [259]), 3, further adjusted for other CVD risk factors such as pack-year of smoking, obesity, and ethanol, 4, further adjusted for weak confounders such as CRP, TG, and eGFR, 5, based on model 3, further adjusted for daidzein, 6, based on model 5, further adjusted for genistein. A p-value <0.05 was considered statistically significant. We also performed logistic regression to evaluate the dose-response relationship between equol and AC>0. We categorized equol as non-EP, low-equol (equol amount lower than median value among EP), high-equol (equol amount higher than median value among EP). We repeated the above analyses with AC≥300 and ≥1000 as the definition of AC presence.

To elucidate the potential modification effect of CVD risk factors in the association between equol producing status and AC>0, we performed logistic regressions in strata of each CVD risk factor. We also performed logistic regression to illustrate the association between log-transformed of total ISFs (defined as daidzein + genistein) and AC>0, AC≥300, and ≥1000. All analyses were performed using R Statistical Software Version 4.0.2 (Foundation for Statistical Computing, Vienna, Austria).

6.4 Results

Among the 979 participants, 50.3% (n=492) were EP. The characteristics of EP and non-EP (n=487) are described in Table 10. There were significant differences between EP and non-EP in age, urinary daidzein and genistein, and AC scores. There was no difference in CVD risk factors such as hypertension, diabetes, hypercholesterolemia, and obesity.

Table 10 Characteristics of SESSA by equol producing status

		Equol-producer (n=492)	Equol-non-producer (n=487)	P-value
Age (year) (mean, SD)		65.0 (9.6)	62.4 (10.0)	<0.001 *
Pack-year of smoking (median, IQR)		24 (3.3 - 44.0)	25 (6 - 43.5)	0.41
Ethanol consumption (g/week) (median, IQR)		98 (7 - 252)	98 (5.9 - 259.3)	0.59
eGFR (ml/min/1.73m2) (mean, SD)		74.4 (9.4)	75.3 (9.8)	0.15
Urinary daidzein (nmol/L) (median, IQR)		3393 (1016 - 9918)	7143 (2429 - 19894)	<0.001 *
Urinary genistein (nmol/L) (median, IQR)		2065 (1102 - 7056)	4369 (1496 - 11709)	<0.001 *
AC score (Agatston unit)	0th percentile	0	0	0.004 *
	25th percentile	46	23	
	50th percentile	381	259	
	75th percentile	1549	1021	
	100th percentile	20007	13463	
AC category (n, %)	0	83 (17%)	79 (16%)	0.01 *
	1 to 9	11 (2%)	23 (5%)	
	10 to 99	60 (12%)	86 (18%)	
	100 to 299	73 (15%)	66 (14%)	
	300 to 999	96 (20%)	110 (23%)	
	≥ 1000	169 (34%)	123 (25%)	
Hypertension (n, %)		270 (55%)	258 (53%)	0.55
Diabetes (n, %)		99 (20%)	104 (21%)	0.75
Hypercholesterolemia (n, %)		221 (40%)	194 (36%)	0.18
Obesity (n, %)		129 (26%)	153 (31%)	0.07

Mean (SD) is shown if the distribution of a continuous variable is normal; median (IQR) is shown if the distribution is skew.

Two-sample t-test was used for normal distributed variables, Mann-Whitney U test was used for skewed variables, chi-square test was used for categorical variables.

SD: standard deviation, IQR: interquartile range, AC: aortic calcification, eGFR: estimated glomerular filtration rate, CT: electron beam computed tomography, EBCT: electron beam computed tomography, MDCT: multi-detector row computed tomography, BMI: body mass index, HDL-C: high-density lipoprotein cholesterol.

Definition: obesity: BMI ≥25, hypercholesterolemia: non-HDL-C ≥170 mg/dL or taking lipid medicine, non-HDL-C = total cholesterol - HDL-C.

In overall population (N=979), EP had significantly lower odds of AC>0 than non-EP in all multivariable models (Table 11). The OR was 0.62 (95%CI: 0.39, 0.98) in the fully-adjusted model (Model 4) which adjusted for age, CT type, hypercholesterolemia, diabetes, hypertension, pack-year of smoking, ethanol, obesity, eGFR, CRP, and triglycerides. When further adjusted for urinary daidzein based on Model 3 which had controlled for the main CVD risk factors, the OR of AC>0 was 0.63 (95%CI: 0.40, 0.99, Model 5). Similarly, among EP and whose equol amount was lower than median value, their odds of AC>0 was 0.47 times (95%CI: 0.28, 0.79, Model 4) that of non-EP (Table 11). Among EP and whose equol amount was higher than median value, their odds of AC>0 was 0.73 times (95%CI: 0.42, 1.25, Model 4) that of non-EP.

Table 11 The ORs (95%CI) of AC>0 for EP vs. non-EP, and low-equol and high-equol vs. non-EP

	EP vs. non-EP			
	OR		95% CI	
Model 1 (CT, age)	0.57		0.37 - 0.88 *	
Model 2 (Model 1 + HC, DM, HT)	0.58		0.37 - 0.89 *	
Model 3 (Model 2 + packyear, ethanol, obesity)	0.61		0.39 - 0.96 *	
Model 4 (Model 3 + CRP, TG, eGFR)	0.62		0.39 - 0.98 *	
Model 5 (Model 3 + daidzein)	0.63		0.40 - 0.99 *	
Model 6 (Model 3 + daidzein + genistein)	0.64		0.40 - 0.9996*	
	Low-equol vs. non-EP		**High-equol vs. non-EP**	
	OR	95% CI	OR	95% CI
Model 1 (CT, age)	0.47	0.28 - 0.77 *	0.73	0.42 - 1.24
Model 2 (Model 1 + HC, DM, HT)	0.47	0.28 - 0.79 *	0.73	0.42 - 1.25
Model 3 (Model 2 + packyear, ethanol, obesity)	0.48	0.28 - 0.81 *	0.82	0.46 - 1.44
Model 4 (Model 3 + CRP, TG, eGFR)	0.47	0.28 - 0.80 *	0.84	0.48 - 1.49
Model 5 (Model 3 + daidzein)	0.49	0.29 - 0.85 *	0.82	0.47 - 1.44
Model 6 (Model 3 + daidzein + genistein)	0.50	0.29 - 0.86 *	0.82	0.47 - 1.44

HT, hypertension; DM, diabetes; HC, hypercholesterolemia; eGFR, estimated glomerular filtration rate; TG, triglycerides; CRP, C-reactive protein.

In multinomial logistic regressions, in the model adjusted for CT, age, hypercholesterolemia, diabetes, hypertension, pack-year of smoking, ethanol, obesity, CRP, triglyceride, and eGFR (Model 3), the log odds of being in the AC category of 1 to 99 compared to not having AC would decrease by 47% (95%CI: 0.32, 0.86) if moving from non-EP to EP (Table 12). In a word, the odds of having a higher AC score will increase if a person is a non-EP instead of an EP. The odds of having other categories of AC were not significant.

Table 12 The ORs (95%CI) of AC categories for EP vs. non-EP

Model	AC category	OR	95% CI
Model 1 (CT, age)	0	1 (ref)	ref
	1 to 99	0.52	0.33 - 0.82 *
	100 to 299	0.83	0.51 - 1.37
	300 to 999	0.58	0.36 - 0.93 *
	≥ 1000	0.79	0.49 - 1.27
Model 2 (Model 1 + HC, DM, HT)	0	1 (ref)	ref
	1 to 99	0.52	0.33 - 0.82 *
	100 to 299	0.83	0.51 - 1.38
	300 to 999	0.59	0.36 - 0.96 *
	≥ 1000	0.80	0.49 - 1.30
Model 3 (Model 2 + packyear, ethanol, obesity, CRP, TG, eGFR)	0	1 (ref)	ref
	1 to 99	0.53	0.32 - 0.86 *
	100 to 299	0.92	0.54 - 1.57
	300 to 999	0.67	0.40 - 1.12
	≥ 1000	0.93	0.55 - 1.57
Model 4 (Model 3 + daidzein)	0	1 (ref)	ref
	1 to 99	0.53	0.32 - 0.86 *
	100 to 299	0.92	0.54 - 1.57
	300 to 999	0.67	0.40 - 1.12
	≥ 1000	0.93	0.55 - 1.57

HT, hypertension; DM, diabetes; HC, hypercholesterolemia; eGFR, estimated glomerular filtration rate; TG, triglycerides; CRP, C-reactive protein.

Because the OR in participants aged 70-79 was very high and the CI was very wide (OR: 8.90, 95%CI: 0.79, 99.99), also because equol seemed to be “harmful” when AC was high (e.g., ≥1000), we further conducted the analyses excluding participants aged 70-79 and whose AC≥1000

(Table 13). EP had non-significant lower odds of AC≥300 and significant lower odds of AC>0 than non-EP.

Table 13 The ORs (95%CI) of AC>0 and AC≥300 and 1000 for EP vs. non-EP among 40-69 years old

	OR	95% CI
AC>0		
Model 1 (+CT, age)	0.49	0.31 - 0.77
Model 2 (Model 1 + HC, DM, HT)	0.50	0.31 - 0.78
Model 3 (Model 2 + packyear, ethanol, obesity, CRP, TG, eGFR)	0.50	0.31 - 0.81
AC≥300		
Model 1 (+CT, age)	0.78	0.51 - 1.20
Model 2 (Model 1 + HC, DM, HT)	0.80	0.52 - 1.23
Model 3 (Model 2 + packyear, ethanol, obesity, CRP, TG, eGFR)	0.81	0.52 - 1.26

In stratified analysis, EP was significantly and negatively associated with AC>0 in participants whose BMI<25 kg/m2 (n=697), ethanol consumption ≥69 g/day (heavy-drinker, n=68), pack-year of smoking ≥40 (heavy-smoker, n=293), had hypertension (n=528), and aged 50-59 years (n=189) (Table 14).

Table 14 The ORs (95%CI) of AC>0 for EP vs. non-EP in strata of CVD risk factors

	OR	95% CI
DM (N=203)	0.51	0.01 - 34.09
non-DM (N=776)	0.64	0.39 - 1.03
HC (N=358)	0.80	0.33 - 1.90
non-HC (n=621)	0.58	0.34 - 1.000
obese (N=282)	0.90	0.38 - 2.09
non-obese (N=697)	0.50	0.29 - 0.88 *
heavy-drinker (ethanol>=69 g/day) (N=68)	0.14	0.03 - 0.67 *
normal-drinker or non-drinker (N=911)	0.64	0.40 - 1.01
heavy-smoker (packyear >=40) (N=293)	0.11	0.02 - 0.68 *
normal-smoker or non-smoker (N=686)	0.70	0.43 - 1.12
HT (N=528)	0.40	0.17 - 0.92 *

non-HT (N=451)	0.72	0.41 - 1.27
age 40-49 (N=119)	0.93	0.38 - 2.31
age 50-59 (N=189)	0.28	0.12 - 0.64 *
age 60-69 (N=386)	0.53	0.24 - 1.15
age 70-79 (N=285)	8.90	0.79 - 99.99

All models are fully-adjusted: age, CT, DM, HTN, HC, obese, packyear, ethanol, TG, CRP, eGFR (except the covariate that was used for stratification)
HT, hypertension; DM, diabetes; HC, hypercholesterolemia; eGFR, estimated glomerular filtration rate; TG, triglycerides; CRP, C-reactive protein.

When using 300 and 1000 as the cut-point of AC prevalence, equol producing status was not significantly associated with AC (Table 15). ISFs were non-significantly associated with AC defined as AC>0, AC≥300, or AC≥1000 (

Table 16).

Table 15 The ORs (95% CI) of AC ≥300 and ≥1000 for EP, low-equol and high-equol vs. non-EP

	EP vs. non-EP			
	OR	95% CI		
AC≥300				
Model 1 (CT, age)	1.004	0.75 - 1.35		
Model 2 (Model 1 + HC, DM, HT)	1.03	0.77 - 1.40		
Model 3 (Model 2 + packyear, ethanol, obesity, CRP, TG, eGFR)	1.07	0.78 - 1.46		
AC≥1000				
Model 1 (CT, age)	1.32	0.98 - 1.79		
Model 2 (Model 1 + HC, DM, HT)	1.35	0.99 - 1.84		
Model 3 (Model 2 + packyear, ethanol, obesity, CRP, TG, eGFR)	1.39	1.01 - 1.91 *		
	Low-equol vs. non-EP		**High-equol vs. non-EP**	
AC≥300				
Model 1 (CT, age)	0.96	0.67 - 1.38	1.05	0.73 - 1.50
Model 2 (Model 1 + HC, DM, HT)	1.01	0.70 - 1.46	1.06	0.74 - 1.53
Model 3 (Model 2 + packyear, ethanol, obesity, CRP, TG, eGFR)	0.99	0.67 - 1.44	1.16	0.79 - 1.70
AC≥1000				
Model 1 (CT, age)	1.35	0.93 - 1.95	1.29	0.90 - 1.86
Model 2 (Model 1 + HC, DM, HT)	1.40	0.96 - 2.04	1.30	0.90 - 1.89

Model 3 (Model 2 + packyear, ethanol, obesity, CRP, TG, eGFR)	1.37	0.93 - 2.02	1.41	0.96 - 2.07

Low-equol is defined as urinary equol lower than median value among equol-producers, high-equol is higher than median value among equol-producers.
HT, hypertension; DM, diabetes; HC, hypercholesterolemia; eGFR, estimated glomerular filtration rate; TG, triglycerides; CRP, C-reactive protein.

Table 16 The ORs (95% CI) of AC >0, ⩾300 or ⩾1000 for log-ISFs

Models	OR	95% CI
AC>0		
Model 1 (age, CT, HC, DM, HT)	1.07	0.77 - 1.50
Model 2 (Model 1 + packyear, ethanol, obesity, CRP, TG, eGFR)	1.18	0.82 - 1.69
AC>=300		
Model 1 (age, CT, HC, DM, HT)	0.81	0.64 - 1.03
Model 2 (Model 1 + packyear, ethanol, obesity, CRP, TG, eGFR)	0.88	0.68 - 1.13
AC>=1000		
Model 1 (age, CT, HC, DM, HT)	0.75	0.58 - 0.96
Model 2 (Model 1 + packyear, ethanol, obesity, CRP, TG, eGFR)	0.79	0.61 - 1.03

ISFs = daidzein + genistein

6.5 Discussion

This population-based cross-sectional study among Japanese men aged 40-79 years showed that EP, which consisted of half of the sampled population, had a significantly lower odds of AC presence and being in a higher AC category compared to non-EP. This association was independent of CVD risk factors as well as ISFs. This study is the first to show that EP had a significantly lower AC burden as compared to non-EP.

Several previous observational studies in humans supported the equol hypothesis. A nested case-control study of CHD in 1130 Chinese women found a significant inverse association of incident CHD with equol (OR: 0.46, 95% CI: 0.24, 0.89, comparing the lowest to highest quartile)

but not with ISFs and their other metabolites [152]. We reported that equol-producing status, but not serum levels of ISFs, was significantly and inversely associated with prevalent CAC (OR: 0.1, 95%CI: 0.01, 0.9) in Japanese men in ERA JUMP [220]. ERA JUMP is a population-based study in middle-aged men that reported that Japanese men in Japan had a significantly lower odds of AC presence compared to White and Japanese Americans despite similar or greater exposure to traditional CVD risk factors in Japanese in Japan [233, 260]. Our previous work suggested that the protective factor for Japanese in Japan was their greater capacity to produce equol than the Americans and this explained the discrepancy of the burden of atherosclerosis between Japanese in Japan and Americans.

RCTs in monkeys also supported the equol hypothesis. The supplementation of ISFs significantly reduced the progression of atherosclerosis in both male and female monkeys than placebo [8, 9]. Given that all monkeys are equol-producers, equol may contribute to the anti-atherogenic property of ISFs in these RCTs. Although secondary analyses of three RCTs of ISFs in Western populations reported no significant association of equol producing status with the progression of atherosclerosis [218, 219, 261], the negative results are very likely to be a lack of statistical power due to low rates of EP in Westerners [219, 261], as well as short duration of intervention [218, 261].

We did not observe dose-response relationship between equol producing status and AC. Thus, we explored the association of equol-producing status and AC in different CVD risk factor strata to examine whether there were any spurious association. Although equol producing status is stable over years, the levels of urinary equol among EP are very likely to be dependent on dietary intake of daidzein. Thus, the one-time measurement of equol may not necessarily reflect long-term exposure of high and low equol. We found that EP had significantly lower odds of AC>0 than

non-EP in subjects who were not obese, had hypertension, aged 50-59 years, consumed ethanol ≥69 g/day, and had pack-year of smoking ≥40. We did not observe negative association between equol producing status and AC≥300 or AC≥1000 in overall participants but when excluding older people and those having large AC values, equol producing status showed an inverse association with AC, although not significant.

Compared to non-EP, the odds of AC>0 was 10 times lower in EP among heavy-smokers, and 7 times lower among heavy-drinker, while the ORs among normal/non-smoker and normal/non-drinker were non-significant. The strong modification effects of smoking and drinking might be due to the strong association between smoking or drinking and atherosclerosis themselves. SESSA group has previously suggested that current-smoker at baseline had 2.47 (95%CI: 1.38, 4.44) times higher the odds of AC progression over 5 years compared to non-smokers [262]. ERA JUMP study, a similar study to SESSA, suggested that heavy-drinkers had 1.67 (95%CI: 1.11, 2.52) times higher AC score compared to non-drinkers [263]. However, the reason for this strong modification was unknown. One possibility was that the anti-inflammatory or anti-oxidative properties of equol was strengthened in heavy-smoker and heavy-drinker and may cause a greater anti-atherosclerotic effect in these subjects. Similarly, we found that the odds of AC>0 were 1.4 times lower in EP than non-EP among hypertensive subjects. Future studies are warranted to explore if smoking, drinking, and hypertensive status can amplify the effect of equol. In addition, after excluding people in the highest age group in SESSA study (aged 70-79) and those with large AC (AC≥1000), EP turned to have lower odds of AC≥300, suggesting that the equol may exert a beneficial effect under the circumstance of the lower burden of atherosclerosis.

Besides having different associations in different strata of CVD risk factors as described above, the association between equol producing status and AC remained similar after adjusting for

traditional risk factors, suggesting that equol may exert its effect through pathways other than the traditional risk factors. These pathways include antioxidation, anti-inflammation, vasorelaxation, and anti-calcification properties of equol [264]. Preclinical studies showed that equol exerts many of these effects through ERβ [264].

We observed non-significant associations between ISFs and the odds of AC>0, AC≥300 and AC≥1000. Previous observational studies of the association of dietary intake of ISFs and CHD in Asian populations showed inconsistent results. Dietary intake of ISFs was significantly inversely associated with CHD in 40462 Japanese in Japan [150] whereas ISFs intake was not associated with CVD risks in 63257 Chinese in Singapore [239]. Much higher dietary intake of ISFs in Japanese than in Singapore Chinese may in part account for this different result. Unfortunately, equol or equol producing status was not reported in these studies.

Although this study examined the association of equol with atherosclerosis, other vascular effects of equol have been reported. A cross-over RCT of equol for 12 weeks among 54 overweight or obese subjects in Japan reported a significant improved cardio-ankle vascular index, a biomarker of arterial stiffness [41]. Three small RCTs of equol in the US and Japan reported significant reduction in vasomotor symptoms and hot flashes in peri- and postmenopausal women [245-247]. We recently reported that among 91 cognitively normal older adults in Japan, equol-producers had significantly lower white matter lesion volume (WML) in the brain, a biomarker of cerebral small vessel disease examined by structural brain magnetic resonance, compared to non-producers. Equol-producing status was determined 6-9 years before the imaging study. As with the current study, however, serum levels of ISFs did not relate to WML [240].

Our study has several limitations. First, we cannot establish causality due to the cross-sectional study design. However, equol producing status is reported to be stable over years [3] and

it is unlikely that the presence of AC affected equol producing status. Second, no optimal cut-off points for EP and prevalent AC have been established. We have tried cut-points for AC and EP in the sensitivity analyses and no dramatic changes have been observed. Third, all the participants were men. While sex may modify the association between equol and AC, further studies in both sexes are needed. Fourth, it is possible that our findings may not be attributed purely to equol but phenotypes related to equol-producing status.

Our study has several strengths. First, this was a community-based study that utilized a random sampling method. Second, compared to our previous analysis which was about equol producing status and AC in the ERA JUMP study (n=304), the sample size in the current study is larger, and therefore we are able to explore the modification effect of age. Third, daidzein was detected in all the participants and thus misclassification of equol producing status was unlikely.

In conclusion, we found that EP had significantly lower burden of AC compared to non-producers in Japanese men aged 40-79. Research on the association of equol producing status and atherosclerosis is almost impossible in Western countries because of the very limited dietary intake of ISFs and the low rate of EP in Western countries. Therefore, additional studies in East Asian countries with larger sample sizes in both sexes are warranted.

7.0 Paper 3 – Effect of equol (soy metabolite) on coronary heart diseases – from molecular mechanisms to epidemiological studies

7.1 Introduction

Coronary heart disease (CHD) is the leading cause of morbidity and mortality worldwide [265]. Soy is a potential nutritional source for preventing CHD [266] and is a standard part of a traditional diet in East Asia [267]. The main components of soy that may exert cardioprotective effects are soy isoflavones (ISFs), mainly daidzein and genistein [1]. ISFs are phytoestrogens and are structurally similar to estradiol [268]. Estradiol exerts its biological action by binding both estrogen receptor α (ERα) expressed in reproductive, central nervous, cardiovascular and other systems [2] and estrogen receptor β (ERβ) expressed in cardiovascular, central nervous and others systems. ISFs, however, preferentially bind to ERβ [3]. Among the flavonoid family of flavones, flavonols, flavanones, and ISFs, ISFs have the highest lipophilicity which could contribute to their greater absorption from the gut [269]. ISFs may lower the risk of CHD by reducing inflammation [270-272] and oxidative stress [14-16]; the latter may prevent the oxidative damage to low-density lipoprotein (LDL) that contributes to atherogenesis [273].

Although there are clear cardiovascular benefits of ISFs in preclinical studies [8, 9, 274, 275], evidence in humans is conflicting [6, 276, 277]. A recent meta-analysis of 23 prospective cohort studies (330,826 participants) showed that dietary intake of ISFs was inversely associated with all-cause and cancer mortality yet not cardiovascular disease (CVD) mortality [277]. Furthermore, ISFs have very small effects on traditional CVD risk factors [24, 178, 278, 279].

A growing hypothesis is that the ability of humans to metabolize daidzein to equol may contribute to the cardioprotective effects of ISFs [38, 153, 280]. A case-control study of myocardial infarction (MI) nested within a prospective cohort study in women in China reported a significant inverse association of incident MI with urinary equol rather than ISFs and their other metabolites [152]. Cell culture and preclinical studies show that equol has a greater affinity for ERβ than its precursor daidzein, a longer half-life, greater bioavailability than daidzein and genistein, and more potent antioxidant activity than any other ISFs [3, 38]. Therefore, equol may be more cardioprotective than ISFs [38, 280].

The mechanistic mode of action of equol is not completely understood. Investigation of the effects of equol has mostly been conducted in the in vitro assays and preclinical studies while several studies in humans explored whether equol production caused the cardioprotective effects previously attributed to ISFs. Therefore, the aim of this systematic review is to summarize the current knowledge about the mechanisms underlying the potential protective effect of equol on atherosclerotic diseases. This review outlines evidence from in vivo, in vitro, and human studies on the anti-inflammatory, antioxidative, and vasodilatory, and other effects of equol, as well as the association of equol with atherosclerosis, arterial stiffness, and CHD.

7.2 Materials and Methods

The PubMed database was searched from inception through July 28, 2021, by 2 investigators (X.Z. and C.V.). Discrepancies were adjudicated with another investigator (A.S.). MeSH terms used for the search were: equol, inflammation, oxidation, endothelial function, vasodilation, atherosclerosis, arterial stiffness, and CHD. We focused on these terms rather than

traditional CHD risk factors because meta-analyses of human randomized controlled trials (RCTs) showed that ISFs had very small effects, if any, on traditional cardiovascular risk factors [281, 282]. A two-stage screening process consisting of a title and abstract scan and a full-text review was used to determine the eligibility of articles. Exclusion criteria were: 1. studies where equol was not reported; 2. human studies that were conducted among equol producers only, instead of between equol producers and non-producers, and thus there was a lack of contrast of the amount of equol; 3. studies where equol was derived from plants other than soy or the source of equol was unknown; 4. studies where the outcomes of the participants were not related to inflammation, oxidation, endothelial function, arterial stiffness, atherosclerosis, or CHD; 5. review articles or proposals; 6. studies that were published in a language other than English.

7.3 Results

The screening process is shown in Figure 9. Of the 231 articles identified, 69 met our criteria for review and were summarized.

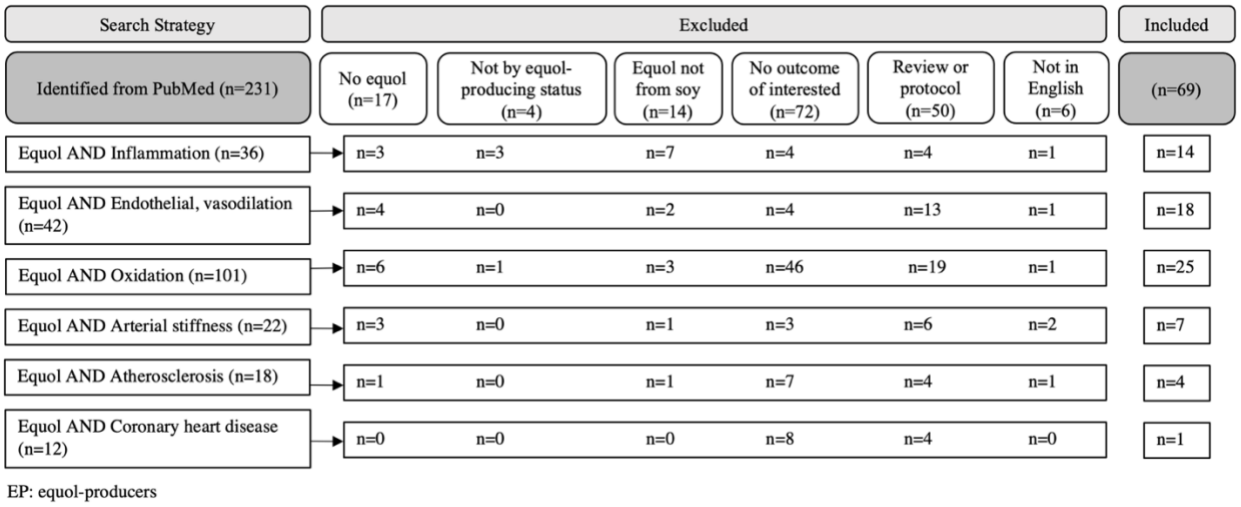


Figure 9 Search strategies in PubMed

Search strategies in PubMed and the literature selection process from inception to 28-July-2021.

7.3.1 Characterization of equol

Equol is a compound that can exist as two isomers, S-equol and R-equol. However, only S-equol is produced in humans and animals. Thus, in this article, we use the term equol to denote S-equol only. Equol is the bioactive metabolite that is metabolized by intestinal bacteria from daidzein (Figure 10) and has been suggested to contribute to the health benefits of ISFs [31, 86, 122, 123, 142]. There is interindividual varia-tion in the ability to produce equol [30, 33]. Although the majority of animals produce equol, 30-70% of the adult human population can produce equol following soy challenge [30] and this capacity is due to the presence of specific gut microflora [283]. Some identified equol producing bacteria are Adlercreutzia equolifaciens, Asaccharobacter celatus, Enterorhabdus mucosicola, Slackia isoflavoniconvertens, and Slackia equolifaciens [280, 284-286]. The bacterial biosynthesis of equol from daidzein proceeds via a series of reduction reactions catalyzed by several reductases, such as nicotinamide adenine dinucleotide phosphate

reductase, dihydrodaidzein reductase, and tetrahydrodaidzein reductase [287-289]. If an individual's ability to produce equol is reported to be stable over 1 to 3 years [290], they can be classified as either "equol producers" or "non-producers".

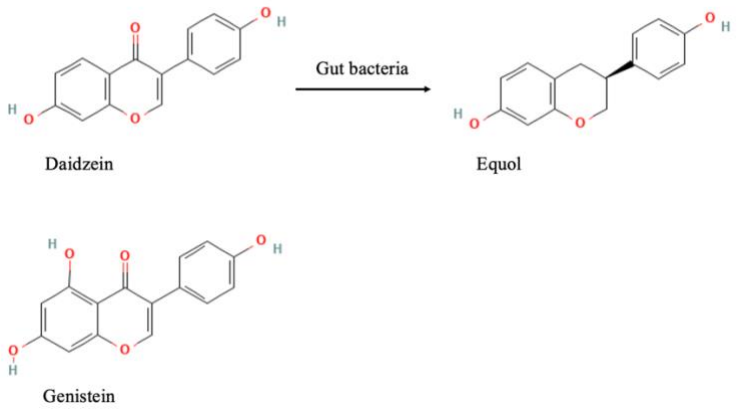


Figure 10 Structures of isoflavones and equol

Structures of genistein, daidzein, and equol [10-12]. Genistein and daidzein are two major ISFs and comprise > 95% of dietary sources. Equol is a metabolite of daidzein, biotransformed by gut bacteria.

Equol has the following characteristics that may grant it with possibly greater cardioprotective properties than ISFs: 1) equol has higher antioxidant activity than ISFs [31, 33, 34] and its antioxidant properties are even greater than vitamins C and E in in vitro studies [36]; 2) the affinity of equol to ERβ is much higher than daidzein, though is similar to genistein [37]; 3) equol is more lipophilic than ISFs, therefore, has a higher absorption rate from the gut than ISFs, resulting in greater bioavailability [269]; 4) equol has a longer plasma half-life than ISFs, being retained in the body for a greater period following soy consumption [33]; 5) equol has a lower ability to bind to serum proteins (i.e., albumin, sex-hormone-binding globulin, and alpha-fetoprotein) than ISFs and thus has a greater availability for receptor occupancy [38].

7.3.2 ERβ

Estrogen receptors (ERs) are intracellular transcription factors whose activity is modulated by estrogens, non-steroidal estrogen antagonists or agonists such as ISFs. ERs belong to the nuclear hormone receptor family with two subtypes, ERα and ERβ [2]. Accumulating evidence has suggested that ERβ may play a more dominant role in mediating the cardioprotective effects of estradiol [2]. ERβ knock-out mice have increased mortality and exacerbated heart failure after MI [291]. Studies of ERβ knock-out mice exhibited poor functional recovery compared to wild-type or ERα knock-out mice in an ex vivo model of global ischemia-reperfusion [292]. Consistently, cardiomyocyte-specific ERβ overexpression improved cardiac function and survival after MI induced by left anterior descending coronary artery ligation [2].

Equol is a selective ERβ agonist [3]. Studies demonstrated that ERβ agonists are cardioprotective. For example, one study demonstrated that a selective ERβ agonist led to significant recovery of cardiac functionality in ovariectomized mice due to the ERβ-dependent upregulation of cardioprotective genes [293]. More-over, the exposure of human macrophages to an ERβ-selective agonist led to a robust decrease in the level of extracellular heat shock protein 27, a biomarker of atherosclerosis [294], while ERα-selective agonists did not show such effect [295]. Subjects receiving ERβ-selective agonists may retain the benefits of hormone therapy without the severe adverse effects.

7.3.3 Potential cardioprotective properties of equol

7.3.3.1 Anti-inflammatory effects

Inflammation contributes to atherosclerosis from its inception to development into thrombotic complications [76, 296]. The accumulation of oxidized lipids triggers atherosclerosis mediated by proinflammatory cytokines such as interleukin-1β, tumor necrosis factor-α (TNF-α), and nuclear factor-κB (NF-κB). Proinflammatory cytokines initiate the expression of vascular cell adhesion molecule-1 (VCAM-1) by endothelial cells (ECs) and allow the attachment of leukocytes, monocytes, and T lymphocytes to the arterial wall. Once adhered to the arterial endothelium, monocytes penetrate the endothelial lining and enter the intima of the vessel wall by diapedesis between ECs, a process triggered by chemokine monocyte chemoattractant protein-1 (MCP-1) [296]. Within the intima, monocytes mature into macrophages, exhibit increased expression of scavenger receptors and engulf modified lipoproteins. Cholesterol esters accumulate in the cytoplasm, and the macrophages be-come foam cells [76, 296].

Equol inhibits the overproduction of inflammatory biomarkers expressed by macrophages, microglia cells, and adipose tissues. In addition, equol ameliorates inflammatory processes important to the pathogenesis of rheumatoid arthritis, metabolic syn-drome and intracranial aneurysm (Table 17, Figure 11).

Table 17 Anti-inflammatory effects of equol

#	Authors	Type	Findings	Has effect
1	Blay [115]	In vitro	Equol (10 μM) significantly inhibited the overproduction of NO and PGE2 induced by LPS plus INF-γ when a pre-treatment was performed or when administered during activation. Moreover, equol regulated gene transcription	Yes

#	Authors	Type	Findings	Has effect
			of cytokines and inflammatory markers. Genistein (20 μM) exerted similar anti-inflammatory effects, but daidzein did not.	
2	Johnson [116]	In vitro	Equol exhibited protective effects against pro-inflammatory cytokines (IL-6 and TNF-α) and NO production in murine microglia cells. Equol also showed greater permeability through artificial gut and blood-brain barriers compared to daidzein.	Yes
3	Obiorah [117]	In vitro	Equol and ISFs induced endoplasmic reticulum stress and inflammatory response stress-related genes in a comparable manner to estrogens. Equol and ISFs induced proliferation of estrogenized breast cancer cells (simulating a perimenopausal state) but induced apoptosis of estrogen-deprived cells (simulating a postmenopausal state).	Yes
4	Nagarajan [118]	In vitro	In an in vitro LPS-induced inflammation model, equol dose-dependently inhibited LPS-induced MCP-1 secretion by macrophages.	Yes
5	Subedi [119]	In vitro	In microglial cells, equol inhibited TLR4 activation, MAPK activation, NF-kB-mediated transcription of inflammatory mediators, production of NO, release of PGE-2, secretion of TNF-α and IL-6, in LPS-activated murine microglia cells.	Yes
6	Moriyama [120]	In vitro	Equol attenuated LPS-induced NO production with a concomitant decrease in expression of iNOS. Equol did not affect LPS-induced increase in intracellular ROS production. Increased NO production is a well-known inflammatory change in astrocytes stimulated by LPS. Attenuation of NO production by equol may mitigate LPS-induced neuroinflammation in astrocytes.	Yes
7	Lin [121]	In vivo	Equol-administered collagen-induced arthritis mice had lower severity of arthritis symptoms. Equol administration suppressed the expression of IL-6 and its receptor in the inflamed area of collagen-induced arthritis mice.	Yes
8	Yokosuka [122]	In vivo	In ovariectomized mice induced to have intracranial aneurysms, equol protected against aneurysm formation; the disruption of the intestinal microbial conversion of daidzein to equol abolished daidzein's protective effect against aneurysm formation. Moreover, mice treated with equol had lower inflammatory cytokines in their cerebral arteries.	Yes

#	Authors	Type	Findings	Has effect
9	van der Velpen [123]	Human	In the adipose tissue of postmenopausal women, expression of inflammation-related genes was upregulated in equol producers but downregulated in non-producers.	Yes
10	Törmälä [124]	Human	ISFs caused a decrease in the VCAM-1 and platelet-selectin. The fall in platelet-selectin was more marked in equol producers. No changes appeared in SHBG, CRP or ICAM-1.	Yes
11	Reverri [125]	Human	Consuming soy improved arterial stiffness as was assessed by the augmentation index, but did not improve the inflammatory biomarkers (CRP, TNF-α, IL-6, IL-18, IL-10). The addition of equol producing status as a covariate did not significantly change these results.	No
12	Nicastro [21]	Human	Equol, while not associated with a decrease in CRP level, was associated with decreased geometric mean WBC counts comparing the highest quartile to the lowest.	Yes
13	Greany [126]	Human	An RCT of 34 postmenopausal women on 44 mg/day of ISFs showed that ISFs did not influence the concentrations of Hcy, CRP, sE-selectin, sVCAM-1, and sICAM-1. Equol producing status did not modify the associations.	No
14	Mangano [127]	Human	In women who received the ISFs intervention, there was no significant differences in percent change in serum inflammatory markers between equol producers and non-producers.	No

Abbreviations: NO, nitric oxide; PGE2, prostaglandin E_2; LPS, lipopolysaccharide; INF-γ, interferon gamma; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractant protein-1; MetS, metabolic syndrome; CRP, C-reactive protein; sICAM, soluble intercellular adhesion molecule; VCAM-1, vascular cell adhesion molecule 1; SHBG, sex hormone binding globulin; ICAM-1, intercellular adhesion molecule 1; IL-8, interleukin-8; IL-10, interleukin-10; WBC, white blood cell; Hcy, homocysteine; sE-selectin, soluble endothelial leukocyte adhesion molecule-1; TLR4, toll-like receptor 4; MAPK, mitogen-activated protein kinase; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; RCT: randomized controlled trial.

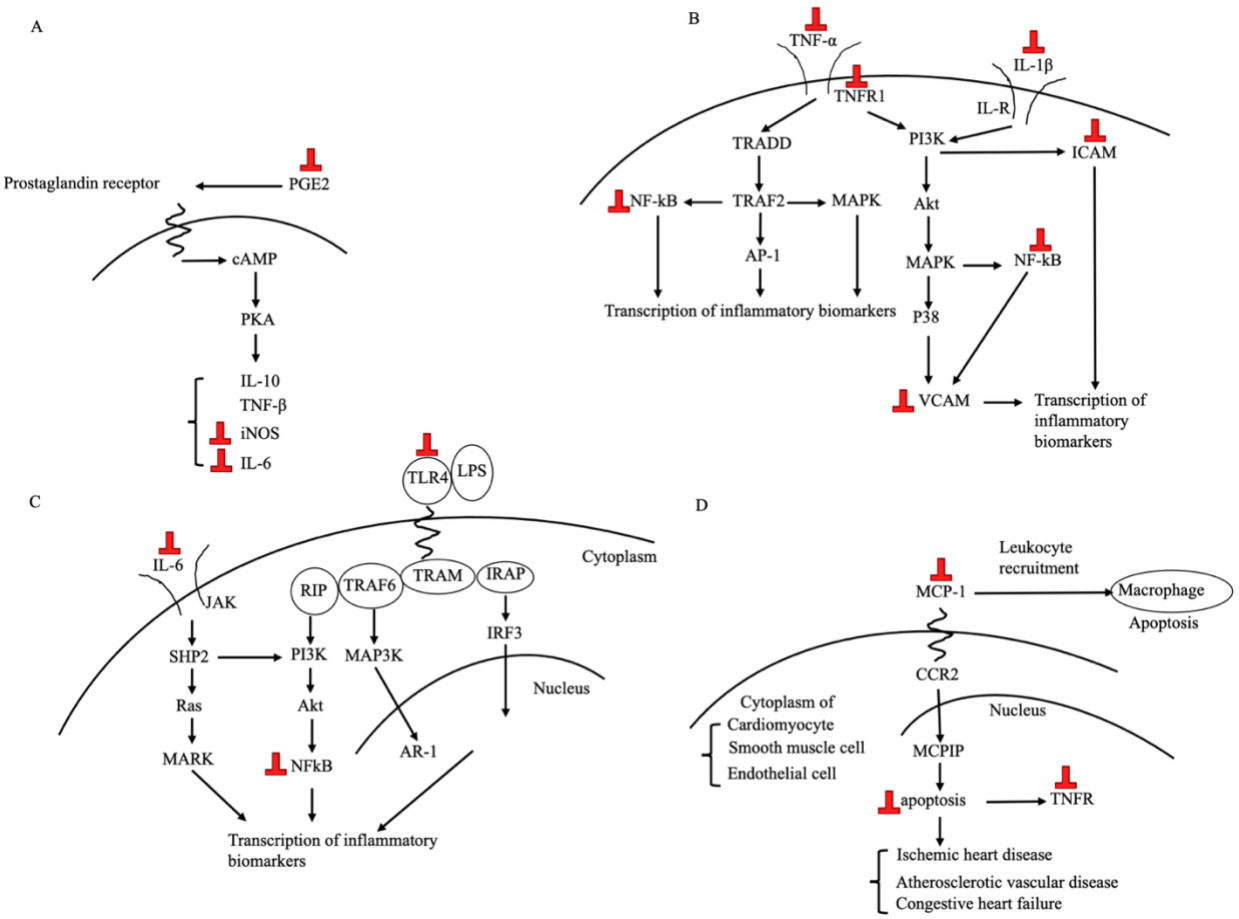


Figure 11 Signaling pathways of anti-inflammatory effects

Signaling and pathways in which equol exerts anti-inflammatory effects. A: PGE2 pathway [115]. B: TNF-α [116] and IL-1 pathway [125]. C: TLR4 [119] and IL-6 pathway [125]. D: MCP-1 pathway [118]. "⊥" indicates the inhibitory effect by equol. PGE2: prostaglandin E2, cAMP: cyclic adenosine monophosphate, PKA: protein kinase A, IL: interleukin, TNF: tumor necrosis factor, iNOS: nitric oxide system, TNFR: TNF receptor, TRADD: TNFR1-associated death domain protein, TRAF2: TNF receptor-associated factor 2, PI3K or Akt: phosphoinositide 3-kinases, MAPK or P38: mitogen-activated protein kinase, AP: activator protein, NF-kB: nuclear factor kappa B, ICAM: intercellular adhesion molecule, VCAM: vascular cell adhesion molecule, IL-R: interleukin receptor, LPS: lipopolysaccharide, TLR: Toll-like receptor, TRIF: Toll/IL-1R domain-containing adaptor-inducing IFN-β, TRAM: TRIF-related adaptor molecule, TRAF: TNFR-associated factor, RIP: receptor-interacting protein, IRF: IFN regulatory factor, IFN: interferon, JAK: janus kinase, SHP: Src homology-2 domain-containing protein tyrosine phosphatase, AR: androgen receptor, MCP: monocyte chemoattractant protein, CCR: Chemokine receptor, MCPIP: monocyte chemoattractant protein-induced protein.

In vitro studies have consistently shown that equol is anti-inflammatory. First, in macrophages, equol down-regulated the expression of genes related to the production of various kinds of cytokines and inflammatory biomarkers [115], and dose-dependently reduced the

secretion of inflammatory biomarkers such as prostaglandin E2 [115] and MCP-1 [118], whereas daidzein did not have such effect [115]. In microglia cells, equol reduced the release of interleukin-6 (IL-6) [116, 119] and TNF-α [116, 119]. These effects may be mediated through the inhibition of Toll-like receptor 4, mitogen-activated protein kinase (MAPK), or NF-κB [119]. In astrocytes, equol attenuated nitric oxide (NO) production and concomitantly decreased the expression of inducible NO synthase [120].

Several in vivo studies in mice reported the effect of equol on inflammatory diseases and inflammation. Equol administration in a rheumatoid arthritis mouse model improved arthritis-induced bone mineral density and suppressed the expression of IL-6 and its receptor in inflamed areas [121]. In ovariectomized mice induced to have intracranial aneurysms, daidzein with equol supplementations protected against aneurysm formation, whereas the disruption of the intestinal microbial conversion of daidzein to equol abolished daidzein's protective effect against aneurysm formation, indicating that equol alone had a protective effect [122]. The same study also suggested that mice treated with equol had lower inflammatory cytokines in their cerebral arteries [122]. All of the above effects were exerted through the activation of ERβ.

In some human RCTs of ISFs, the anti-inflammatory effects of ISFs were more pronounced in equol producers than non-producers, while in other RCTs, the anti-inflammatory effects of ISFs were only observed in equol producers. One RCT of ISFs among 110 postmenopausal women on tibolone showed that equol producers had a larger reduction in platelet-selectin levels than non-producers after being treated with ISFs [128]. Platelet-selectin is a cell adhesion molecule on the surface of activated ECs [129]. In the peripheral blood mononuclear cells in 30 equol-producing postmenopausal women, ISFs intervention down-regulated clusters of genes that were involved in inflammation, oxidative phosphorylation, and cell cycle regulation, as was suggested by the

analysis in the whole-genome gene expression profiles [130]. Further analysis in the same group of participants later reported that ISF supplements upregulated the expression of anti-inflammatory genes in the adipose tissue of equol producers but down-regulated the expression in non-producers [123].

An RCT of ISFs with 34 women found that neither ISFs nor equol-producing status was associated with CRP, VCAM-1, and ICAM-1 [126]. This null finding may be due to the small number of participants.

7.3.3.2 Antioxidative effect

Progression of atherosclerotic plaque is caused by molecular changes induced by cytokines and reactive oxidant species (ROS) [63, 64]. Myeloperoxidase (MPO) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase serve as enzymatic sources of oxidant species to generate oxidized LDL (oxLDL) [65, 297]. Inhibition of NADPH oxidase activity reduced atherosclerotic lesions in animal models [298, 299]. In humans, higher levels of oxidative biomarkers, such as oxLDL, MPO and F2-isoprostanes, were associated with an increased risk of CHD [66, 300-304]. In addition, individuals with MPO deficiency were found to have a very low incidence of CVD [71].

Cell culture studies show that equol is a potent antioxidant in numerous models (Table 18, Figure 12). Equol protected macrophages from oxidative stress induced by L-lactate dehydrogenase or oxLDL by reducing lipid peroxidation product malondialdehyde (MDA), enhancing antioxidant glutathione, or increasing the activities of the antioxidant enzyme superoxide dismutase (SOD) [93, 94]. Equol treatment on neutrophils that were activated with various stimulants to produce ROS caused reduced toxic action of ROS, for example, equol decreased phosphorylation of proteins regulating NADPH oxidase [95]. Phagocytic cells, after

being incubated with equol for 1 hour, showed a significant reduction in the intracellular production of superoxide anion and hydrogen peroxide [96]. In the intestinal epithelial cells with oxidative stress induced by hydrogen peroxide, equol promoted the expression of antioxidant genes, increased the activities of SOD, and in-creased the abundance of nuclear factor erythroid 2 (Nrf2) transcripts [97]. Finally, equol decreased the ratio of reduced/oxidized glutathione in primary cortical neuron cells [98]. Although some antioxidant effects are independent of ERβ [99], these effects are generally considered to be mediated through ERβ.

Table 18 Antioxidative effects of equol

#	Authors	Type	Findings	Has effect
1	Lin [97]	In vitro	Equol was shown to protect chicken intestinal epithelial cells from oxidative damage by promoting the expression of antioxidant genes, increasing the activities of antioxidant enzymes, and enhancing antioxidant capacity. Equol significantly enhanced total SOD activity and the Nrf2 transcript.	Yes
2	Pereboom [96]	In vitro	Equol decreased the intracellular production of the superoxide anion and hydrogen peroxide content of phagocytic cells.	Yes
3	Hwang [100]	In vitro	Equol and ascorbic acid interacted synergistically in preventing LDL oxidation. All phases of LDL oxidation were affected by these compounds, which is atypical of the behavior of antioxidants that are consumed during the early phases. Equol was more potent than daidzein and genistein because of its absence of a carbonyl group, C2-C3 double bond and flanking hydroxyl groups in the pyran ring.	Yes
4	Pažourekov á [95]	In vitro	Upon activation by ROS, neutrophils treated by equol produced less p40 phox (a component of NADPH oxidase, responsible for the assembly of functional oxidase in intracellular membranes) both extra- and intracellularly to the control.	Yes
5	Choi [101]	In vitro	Equol pretreatment significantly decreased levels of oxidative stress biomarkers such as thiobarbituric acid-reactive substances, carbonyl content and serum 8-hydroxy-2-deoxyguanosine. Moreover, equol increased	Yes

#	Authors	Type	Findings	Has effect
			the activity of CAT, superoxide dismutase, GPx and glutathione reductase. In addition, equol possessed anticancer activity through acting as an antioxidant therefore reduced apoptosis.	
6	Wei [102]	In vitro	Low doses of equol could prevent skeletal muscle cell damage induced by hydrogen peroxide. Equol increased cell viability, the concentration of MDA content, and LDH activity.	Yes
7	Kamiyama [103]	In vitro	Equol might contribute to a reduced level of oxLDL-stimulated apoptosis linked to the reduced generation of intracellular ROS in human umbilical vein endothelial cells.	Yes
8	Sierens [104]	In vitro	Equol was able to function as an antioxidant, scavenging potentially harmful free radicals. Equol protected against oxidative-induced DNA damage. Pretreatment of a physiological range of equol offered protection against the hydrogen peroxide-mediated DNA damage in human lymphocytes cells. This protection was greater than that offered by the addition of antioxidant vitamins ascorbic acid and alpha-tocopherol, or the compounds 17β-estradiol and tamoxifen, which have similar structures to ISFs and are known to have moderate antioxidant activity.	Yes
9	Rüfer [105]	In vitro	Equol exhibited higher antioxidant activity than daidzein and about the same antioxidant capacity as the oxidative metabolites of daidzein and genistein despite the lack of the 2,3-double bond with the 4-oxo group and a 5,7-dihydroxyl structure. The antioxidative effect was tested by an ORAC assay which determined the ability of compounds to scavenge peroxyl radicals.	Yes
10	Hwang [34]	In vitro	Equol inhibited LDL oxidation in vitro and LDL oxidative modification by monocyte/macrophages. The antioxidant effect of equol was found to be mediated by inhibition of superoxide radical production and manifested through enhanced levels of free NO. Equol had a greater antioxidant activity than genistein and daidzein.	Yes
11	Sierens [106]	In vitro	Pretreatment with equol significantly protected sperm DNA against oxidative damage. Compared with ascorbic acid and alpha-tocopherol, being added at physiological concentrations, genistein was the most potent antioxidant, followed by equol, ascorbic acid, and alpha-tocopherol. Equol might have a role to play in antioxidant protection against male infertility.	Yes

#	Authors	Type	Findings	Has effect
12	Arora [107]	In vitro	Compared to genistein and daidzein with their glycosylated and methoxylated derivatives, equol and its 4-hydroxy and 5-hydroxy derivatives were more potent antioxidants, suggesting that the absence of the 2, 3-double bond and the 4-oxo group on the ISF nucleus enhanced antioxidant activity.	Yes
13	Turner [108]	In vitro	Equol inhibited the oxidation of LDL 2.65-fold more than its parent compound daidzein.	Yes
14	Choi [98]	In vitro	Equol acted as an antioxidant in the brain of rats. The ratio of GSH/GSSG in primary cortical neuron cells exposed equol for 24 and 72 hours significantly decreased in a time- and dose-dependent manner. Moreover, equol treatment was significantly increased the LDH release in a time-and dose-dependent manner.	Yes
15	Gou [94]	In vitro	Equol protected chicken macrophages from oxidative stress induced by lipopolysaccharide through reducing lipid peroxidation products such as MDA and enhancing contents of antioxidants such as glutathione, and activities of relevant antioxidase enzymes such as total SOD; effects were also seen in gene expression related to the immune response and increased contents of cytokines.	Yes
16	Liu [109]	In vitro	Equol elevated brain antioxidant activity by increasing SOD, CAT and GPx levels. MDA levels and AChE activity were decreased in hypertensive and vascular dementia rats. Equol further improved the long- and short-term memory of the rats.	Yes
17	Vedavanam [110]	In vivo	The order of the half-maximal inhibitory concentration values, the indication of the potency of inhibiting glucose-induced LDL lipid peroxidation observed for the compounds was equol > genistein > daidzein.	Yes
18	Choi [93]	In vivo	Equol might act as an antioxidant through an inhibition of oxidative stress and stimulation of CAT and SOD, but could also cause pro-oxidant effects, such as reduction of the GSH/GSSG ratio, depending on the treatment period. Study in mice showed that equol administration significantly inhibited biomarkers of oxidative stress (thiobarbituric acid-reactive substances value, carbonyl content, and serum 8-hydroxydeoxyguanosine). Moreover, CAT and total SOD activities and their transcripts were significantly increased by equol. Although equol increased the	Yes

#	Authors	Type	Findings	Has effect
			glutathione peroxidase activity in mice treated with equol for 1-week, long-term administration of equol (7 weeks) caused a decrease in the ratio of GSH/GSSG and the activities of GPx and glutathione reductase .	
19	Ma [111]	In vivo	A study in male and ovariectomized female rats with transient middle cerebral artery occlusion revealed that pretreatment of equol significantly reduced infarct size in both sexes. This neuroprotection was accompanied by a decrease in NADPH oxidase activity and superoxide levels in the brain. In addition, equol reduced plasma thiobarbituric acid reactive substances and neurological deficits up to 7 days after injury.	Yes
20	Horiuchi [112]	In vitro	The study demonstrated that equol had suppressive effects against oxidative stress in pancreatic β-cells in a dose-dependent manner and presumably through activating PKA signaling.	Yes
21	Jackman [31]	In vivo	Equol exerted weak antioxidant effects in cerebral arteries, whereas effects of daidzein were insignificant. Antioxidant activity was assessed as the reduction of NADPH-induced superoxide levels.	Yes
22	Widyarini [113]	In vivo	In addition to the activation of estrogenic signaling pathways for photoprotection, equol also provided UV-protective antioxidant effects that depend partially on HO-1 induction. Equol dose-dependently inhibited the oxidative stress measured as UVA-induced lipid peroxidation on mouse skin. A component of equol lipid protection capacity is attributed to endogenous cutaneous antioxidant enzymes including the inducible stress protein HO-1.	Yes
23	Nhan [114]	Human	Urinary equol was not associated with the secretion of urinary F2 isoprostane,a measure of cellular lipid peroxidation, after ISF treatment in postmenopausal women. But the observations on the effect of equol were limited because only 2 of the 8 subjects were equol producers, one of whom experienced a large increase in the biomarker excretion, whereas the other experienced small decreases.	No
24	Hidayat [86]	Human	The level of MDA, an oxidative stress marker, was lower in equol producers than non-producers. This RCT was conducted with 190 postmenopausal women aged 47-60 and who received 100 mg ISFs for 6 months. The random allocation of ISFs intervention was carried out separately by equol producing status.	Yes

#	Authors	Type	Findings	Has effect
25	Richardson [99]	In vitro	Equol might have a beneficial effect in delaying the onset and decreasing the severity of symptoms in Friedreich's ataxia patients by an antioxidant mechanism such as reducing ROS-induced modification of proteins and lipids and impaired mitochondrial function. These effects were independent of ERβ.	Yes

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; MDA, malondialdehyde; AChE, acetylcholinesterase; PKA, protein kinase A; Nrf2, nuclear factor erythroid 2–related factor 2; NADPH, nicotinamide adenine dinucleotide phosphate; LDH, L-lactate dehydrogenase; ORAC, oxygen radical absorbance capacity; GSH/GSSG, reduced/oxidized glutathione; DNA, deoxyribonucleic acid; HO-1, heme oxygenase-1; NQO1, NADPH-quinone oxidoreductase 1; UV, ultraviolet.

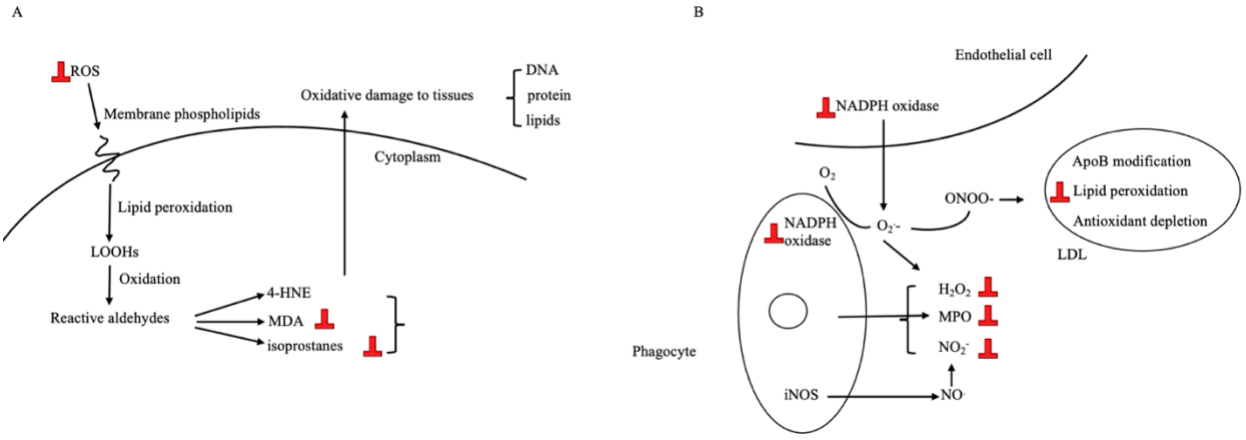


Figure 12 Signaling pathways of antioxidative effect

Signaling and pathways on which equol exerts antioxidative effect. A: ROS pathway [103]. B. NADPH pathway [95]. "⊥" indicates the inhibitory effect by equol. MDA: malondialdehyde, 4-HNE: dyhidroxynonel, LOOHs: lipid hydroperoxides, ROS: reactive oxygen, DNA: deoxyribonucleic acid, ONOO-: peroxynitrite, NO2: nitrogen dioxide, O2-: superoxide, MPO: myeloperoxidase, NADPH: nicotinamide adenine dinucleotide phosphate, apoB: apolipoprotein B, iNOS: inducible nitric oxide synthase, H2O2: hydrogen peroxide.

Animal studies also demonstrated the antioxidant properties of equol. Two mice studies demonstrated that equol improved vascular health in the brain [31, 109]. It was suggested that equol elevated the activities of SOD, catalase, acetylcholinesterase, and glutathione peroxidase, and decreased MDA levels in mice that had deoxycorticosterone acetate salt-induced hypertension and associated vascular dementia [109]. Equol also reduced NADPH-induced superoxide

production in the cerebral arteries of normotensive rats and hypertensive rats that were induced by angiotensin II [31]. However, the antioxidant effect was not observed in daidzein [31]. One study showed that equol suppressed tumor formation in rats, presumably through decreasing the concentrations of thiobarbituric acid-reactive substances and 8-hydroxy-2-deoxyguanosine, and increasing the activity of catalase, SOD, glutathione peroxidase [101].

One RCT in humans demonstrated that equol-producing individuals could receive the antioxidant benefits from ISFs but non-producers could not. An RCT of 190 post-menopausal women found that after 6 months of supplementation with ISFs, blood MDA concentrations were significantly lower in the equol producers compared with non-producers in the ISFs group [86].

At the normal physiological concentrations, equol is a more potent antioxidant compared to ISFs probably due to the absence of 2,3-double bond, the 4-oxo group, and a 5,7-dihydroxyl [100, 105-107, 110]. The antioxidant property of equol is even more potent than ascorbic acid, alpha-tocopherol, and 17β-estradiol. Comparative studies found that the potency of inhibiting LDL lipid peroxidation was in such order: equol > genistein > daidzein [100, 110].

7.3.3.3 Endothelial function and vasodilation

The endothelium regulates vascular tone, carefully balancing vasoconstriction and vasodilation to provide adequate perfusion to target organs [305]. All of the above inflammatory and oxidative processes cause damage to the vascular endothelium, leading to the formation of arteriosclerotic plaques [87]. Flow-mediated dilatation (FMD) of the brachial arteries provides a non-invasive measurement of endothelial function [306].

It has been observed in several categories of vessels (umbilical vein, aorta, pulmonary artery, and cerebral basilar artery) that equol stimulated endothelial redox signaling and increased NO production in the ECs [35, 131, 132] (Table 19). Studies indicated that equol, by binding to

ERβ, could rapidly stimulate phosphorylation of extracellular signal-regulated protein kinase 1/2 and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), leading to the activation of endothelial NO synthase (eNOS) [131, 133]. NO can react with superoxide anions to form peroxynitrite. NO and peroxynitrite then, in turn, enhance the nuclear accumulation of Nrf2, which binds to an antioxidant response element in target genes to enhance the transcription of the phase II antioxidant defense enzymes, such as superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase, and heme oxygenase-1 [134]. A second pathway by which equol improved endothelial function was through transactivation of the epidermal growth factor receptor kinase, resulting in a reorganized F-actin cytoskeleton [135]. A third pathway in which equol was involved was through the direct upregulation of eNOS, resulting in reduced oxidative stress in ECs [35, 136]. The target gene eNOS contains an estrogen-response element. Thus, it can be reasoned that the binding of equol to ERβ may be responsible for enhanced eNOS expression. In a fourth pathway, equol could increase the expression of phospho-p38 MAPK and B-cell lymphoma-2 to reduce intracellular ROS production in ECs [137]. Furthermore, equol directly inhibited apoptosis of ECs [138], presumably through stimulating the thymidine incorporation which is important for the deoxyribonucleic acid (DNA) synthesis of ECs [138]. This stimulatory effect on cell growth was not observed for daidzein or genistein [138].

Table 19 Endothelial function improvement effects of equol

#	Authors	Type	Findings	Has effect
1	Joy [133]	In vitro	Nutritionally relevant plasma concentrations of equol rapidly stimulated phosphorylation of ERK1/2 and PI3K/Akt, leading to the activation of NOS and increased NO production at resting cytosolic Ca^{2+} levels.	Yes

#	Authors	Type	Findings	Has effect
2	Rowlands [135]	In vitro	Equol-stimulated mitochondrial ROS modulated endothelial redox signaling and NO release through transactivation of epidermal growth factor receptor kinase and reorganization of the F-actin cytoskeleton.	Yes
3	Cheng [35]	In vitro	Equol prevented oxidative damage to vascular function in pulmonary cells via downregulating eNOS and oxidative stress.	Yes
4	Zhang [131]	In vitro	In HUVEC, equol increased Nrf2 mRNA as well as mRNA of gene products of HO-1 and NQO1. Pretreatment of cells with specific endoplasmic reticulum inhibitors or PI3K/Akt increased Nrf2, HO-1, and NQO1 protein.	Yes
5	Chung [137]	In vitro	Equol had a significant antioxidant effect on the bAECs that were exposed to hydrogen peroxide. Equol pretreatment effectively inhibited the hydrogen peroxide-induced cell death by the reduction of intracellular ROS production, probably through increasing phospho-p38 MAPK.	Yes
6	Zhang [139]	In vitro	The improvement of atherosclerosis by equol through attenuation of endoplasmic reticulum stress is mediated by activating the Nrf2 signaling pathway. Equol treatment inhibited cell apoptosis and attenuated upregulation of endoplasmic reticulum stress markers in HUVECs. In an oxidative stress environment, equol treatment dose-dependently activated the Nrf2 signaling pathway.	Yes
7	Somjen [138]	In vitro	Equol, but not daidzein and genistein, had a monophasic stimulatory effect on thymidine incorporation, which boosts DNA synthesis. In human endothelial cells, equol, daidzein, and genistein stimulated DNA synthesis in a dose-dependent manner. The administration of equol, daidzein, and genistein to immature and ovariectomized female rats resulted in increased creatine phosphokinase in the aorta and in the left ventricle of the heart.	Yes
8	Kim [140]	In vitro	Equol had a vasodilatory effect on human uterine arteries vascular smooth muscle, which was mediated through antagonistic action for receptor-dependent Ca^{2+} channel.	Yes
9	Johnson [116]	In vitro	Equol exhibited protective effects against NO production in murine microglial cells. Equol also showed greater permeability through artificial gut and blood-brain barriers compared to daidzein.	Yes

#	Authors	Type	Findings	Has effect
10	Chin-Dusting [25]	In vivo	Equol had a dose-dependent inhibition of the contractile responses to noradrenaline in rat isolated aortic rings. Equol independently increased the release of a vasoconstrictor proteinoid, such as thromboxane.	Yes
11	Jackman [31]	In vivo	In normotensive rats, equol displayed vasorelaxant activity similar to daidzein. The relaxant effect of equol was independent of intact endothelium, NOS activity, K^+ channels and gender. In the basilar artery, where superoxide levels are higher, equol exerted weak antioxidant effects, whereas effects of daidzein were insignificant. During hypertension, equol-induced vasorelaxation was preserved, whereas relaxant responses to daidzein were impaired.	Yes
12	Matsumoto [141]	In vivo	Contractions induced by a selective 5-HT receptor agonist increased with insulin treatment, but less so with equol + insulin. In the endothelium-denuded preparations, 5-HT-induced contractions were augmented with insulin treatment but less so by equol + insulin treatment. These differences in 5-HT-induced contractions were eliminated by a large-conductance Ca^{2+}-activated K^+ channel inhibitor.	Yes
13	Yu [132]	In vivo	Equol significantly increased regional cerebral blood flow in rats and produced an endothelium-independent relaxation in rat cerebral basilar arteries. Selective Ca^{2+}-activated K^+ channel blockers significantly inhibited equol-induced vasodilation in cerebral arteries.	Yes
14	Ohkura [136]	In vivo	Ovariectomized rats were assigned to 1) ISF-deficient but equol-sufficient group, 2) ISFs-deficient and equol-deficient group. In the thoracic artery, endothelium-dependent relaxation, cyclic guanosine monophosphate levels in the tissue, and eNOS synthase expression and phosphorylation were significantly higher in the first group compared to the second group.	Yes
15	Törmälä [128]	Human	Before ISF intervention, women with a 4-fold elevation in equol levels had a lower endothelial function index compared to women without this capacity. Soy supplementation had no effect on arterial stiffness or endothelial function in either group.	Yes

#	Authors	Type	Findings	Has effect
16	Kreijkamp-Kaspers [142]	Human	This RCT did not support the hypothesis that ISFs have beneficial effects on endothelial function in older postmenopausal women. However, in the soy-only group, systolic and diastolic blood pressure decreased and endothelial function improved in the equol producers, whereas blood pressure increased and endothelial function deteriorated in the non-producers.	Yes
17	Hidayat [86]	Human	ISFs did not improve endothelial functions in both equol producer and non-producers. The VCAM-1 and NO did not differ by equol-producing status.	No
18	Clerici [143]	Human	After ISFs treatment, brachial artery flow-mediated vasodilatation was improved more obviously in equol producers.	Yes

Abbreviations: ERK1/2, extracellular signal-regulated protein kinases 1 and 2; PI3K/Akt, protein kinase 1/2 and phosphatidylinositol 3-kinase/protein kinase B; NOS, nitric oxide synthase; NO, nitric oxide; ROS, reactive oxygen species; HUVEC, human umbilical vein endothelial cell; eNOS, endothelial nitric oxide synthase; HO-1, heme oxygenase-1; NQO1, NADPH-quinone oxidoreductase 1; Nrf2, nuclear factor-erythroid 2-related factor 2; bAECs, bovine aortic endothelial cell; MAPK, mitogen-activated protein kinase; HUVECs, human umbilical vein endothelial cells; cfPWV, carotid-femoral pulse wave velocity; VCAM-1, vascular cell adhesion molecule-1; 5-HT, 5-hydroxytryptamine; Ca, calcium; K, potassium.

Independent of an effect on the intact endothelium and NOS activity, equol exhibited vasodilator activity in vascular smooth muscle cells of various of arteries which may be induced by antagonistic of the Ca2+-activated K+ channel [31, 132, 140, 141]. This vasodilatory effect was not observed with daidzein [31].

Equol-producing status in humans may be critical in unlocking the endothelial benefits of ISFs. In a long-term RCT of ISFs among 202 older postmenopausal women, endothelial function was significantly improved in equol producers whereas it deteriorated in non-producers, when both groups were treated with ISFs [142]. Secondary analysis of RCT of ISFs with 110 women [128] reported significantly improved endothelial function in equol producers as compared to controls whereas ISFs themselves had no significant effects on endothelial function. An-other RCT of ISFs in 62 adults with hypercholesterolemia observed the vasodilatory effect of ISFs on brachial artery, and determined that improvement was larger in equol producers [143].

7.3.3.4 Arterial stiffness

A healthy aorta normally exerts a powerful cushioning function, which delivers a nearly steady flow of blood to the end organs [307]. Arterial stiffness impairs this cushioning function and is now recognized as a significant predictor of future cardiovascular events independent of traditional risk factors [308-310]. Arterial stiffness is closely associated with endothelial function and vasodilation [311] described in the previous section. Arterial stiffness can be determined by measuring carotid-femoral pulse wave velocity (cfPWV), brachial-ankle PWV (baPWV) and cardio-ankle vascular index (CAVI) [308].

Many nutrients have been hypothesized to improve arterial stiffness, potentially through modulation of endothelial function, and reduce oxidative stress and inflammatory processes [312]. Pase et al. conducted a systematic review of 38 RCTs of nutrients in 2011 and concluded that supplementation of ISFs and marine-derived omega-3 fatty acids improved arterial stiffness [312]. We recently updated this systematic review of RCTs of ISFs and conducted a meta-analysis. We showed that supplementation of ISFs significantly improved arterial stiffness [313]. The effect of ISFs on arterial stiffness is independent of blood pressure. However, the duration of intervention in these RCTs was relatively short (23 hours to 12 weeks) and the effects of ISFs in these RCTs are likely to result in functional rather than structural changes.

Equol supplements relieve the severity of arterial stiffness in the human body, as is suggested by RCTs (Table 20). Usui et al. conducted a crossover RCT of 10 mg/day equol on arterial stiffness assessed by CAVI in 54 overweight or obese middle-aged men and women in Japan and showed that supplementation of equol significantly improved arterial stiffness [41]. However, the sample size was small and the duration of intervention was short (12 weeks). Several sources of evidence support the hypothesis that equol may have a long-term effect on arterial

stiffness. A 12-month RCT of ISFs plus epicatechin in 35 patients with diabetes (mean age of 62) showed ISFs plus epicatechin significantly improved arterial stiffness assessed by cfPWV. Although the effect was not solely attributed to ISFs, the improvement was more pronounced in equol producers, suggesting that long-term exposure to equol may improve arterial stiffness [261]. Furthermore, administration of 10 mg/day equol in 74 women in Japan (mean age of 55) for 12 months significantly reduced arterial stiffness assessed by baPWV [314]. This study was not placebo-controlled; thus, the interpretation of the result is limited. In men prospectively recruited according to equol-producing status, acute (24h) ISF treatment improved cfPWV in equol producers but had no effect in non-producers [315].

Table 20 Arterial stiffness preventive effects of equol

#	Authors	Type	Findings	Has effect
1	Usui [41]	Human	Compared with the placebo group, intervention with natural equol led to a significant decrease in HbA1c, serum LDL-C levels and CAVI score. Furthermore, the effect was more prominent in a subgroup of female equol non-producers.	Yes
2	Curtis [261]	Human	Overall, the ISF intervention did not significantly change common carotid artery or augmentation index, but pulse pressure variability improved. Equol producers had larger reductions in mean arterial pressure and PWV compared with non–producers.	Yes
3	Hazim [315]	Human	In an RCT, acute ISF treatment (24h) improved cfPWV in equol producers but had no effect on endothelial function and NO in non-producers.	Yes
4	Yoshikata [314]	Human	Reduction in arterial stiffness was observed after 12 months of equol supplementation. Significant reductions in respective parameters were observed in women with moderate to high risk for arteriosclerosis, hypertriglyceridemia, bone resorption risk, and bone fracture risk.	Yes

#	Authors	Type	Findings	Has effect
5	Yoshikata [316]	Human	Equol-producing women in their 50s showed significantly lower PWV. In a multivariate logistic regression, for women in their 50s, equol production was significantly associated with lower arterial stiffness.	Yes
6	Reverri [125]	Human	Consuming soy nuts improved arterial stiffness as assessed by the augmentation index using peripheral arterial tonometry. Addition of equol producing status as a covariate did not significantly change the result.	No
7	Törmälä [128]	Human	Soy supplementation had no effect on arterial stiffness in either equol producers or non-producers. At baseline (before ISF treatment), women with 4-fold elevation in equol level had lower augmentation index compared to women without this capacity.	Yes

Abbreviations: LDL-C, low-density lipoprotein cholesterol; CAVI, cardio-ankle vascular index; cfPWV, carotid-femoral pulse wave velocity; HbA1c, glycated hemoglobin; ISF, isoflavones; PWV, pulse wave velocity; NO, nitric oxide.

Observational studies in humans also show that equol-producing status or serum equol levels were associated with a lower degree of arterial stiffness. In a cross-sectional study of Japanese women aged 21-89 years, 67 equol-producing women in their 50s showed significantly lower PWV than 147 non-producers [316]. Although soy had no effect on arterial stiffness measured by augmentation index, women with 4-fold elevation in equol level cross-sectionally had lower augmentation index compared to women without the capacity of producing equol [128]. It is worth noting that because the Japanese RCTs comprise a population with the habitual intake of soy ISFs and a high prevalence of equol producers, studies that showed a beneficial effect of ISFs may be attributed to equol. A cross-sectional study of 652 men in Japan (mean age of 49) showed that dietary intake of ISFs was inversely associated with arterial stiffness assessed by baPWV even after adjusting for age and traditional cardiovascular risk factors [317]. Although this study was cross-sectional, the result implies that long-term exposure to equol may improve

arterial stiffness. On the contrary, in one RCT, equol-producing status did not modify the effect of ISFs on arterial stiffness, measured by peripheral arterial tonometry [125].

7.3.4 Atherosclerosis and CHD

An RCT in mice suggested that equol had anti-atherogenic effects, where equol intervention reduced atherosclerotic lesions in the aorta in apolipoprotein E-deficient mice fed a high-fat diet [139] (Table 21).

Table 21 Anti-atherosclerotic and CHD preventive effects of equol

#	Authors	Type	Findings	Has effect
1	Eyster [318]	In vivo	Equol did not impact atherosclerotic lesions. Similar responses of genes to both equol and estradiol might reflect that equol served as a natural selective estrogen receptor modulator in the arteries. Equol modulated the expression of 10 genes in the atherosclerosis model that estradiol did not.	No
2	Zhang [139]	In vivo	Equol intervention reduced atherosclerotic lesions in the aorta in high-fat diet treated apolipoprotein E-deficient mice. Plasma lipid analysis showed that equol intervention reduced triglycerides, TC and LDL-C and increased HDL-C.	Yes
3	Ahuja [220]	Human	In multivariable model, the odds ratio for the presence of CAC in equol producers compared with equol non-producers was 0.10 (95 % confidence interval: 0.01, 0.90, P<0.04). However, serum ISFs were not related to CAC.	Yes
4	Zuo [226]	Human	An 8.8-year prospective study including 2,572 subjects (40 to 75 years old) found that ISFs and equol were associated with reduced progression of carotid intima-media thickness. Path analyses indicated that the association of serum equol with atherosclerosis was mediated by increased SHBG and decreased blood pressure but not lipids.	Yes
5	Zhang [152]	Human	Urinary levels of ISFs and other metabolites of ISFs were not associated with incident CHD while urinary equol were significantly associated with CHD. The adjusted odds ratios (95% confidence intervals) for CHD across increasing quartiles of equol levels in women were 1 (reference), 0.61	Yes

#	Authors	Type	Findings	Has effect
			(0.32, 1.15), 0.51 (0.26, 0.98) and 0.46 (0.24, 0.89) (P = 0.02 for trend).	

Abbreviations: apoE, apolipoprotein E, cIMT, carotid intima–media thickness; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBG, systolic blood pressure; SHBG, sex hormone–binding globulin; TC, total cholesterol; CAC, coronary artery calcium; SHBG, sex hormone binding globulin; CHD, coronary heart disease.

Observational studies in humans have shown that equol producers as compared to non-producers had significantly lower levels of atherosclerosis. Our cross-sectional study in 272 Japanese men aged 40–49 years found that equol producers had 9-fold lower odds of coronary artery calcification (CAC) presence compared to non-producers but serum ISFs were not significantly associated with CAC [220]. The Guangzhou Nutrition and Health Study of 2,572 middle-aged and older adults found that the serum level of equol was prospectively associated with reduced progression of carotid intima-media thickness over 8.8 years [226].

The first and the only observational study that ever explored the association between the equol level and CHD incidence was a nested case-control study within a cohort in China (377 cases and 753 controls). Total urinary ISFs or their metabolites other than equol were not associated with CHD in either women or men. However, urinary equol excretion showed an inverse association with CHD in women. The adjusted hazard ratio (95% confidence intervals) for CHD across increasing quartiles of equol levels were 1.00 (reference), 0.61 (0.32, 1.15), 0.51 (0.26, 0.98) and 0.46 (0.24, 0.89) (P = 0.02) [152].

7.4 Discussion

Evidence in this present systematic review suggests that equol has anti-atherogenic properties which are more potent than ISFs. Previous studies investigating the association of

dietary intake of ISFs with CHD have observed controversial results. Our review suggests that potential protective effects of ISFs on CHD may require the intestinal conversion of daidzein to equol. This systematic review demonstrates that the potential cardioprotective effects of equol as potentially through its anti-inflammatory, vasodilatory, antioxidative properties as well as its association with improvement in endothelial functions and arterial stiffness.

In general, cell culture and preclinical studies show that equol is more efficacious than ISFs. Some effects, such as reducing the secretion of inflammatory biomarkers in macrophage [115], reducing NADPH-induced superoxide levels [31], stimulating endothelial cell growth [138], and improving vasodilation [31] were observed with equol treatment only and not daidzein or genistein. Moreover, the disruption of the intestinal microbial conversion of daidzein to equol abolished daidzein's protective effect against aneurysm formation [122]. In addition, observational studies showed that equol rather than ISFs was significantly and inversely associated with CHD incidence [226] as well as the progression of atherosclerosis [220]. In RCTs of ISFs in humans, the cardioprotective effects of ISFs were either more pronounced in equol producers than non-producers [128] or were only present in equol producers [86, 123]. These observations strongly support the equol hypothesis that equol rather than ISFs are cardioprotective.

Observational studies showed that dietary intake of antioxidants is significantly and inversely associated with the risk of CVD whereas RCTs of vitamins E and other anti-oxidants reported little or no observed risk reduction among participants of a variety of characteristics [78-84]. One explanation for this discrepancy is that synthetic versions of the antioxidants administered in these trials may not completely mimic the natural forms of the antioxidants [319]. Equol is available as dietary supplement and can be administered as a natural form because it is developed by fermentation of soy germ with Lactococcus [41].

A large number of studies in this systematic review focused on an estro-gen-deprivation situation, e.g., cell models simulating a postmenopausal cellular environment and human studies among postmenopausal women. The effects of estrogen therapy on CVD are controversial. Generally, when blood vessels are healthy, estrogen appears to protect them from the development of atherosclerotic plaques [320]. However, if atherosclerosis is well-established, estrogen does not appear to be beneficial but rather increases the risk of clinical events [321, 322]. Because equol has a greater affinity for ERβ than for ERα, equol may not have adverse effects associated with estrogen supplementation, which is considered to target ERα [323].

Overall, we thoroughly reviewed the potential protective effects of equol on CHD from a comprehensive collection of studies including transcriptomics, whole-genome gene expression, in vitro, animal, and observational and clinical studies. Based on this systematic review, clinical studies of equol on atherosclerosis and CHD are warranted.

8.0 Discussion

8.1 Public Health Significance

Finding the underlying pathology of the variations in rates of diseases among populations with a substantial lifestyle difference is a major component of epidemiology. Studies have shown that Japan had the lowest rate of CHD mortality among developed countries [324]. The low CHD mortality in Japan was unlikely to be due to genetic susceptibility because migrant studies of Japanese to the U.S. documented a dramatic rise in CHD rates [243]. In addition, we previously found that Japanese men in Japan had a significantly lower prevalence of atherosclerosis compared to White and Japanese Americans, despite similar or greater exposure to traditional cardiovascular risk factors (hypertension, diabetes, hypercholesterolemia, and especially smoking) in Japanese in Japan [233]. This discrepancy may suggest unique cardioprotective factors in the Japanese population. Meanwhile, the Japanese diet is characterized by a very high dietary intake of ISFs [4]. Besides, the Japanese population, in general, has a much higher ability to produce equol through the gut microbiome after consuming ISFs than Americans do [325]. Up to now, there has been no study exploring if the equol-producing status is directly associated with atherosclerosis. The lack of such information is a limiting factor in identifying a unique lifestyle that contributes to the risk of CHD. In our present studies, we filled this gap by confirming that the stronger equol producing capacity in the Japanese population grants them a lower atherosclerotic burden than Western populations. We justified this by first conducting a literature review that proved that equol has a strong property to alleviate all of the risk factors involved in atherosclerosis's pathogenesis, based on cell, animal, and human studies. Furthermore, we conducted two observational studies in

Japanese, which further proved our hypothesis that equol producing status is the key to the anti-atherosclerotic of ISFs. Consequently, the difference in the equol-producing status between Japanese and Americans largely explained their difference in the rates of CHD.

The present work also supports the contribution of biometabolites to the health benefits of polyphenols, thus offering a better perspective in understanding the role of ISFs in cardiovascular health. Although literature abounds with studies reporting the *in vitro* and *in vivo* bioactivity of ISFs [8, 9, 274, 275], very little is known about the mechanisms underlying their health effects as they have a low oral bioavailability. Large and prospective cohort studies have shown that dietary intake of ISFs was not associated with CVD mortality [277], and ISFs have minimal effects on traditional CVD risk factors [24, 178, 278, 279]. A hypothesis is that the ability of humans to metabolize daidzein to equol may contribute to the cardioprotective effects of ISFs [38, 153, 280]. This hypothesis has been suggested by one prospective cohort study in China [152]. Yet, no observational studies have ever been conducted in Japan, and no literature review has systematically summarized the mechanisms through which equol is improving the biomarkers of atherosclerosis. This dissertation is the first comprehensive analysis that proves the cardioprotective effect of equol, and this effect is more substantial than its precursor, ISF. Recently, our group also showed that equol-producing status was associated with a lower burden of cerebral small vessel disease but ISFs did not have such effect [240]. Together with this dissertation, these studies set the stage for future investigation of the effect of equol on other health outcomes.

8.2 Strength and limitation

Our studies have several strengths. First, our two studies were community-based studies that utilized a random sampling method. Second, daidzein was detected in all the participants. Therefore, misclassification of equol-producing status was unlikely. Third, we collected both serum and urinary equol and compared these two biomarkers, thus greatly increasing the credibility of our work. Fourth, we found considerable differences in AC scores between equol-producers and non-producers, suggesting a substantial improvement in atherosclerosis. Motivated by these findings, additional equol-focused RCTs should be conducted to justify the effect of equol in all populations.

The following limitations should be noted. First, due to the cross-sectional study designs, a causal association between equol-producing status and cardiovascular risk factors cannot be established. Nevertheless, equol producing status has been found to be stable over the years, and the presence of calcification is unlikely to affect equol producing status. Second, the generalizability of this dissertation is limited because all participants were middle-aged and older men. Given that sex and age may modify the association between equol and atherosclerosis, further studies in women and young adults are needed. Third, our findings may not be attributed purely to equol, but phenotypes related to the equol-producing status, as well.

8.3 Future works

We first propose an RCT to further confirm the effect of supplementation of equol on the Japanese population, then discuss the application in the American populations.

8.3.1 Study protocol of RCT

The findings of this dissertation work support the need for future research to examine the equol and CVD relationship. From our work, we first propose the importance of a future RCT to confirm the effect of supplementation of equol on Japanese population, then discuss the application in the American populations. Such a study could be envisioned as a single-center, randomized, placebo-controlled, double-blind, cross-over trial in the Japanese population. The methods would involve a stratified randomization of 30 equol-producers and 30 non-producers. 30 equol-producers would be randomly assigned to 3-month, 20 mg/day of equol supplementation group or the placebo group, and 30 equol-non-producers would be randomly assigned to treatment and placebo group. We aim to understand if original equol producing status would influence the benefits obtained from the intervention of equol. Usui et al. has suggested that 10 mg/day of equol over 3-month can successfully lower the progression of arterial stiffness and the level of LDL-C and HbA1c [41], so the 3-month intervention duration is chosen. However, we double the dose of equol because Usui *et al.* did not observe the effect of 10 mg/day of equol on blood pressure, fasting glucose, total cholesterol, HDL-C, triglyceride, CRP, adiponectin, and leptin, because a higher dose might reveal a more pronounced change in these biomarkers. Participants, investigators, study staff, and laboratory technicians are blinded to group assignment until the final analysis of the trial. Compliance is assessed by interview and counting returned empty supplement packs at the end of the intervention. Several CVD risk factors will be examined at the endpoint, including CRP, baPWV, MDA, VCAM, ICAM, LDL-C, HDL-C, total cholesterol, triglycerides, blood pressure, fasting blood glucose, and HbA1c. We do not plan to assess AC and CAC because these parameters is unlikely to change over three months.

We hypothesize that equol treatment will improve all CVD biomarkers over the intervention period; original equol-producers will not have a larger improvement of levels of CVD biomarkers compared to non-producers. The objective is to confirm that using equol supplementation as treatment will partially bridge the gap led by the different equol producing status. In other words, the subject could benefit from equol consumption regardless of their equol producing status.

To determine the equol-producing status, participants will consume soy food products containing 50 mg ISFs twice in one day, and urine samples are collected the following morning. Participants with detectable equol in their urine will be classified as equol-producers. T-test will be used to compare the means between the two groups at baseline and after, or differences between the baseline and after values. Changes from the baseline to post-treatment will be evaluated by a paired t-test.

8.3.2 Public health implication in the US

The advocation of eating soy may be meaningful in the U.S. Because even though the rate of equol-producers is very low, there are still some people that are equol-producers in America. In addition, the rate of equol-producers is higher in vegetarians than the general people [326]. A diet high in fiber and low in animal fat can change the gut metabolite and consequently enhance the capacity of producing equol. Some population groups, such as vegetarians, solely rely on soy products as the main protein source. Therefore, vegetarians can benefit from soy in at least the following two ways. First, soy products offer the high-quality protein that is necessary for their body [327], which is widely recognized. Second, the equol-producers among the vegetarians will be directly benefited from the additional nutritional benefits of ISFs. Non-producers might

gradually develop their capacity of producing equol and thus could subsequently benefit from ISFs.

Another future study to propose is a randomized, placebo-controlled, double-blind, cross-over trial in the American populations to see if the equol supplementation also has an effect on CVD risk factors in American populations. 15 whites and 15 blacks would be randomly assigned to 3-month, 20 mg/day of equol supplementation group or the placebo group. CRP, baPWV, MDA, VCAM, ICAM, LDL-C, HDL-C, total cholesterol, triglycerides, blood pressure, fasting blood glucose, and HbA1c are measured at baseline and end point. Mean outcomes between the two groups would be compared at baseline and after, or differences between the baseline and after values. Changes from the baseline to post-treatment would also be measured. We hypothesize that equol treatment will improve some or all CVD biomarkers over the intervention period. A secondary goal is to investigate whether the rate of equol-producer is changed after equol treatment. In the trial, to determine the equol-producing status, participants will consume soy food products containing 50 mg ISFs twice in one day, and urine samples are collected the following morning. Participants with detectable equol in their urine will be classified as equol-producers.

Findings from our proposed trial can inform how broader population groups, regardless of their original equol-producing capacities, could benefit from dietary equol supplements to improve their cardiovascular health. Additionally, if equol-producing status can be proved to be modifiable through dietary invention or fecal microbiota transplantation, the Western population might greatly benefit from the cardioprotective effect from soy product, similar to the Asia population.

Bibliography

1. Setchell, K.D., *Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones.* The American journal of clinical nutrition, 1998. 68(6): p. 1333S-1346S.

2. Nilsson, S., K.F. Koehler, and J.-Å. Gustafsson, *Development of subtype-selective oestrogen receptor-based therapeutics.* Nature reviews Drug discovery, 2011. 10(10): p. 778-792.

3. Setchell, K.D. and C. Clerici, *Equol: history, chemistry, and formation.* The Journal of nutrition, 2010. 140(7): p. 1355S-1362S.

4. Klein, M.A., et al., *Guidance from an NIH workshop on designing, implementing, and reporting clinical studies of soy interventions.* The Journal of nutrition, 2010. 140(6): p. 1192S-1204S.

5. Anderson, J.W., B.M. Johnstone, and M.E. Cook-Newell, *Meta-analysis of the effects of soy protein intake on serum lipids.* N Engl J Med, 1995. 333(5): p. 276-82.

6. Sacks, F.M., et al., *Soy protein, isoflavones, and cardiovascular health: an American Heart Association Science Advisory for professionals from the Nutrition Committee.* Circulation, 2006. 113(7): p. 1034-44.

7. Mehta, J.L., T.G. Saldeen, and K. Rand, *Interactive role of infection, inflammation and traditional risk factors in atherosclerosis and coronary artery disease.* Journal of the American College of Cardiology, 1998. 31(6): p. 1217-1225.

8. Clarkson, T.B., M.S. Anthony, and T.M. Morgan, *Inhibition of postmenopausal atherosclerosis progression: a comparison of the effects of conjugated equine estrogens and soy phytoestrogens.* The Journal of Clinical Endocrinology & Metabolism, 2001. 86(1): p. 41-47.

9. Anthony, M.S., et al., *Soy protein versus soy phytoestrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys.* Arterioscler Thromb Vasc Biol, 1997. 17(11): p. 2524-31.

10. Information., N.C.f.B., *"PubChem Compound Summary for CID 5280961, Genistein"* PubChem, 2021. https://pubchem.ncbi.nlm.nih.gov/compound/Genistein.

11. Information, N.C.f.B., *PubChem Compound Summary for CID 5281708, Daidzein.* PubChem, 2021. https://pubchem.ncbi.nlm.nih.gov/compound/Daidzein.

12. Information, N.C.f.B., *PubChem Compound Summary for CID 91469, Equol.* PubChem, 2021. https://pubchem.ncbi.nlm.nih.gov/compound/Equol.

13. Ruiz-Larrea, M.B., et al., *Antioxidant activity of phytoestrogenic isoflavones.* Free Radic Res, 1997. 26(1): p. 63-70.

14. Ibrahim, W.H., et al., *Isoflavone-rich soy isolate reduces lipid peroxidation in mouse liver.* International journal for vitamin and nutrition research, 2008. 78(45): p. 217-222.

15. Liu, J., S.K. Chang, and D. Wiesenborn, *Antioxidant properties of soybean isoflavone extract and tofu in vitro and in vivo.* Journal of Agricultural and Food Chemistry, 2005. 53(6): p. 2333-2340.

16. Barbosa, A.C., F.M. Lajolo, and M.I. Genovese, *Effect of free or protein-associated soy isoflavones on the antioxidant status in rats.* Journal of the Science of Food and Agriculture, 2011. 91(4): p. 721-731.

17. Jenkins, D.J., et al., *Effect of soy-based breakfast cereal on blood lipids and oxidized low-density lipoprotein.* Metabolism-Clinical and Experimental, 2000. 49(11): p. 1496-1500.

18. Wiseman, H., et al., *Isoflavone phytoestrogens consumed in soy decrease F2-isoprostane concentrations and increase resistance of low-density lipoprotein to oxidation in humans.* The American Journal of Clinical Nutrition, 2000. 72(2): p. 395-400.

19. Jenkins, D.J., et al., *Effects of high- and low-isoflavone soyfoods on blood lipids, oxidized LDL, homocysteine, and blood pressure in hyperlipidemic men and women.* Am J Clin Nutr, 2002. 76(2): p. 365-72.

20. Beavers, K.M., S.S. Jonnalagadda, and M.J. Messina, *Soy consumption, adhesion molecules, and pro-inflammatory cytokines: a brief review of the literature.* Nutr Rev, 2009. 67(4): p. 213-21.

21. Nicastro, H.L., et al., *Associations between urinary soy isoflavonoids and two inflammatory markers in adults in the United States in 2005–2008.* Cancer Causes & Control, 2013. 24(6): p. 1185-1196.

22. Wu, S.H., et al., *Soy food intake and circulating levels of inflammatory markers in Chinese women.* Journal of the Academy of Nutrition and Dietetics, 2012. 112(7): p. 996-1004.

23. Peluso, I., A. Raguzzini, and M. Serafini, *Effect of flavonoids on circulating levels of TNF-α and IL-6 in humans: A systematic review and meta-analysis.* Molecular Nutrition & Food Research, 2013. 57(5): p. 784-801.

24. Khodarahmi, M., et al., *A systematic review and meta-analysis of the effects of soy on serum hs-CRP.* Clinical nutrition, 2019. 38(3): p. 996-1011.

25. Chin‐Dusting, J.P., et al., *The vascular activity of some isoflavone metabolites: implications for a cardioprotective role.* British journal of pharmacology, 2001. 133(4): p. 595-605.

26. Jiang, C., et al., *Acute effect of 17 beta-estradiol on rabbit coronary artery contractile responses to endothelin-1.* American Journal of Physiology-Heart and Circulatory Physiology, 1992. 263(1): p. H271-H275.

27. Nevala, R., R. Korpela, and H. Vapaatalo, *Plant derived estrogens relax rat mesenteric artery in vitro.* Life Sci, 1998. 63(6): p. PL 95-100.

28. Kostelac, D., G. Rechkemmer, and K. Briviba, *Phytoestrogens modulate binding response of estrogen receptors α and β to the estrogen response element.* Journal of agricultural and food chemistry, 2003. 51(26): p. 7632-7635.

29. Axelson, M., et al., *The identification of the weak oestrogen equol [7-hydroxy-3-(4′-hydroxyphenyl) chroman] in human urine.* Biochemical Journal, 1982. 201(2): p. 353-357.

30. Setchell, K.D., et al., *The pharmacokinetics of S-(-) equol administered as SE5-OH tablets to healthy postmenopausal women.* The Journal of nutrition, 2009. 139(11): p. 2037-2043.

31. Jackman, K.A., et al., *Vasorelaxant and antioxidant activity of the isoflavone metabolite equol in carotid and cerebral arteries.* Brain research, 2007. 1141: p. 99-107.

32. Joy, S., et al., *The isoflavone Equol mediates rapid vascular relaxation: Ca2+-independent activation of endothelial nitric-oxide synthase/Hsp90 involving ERK1/2 and Akt phosphorylation in human endothelial cells.* J Biol Chem, 2006. 281(37): p. 27335-45.

33. Setchell, K.D., N.M. Brown, and E. Lydeking-Olsen, *The clinical importance of the metabolite equol—a clue to the effectiveness of soy and its isoflavones.* The Journal of nutrition, 2002. 132(12): p. 3577-3584.

34. Hwang, J., et al., *The phytoestrogen equol increases nitric oxide availability by inhibiting superoxide production: an antioxidant mechanism for cell-mediated LDL modification.* Free Radic Biol Med, 2003. 34(10): p. 1271-82.

35. Cheng, C., et al., *The soybean isoflavonoid equol blocks ritonavir-induced endothelial dysfunction in porcine pulmonary arteries and human pulmonary artery endothelial cells.* J Nutr, 2010. 140(1): p. 12-7.

36. Mitchell, J.H., et al., *Antioxidant efficacy of phytoestrogens in chemical and biological model systems.* Archives of biochemistry and biophysics, 1998. 360(1): p. 142-148.

37. Muthyala, R.S., et al., *Equol, a natural estrogenic metabolite from soy isoflavones: convenient preparation and resolution of R- and S-equols and their differing binding and*

biological activity through estrogen receptors alpha and beta. Bioorg Med Chem, 2004. 12(6): p. 1559-67.

38. Setchell, K.D. and C. Clerici, *Equol: pharmacokinetics and biological actions.* The Journal of nutrition, 2010. 140(7): p. 1363S-1368S.

39. Sekikawa, A., et al., *Effect of S-equol and soy isoflavones on heart and brain.* Current Cardiology Reviews, 2019. 15(2): p. 114-135.

40. Nagata, C., et al., *Dietary and lifestyle correlates of urinary excretion status of equol in Japanese women.* Nutr Cancer, 2008. 60(1): p. 49-54.

41. Usui, T., et al., *Effects of natural S‐equol supplements on overweight or obesity and metabolic syndrome in the Japanese, based on sex and equol status.* Clinical endocrinology, 2013. 78(3): p. 365-372.

42. Yuan, J.-P., J.-H. Wang, and X. Liu, *Metabolism of dietary soy isoflavones to equol by human intestinal microflora–implications for health.* Molecular nutrition & food research, 2007. 51(7): p. 765-781.

43. Hall, M.C., B. O'Brien, and T. McCormack, *Equol producer status, salivary estradiol profile and urinary excretion of isoflavones in Irish Caucasian women, following ingestion of soymilk.* Steroids, 2007. 72(1): p. 64-70.

44. Guo, K., et al., *Daidzein-metabolising phenotypes in relation to serum lipids and uric acid in adults in Guangzhou, China.* Br J Nutr, 2010. 104(1): p. 118-24.

45. Liu, B., et al., *Prevalence of the equol-producer phenotype and its relationship with dietary isoflavone and serum lipids in healthy Chinese adults.* J Epidemiol, 2010. 20(5): p. 377-84.

46. Tanaka, M., et al., *Isoflavone supplements stimulated the production of serum equol and decreased the serum dihydrotestosterone levels in healthy male volunteers.* Prostate Cancer Prostatic Dis, 2009. 12(3): p. 247-52.

47. Wiseman, H., et al., *Influence of 10 wk of soy consumption on plasma concentrations and excretion of isoflavonoids and on gut microflora metabolism in healthy adults.* Am J Clin Nutr, 2004. 80(3): p. 692-9.

48. Atkinson, C., C.L. Frankenfeld, and J.W. Lampe, *Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health.* Exp Biol Med (Maywood), 2005. 230(3): p. 155-70.

49. Setchell, K.D. and S.J. Cole, *Method of defining equol-producer status and its frequency among vegetarians.* The Journal of nutrition, 2006. 136(8): p. 2188-2193.

50. Peeters, P.H., et al., *Variations in plasma phytoestrogen concentrations in European adults.* J Nutr, 2007. 137(5): p. 1294-300.

51. Rowland, I.R., et al., *Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora.* Nutr Cancer, 2000. 36(1): p. 27-32.

52. Lampe, J.W., et al., *Urinary equol excretion with a soy challenge: influence of habitual diet.* Proc Soc Exp Biol Med, 1998. 217(3): p. 335-9.

53. Frankenfeld, C.L., et al., *Familial correlations, segregation analysis, and nongenetic correlates of soy isoflavone-metabolizing phenotypes.* Exp Biol Med (Maywood), 2004. 229(9): p. 902-13.

54. Bolca, S., et al., *Microbial and dietary factors are associated with the equol producer phenotype in healthy postmenopausal women.* J Nutr, 2007. 137(10): p. 2242-6.

55. Teas, J., et al., *Dietary seaweed modifies estrogen and phytoestrogen metabolism in healthy postmenopausal women.* J Nutr, 2009. 139(5): p. 939-44.

56. Song, K.B., et al., *Prevalence of daidzein-metabolizing phenotypes differs between Caucasian and Korean American women and girls.* J Nutr, 2006. 136(5): p. 1347-51.

57. Tseng, M., et al., *Equol-producing status, isoflavone intake, and breast density in a sample of U.S. Chinese women.* Cancer Epidemiol Biomarkers Prev, 2013. 22(11): p. 1975-83.

58. Fujimoto, K., et al., *Age-stratified serum levels of isoflavones and proportion of equol producers in Japanese and Korean healthy men.* Prostate Cancer Prostatic Dis, 2008. 11(3): p. 252-7.

59. Cai, Y., et al., *Soya isoflavone consumption in relation to carotid intima-media thickness in Chinese equol excretors aged 40-65 years.* Br J Nutr, 2012. 108(9): p. 1698-704.

60. Morton, M.S., et al., *Phytoestrogen concentrations in serum from Japanese men and women over forty years of age.* J Nutr, 2002. 132(10): p. 3168-71.

61. Arai, Y., et al., *Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake.* Journal of Epidemiology, 2000. 10(2): p. 127-135.

62. Violi, F., et al., *Atherothrombosis and oxidative stress: mechanisms and management in elderly.* Antioxidants & redox signaling, 2017. 27(14): p. 1083-1124.

63. Pignatelli, P., et al., *Oxidative stress and cardiovascular disease: new insights.* Kardiol Pol, 2018. 76(4): p. 713-722.

64. Bonomini, F., et al., *Atherosclerosis and oxidative stress.* Histol Histopathol, 2008. 23(3): p. 381-90.

65. Daugherty, A., et al., *Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions.* The Journal of clinical investigation, 1994. 94(1): p. 437-444.

66. Vassalle, C., et al., *Oxidative stress and its association with coronary artery disease and different atherogenic risk factors.* Journal of internal medicine, 2004. 256(4): p. 308-315.

67. Arca, M., et al., *C242T polymorphism of NADPH oxidase p22phox and recurrence of cardiovascular events in coronary artery disease.* Arteriosclerosis, thrombosis, and vascular biology, 2008. 28(4): p. 752-757.

68. Azumi, H., et al., *Expression of NADH/NADPH oxidase p22phox in human coronary arteries.* Circulation, 1999. 100(14): p. 1494-1498.

69. Rimm, E.B., et al., *Vitamin E consumption and the risk of coronary heart disease in men.* New England Journal of Medicine, 1993. 328(20): p. 1450-1456.

70. Shardell, M.D., et al., *Low-serum carotenoid concentrations and carotenoid interactions predict mortality in US adults: the Third National Health and Nutrition Examination Survey.* Nutrition research, 2011. 31(3): p. 178-189.

71. Kutter, D., et al., *Consequences of total and subtotal myeloperoxidase deficiency: risk or benefit?* Acta haematologica, 2000. 104(1): p. 10-15.

72. Dharma, R., et al., *Effect of soy isoflavone supplementation on vascular endothelial function and oxidative stress in postmenopausal women: a community randomized controlled trial.* Asia Pacific journal of clinical nutrition, 2013. 22(3): p. 357.

73. Vega-López, S., et al., *Plasma antioxidant capacity in response to diets high in soy or animal protein with or without isoflavones.* The American journal of clinical nutrition, 2005. 81(1): p. 43-49.

74. Mittal, R., et al., *Antioxidant effect of isoflavones: a randomized, double-blind, placebo controlled study in oophorectomized women.* International Journal of Applied and Basic Medical Research, 2014. 4(1): p. 28.

75. Sen, C., et al., *Soy foods and urinary isoprostanes: results from a randomized study in premenopausal women.* Food & function, 2012. 3(5): p. 517-521.

76. Libby, P., *Inflammation and cardiovascular disease mechanisms.* The American journal of clinical nutrition, 2006. 83(2): p. 456S-460S.

77. Sever, P.S., et al., *Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial—Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial.* The Lancet, 2003. 361(9364): p. 1149-1158.

78. Lee, I.-M., et al., *Vitamin E in the primary prevention of cardiovascular disease and cancer: the Women's Health Study: a randomized controlled trial.* Jama, 2005. 294(1): p. 56-65.

79. Cook, N.R., et al., *A randomized factorial trial of vitamins C and E and beta carotene in the secondary prevention of cardiovascular events in women: results from the Women's Antioxidant Cardiovascular Study.* Archives of internal medicine, 2007. 167(15): p. 1610-1618.

80. Albert, C.M., et al., *Effect of folic acid and B vitamins on risk of cardiovascular events and total mortality among women at high risk for cardiovascular disease: a randomized trial.* Jama, 2008. 299(17): p. 2027-2036.

81. Ebbing, M., et al., *Mortality and cardiovascular events in patients treated with homocysteine-lowering B vitamins after coronary angiography: a randomized controlled trial.* Jama, 2008. 300(7): p. 795-804.

82. Galan, P., et al., *Effects of B vitamins and omega 3 fatty acids on cardiovascular diseases: a randomised placebo controlled trial.* Bmj, 2010. 341.

83. Sesso, H.D., et al., *Multivitamins in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial.* Jama, 2012. 308(17): p. 1751-1760.

84. Myung, S.-K., et al., *Efficacy of vitamin and antioxidant supplements in prevention of cardiovascular disease: systematic review and meta-analysis of randomised controlled trials.* Bmj, 2013. 346.

85. Yari, Z., et al., *Effects of soy isoflavones on serum systemic and vascular inflammation markers and oxidative stress in peritoneal dialysis patients: A randomized controlled trial.* Phytotherapy Research, 2020. 34(11): p. 3011-3018.

86. Hidayat, A., *Effect of soy isoflavone supplementation on endothelial dysfunction and oxidative stress in equol-producing postmenopausal women.* Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders), 2015. 15(1): p. 71-79.

87. Blankenberg, S., S. Barbaux, and L. Tiret, *Adhesion molecules and atherosclerosis.* Atherosclerosis, 2003. 170(2): p. 191-203.

88. Beavers, D., et al., *Exposure to isoflavone-containing soy products and endothelial function: a Bayesian meta-analysis of randomized controlled trials.* Nutrition, Metabolism and Cardiovascular Diseases, 2012. 22(3): p. 182-191.

89. Corretti, M.C., et al., *Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force.* Journal of the American College of Cardiology, 2002. 39(2): p. 257-265.

90. Li, S.-H., et al., *Effect of oral isoflavone supplementation on vascular endothelial function in postmenopausal women: a meta-analysis of randomized placebo-controlled trials–.* The American journal of clinical nutrition, 2010. 91(2): p. 480-486.

91. Toma, C., et al., *Effects of tyrosine kinase inhibitors on the contractility of rat mesenteric resistance arteries.* British journal of pharmacology, 1995. 114(6): p. 1266-1272.

92. Zhang, H.-p., et al., *Genistein ameliorated endothelial nitric oxidase synthase uncoupling by stimulating sirtuin-1 pathway in ox-LDL-injured HUVECs.* Environmental toxicology and pharmacology, 2016. 42: p. 118-124.

93. Choi, E., *Evaluation of Equol Function on Anti‐or Prooxidant Status in vivo.* Journal of food science, 2009. 74(2): p. H65-H71.

94. Gou, Z., et al., *Equol inhibits LPS-induced oxidative stress and enhances the immune response in chicken HD11 macrophages.* Cellular Physiology and Biochemistry, 2015. 36(2): p. 611-621.

95. Pažoureková, S., et al., *Equol effectively inhibits toxic activity of human neutrophils without influencing their viability.* Pharmacology, 2016. 97(3-4): p. 138-145.

96. PEREBOOM, D., et al., *Antioxidant intracellular activity of genistein and equol.* Journal of medicinal food, 1999. 2(3-4): p. 253-256.

97. Lin, X., et al., *Effects of equol on H2 O2-induced oxidative stress in primary chicken intestinal epithelial cells.* Poultry Science, 2016. 95(6): p. 1380-1386.

98. Choi, E.J., *The prooxidant, rather than antioxidant, acts of daidzein in vivo and in vitro: Daidzein suppresses glutathione metabolism.* European journal of pharmacology, 2006. 542(1-3): p. 162-169.

99. Richardson, T.E. and J.W. Simpkins, *R-and S-Equol have equivalent cytoprotective effects in Friedreich's Ataxia.* BMC Pharmacology and Toxicology, 2012. 13(1): p. 1-6.

100. Hwang, J., et al., *Synergistic inhibition of LDL oxidation by phytoestrogens and ascorbic acid.* Free Radical Biology and Medicine, 2000. 29(1): p. 79-89.

101. Choi, E.J. and G.-H. Kim, *Anticancer mechanism of equol in 7, 12-dimethylbenz (a) anthracene-treated animals.* International journal of oncology, 2011. 39(3): p. 747-754.

102. Wei, X.-J., et al., *Antioxidant effect of a phytoestrogen equol on cultured muscle cells of embryonic broilers.* In Vitro Cellular & Developmental Biology-Animal, 2011. 47(10): p. 735-741.

103. Kamiyama, M., et al., *Effects of equol on oxidized low-density lipoprotein-induced apoptosis in endothelial cells.* Journal of atherosclerosis and thrombosis, 2009. 16(3): p. 239-249.

104. Sierens, J., et al., *Effect of phytoestrogen and antioxidant supplementation on oxidative DNA damage assessed using the comet assay.* Mutation Research/DNA Repair, 2001. 485(2): p. 169-176.

105. Rüfer, C.E. and S.E. Kulling, *Antioxidant activity of isoflavones and their major metabolites using different in vitro assays.* Journal of Agricultural and Food Chemistry, 2006. 54(8): p. 2926-2931.

106. Sierens, J., et al., *In vitro isoflavone supplementation reduces hydrogen peroxide‐induced DNA damage in sperm.* Teratogenesis, carcinogenesis, and mutagenesis, 2002. 22(3): p. 227-234.

107. Arora, A., M.G. Nair, and G.M. Strasburg, *Antioxidant activities of isoflavones and their biological metabolites in a liposomal system.* Archives of biochemistry and biophysics, 1998. 356(2): p. 133-141.

108. Turner, R., et al., *Effect of circulating forms of soy isoflavones on the oxidation of low density lipoprotein.* Free Radical Research, 2004. 38(2): p. 209-216.

109. Liu, T.-H. and T.-Y. Tsai, *Effects of equol on deoxycorticosterone acetate salt-induced hypertension and associated vascular dementia in rats.* Food & function, 2016. 7(8): p. 3444-3457.

110. Vedavanam, K., et al., *Antioxidant action and potential antidiabetic properties of an isoflavonoid‐containing soyabean phytochemical extract (SPE).* Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 1999. 13(7): p. 601-608.

111. Ma, Y., J.C. Sullivan, and D.A. Schreihofer, *Dietary genistein and equol (4′, 7 isoflavandiol) reduce oxidative stress and protect rats against focal cerebral ischemia.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2010. 299(3): p. R871-R877.

112. Horiuchi, H., et al., *S-equol enantioselectively activates cAMP-protein kinase A signaling and reduces alloxan-induced cell death in INS-1 pancreatic β-cells.* Journal of nutritional science and vitaminology, 2014. 60(4): p. 291-296.

113. Widyarini, S., et al., *Photoimmune protective effect of the phytoestrogenic isoflavonoid equol is partially due to its antioxidant activities*. Photochemical & Photobiological Sciences, 2012. 11(7): p. 1186-1192.

114. Nhan, S., et al., *Effect of a soymilk supplement containing isoflavones on urinary F2 isoprostane levels in premenopausal women.* Nutrition and cancer, 2005. 53(1): p. 73-81.

115. Blay, M., et al., *Isoflavone effect on gene expression profile and biomarkers of inflammation.* Journal of pharmaceutical and biomedical analysis, 2010. 51(2): p. 382-390.

116. Johnson, S.L., et al., *Polyphenol microbial metabolites exhibit gut and blood–brain barrier permeability and protect murine microglia against LPS-induced inflammation.* Metabolites, 2019. 9(4): p. 78.

117. Obiorah, I.E., P. Fan, and V.C. Jordan, *Breast cancer cell apoptosis with phytoestrogens is dependent on an estrogen-deprived state.* Cancer prevention research, 2014. 7(9): p. 939-949.

118. Nagarajan, S., et al., *Dietary soy protein isolate ameliorates atherosclerotic lesions in apolipoprotein E-deficient mice potentially by inhibiting monocyte chemoattractant protein-1 expression.* The Journal of nutrition, 2008. 138(2): p. 332-337.

119. Subedi, L., et al., *Equol, a dietary daidzein gut metabolite attenuates microglial activation and potentiates neuroprotection in vitro.* Nutrients, 2017. 9(3): p. 207.

120. Moriyama, M., et al., *-Equol, a Major Isoflavone from Soybean, Inhibits Nitric Oxide Production in Lipopolysaccharide-Stimulated Rat Astrocytes Partially via the GPR30-Mediated Pathway.* International journal of inflammation, 2018. 2018.

121. Lin, I.-C., et al., *Equol suppresses inflammatory response and bone erosion due to rheumatoid arthritis in mice.* The Journal of nutritional biochemistry, 2016. 32: p. 101-106.

122. Yokosuka, K., et al., *Roles of Phytoestrogen in the Pathophysiology of Intracranial Aneurysm.* Stroke, 2020: p. STROKEAHA. 120.032042.

123. van der Velpen, V., et al., *Isoflavone supplement composition and equol producer status affect gene expression in adipose tissue: a double-blind, randomized, placebo-controlled crossover trial in postmenopausal women.* Am J Clin Nutr, 2014. 100(5): p. 1269-77.

124. Törmälä, R., et al., *Impact of soy supplementation on sex steroids and vascular inflammation markers in postmenopausal women using tibolone: role of equol production capability.* Climacteric, 2008. 11(5): p. 409-415.

125. Reverri, E.J., et al., *Soy provides modest benefits on endothelial function without affecting inflammatory biomarkers in adults at cardiometabolic risk.* Molecular nutrition & food research, 2015. 59(2): p. 323-333.

126. Greany, K., et al., *Consumption of isoflavone-rich soy protein does not alter homocysteine or markers of inflammation in postmenopausal women.* European journal of clinical nutrition, 2008. 62(12): p. 1419-1425.

127. Mangano, K.M., et al., *Soy proteins and isoflavones reduce interleukin-6 but not serum lipids in older women: a randomized controlled trial.* Nutrition research, 2013. 33(12): p. 1026-1033.

128. Törmälä, R., et al., *Equol production capability is associated with favorable vascular function in postmenopausal women using tibolone; no effect with soy supplementation.* Atherosclerosis, 2008. 198(1): p. 174-178.

129. Delfino, R.J., et al., *Circulating biomarkers of inflammation, antioxidant activity, and platelet activation are associated with primary combustion aerosols in subjects with coronary artery disease.* Environmental health perspectives, 2008. 116(7): p. 898-906.

130. van der Velpen, V., et al., *Estrogen receptor–mediated effects of isoflavone supplementation were not observed in whole-genome gene expression profiles of peripheral blood mononuclear cells in postmenopausal, equol-producing women.* The Journal of nutrition, 2013. 143(6): p. 774-780.

131. Zhang, T., et al., *Estrogen receptor and PI3K/Akt signaling pathway involvement in S-(-)equol-induced activation of Nrf2/ARE in endothelial cells.* PLoS One, 2013. 8(11): p. e79075.

132. Yu, W., et al., *Equol increases cerebral blood flow in rats via activation of large-conductance Ca2+-activated K+ channels in vascular smooth muscle cells.* Pharmacological research, 2016. 107: p. 186-194.

133. Joy, S., et al., *The isoflavone equol mediates rapid vascular relaxation: Ca2+-independent activation of endothelial nitric-oxide synthase/Hsp90 involving ERK1/2 and Akt phosphorylation in human endothelial cell.* Journal of Biological Chemistry, 2006. 281(37): p. 27335-27345.

134. Priestley, J.R., et al., *The NRF2 knockout rat: a new animal model to study endothelial dysfunction, oxidant stress, and microvascular rarefaction.* American Journal of Physiology-Heart and Circulatory Physiology, 2016. 310(4): p. H478-H487.

135. Rowlands, D.J., et al., *Equol-stimulated mitochondrial reactive oxygen species activate endothelial nitric oxide synthase and redox signaling in endothelial cells: roles for F-actin and GPR30.* Hypertension, 2011. 57(4): p. 833-840.

136. Ohkura, Y., et al., *S-equol partially restored endothelial nitric oxide production in isoflavone-deficient ovariectomized rats.* Journal of Cardiovascular Pharmacology, 2015. 65(5): p. 500-507.

137. Chung, J.-E., et al., *Antioxidant effects of equol on bovine aortic endothelial cells.* Biochemical and biophysical research communications, 2008. 375(3): p. 420-424.

138. Somjen, D., et al., *Effects of phytoestrogens on DNA synthesis and creatine kinase activity in vascular cells.* American journal of hypertension, 2001. 14(12): p. 1256-1262.

139. Zhang, T., et al., *Equol attenuates atherosclerosis in apolipoprotein E-deficient mice by inhibiting endoplasmic reticulum stress via activation of Nrf2 in endothelial cells.* PLoS One, 2016. 11(12): p. e0167020.

140. Kim, J.Y., M.Y. Lee, and H.M. Park, *The effect of eqoul, a metabolite of isoflavone, on endothelial cell-independent vasodilatation of human uterine artery in vitro*. Journal of bone metabolism, 2015. 22(2): p. 57-69.

141. Matsumoto, T., et al., *Effect of equol on vasocontractions in rat carotid arteries treated with high insulin.* Biological and Pharmaceutical Bulletin, 2019. 42(6): p. 1048-1053.

142. Kreijkamp-Kaspers, S., et al., *Randomized controlled trial of the effects of soy protein containing isoflavones on vascular function in postmenopausal women.* The American journal of clinical nutrition, 2005. 81(1): p. 189-195.

143. Clerici, C., et al., *Pasta naturally enriched with isoflavone aglycons from soy germ reduces serum lipids and improves markers of cardiovascular risk.* The Journal of nutrition, 2007. 137(10): p. 2270-2278.

144. Wang, J., et al., *Equol promotes rat osteoblast proliferation and differentiation through activating estrogen receptor.* Genet. Mol. Res, 2014. 13(3): p. 5055-5063.

145. Demer, L.L. and Y. Tintut, *Vascular calcification: pathobiology of a multifaceted disease.* Circulation, 2008. 117(22): p. 2938-2948.

146. Ho, S.-Y., et al., *Soy consumption and mortality in Hong Kong: proxy-reported case–control study of all older adult deaths in 1998.* Preventive medicine, 2006. 43(1): p. 20-26.

147. Yamori, Y., et al., *Different associations of blood pressure with 24-hour urinary sodium excretion among pre- and post-menopausal women. WHO Cardiovascular Diseases and Alimentary Comparison (WHO-CARDIAC) Study.* J Hypertens, 2001. 19(3 Pt 2): p. 535-8.

148. Yamori, Y., *Food factors for atherosclerosis prevention: Asian perspective derived from analyses of worldwide dietary biomarkers.* Exp Clin Cardiol, 2006. 11(2): p. 94-8.

149. Zhang, X., et al., *Soy food consumption is associated with lower risk of coronary heart disease in Chinese women.* The Journal of nutrition, 2003. 133(9): p. 2874-2878.

150. Kokubo, Y., et al., *Association of Dietary Intake of Soy, Beans, and Isoflavones With Risk of Cerebral and Myocardial Infarctions in Japanese Populations.* Circulation, 2007. 116: p. 553-2562.

151. van der Schouw, Y.T., et al., *Prospective study on usual dietary phytoestrogen intake and cardiovascular disease risk in Western women.* Circulation, 2005. 111(4): p. 465-471.

152. Zhang, X., et al., *Urinary isoflavonoids and risk of coronary heart disease.* International journal of epidemiology, 2012. 41(5): p. 1367-1375.

153. Birru, R.L., et al., *The impact of equol-producing status in modifying the effect of soya isoflavones on risk factors for CHD: a systematic review of randomised controlled trials.* Journal of nutritional science, 2016. 5.

154. Taku, K., et al., *Soy isoflavones lower serum total and LDL cholesterol in humans: a meta-analysis of 11 randomized controlled trials.* Am J Clin Nutr, 2007. 85(4): p. 1148-56.

155. Yang, B., et al., *Systematic review and meta-analysis of soy products consumption in patients with type 2 diabetes mellitus.* Asia Pac J Clin Nutr, 2011. 20(4): p. 593-602.

156. Tokede, O.A., et al., *Soya products and serum lipids: a meta-analysis of randomised controlled trials.* Br J Nutr, 2015. 114(6): p. 831-43.

157. Taku, K., et al., *Effects of extracted soy isoflavones alone on blood total and LDL cholesterol: Meta-analysis of randomized controlled trials.* Ther Clin Risk Manag, 2008. 4(5): p. 1097-103.

158. Balk, E., et al., *Effects of soy on health outcomes.* Evid Rep Technol Assess (Summ), 2005(126): p. 1-8.

159. Acharjee, S., et al., *Effect of soy nuts and equol status on blood pressure, lipids and inflammation in postmenopausal women stratified by metabolic syndrome status.* Metabolism, 2015. 64(2): p. 236-43.

160. Hall, W.L., et al., *Soy-isoflavone-enriched foods and markers of lipid and glucose metabolism in postmenopausal women: interactions with genotype and equol production.* The American journal of clinical nutrition, 2006. 83(3): p. 592-600.

161. Meyer, B.J., et al., *Limited lipid-lowering effects of regular consumption of whole soybean foods.* Annals of nutrition and metabolism, 2004. 48(2): p. 67-78.

162. Pipe, E.A., et al., *Soy protein reduces serum LDL cholesterol and the LDL cholesterol: HDL cholesterol and apolipoprotein B: apolipoprotein AI ratios in adults with type 2 diabetes.* The Journal of nutrition, 2009. 139(9): p. 1700-1706.

163. McVeigh, B.L., et al., *Effect of soy protein varying in isoflavone content on serum lipids in healthy young men.* The American journal of clinical nutrition, 2006. 83(2): p. 244-251.

164. Wong, J.M., et al., *Equol status and blood lipid profile in hyperlipidemia after consumption of diets containing soy foods.* The American journal of clinical nutrition, 2012. 95(3): p. 564-571.

165. Greany, K.A., et al., *Probiotic consumption does not enhance the cholesterol-lowering effect of soy in postmenopausal women.* The Journal of nutrition, 2004. 134(12): p. 3277-3283.

166. Badeau, R., et al., *Effect of isolated isoflavone supplementation on ABCA1-dependent cholesterol efflux potential in postmenopausal women.* Menopause, 2007. 14(2): p. 293-299.

167. Gardner, C.D., et al., *Effect of two types of soy milk and dairy milk on plasma lipids in hypercholesterolemic adults: a randomized trial.* Journal of the American College of Nutrition, 2007. 26(6): p. 669-677.

168. Nestel, P., et al., *A biochanin-enriched isoflavone from red clover lowers LDL cholesterol in men.* European journal of clinical nutrition, 2004. 58(3): p. 403-408.

169. Qin, Y., et al., *Daidzein supplementation decreases serum triglyceride and uric acid concentrations in hypercholesterolemic adults with the effect on triglycerides being greater in those with the GA compared with the GG genotype of ESR-β Rsa I.* The Journal of nutrition, 2014. 144(1): p. 49-54.

170. Thorp, A.A., et al., *Soy food consumption does not lower LDL cholesterol in either equol or nonequol producers.* The American journal of clinical nutrition, 2008. 88(2): p. 298-304.

171. He, F.J., J. Li, and G.A. Macgregor, *Effect of longer term modest salt reduction on blood pressure: Cochrane systematic review and meta-analysis of randomised trials.* BMJ, 2013. 346: p. f1325.

172. Taku, K., et al., *Effects of soy isoflavone extract supplements on blood pressure in adult humans: systematic review and meta-analysis of randomized placebo-controlled trials.* Journal of hypertension, 2010. 28(10): p. 1971-1982.

173. Liu, X.X., et al., *Effect of soy isoflavones on blood pressure: a meta-analysis of randomized controlled trials.* Nutr Metab Cardiovasc Dis, 2012. 22(6): p. 463-70.

174. Kou, T., et al., *Effect of soybean protein on blood pressure in postmenopausal women: a meta-analysis of randomized controlled trials.* Food Funct, 2017. 8(8): p. 2663-2671.

175. Welty, F.K., et al., *Effect of soy nuts on blood pressure and lipid levels in hypertensive, prehypertensive, and normotensive postmenopausal women.* Archives of internal medicine, 2007. 167(10): p. 1060-1067.

176. Ding, M., et al., *Consumption of soy foods and isoflavones and risk of type 2 diabetes: a pooled analysis of three US cohorts.* European journal of clinical nutrition, 2016. 70(12): p. 1381-1387.

177. Li, W., et al., *Soy and the risk of type 2 diabetes mellitus: A systematic review and meta-analysis of observational studies.* Diabetes Res Clin Pract, 2018. 137: p. 190-199.

178. Fang, K., et al., *Soy isoflavones and glucose metabolism in menopausal women: A systematic review and meta-analysis of randomized controlled trials.* Mol Nutr Food Res, 2016. 60(7): p. 1602-14.

179. Ricci, E., et al., *Effects of soy isoflavones and genistein on glucose metabolism in perimenopausal and postmenopausal non-Asian women: a meta-analysis of randomized controlled trials.* Menopause, 2010. 17(5): p. 1080-1086.

180. Goff, D.C., Jr., et al., *2013 ACC/AHA guideline on the assessment of cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines.* Circulation, 2014. 129(25 Suppl 2): p. S49-73.

181. Authors/Task Force, M., et al., *2016 European Guidelines on cardiovascular disease prevention in clinical practice: The Sixth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of 10 societies and by invited experts) Developed with the special contribution of the European Association for Cardiovascular Prevention & Rehabilitation (EACPR).* Atherosclerosis, 2016. 252: p. 207-274.

182. Anderson, K.M., et al., *Cardiovascular disease risk profiles.* American heart journal, 1991. 121(1): p. 293-298.

183. Gonçalves, F.B., et al., *Calcification of the abdominal aorta as an independent predictor of cardiovascular events: a meta-analysis.* Heart, 2012. 98(13): p. 988-994.

184. Rumberger, J.A., et al., *Coronary artery calcium area by electron-beam computed tomography and coronary atherosclerotic plaque area. A histopathologic correlative study.* Circulation, 1995. 92(8): p. 2157-62.

185. Folsom, A.R., et al., *Coronary artery calcification compared with carotid intima-media thickness in the prediction of cardiovascular disease incidence: the Multi-Ethnic Study of Atherosclerosis (MESA).* Arch Intern Med, 2008. 168(12): p. 1333-9.

186. Kavousi, M., et al., *Evaluation of newer risk markers for coronary heart disease risk classification: a cohort study.* Ann Intern Med, 2012. 156(6): p. 438-44.

187. Yeboah, J., et al., *Comparison of novel risk markers for improvement in cardiovascular risk assessment in intermediate-risk individuals.* JAMA, 2012. 308(8): p. 788-95.

188. Park, H.E., et al., *Clinical and imaging parameters to predict cardiovascular outcome in asymptomatic subjects.* Int J Cardiovasc Imaging, 2013. 29(7): p. 1595-602.

189. Tintut, Y., et al., *Multilineage potential of cells from the artery wall.* Circulation, 2003. 108(20): p. 2505-10.

190. Tyson, K.L., et al., *Osteo/chondrocytic transcription factors and their target genes exhibit distinct patterns of expression in human arterial calcification.* Arteriosclerosis, thrombosis, and vascular biology, 2003. 23(3): p. 489-494.

191. Bear, M., M. Butcher, and S.G. Shaughnessy, *Oxidized low-density lipoprotein acts synergistically with beta-glycerophosphate to induce osteoblast differentiation in primary cultures of vascular smooth muscle cells.* J Cell Biochem, 2008. 105(1): p. 185-93.

192. Li, H., et al., *Cross talk between the bone and immune systems: osteoclasts function as antigen-presenting cells and activate CD4+ and CD8+ T cells.* Blood, The Journal of the American Society of Hematology, 2010. 116(2): p. 210-217.

193. Chen, N.X., et al., *High glucose increases the expression of Cbfa1 and BMP-2 and enhances the calcification of vascular smooth muscle cells.* Nephrol Dial Transplant, 2006. 21(12): p. 3435-42.

194. Wang, C.C., et al., *Insulin attenuates vascular smooth muscle calcification but increases vascular smooth muscle cell phosphate transport.* Atherosclerosis, 2007. 195(1): p. e65-75.

195. Luo, X.H., et al., *Development of arterial calcification in adiponectin-deficient mice: adiponectin regulates arterial calcification.* J Bone Miner Res, 2009. 24(8): p. 1461-8.

196. Budoff, M.J., et al., *Progression of coronary artery calcium predicts all-cause mortality.* JACC: Cardiovascular Imaging, 2010. 3(12): p. 1229-1236.

197. McClelland, R.L., et al., *Distribution of coronary artery calcium by race, gender, and age: results from the Multi-Ethnic Study of Atherosclerosis (MESA).* Circulation, 2006. 113(1): p. 30-7.

198. Yano, Y., et al., *Association of Coronary Artery Calcium Score vs Age With Cardiovascular Risk in Older Adults: An Analysis of Pooled Population-Based Studies.* JAMA Cardiol, 2017. 2(9): p. 986-994.

199. Arad, Y., et al., *Coronary calcification, coronary disease risk factors, C-reactive protein, and atherosclerotic cardiovascular disease events: the St. Francis Heart Study.* J Am Coll Cardiol, 2005. 46(1): p. 158-65.

200. Kavousi, M., et al., *Prevalence and Prognostic Implications of Coronary Artery Calcification in Low-Risk Women: A Meta-analysis.* JAMA, 2016. 316(20): p. 2126-2134.

201. Desai, M.Y., P.C. Cremer, and P. Schoenhagen, *Thoracic aortic calcification: diagnostic, prognostic, and management considerations.* JACC: Cardiovascular Imaging, 2018. 11(7): p. 1012-1026.

202. Lanzer, P., et al., *Medial vascular calcification revisited: review and perspectives.* Eur Heart J, 2014. 35(23): p. 1515-25.

203. Group, P.D.o.A.i.Y.R., *Natural history of aortic and coronary atherosclerotic lesions in youth: findings from the PDAY Study.* Arterioscler Thromb, 1993. 13: p. 1291-1298.

204. Chuang, M.L., et al., *Prevalence and distribution of abdominal aortic calcium by gender and age group in a community-based cohort (from the Framingham Heart Study).* Am J Cardiol, 2012. 110(6): p. 891-6.

205. Criqui, M.H., et al., *Risk factor differences for aortic versus coronary calcified atherosclerosis: the multiethnic study of atherosclerosis.* Arteriosclerosis, thrombosis, and vascular biology, 2010. 30(11): p. 2289-2296.

206. Onuma, O.K., et al., *Relation of risk factors and abdominal aortic calcium to progression of coronary artery calcium (from the Framingham Heart Study).* The American journal of cardiology, 2017. 119(10): p. 1584-1589.

207. Allison, M.A., P. Pavlinac, and C.M. Wright, *The differential associations between HDL, non-HDL and total cholesterols and atherosclerotic calcium deposits in multiple vascular beds.* Atherosclerosis, 2007. 194(2): p. e87-e94.

208. Jensky, N.E., et al., *Blood pressure and vascular calcification.* Hypertension, 2010. 55(4): p. 990-997.

209. Kim, E.-D., et al., *Association of abdominal aortic calcification with lifestyle and risk factors of cardiovascular disease.* Korean journal of family medicine, 2013. 34(3): p. 213.

210. Hoffmann, U., et al., *Cardiovascular event prediction and risk reclassification by coronary, aortic, and valvular calcification in the Framingham Heart Study.* Journal of the American Heart Association, 2016. 5(2): p. e003144.

211. Chen, H.-C., et al., *Abdominal aortic calcification score can predict future coronary artery disease in hemodialysis patients: a 5-year prospective cohort study.* BMC nephrology, 2018. 19(1): p. 1-7.

212. Van der Meer, I.M., et al., *Predictive value of noninvasive measures of atherosclerosis for incident myocardial infarction: the Rotterdam Study.* Circulation, 2004. 109(9): p. 1089-1094.

213. Levitzky, Y.S., et al., *Prediction of intermittent claudication, ischemic stroke, and other cardiovascular disease by detection of abdominal aortic calcific deposits by plain lumbar radiographs.* The American journal of cardiology, 2008. 101(3): p. 326-331.

214. Hendriks, E.J., et al., *Annularity of aorto-iliac arterial calcification and risk of all-cause and cardiovascular mortality.* JACC: Cardiovascular Imaging, 2018. 11(11): p. 1718-1719.

215. Criqui, M.H., et al., *Abdominal aortic calcium, coronary artery calcium, and cardiovascular morbidity and mortality in the Multi-Ethnic Study of Atherosclerosis.* Arteriosclerosis, thrombosis, and vascular biology, 2014. 34(7): p. 1574-1579.

216. Papanas, N., et al., *Aortic arch calcification predicts the extent of coronary atherosclerosis in patients with or without type 2 diabetes.* Acta Clinica Belgica, 2007. 62(1): p. 52-55.

217. O'Connor, S.D., et al., *Does nonenhanced CT-based quantification of abdominal aortic calcification outperform the Framingham risk score in predicting cardiovascular events in asymptomatic adults?* Radiology, 2019. 290(1): p. 108-115.

218. Liu, Z.-m., et al., *Whole soy, but not purified daidzein, had a favorable effect on improvement of cardiovascular risks: a 6-month randomized, double-blind, and placebo-controlled trial in equol-producing postmenopausal women.* Molecular nutrition & food research, 2014. 58(4): p. 709-717.

219. Hodis, H.N., et al., *Isoflavone soy protein supplementation and atherosclerosis progression in healthy postmenopausal women: a randomized controlled trial.* Stroke, 2011. 42(11): p. 3168-3175.

220. Ahuja, V., et al., *Significant inverse association of equol-producer status with coronary artery calcification but not dietary isoflavones in healthy Japanese men.* British Journal of Nutrition, 2017. 117(2): p. 260-266.

221. Touboul, P.J., et al., *Mannheim carotid intima-media thickness and plaque consensus (2004-2006-2011). An update on behalf of the advisory board of the 3rd, 4th and 5th watching the risk symposia, at the 13th, 15th and 20th European Stroke Conferences, Mannheim, Germany, 2004, Brussels, Belgium, 2006, and Hamburg, Germany, 2011.* Cerebrovasc Dis, 2012. 34(4): p. 290-6.

222. Salonen, J.T. and R. Salonen, *Ultrasonographically assessed carotid morphology and the risk of coronary heart disease.* Arterioscler Thromb, 1991. 11(5): p. 1245-9.

223. Lorenz, M.W., et al., *Carotid intima-media thickening indicates a higher vascular risk across a wide age range: prospective data from the Carotid Atherosclerosis Progression Study (CAPS).* Stroke, 2006. 37(1): p. 87-92.

224. Magyar, M.T., et al., *Early-onset carotid atherosclerosis is associated with increased intima-media thickness and elevated serum levels of inflammatory markers.* Stroke, 2003. 34(1): p. 58-63.

225. Lorenz, M.W., et al., *Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis.* Circulation, 2007. 115(4): p. 459-67.

226. Zuo, L.-S.-Y., et al., *Isoflavone biomarkers are inversely associated with atherosclerosis progression in adults: a prospective study.* The American Journal of Clinical Nutrition, 2021.

227. Bots, M.L., et al., *Carotid intima-media thickness measurements in intervention studies: design options, progression rates, and sample size considerations: a point of view.* Stroke, 2003. 34(12): p. 2985-2994.

228. Hodis, H.N., et al., *Hormone therapy and the progression of coronary-artery atherosclerosis in postmenopausal women.* N Engl J Med, 2003. 349(6): p. 535-45.

229. Hodis, H.N., et al., *Estrogen in the prevention of atherosclerosis. A randomized, double-blind, placebo-controlled trial.* Ann Intern Med, 2001. 135(11): p. 939-53.

230. Ananth, C.V. and J.A. Lavery, *Biases in secondary analyses of randomised trials: recognition, correction, and implications.* BJOG, 2016. 123(7): p. 1056-9.

231. McClelland, R.L., et al., *10-year coronary heart disease risk prediction using coronary artery calcium and traditional risk factors: derivation in the MESA (Multi-Ethnic Study of Atherosclerosis) with validation in the HNR (Heinz Nixdorf Recall) study and the DHS (Dallas Heart Study).* Journal of the American College of Cardiology, 2015. 66(15): p. 1643-1653.

232. Budoff, M.J., et al., *Thoracic aortic calcification and coronary heart disease events: the multi-ethnic study of atherosclerosis (MESA).* Atherosclerosis, 2011. 215(1): p. 196-202.

233. Sekikawa, A., et al., *A "natural experiment" in cardiovascular epidemiology in the early 21st century.* Heart, 2003. 89(3): p. 255-257.

234. McGill Jr, H.C., et al., *Effects of serum lipoproteins and smoking on atherosclerosis in young men and women.* Arteriosclerosis, thrombosis, and vascular biology, 1997. 17(1): p. 95-106.

235. Sekikawa, A., et al., *Marine-derived n-3 fatty acids and atherosclerosis in Japanese, Japanese-American, and white men: a cross-sectional study.* Journal of the American College of Cardiology, 2008. 52(6): p. 417-424.

236. Pumford, S.L., et al., *Determination of the isoflavonoids genistein and daidzein in biological samples by gas chromatography-mass spectrometry.* Annals of clinical biochemistry, 2002. 39(3): p. 281-292.

237. Agatston, A.S., et al., *Quantification of coronary artery calcium using ultrafast computed tomography.* Journal of the American College of Cardiology, 1990. 15(4): p. 827-832.

238. Tobin, J., *Estimation of relationships for limited dependent variables.* Econometrica: journal of the Econometric Society, 1958: p. 24-36.

239. Talaei, M., et al., *Dietary soy intake is not associated with risk of cardiovascular disease mortality in Singapore Chinese adults.* The Journal of nutrition, 2014. 144(6): p. 921-928.

240. Sekikawa, A., et al., *Associations of equol‐producing status with white matter lesion and amyloid‐β deposition in cognitively normal elderly Japanese.* Alzheimer's & Dementia: Translational Research & Clinical Interventions, 2020. 6(1): p. e12089.

241. Yamamoto, S., et al., *Validity and reproducibility of a self-administered food-frequency questionnaire to assess isoflavone intake in a Japanese population in comparison with dietary records and blood and urine isoflavones.* The Journal of nutrition, 2001. 131(10): p. 2741-2747.

242. Tang, J., et al., *Legume and soy intake and risk of type 2 diabetes: A systematic review and meta-analysis of prospective cohort studies.* The American journal of clinical nutrition, 2020. 111(3): p. 677-688.

243. Sekikawa, A., et al., *Continuous decline in mortality from coronary heart disease in Japan despite a continuous and marked rise in total cholesterol: Japanese experience after the Seven Countries Study.* International journal of epidemiology, 2015. 44(5): p. 1614-1624.

244. Sekikawa, A., et al., *Long chain n-3 polyunsaturated fatty acids and incidence rate of coronary artery calcification in Japanese men in Japan and white men in the USA: population based prospective cohort study.* Heart, 2014. 100(7): p. 569-573.

245. Aso, T., *Equol improves menopausal symptoms in Japanese women.* The Journal of nutrition, 2010. 140(7): p. 1386S-1389S.

246. Ishiwata, N., et al., *New equol supplement for relieving menopausal symptoms: randomized, placebo-controlled trial of Japanese women.* Menopause, 2009. 16(1): p. 141-148.

247. Jenks, B.H., et al., *A pilot study on the effects of S-equol compared to soy isoflavones on menopausal hot flash frequency.* Journal of women's health, 2012. 21(6): p. 674-682.

248. Ueshima, H., *Explanation for the Japanese paradox: prevention of increase in coronary heart disease and reduction in stroke.* J Atheroscler Thromb, 2007. 14(6): p. 278-86.

249. Okamura, T., et al., *Specific alcoholic beverage and blood pressure in a middle-aged Japanese population: the High-risk and Population Strategy for Occupational Health Promotion (HIPOP-OHP) Study.* Journal of human hypertension, 2004. 18(1): p. 9-16.

250. Collaboration, A.P.C.S., *The burden of overweight and obesity in the Asia–Pacific region.* Obesity Reviews, 2007. 8(3): p. 191-196.

251. Havel, R.J. and E. Rapaport, *Management of primary hyperlipidemia.* New England Journal of Medicine, 1995. 332(22): p. 1491-1498.

252. Kashiwagi, A., et al., *International clinical harmonization of glycated hemoglobin in Japan: from Japan Diabetes Society to National Glycohemoglobin Standardization Program values.* Diabetology international, 2012. 3(1): p. 8-10.

253. Sekikawa, A., et al., *Association of blood levels of marine omega-3 fatty acids with coronary calcification and calcium density in Japanese men.* European journal of clinical nutrition, 2019. 73(5): p. 783-792.

254. Fujiyoshi, A., et al., *Proteinuria and reduced estimated glomerular filtration rate are independently associated with lower cognitive abilities in apparently healthy community-dwelling elderly men in Japan: a cross-sectional study.* Journal of epidemiology, 2020. 30(6): p. 244-252.

255. Horio, M., et al., *Modification of the CKD epidemiology collaboration (CKD-EPI) equation for Japanese: accuracy and use for population estimates.* American Journal of Kidney Diseases, 2010. 56(1): p. 32-38.

256. Fujiyoshi, A., et al., *Cross-sectional comparison of coronary artery calcium scores between Caucasian men in the United States and Japanese men in Japan: the multi-ethnic study of atherosclerosis and the Shiga epidemiological study of subclinical atherosclerosis.* American journal of epidemiology, 2014. 180(6): p. 590-598.

257. Kälsch, H., et al., *Prevalence of thoracic aortic calcification and its relationship to cardiovascular risk factors and coronary calcification in an unselected population-based cohort: the Heinz Nixdorf Recall Study.* The international journal of cardiovascular imaging, 2013. 29(1): p. 207-216.

258. Confavreux, C.B., et al., *Higher serum osteocalcin is associated with lower abdominal aortic calcification progression and longer 10-year survival in elderly men of the MINOS cohort.* The Journal of Clinical Endocrinology & Metabolism, 2013. 98(3): p. 1084-1092.

259. Greenland, P., et al., *Major risk factors as antecedents of fatal and nonfatal coronary heart disease events.* Jama, 2003. 290(7): p. 891-897.

260. El-Saed, A., et al., *The prevalence of aortic calcification in Japanese compared to white and Japanese-American middle-aged men is confounded by the amount of cigarette smoking.* International journal of cardiology, 2013. 167(1): p. 134-139.

261. Curtis, P.J., et al., *Vascular function and atherosclerosis progression after 1 y of flavonoid intake in statin-treated postmenopausal women with type 2 diabetes: a double-blind randomized controlled trial.* The American of Clinical Nutrition, 2013. 97(5): p. 936-942.

262. Pham, T., et al., *Smoking habits and progression of coronary and aortic artery calcification: A 5-year follow-up of community-dwelling Japanese men.* International journal of cardiology, 2020. 314: p. 89-94.

263. Mahajan, H., et al., *Association of alcohol consumption and aortic calcification in healthy men aged 40–49 years for the ERA JUMP Study.* Atherosclerosis, 2018. 268: p. 84-91.

264. Zhang, X., et al., *Potential Protective Effects of Equol (Soy Isoflavone Metabolite) on Coronary Heart Diseases—From Molecular Mechanisms to Studies in Humans.* Nutrients, 2021. 13(11): p. 3739.

265. (WHO)., W.H.O., *Global Health Estimates 2016: deaths by cause, age, sex, by country and by region, 2000–2016.*

266. Clarkson, T.B., *Soy, soy phytoestrogens and cardiovascular disease.* The Journal of nutrition, 2002. 132(3): p. 566S-569S.

267. Messina, M., C. Nagata, and A.H. Wu, *Estimated Asian adult soy protein and isoflavone intakes.* Nutr Cancer, 2006. 55(1): p. 1-12.

268. Setchell, K.D. and A. Cassidy, *Dietary isoflavones: biological effects and relevance to human health.* The journal of nutrition, 1999. 129(3): p. 758S-767S.

269. Rothwell, J.A., A.J. Day, and M.R. Morgan, *Experimental determination of octanol–water partition coefficients of quercetin and related flavonoids.* Journal of agricultural and food chemistry, 2005. 53(11): p. 4355-4360.

270. Verdrengh, M., et al., *Genistein as an anti-inflammatory agent.* Inflammation Research, 2003. 52(8): p. 341-346.

271. Paradkar, P.N., et al., *Dietary isoflavones suppress endotoxin-induced inflammatory reaction in liver and intestine.* Cancer letters, 2004. 215(1): p. 21-28.

272. Duan, W., et al., *Antiinflammatory effects of genistein, a tyrosine kinase inhibitor, on a guinea pig model of asthma.* American journal of respiratory and critical care medicine, 2003. 167(2): p. 185-192.

273. Steinberg, D. and J.L. Witztum, *Oxidized low-density lipoprotein and atherosclerosis.* Arteriosclerosis, thrombosis, and vascular biology, 2010. 30(12): p. 2311-2316.

274. Adams, M.R., et al., *Soy protein containing isoflavones reduces the size of atherosclerotic plaques without affecting coronary artery reactivity in adult male monkeys.* The Journal of nutrition, 2005. 135(12): p. 2852-2856.

275. Kondo, K., et al., *Genistein, an isoflavone included in soy, inhibits thrombotic vessel occlusion in the mouse femoral artery and in vitro platelet aggregation.* European journal of pharmacology, 2002. 455(1): p. 53-57.

276. Marventano, S., et al., *Legume consumption and CVD risk: a systematic review and meta-analysis.* Public Health Nutr, 2017. 20(2): p. 245-254.

277. Nachvak, S.M., et al., *Soy, Soy Isoflavones, and Protein Intake in Relation to Mortality from All Causes, Cancers, and Cardiovascular Diseases: A Systematic Review and Dose–Response Meta-Analysis of Prospective Cohort Studies.* Journal of the Academy of Nutrition and Dietetics, 2019. 119(9): p. 1483-1500. e17.

278. Zhan, S. and S.C. Ho, *Meta-analysis of the effects of soy protein containing isoflavones on the lipid profile.* The American journal of clinical nutrition, 2005. 81(2): p. 397-408.

279. Liu, X., et al., *Effect of soy isoflavones on blood pressure: a meta-analysis of randomized controlled trials.* Nutrition, Metabolism and Cardiovascular Diseases, 2012. 22(6): p. 463-470.

280. Mayo, B., L. Vazquez, and A.B. Florez, *Equol: A Bacterial Metabolite from The Daidzein Isoflavone and Its Presumed Beneficial Health Effects.* Nutrients, 2019. 11(9).

281. Gil-Izquierdo, A., et al., *Soy isoflavones and cardiovascular disease epidemiological, clinical and-omics perspectives.* Current pharmaceutical biotechnology, 2012. 13(5): p. 624-631.

282. Cano, A., M.Á. García-Pérez, and J.J. Tarín, *Isoflavones and cardiovascular disease.* Maturitas, 2010. 67(3): p. 219-226.

283. Atkinson, C., et al., *In vitro incubation of human feces with daidzein and antibiotics suggests interindividual differences in the bacteria responsible for equol production.* The Journal of nutrition, 2004. 134(3): p. 596-599.

284. Maruo, T., et al., *Adlercreutzia equolifaciens gen. nov., sp. nov., an equol-producing bacterium isolated from human faeces, and emended description of the genus Eggerthella.* International Journal of Systematic and Evolutionary Microbiology, 2008. 58(5): p. 1221-1227.

285. Jin, J.-S., et al., *Slackia equolifaciens sp. nov., a human intestinal bacterium capable of producing equol.* International journal of systematic and evolutionary microbiology, 2010. 60(8): p. 1721-1724.

286. Matthies, A., et al., *Conversion of daidzein and genistein by an anaerobic bacterium newly isolated from the mouse intestine.* Applied and environmental microbiology, 2008. 74(15): p. 4847-4852.

287. Shimada, Y., et al., *Cloning and expression of a novel NADP (H)-dependent daidzein reductase, an enzyme involved in the metabolism of daidzein, from equol-producing Lactococcus strain 20-92.* Applied and environmental microbiology, 2010. 76(17): p. 5892-5901.

288. Shimada, Y., et al., *Identification of two novel reductases involved in equol biosynthesis in Lactococcus strain 20–92.* Journal of molecular microbiology and biotechnology, 2011. 21(3-4): p. 160-172.

289. Shimada, Y., et al., *Identification of a novel dihydrodaidzein racemase essential for biosynthesis of equol from daidzein in Lactococcus sp. strain 20-92.* Applied and environmental microbiology, 2012. 78(14): p. 4902-4907.

290. Frankenfeld, C.L., et al., *High concordance of daidzein-metabolizing phenotypes in individuals measured 1 to 3 years apart.* British journal of nutrition, 2005. 94(6): p. 873-876.

291. Pelzer, T., et al., *Increased mortality and aggravation of heart failure in estrogen receptor-β knockout mice after myocardial infarction.* Circulation, 2005. 111(12): p. 1492-1498.

292. Gabel, S.A., et al., *Estrogen receptor beta mediates gender differences in ischemia/reperfusion injury.* Journal of molecular and cellular cardiology, 2005. 38(2): p. 289-297.

293. Nikolic, I., et al., *Treatment with an estrogen receptor-beta-selective agonist is cardioprotective.* Journal of molecular and cellular cardiology, 2007. 42(4): p. 769-780.

294. Martin-Ventura, J.L., et al., *Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis.* Circulation, 2004. 110(15): p. 2216-2219.

295. Rayner, K., et al., *Heat shock protein 27 protects against atherogenesis via an estrogen-dependent mechanism: role of selective estrogen receptor beta modulation.* Arteriosclerosis, thrombosis, and vascular biology, 2009. 29(11): p. 1751-1756.

296. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease.* New England Journal of Medicine, 2005. 352(16): p. 1685-1695.

297. Violi, F. and P. Pignatelli, *Clinical application of NOX activity and other oxidative biomarkers in cardiovascular disease: a critical review.* Antioxidants & redox signaling, 2015. 23(5): p. 514-532.

298. Liu, Y., et al., *Molecular imaging of inflammation and platelet adhesion in advanced atherosclerosis effects of antioxidant therapy with NADPH oxidase inhibition.* Circulation: Cardiovascular Imaging, 2013. 6(1): p. 74-82.

299. Quesada, I.M., et al., *Selective inactivation of NADPH oxidase 2 causes regression of vascularization and the size and stability of atherosclerotic plaques.* Atherosclerosis, 2015. 242(2): p. 469-475.

300. Cipollone, F., et al., *Oxidant stress and aspirin-insensitive thromboxane biosynthesis in severe unstable angina.* Circulation, 2000. 102(9): p. 1007-1013.

301. Kim, J.Y., et al., *Lipoprotein-associated phospholipase A2 activity is associated with coronary artery disease and markers of oxidative stress: a case-control study.* The American journal of clinical nutrition, 2008. 88(3): p. 630-637.

302. Wang, B., et al., *Associations of plasma 8-isoprostane levels with the presence and extent of coronary stenosis in patients with coronary artery disease.* Atherosclerosis, 2006. 184(2): p. 425-430.

303. Holvoet, P., et al., *Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease.* Circulation, 1998. 98(15): p. 1487-1494.

304. Ehara, S., et al., *Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes.* Circulation, 2001. 103(15): p. 1955-1960.

305. Widmer, R.J. and A. Lerman, *Endothelial dysfunction and cardiovascular disease.* Global Cardiology Science and Practice, 2014. 2014(3): p. 43.

306. Alexander, Y., et al., *Endothelial function in cardiovascular medicine: a consensus paper of the European Society of Cardiology Working Groups on Atherosclerosis and Vascular Biology, Aorta and Peripheral Vascular Diseases, Coronary Pathophysiology and Microcirculation, and Thrombosis.* Cardiovascular research, 2021. 117(1): p. 29-42.

307. Chirinos, J.A., et al., *Large-artery stiffness in health and disease: JACC state-of-the-art review.* Journal of the American College of Cardiology, 2019. 74(9): p. 1237-1263.

308. Townsend, R.R., et al., *Recommendations for improving and standardizing vascular research on arterial stiffness: a scientific statement from the American Heart Association.* Hypertension, 2015. 66(3): p. 698-722.

309. Vlachopoulos, C., K. Aznaouridis, and C. Stefanadis, *Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis.* Journal of the American College of Cardiology, 2010. 55(13): p. 1318-1327.

310. Vlachopoulos, C., et al., *Prediction of cardiovascular events and all-cause mortality with brachial-ankle elasticity index: a systematic review and meta-analysis.* Hypertension, 2012. 60(2): p. 556-562.

311. Marti, C.N., et al., *Endothelial dysfunction, arterial stiffness, and heart failure.* Journal of the American College of Cardiology, 2012. 60(16): p. 1455-1469.

312. Pase, M.P., N.A. Grima, and J. Sarris, *The effects of dietary and nutrient interventions on arterial stiffness: a systematic review.* The American journal of clinical nutrition, 2011. 93(2): p. 446-454.

313. Man, B., et al., *The effect of soy isoflavones on arterial stiffness: A systematic review and meta-analysis of randomized controlled trials.* European Journal of Nutrition, 2021. 60(2): p. 603-614.

314. Yoshikata, R., K.Z.Y. Myint, and H. Ohta, *Effects of equol supplement on bone and cardiovascular parameters in middle-aged Japanese women: A prospective observational study.* The Journal of Alternative and Complementary Medicine, 2018. 24(7): p. 701-708.

315. Hazim, S., et al., *Acute benefits of the microbial-derived isoflavone metabolite equol on arterial stiffness in men prospectively recruited according to equol producer phenotype: a double-blind randomized controlled trial.* The American journal of clinical nutrition, 2016. 103(3): p. 694-702.

316. Yoshikata, R., K.Z. Myint, and H. Ohta, *Relationship between equol producer status and metabolic parameters in 743 Japanese women: Equol producer status is associated with antiatherosclerotic conditions in women around menopause and early postmenopause.* Menopause, 2017. 24(2): p. 216-224.

317. Uemura, H., et al., *Inverse association between soy food consumption, especially fermented soy products intake and soy isoflavone, and arterial stiffness in Japanese men.* Scientific reports, 2018. 8(1): p. 1-9.

318. Eyster, K., et al., *Effects of equol on gene expression in female cynomolgus monkey iliac arteries.* Nutrition, Metabolism and Cardiovascular Diseases, 2014. 24(4): p. 423-427.

319. Arad, Y., et al., *Treatment of asymptomatic adults with elevated coronary calcium scores with atorvastatin, vitamin C, and vitamin E: the St. Francis Heart Study randomized clinical trial.* Journal of the American College of Cardiology, 2005. 46(1): p. 166-172.

320. Miller, V.M. and S.P. Duckles, *Vascular actions of estrogens: functional implications.* Pharmacological reviews, 2008. 60(2): p. 210-241.

321. Clarkson, T.B., *Estrogen effects on arteries vary with stage of reproductive life and extent of subclinical atherosclerosis progression.* Menopause, 2007. 14(3): p. 373-384.

322. Dubey, R.K., et al., *Vascular consequences of menopause and hormone therapy: importance of timing of treatment and type of estrogen.* Cardiovascular research, 2005. 66(2): p. 295-306.

323. Brzezinski, A. and A. Debi, *Phytoestrogens: the "natural" selective estrogen receptor modulators?* European Journal of Obstetrics & Gynecology and Reproductive Biology, 1999. 85(1): p. 47-51.

324. Verschuren, W.M., et al., *Serum total cholesterol and long-term coronary heart disease mortality in different cultures: Twenty-five—year follow-up of the seven countries study.* Jama, 1995. 274(2): p. 131-136.

325. Sekikawa, A., et al., *Effect of S-equol and Soy Isoflavones on Heart and Brain.* Curr Cardiol Rev, 2019. 15(2): p. 114-135.

326. Setchell, K.D., et al., *Dietary factors influence production of the soy isoflavone metabolite s-(-)equol in healthy adults.* J Nutr, 2013. 143(12): p. 1950-8.

327. Potter, S.M., *Soy protein and cardiovascular disease: the impact of bioactive components in soy.* Nutrition reviews, 1998. 56(8): p. 231-235.