

**CARDIOVASCULAR AND MENTAL HEALTH BENEFITS OF SOY CONSUMPTION:
ROLE OF SOY ISOFLAVONES**

Alicia A Thorp

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1.0 Introduction

This thesis addresses potential cardiovascular (CV) and mental health benefits from soy consumption. It focuses on the role of isoflavones (ISO) and, in particular, the soy metabolite equol in mediating improvements of CV risk factors and enhancing cognitive performance.

1.1 Overview

Soy research has intensified in the last twenty years due to epidemiological evidence and the release of an official health claim by the US Food and Drug Administration (FDA) stating regular soy consumption can reduce risk of coronary heart disease (CHD) (1). Whilst soy protein (SP) is specifically credited in the health claim for mediating this benefit via the ability to reduce cholesterol, dietary interventions delivering the recommended intake (≥ 25 g SP/day) do not always report a favourable effect (2, 3). It has been suggested that discrepancies in observations may be attributed in part to the inclusion of ISOs which, depending on the method used to process SP, may also be present to a variable extent. Indeed studies which concurrently deliver ISOs with SP tend to show greater reductions in cholesterol than is possible with SP alone (4-6). Interestingly, supplementing the diet with isolated ISOs fails to lower blood cholesterol (7-10) indicating a certain amount of SP is necessary for an effect.

Equol, an exclusive metabolite of the ISO daidzein (DAZ), has been postulated to mediate the favourable effects observed when delivering ISOs with SP on blood cholesterol. This is due to equol possessing significantly greater estrogenic activity than DAZ and genistein (GEN), the other major ISO in soy. As its production is limited to only one third of the population, the phenomenon of equol could explain why health benefits following soy supplementation are so inconsistent. Whilst studies such as that by Meyer *et al* (11) strengthen the argument that equol producers benefit far greater from soy consumption, it is not clear whether those who are producers need to consume the Health Claim's recommended intake of 25g SP/ day to achieve a cholesterol benefit or whether a more modest intake of SP delivered in combination with ISO would suffice.

Independent of their potential contribution in cholesterol reduction, there is increasing evidence

that the estrogenic activity of ISOs can improve circulatory function. As alterations to vascular homeostasis are associated with the underlying aetiology of CVD, ISOs may offer an additional mechanism by which to reduce CVD risk. Indeed studies that identify equol producers within their cohorts have shown these individuals gain greater vascular benefits from soy consumption, specifically with respect to BP (12) and endothelial function (13).

The discovery that cerebral vascular function may influence cognitive performance and evidence that ISOs possess potent neurological properties, has additionally led researchers to investigate potential mental health benefits from soy consumption. Specifically under investigation is the ability of ISOs to improve performance in cognitive tasks that may be regulated by estrogen.

This chapter provides a comprehensive overview of the physiological and pharmacological properties of ISOs and a rationale as to why they may play a functional role in improving CVD risk and cognitive function. Moreover, the importance of equol which is known to possess enhanced health properties beyond that of ISOs, is discussed as the potential mediator of these benefits.

1.2 Health Benefits of Soy Consumption - Epidemiological Evidence

Epidemiological evidence indicates Asians have a lower incidence of CVD (14), hormone-dependent cancers of the breast (15) and prostate (16), colon cancer (17), menopausal symptoms (18) and osteoporosis (19, 20) compared to Western populations. Diet, lifestyle and genetic factors have all been suggested as playing a role in the aetiology of these diseases. Although Asians typically consume less dietary fat and red meat, eat more fish and rice, and engage in higher levels of physical activity than Westerners, much attention has been given to their greater consumption of soy and soy-based foods which has been a staple in their diets for more than 5000 years. Further support for a potential benefit from soy consumption comes from the reported increase in the incidence of hormone dependent cancers (21, 22) and CVD (23) which occur when Asians migrate to Western countries. Substantial research has been conducted to identify properties of the soybean's composition which may afford some protection against these conditions. It is now apparent that the physiological actions SP and more notably ISOs, mediate many of soy's reported health effects.

1.3 Soybeans

1.3.1 Composition

Soy (*Glycine max*) is a plant derived legume with a unique macronutrient profile. Unlike other legumes, soy contains a range of phytochemicals in addition to proteins, lipids, carbohydrates, vitamins and minerals which can offer health benefits beyond that of basic nutrition. Phytochemicals present in soy include ISOs, saponins, phytosterols, phytate (inositol hexaphosphate) and protease inhibitors (24). Whilst the health benefits of ISOs will be discussed in detail later in this chapter, it has been recognised that soy protease inhibitors can slow the rate of cancer division in cells, saponins may prevent cells from multiplying and phytosterols can block the action of estrogen.

With respect to its macronutrient composition, soybeans are distinctly higher in both protein and fat than other legumes, deriving 35-38% and 40% of calories from protein and fat respectively, compared to 20-30 % and 2-14% in other legumes. Soybeans also contain seven of the essential amino acids (deficient only in methionine) which are comparable to animal protein. Two peptides; β -conglycinin (7S globulin) and glycinin (11S globulin) make up to 90% of the total protein content of soy and have been recognised as having potential cholesterol lowering properties (25). The fat profile of soybeans is predominately unsaturated; polyunsaturated (63%), monounsaturated (23%) and saturated (14%). Soy's polyunsaturated content also contains two essential fatty acids, linoleic acid (50%) and α -linolenic acid (8%), which have their own recognised health benefits. Soluble and insoluble carbohydrates constitute 30% of the soybean. Primary soluble carbohydrates make up approximately 10% and are present as sugars; stachyose, raffinose and sucrose. Insoluble carbohydrates, or dietary fibre, come from the outer soybean hull and are composed primarily of cellulose, hemicellulose and pectin. In addition to providing high-quality protein, fat and carbohydrate, soybeans are also a rich source of micronutrients. Whilst their content can vary depending on soil and growing conditions, the major minerals present in soy are potassium, sodium, calcium, magnesium, sulphur and phosphorus. Soybeans are also a rich source of water soluble vitamins such as thiamine, riboflavin, niacin, pantothenic acid, biotin, folic acid, inositol and choline as well as fat soluble vitamins, viz. Vitamin A and E.

1.3.2 Methods of Processing Soybeans

Whole soybeans can be manufactured into three main soy products; soybean meal, SP concentrate (SPC) and SP isolate (SPI). Each of these products is shown to differ in their SP and ISO content as a consequence of processing. Figure 1.1 describes the steps involved in manufacturing each of these products. After removing the antinutritional content of the whole soybean (i.e. trypsin inhibitor content) via steaming/cooking (26), the beans are cleaned, dried and dehulled to produce a high-protein end product which is then fed into an extruder to produce full fat flakes. Immersing flakes in a solvent bath extracts the crude soybean oil and enables them to be toasted and ground into soybean meal which forms the basis of all soy flours, SPC and SPI. Soy flour is composed of approximately 50% protein by weight and contains the highest amount of ISOs, typically in the range of 5.5 $\mu\text{g}/\text{g}$ SP. It also contains the carbohydrate components of the soybean sugars (oligosaccharides) and fibre. SPC is made by selectively removing the soluble carbohydrates (sugars) from defatted soy flakes to produce a product that contains at least 65% protein. Whilst it remains high in fibre, it contains the lowest ISO content of all soy products, typically in the range of 0.3 $\mu\text{g}/\text{g}$ SP. There are three common processes used to extract the sugars, while not solubilising the protein portion and they include; extraction with aqueous ethyl alcohol (20 to 80%), acid washing with water near the isoelectric point (pH 4-5), denaturing the protein with moist heat and water extraction. Aqueous ethyl alcohol extraction not only removes soluble carbohydrates and ISOs but the peptides, glycinin and β -conglycinin which are postulated to mediate cholesterol lowering effects (refer to section 1.9.3 for detailed description). SPI is prepared by adding defatted soy flakes to deionised water. In this process, the protein is initially solubilised by keeping the mixture at a pH of 8.5-9.5 using sodium hydroxide and heat (55°C). The protein solution is then separated from the flakes by centrifugation. The solid soy flake residue is then re-extracted under the same conditions, whilst the proteins in the solution are precipitated out as a curd by hydrochloric acid. The curd is then washed with water and concentrated by centrifugation where the separated solid is neutralised and spray dried to produce protein isolates. This end product is almost free of carbohydrates and fat and contains 90% SP. What's more, SPI retains a significant amount of ISOs, typically in the range of 2.2 $\mu\text{g}/\text{g}$ SP.

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This figure is included on page 5 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.1: Processing methods for soybean products. Source: Peisker, M (27).

Understanding how soybeans are processed enables one to realise previous studies which report health benefits from SP may have underestimated the contribution of ISOs. This could be particularly relevant in respect to SP's cholesterol lowering benefit, as interventions which supplemented SP in the form of a SPI or from foods manufactured from soy flour would have also been delivering a certain amount of ISOs.

1.3.3 Food Products Manufactured from Soybeans

Approximately 10% of the world's soybean crop is directly used to manufacture human foods, with 95% of these products consumed by Asian nations. Whilst the high consumption of soy foods in Asia stems from their long standing traditional eating patterns, consumption of soy is a new development which is rapidly gaining acceptance in Western nations. Over the last nine years, consumer awareness of soy has dramatically increased with 85% of western cultures recognising it as a healthy food source. Today, there are thousands of soy foods and beverages available, with some utilising the whole soybean and others made with a variety of SP ingredients (SPI, SPC and soy flour).

Soy foods which are produced from the whole soybean are typically divided into two categories: non-fermented and fermented. Traditional non-fermented soy foods include fresh green soybeans, whole dry soybeans, soy nuts, soy sprouts, whole-fat soy flour, soymilk and soymilk products, tofu, okra and yuba. Traditional fermented soy foods include tempeh, miso, soy sauces, natto and fermented tofu

and soymilk products. A myriad of “second generation” soy foods have also been developed to accommodate western palates and include tofu hot dogs, tofu ice cream, soy veggie burgers, tempeh burgers, soymilk yoghurt, soymilk cheese and soy flour pancake mix. Thanks to developments in food processing techniques, many of the undesirable attributes associated with soy, such as its “beany taste” and smell, have been eliminated, while potentially harmful toxins such as trypsin inhibitors (found in whole soybeans) (26) have also been removed using heat and pressurised steaming techniques.

1.4 Soybean Isoflavones

1.4.1 Variability of Isoflavone Content in Soy foods

In whole soybeans, ISOs are present in concentrations of 0.2-3.0mg/g with levels strongly influenced by genetic (variety) and environmental factors (geographic location, soil type and seasonal duration) (28-30). Taiwan soybeans for example are shown to have a 40% higher concentration of ISOs than that found in soybeans from Korea (30). The method of preparing soybeans for use in traditional and commercial foods can also cause significant variability in ISO content (29, 31). Evidence for shifting quantities of ISOs in differently prepared forms of soy flour is described in Figure 1.2. Traditional soy foods per serve provide about 0.25 - 40mg ISO/g SP (28, 32). Generally speaking, minimally processed soy foods that contain most of the bean, like full fat soy flour, textured SP, roasted soybeans, tofu and soymilk retain the second highest content of ISO of about 0.1-5 mg/g SP (30, 33). By comparison SPI, the most concentrated form of SP (90%), contains substantially reduced amounts of ISO (up to 53% less) as a result of losses during the extraction process with organic solvents (34). Listed below in Table 1.1 are the average ISO concentrations of common soy foods.

Figure 1.2: The effect of processing on the average mg concentration of isoflavone/100g of soy flour.

Source: USDA-Iowa State University Database on the Isoflavone Content of Foods (30).

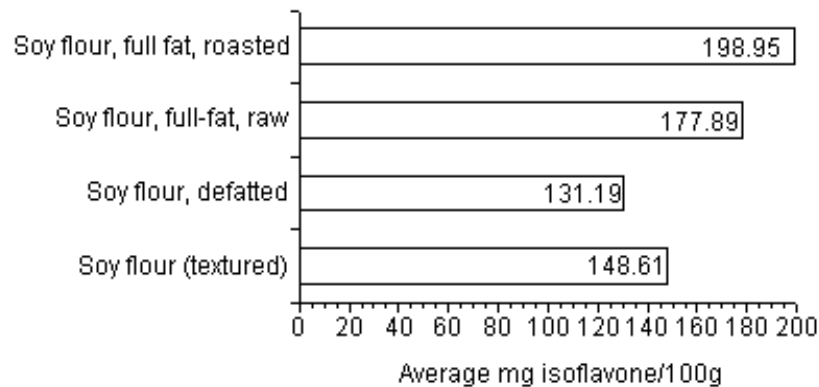


Table 1.1: Typical isoflavone concentrations of commercial soy products.

Food	Serving size	ISO (mg/serve)
canned soy beans	1/2 cup (95g)	40-75
soy flour	1/4 cup (25g)	45-69
soy milk	1 glass (250ml)	15-60
textured vegetable protein	1 tablespoon (22g) dry	24-54
Tofu	1 block (115g)	13-43
tempeh (fermented soybeans)	1 block (110g)	41
powdered soy drink mix	1 tablespoon (25g)	19-34
soy grits	1 tablespoon (15g)	25-32
soy flakes	1.5 tablespoons (15g)	15-26
tofu yoghurt	1 tub (200g)	26
soy and linseed bread	2 slices (80g)	7-15
miso (fermented soybean paste)	1 teaspoon (6g)	6
soy bacon	2 rashers (30g)	3
soy cheese	1 cube (25g)	1
soy sauce	1/2 teaspoon	0.2-1.2
white bread	2 slices (80g)	0.1-0.3
soy oil	1 tablespoon	0
SP concentrate	100 g	1-2

Sources: USDA-Iowa State University Database on the Isoflavone content of foods (35) and King and Bignell (36).

1.4.2 Sources of Isoflavones

Soybeans are the only dietary source of ISOs with levels that are nutritionally relevant (37). However, there are approximately 230 other foods which contain a small amount of ISOs, including certain vegetables, grains, nuts and legumes. For instance, navy, pinto, red, fava, garbanza and small white beans contain 0.10 to 0.74 mg/100g ISOs whereas peanuts contain 0.26 mg ISO/100g (38).

In western populations it is generally these alternate food sources; beans and peas (45%), tea and coffee (25%) and nuts (10%) that are the main dietary source of ISOs (39). Alternate sources include dietary supplements extracted from red clover, containing formononetin and biochanin A which can be metabolised to form soy ISOs. The pharmaceutical compound ipriflavone (chemical structure: 7-isopropoxyisoflavone) is a synthetic source of soy ISO.

1.4.3 Typical Dietary Soy Isoflavone Intakes

The intake level of ISOs required to provide optimal health benefits is still not known. The American Heart Association (AHA) currently recommends a daily consumption between 30-50 mg to offer health benefits. This amount is based on observations that a daily intake of 45 mg/ISO can influence endocrine regulation of the menstrual cycle of premenopausal women (40). To achieve the AHA's recommendation, an individual would have to consume 20-150 g of soy foods or 3 cups of soymilk each day. Whilst the health effects from higher consumptions of ISOs are still not clear, the Italian Health Authority have taken the unprecedented action of advising their public to maintain a daily intake of ISOs lower than 80 mg/day (41, 42) after evidence that higher levels can have undesirable effects in children and infants (10, 43).

In an archetypal Asian diet, SP intake is estimated to be 4-8 g/day (44, 45) which would provide an average ISO intake of 20-50 mg/day (46, 47). In China and Japan, ISO consumption is estimated at 30-40 mg/day (48, 49), whereas in Korea, it is 15 mg/day (50). Despite increased trends in soy food consumption, average ISO intakes reported for the U.S are substantially lower; ranging from 0.15 µg- 3 mg/day (38, 39), with intakes in European countries even lower at 0.5-1 mg/day (51).

1.5 Properties of Isoflavones

1.5.1 Phytoestrogens

A phytoestrogen is defined as any non-steroidal, diphenolic structure that comes from a plant source (i.e. fruits, vegetables, legumes, whole grains and soybeans) which is able to mimic the chemical and structural properties of estrogens. Hundreds of molecules fall under this category. However, the three main groups of biological interest are; ISOs, lignans and coumestans.

As illustrated in Figure 1.3, all phytoestrogens have a phenolic ring structure and hydroxyl group which enables them to bind to the estrogen receptor (ER). Soy derived ISOs such as GEN are the most biologically active of all the phytoestrogens because the distance between the two aromatic hydroxyl groups is almost identical to the distance between the two hydroxyl groups of estradiol (see Figure

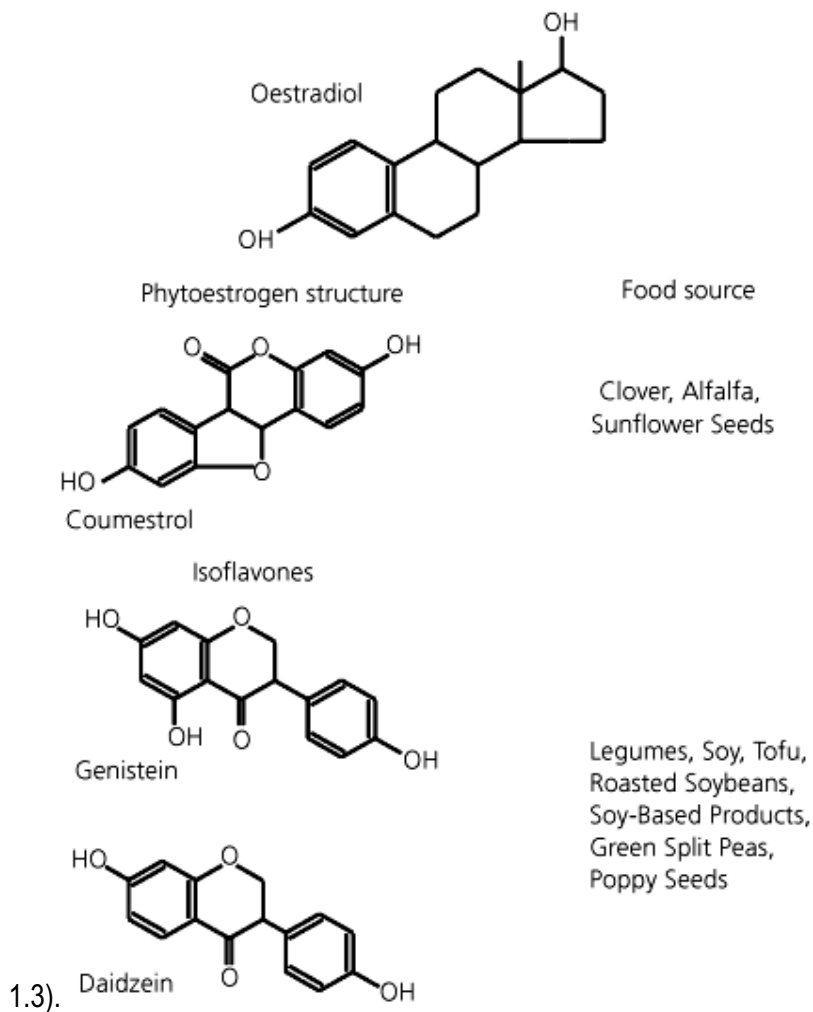


Figure 1.3: Structure and source of notable phytoestrogens. Source: Patiala et al (52).

1.5.2 Isoflavones and their Biosynthesis

ISOs are naturally occurring polyphenolic compounds produced almost exclusively by the members of the *Fabaceae/ Leguminosae* (bean) family. They represent a class of phenylpropanoids, called isoflavonoids due to their biosynthesis from the amino acid phenylalanine. Soybeans are the richest source of the ISO; DAZ (4',7-dihydroxyISO), GEN (4',5,7-trihydroxyISO) and glycitein (GLY;4'7-dihydroxy-6-methoxyISO). All ISOs have a diphenolic structure in which the B ring is attached at the carbon 3 position of phenolic C ring. This configuration differentiates them from other phenylpropanoids, such as flavonoids, which have their B ring attached typically at the carbon 2 position (53).

The biosynthetic pathway for free ISOs and their relationship with several other classes of phenylpropanoids is presented in Figure 1.4. The pathway begins from phenylalanine and has multiple branches which are common to legumes, as well as non-legumes, for synthesising different compounds e.g. flavones, lignans, anthocyanins, and certain classes of phytoalexins. ISO synthesis is dependent on the presence of the enzyme *Isoflavone synthase* which redirects intermediates of the phenylpropanoid pathway from flavonoids to isoflavonoids. Legumes and few other species possess this unique enzymatic function that carries out a 2, 3 migration of the B-ring of naringenin and liquiritigenin, to produce GEN and DAZ respectively.

Other ISOs, like formononetin and biochanin A are also formed from the phenylpropanoid pathway (details not shown in figure) and serve as the precursors for DAZ and GEN respectively. Rich sources of these non-soy ISO are red clover seeds, alfalfa sprouts and chick peas. Figure 1.5 shows the structures of the ISOs produced from this pathway.

Biosynthesis occurs as a result of plant-microbial interactions to enhance survival and growth. As a result, ISOs possess antifungal, antimicrobial and antioxidant properties, as well as the ability to stimulate nodulation genes in soil bacteria, to provide a source of nitrogen for growth (54, 55). This explains the variation seen in ISO concentrations for different soybeans effected by poor soil conditions, high temperatures and pollution (29, 56, 57).

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This figure is included on page 11 of the print copy of
the thesis held in the University of Adelaide Library.

Figure 1.4: A partial diagram of the phenylpropanoid pathway showing intermediates and enzymes involved in isoflavone synthesis, as well as some branch pathways. Dotted arrows represent multiple steps. Enzymes are indicated in italics. Source: Plant Physiology 2000 (58).

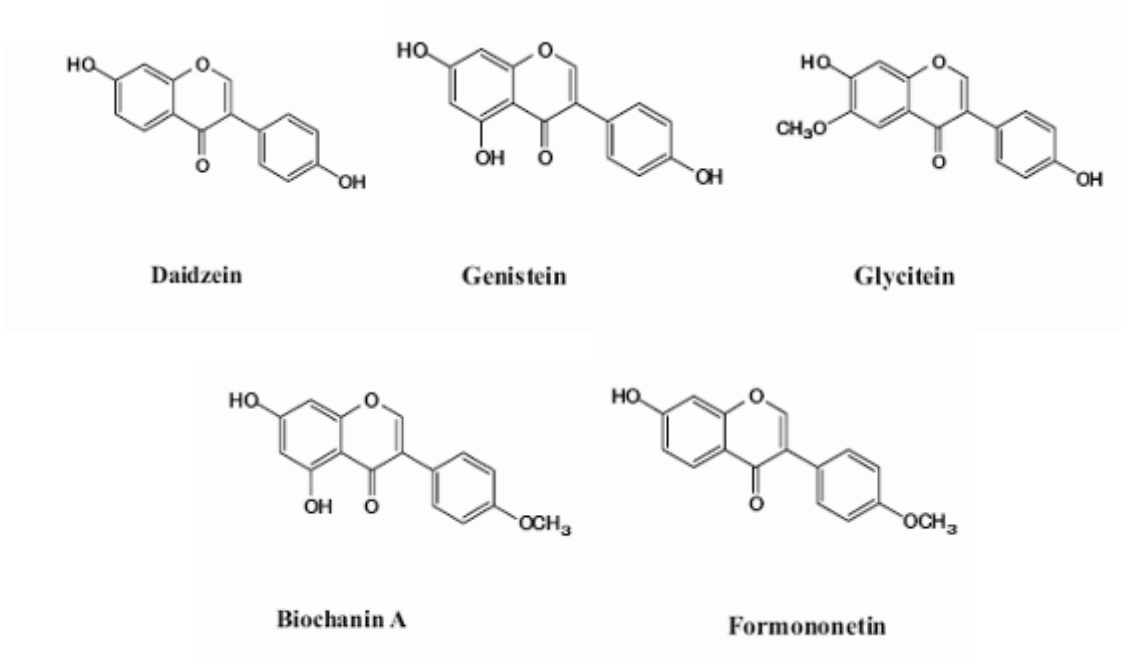


Figure 1.5: Structure of soy and red clover derived isoflavones. Source: Goetzl et al (59).

1.5.3 Soy Isoflavones

As mentioned, soybeans contain three forms of ISOs; DAZ, GEN and GLY which can exist as four isomeric structures; as aglycones and three glucoside conjugates, beta-, acetyl- and malonyl (see Figure 1.6).

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This figure is included on page 12 of the print copy of
the thesis held in the University of Adelaide Library.

Figure 1.6: Four chemical forms of three analogues of isoflavones found in soybeans.

Source: Adapted from King and Bursill (36).

The chemical structure of aglycone ISOs (DAZ, GEN and GLY) enables them to be biologically active at ERs. However, they rarely accumulate to high levels in soybeans constituting only 2-3% of total ISO content. Instead soy ISOs are usually present as their esterified conjugates, predominately as 6"-O-malonylglucoside conjugates with the β -glucoside isomers (daidzin and genistin) being the second most abundant (60).

1.5.3.1 Distribution of Isoflavones in the Soybean

In soybeans, ISOs are predominately located in the cotyledon and hypocotyl regions (99%) which form the embryo and stem respectively within the soybean. Early analysis of soybean fractions has revealed a total ISO concentration of 1405-1750 mg/100g in the hypocotyl, 319-808 mg/100g in the cotyledon and 10-20 mg/100g in the hull. GLY and its glucoside derivatives occur exclusively in the

hypocotyl of the soybean, whereas GEN and its derivatives coexist in both the hypocotyl and the cotyledon. Only DAZ is distributed in all areas (61). In soybeans, GEN is generally present in higher concentrations than DAZ and GLY (62) with levels reaching up to 1600 µg/g compared with up to 1100 µg/g for DAZ and 600 µg/g for GLY.

1.5.4 Bioavailability of Isoflavones in Humans

As endogenous ISOs are a distinctly unique constitute of soybeans, plasma and urine serve as useful markers for their bioavailability in humans. Urine is a particularly good marker because it is easy to collect, contains 100-fold higher concentrations of ISO than plasma and maximum excretion occurs within 24 hours of soy consumption (63, 64).

Early studies measuring the urinary excretion of ISO, following a single dose of soy, found substantially greater recoveries of DAZ compared to GEN, suggesting the latter has greater bioavailability in the systemic circulation (65-70). However, in these studies quantities excreted in urine were shown to vary quite considerably, with levels of DAZ ranging from 16-62% and for GEN 9-37%. It is generally accepted that up to 30% of an ingested dose of ISO in humans is accounted for in urine and plasma (67, 71, 72), 5% in faeces (66-68, 70) and the remainder subject to further metabolism to unidentified phenols or degradation by gut bacteria. Only one study has looked at the bioavailability of biochanin A and formononetin after chronic supplementation and found them to be equally low, with only 1-5% excreted in urine after a 24 hour collection (73)

Although it was concluded that DAZ had a greater bioavailability, based on urinary excretion rates, more recent pharmacokinetic studies comparing plasma ISO concentrations suggest otherwise. Studies involving both animals (74) and humans (70) report higher concentrations of GEN in plasma following soy consumption, indicating greater systemic bioavailability. Furthermore, there is evidence which shows they possess similar bioavailability (63, 65). In what may be the most eloquent study to date investigating the bioavailability of ISO, Setchell *et al* (64) employed the use of stable-isotope-labelled analogues of DAZ and GEN (which were chemically and metabolically inert) to investigate their pharmacokinetics independent of naturally occurring ISOs. Based on serum [¹³C]DAZ and [¹³C]GEN levels, it was concluded that GEN has the greater bioavailability.

1.5.4.1 Factors that Influence Isoflavone Bioavailability

ISO bioavailability varies between individuals, with evidence suggesting around a ten fold difference (66). Bacterial populations in the gut are thought to underlie much of this variation since the intestinal micro flora play an integral role in mediating the absorption and subsequent metabolism of ISOs (75).

Other factors like age, gender, food matrix, chemical isoform and background diet are also postulated to influence ISO bioavailability. As mentioned in section 1.5.3, ISOs are predominately present in soy foods as glucoside conjugates which are biologically inactive. Only soy foods (e.g. miso or tempeh) that have undergone fermentation with bacterial cultures or been exposed to extreme heat contain substantial levels of aglycones (60, 76, 77). Furthermore, ISO glucosides require hydrolysis in the intestine before they are absorbed into the circulation (78) and can elicit a physiological effect. Systemic bioavailability of ISOs should therefore be greatest when consumed as aglycones. However, the evidence to date is inconsistent, with some studies suggesting that aglycones are more bioavailable than their corresponding glucoside conjugates (68, 70, 79), others reporting glucosides are more bioavailable (63) and others reporting no difference (68, 69, 80, 81).

Gender differences in bioavailability have been observed in some studies (82, 83) despite others reporting no such effect (84, 85). The mechanism behind gender differences is not known. However, differences in background diet between men and women have been implicated. Dietary intake of fibre, certain oligosaccharides and resistant starch can all alter micro flora populations of the bowel and more specifically, individual bacterial species which affect intestinal hydrolysis of ISOs. Increased dietary fibre can also influence the transit time of ISOs in the intestine and their potential to be absorbed into the circulation.

The influence of soy food types on ISO bioavailability compared with pure ISO compounds has previously been investigated (79). Faughnan *et al* (82) was the first to address what influence a food matrix delivering ISOs had on bioavailability. In this particular study, ISOs delivered in a solid food matrix yielded higher quantities of the DAZ metabolite, equol, than a liquid matrix. This led the authors to conclude a solid food matrix could protect ISOs from enzyme degradation in the gastrointestinal tract.

1.5.5 Absorption of Isoflavones

Human and animal studies both show that glucoside ISOs are poorly absorbed (86, 87), possibly not at all (78) in the small intestine following their consumption as either a soy food or supplement. Only once they have been deconjugated into their hydrophilic aglycone form do they appear to be rapidly absorbed across the intestinal wall via passive diffusion. In order to convert ISOs into their free aglycone form, esterified ISOs must be decarboxylated and deacetylated by gastric enzymes. β -glucosidase enzymes then cleave the sugar moiety from the newly formed glucoside isomer resulting in a free aglycone. Figure 1.7 shows the formation of DAZ into an aglycone which is ready for absorption across the luminal wall.

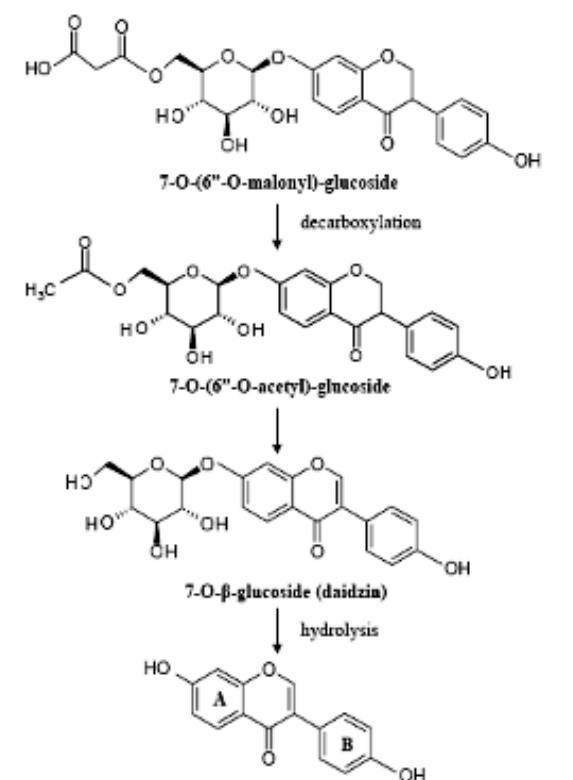


Figure 1.7: Transformation of esterified malonyl glucoside into an aglycone. Source: Wang and Murphy (34).

β -glucosidase enzymes can be both mammalian and bacterial in origin, as shown by absorption of ISOs in both the small and large intestine following consumption and subsequent breakdown of the food matrix. In the small intestine the two mammalian β -glucosidase enzymes that have been identified in the deconjugation of ISOs are the broad specificity cytosolic β -glucosidase enzyme and lactase phlorizin hydrolase (LPH) enzyme. Human cytosolic β -glucosidase enzyme is

present in enterocytes of the small intestine(88), as well as the liver and kidney. However, LPH is present in the luminal side of the brush boarder membrane of the small intestine (89). Those glucoside ISOs that are not deconjugated and absorbed into the circulation from the small intestine are subsequently hydrolysed by bacterial β - glucosidase enzymes in the large intestine.

1.5.6 Metabolism of Isoflavones

Following passive diffusion across enterocytes in the intestine, free aglycones are transformed by the enzyme UDP-glucuronosyltransferase into glucuronide conjugates. Small proportions are also converted into sulphate conjugates by the enzyme sulphotransferase. However, this is thought to occur mostly in the liver. Less than 10% of ISOs remain unconjugated in plasma and urine (90) with hydroxyl groups on the aglycones providing the sites for conjugation.

The wall of the gastrointestinal tract is the major site for glucuronidation of ISOs (74, 91-93). However for those free aglycones (or the few percent of glucosides) that do enter the circulation unconjugated, the liver provides an alternative site (67, 90). Conjugated ISOs undergo classic enterohepatic circulation where they can either be; transported in the systemic circulation to tissues and excreted via the kidneys, or secreted in bile and returned to the intestine (66) . There is also evidence that conjugated ISOs are directly excreted back into the intestinal lumen (94). Upon re-entering the intestine, ISOs are deconjugated by gut bacteria and can either be reabsorbed, returning to the liver via the portal vein, or undergo further metabolism. Figure 1.8 describes the integral steps of human ISO absorption and metabolism.

NOTE:

This figure is included on page 16 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.8: Mechanism of intestinal absorption and metabolism of isoflavone glycosides. BLM, basolateral membrane; BBM, brush border membrane. Source: Satchel et al (78).

1.5.6.1 Formation of Isoflavone Metabolites

The metabolism of ISOs in humans is a multi step process with intestinal bacteria shown to play a major role (95). After deconjugation with glucuronidase/sulfatase, specific bacterial strains in the large bowel can structurally modify and degrade the resulting aglycones into a series of metabolites through dehydroxylation, demethylation and reduction of the double bond of the central C-ring. Some of these newly formed metabolites have been shown to exhibit greater biological activity than their aglycone derivatives. As shown in Figure 1.9, the major metabolites of DAZ produced via the intermediates dihydroequol and dihydrodaidzein, are equol and *O*-desmethylangolensin (ODMA) (96, 97). For GEN, the intermediate dihydrogenistein produces 6'-hydroxy-*O*-desmethylangolensin which can be further metabolised to *p*-ethyl-phenol (not shown)(98). Metabolites of GLY have only cautiously been identified and include 5'-OH- *O*-desmethylangolensin and 5'-methoxy- *O*-desmethylangolensin.

Many studies have demonstrated a significant difference in the biological activities of ISOs and their bacterial metabolites. *O*-DMA for example is shown to have a much weaker affinity for ERs than GEN or DAZ (99, 100). Furthermore equol possess significantly greater biological properties than its precursor which will be described later in this chapter.

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This figure is included on page 17 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.9: Main metabolites and respective intermediates produced from daidzein and genistein.

Source: Kurzer and Xu (101).

1.5.6.2 Factors that Influence Isoflavone Metabolite Formation

Several factors are recognised as enhancing and conversely inhibiting the metabolic conversion of aglycone ISOs. Differences in sub populations of micro flora are probably the most powerful determinant followed by dietary influences.

Certain bacterial strains have been identified in the metabolism of DAZ and GEN (102-104) and these appear to exist only during adulthood, with it well established that infants are unable to metabolise ISOs (105). Disruption to these specific bacteria through either the use of antibiotics, bowel disease, surgery and host immunity is shown to hinder metabolite production (106-108). This is most notable for metabolites of DAZ (95, 97, 109, 110).

Diet can also alter ISO metabolism with carbohydrate and fat intake both shown to cause considerable effects. Increased carbohydrates and more specifically, dietary fibre, is shown to positively correlate with increased production of DAZ metabolites (5, 84, 111, 112) possibly by promoting an environment in which bacteria can grow. Figure 1.10 demonstrates how a high carbohydrate diet promotes production of the metabolite equol. Increased fat consumption is also shown to be negatively correlated with equol production (112).

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This figure is included on page 18 of the print copy of
the thesis held in the University of Adelaide Library.

Figure 1.10: In vitro metabolism of daidzein in a colonic model of fermentation of human faecal flora showing the influence of a high carbohydrate milieu on the rate of conversion of daidzein to the intestinal bacterially derived metabolite equol. Source: Setchell and Cassidy (5).

1.5.7 Pharmacokinetics of Isoflavones

Peak plasma concentrations of DAZ and GEN typically range from 50-800 ng/ mL (113) and occur within 6-8 hours after soy consumption in adults (65-67, 69, 114, 115). Plasma ISOs are conversely detectable within the first 30 minutes following a soy meal with levels reaching 40% of peak value after one hour (65). This rapid appearance in the circulation is probably the result of aglycone ISOs present in the soy meal itself being directly absorbed across the luminal wall in the small intestine.

Pharmacokinetics for ISOs show DAZ reaches peak plasma concentrations before GEN. However it has a faster clearance rate and a lower maximum concentration (65, 71). Most ISOs in plasma are present in their glucuronide form (113), but small amounts do occur as sulphates and can also be detected in even smaller quantities in urine (116). As far as the pharmacokinetics of glucuronide and sulphate conjugates, DAZ conjugates are cleared faster than those for GEN from plasma and urine (117). As one might expect, urinary ISO excretion is dose dependent (118). In urine, DAZ is excreted at far greater concentrations than GEN (67) and at a peak excretion rate 2-3 fold that of GEN. This suggests it undergoes reduced excretion via the bile duct (65, 119).

1.5.8 Discovery of Equol

The chemical structure of equol was first elucidated in 1932 after it was discovered in the urine of pregnant mares (120). At the time, its presence was determined to be a seasonal occurrence rather than a direct result of pregnancy, and was not investigated further. By the 1940's there was renewed scientific interest in equol after it was found to be present in the plasma of sheep that had developed debilitating reproductive and fertility problems. Scientists concluded that equol was the causative agent behind the condition known as "Clover Disease" as a result of being present in the *Trifolium* (red clover) plant (121, 122). It was latter established that red clover contained the methoxylated precursor of DAZ, formononetin. Figure 1.11 describes the formation of equol from formononetin via the intermediate dihydrodaidzein. Equol was later identified in the urine and plasma of adults consuming soy foods in 1982 (96) with its structure fully elucidated by mass spectrometry and nuclear magnetic resonance spectroscopy.

NOTE:

This figure is included on page 20 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.11: Pathway for equol formation after hydrolysis of the glycoside conjugates daidzein from soy, and the methoxylated isoflavone, formononetin found in clover. Source: Setchell et al (123).

1.5.9 Diversity of Equol Production

Almost every animal species studied is capable of producing equol when fed a soy rich diet (123). However, humans are far less efficient, with only 20-35% of Caucasians able to produce the metabolite in detectible quantities (84, 95, 112). The extent to which DAZ is converted to equol is presumably due to differences in the composition of intestinal micro flora (124). Interestingly, the prevalence of equol producers in Asian populations (50%) (70), vegetarians (60%)(125) and males (66%)(125) is shown to be much higher indicating cultural, gender and genetic differences influence its production.

All humans produce equol to some degree, as reflected by the 600-800 fold variation seen in urinary excretions (112, 124, 126). The demarcation of two distinct populations; equol producers and non- producers, has been of particular interest to scientists in attempts to establish health benefits associated with its production. An individual is typically deemed to be an “equol producer” if they have absolute plasma equol concentrations $> 20 \mu\text{g/L}$ ($>83 \text{ nmol/L}$), or urinary concentrations $> 1000 \text{ nmol/L}$ (123) after completing a dietary soy challenge. These threshold concentrations are based upon the distinct clustering of subjects with low/ negligible levels from those with significantly high levels. It is a consistent observation that those individuals who are “equol producers” remain so over time (64, 123) and production can be enhanced dose dependently (127). Attempts have been made to try and convert

non-producers using diets high in unrefined carbohydrates which correlate well with equol production (128). However they have yielded no success (127, 129) indicating intestinal micro flora is relatively stable and resistant to change.

1.5.10 Biological and Structural Properties of Equol

Equol is not a phytoestrogen; rather it is a nonsteroidal estrogen of the ISO class that is an exclusive metabolic product of intestinal bacterial metabolism. Evidence to support the role of bacteria in its generation comes from the inability of animals and humans with compromised gut micro flora, such as germ free rats (110, 130) and young infants(131), to excrete equol when fed soy. Also symptomatic of its formation in the large intestine and colon by gut bacteria, is equol's belated appearance in plasma, compared to DAZ and GEN, with negligible levels only shown >4 hours post consumption (132). Maximum plasma concentrations for equol typically occur after 24 hours, with some adults taking up to 36 hours. Once in the circulation, equol displays a much longer half life and slower excretion rate than DAZ (81). Approximately 50% of equol exists in plasma unconjugated compared to 19% for DAZ and 5% for oestradiol (133). Equol's biological activity is significantly enhanced by its ability to circulate unbound to serum proteins for extended periods after its production; making it more accessible to occupy ER sites.

There is no conclusive evidence that one specific strain of bacteria is responsible for the metabolic conversion of DAZ into equol. In fact, several strains have been identified as being able to carry out the process including, the gram negative *Bacteroides ovatus* spp. and the gram positive *Streptococcus intermedius* spp. and *Ruminococcus productus* spp. (123, 129, 134). New evidence has also emerged using human liver microsomes to suggest equol can be further converted via hydroxylation into metabolites that act as substrates for CYP450 enzymes (135).

In comparison to estrogen, the chemical structure of equol is strikingly similar with Figure 1.12 highlighting their virtually identical planar spatial arrangements.

NOTE:

This figure is included on page 22 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.12: Comparison of the structure of the isoflavone metabolite equol with that of estradiol

Source: Setchell and Cassidy (5).

Unlike its precursor DAZ (109), equol is unique in that it has a chiral carbon atom at position C-3 due to the lack of a double bond in its heterocyclic C-ring. Equol is thus capable of existing in two distinct optically active enantiomeric forms; as S-equol and R-equol (see Figure 1.13). There is no difference in the pharmacokinetics of the equol enantiomers. However because they both differ in their conformations, they do express different ER binding potencies and transcriptional activities.

NOTE:

This figure is included on page 22 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.13: Comparison of the chemical structures of the diastereoisomers of equol to estradiol, showing the site position of the chiral carbon centre. Source: Setchell *et al* (123).

1.6 Mechanism of Action of Isoflavones

1.6.1 Estrogen Receptors- Structure, Location and Activation

ERs belong to the nuclear receptor super family of ligand inducible transcription factors whose members also include the steroid, thyroid hormone, retinoic acid, Vitamin D and nuclear orphan

receptors (136-138). There was only thought to be one ER in the body, with its structure elucidated in 1987 (139). However a second ER, termed ER β was discovered in rat prostate and its cDNA cloned in 1996 (140, 141). The original ER was consequently renamed ER α . ER α and ER β mRNA are transcribed from two distinct genes; the human ER α gene is localized on the long arm of chromosome 6, whilst the human ER β gene is mapped chromosome 14q22-24 (142).

Binding, immunochemistry and *in situ* hybridization studies (142-147) reveal distinct distribution patterns of mRNA for ER α and ER β in the body. As seen in Figure 1.14, ER α is highly expressed in the pituitary, kidney, epididymis and adrenal glands whilst ER β predominates in the prostate, lung and brain. Both ERs are co-localised in the vasculature, breast, ovary and uterus (145). Different isoforms of ER α (148, 149) and ER β (143, 150-153) have subsequently been discovered, but will not be discussed.

NOTE:

This figure is included on page 23 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.14: Anatomical distribution of estrogen receptors; ER α and ER β in males and females.

Source: Setchell and Cassidy (5).

Whilst ER α and ER β subtypes share some homology in their structure, they are genetically and functionally distinct. As shown in Figure 1.15, ERs consist of five structural domains; the N-terminal domain (A/B), the DNA-binding domain (C), a short hinge region (D), a large ligand binding domain (E) and a short C-terminal domain (F). ER α and ER β share virtually identical DNA binding domains (95%) which enable them to dimerise to the same hormone response elements as either homo- and heterodimer complexes (i.e. ER α /ER α , ER β /ER β or ER α /ER β) and mediate transcriptional events (154). ERs do display differences in their ligand binding domains (LBD) (60%) which accounts for differences seen in their binding affinities and ligand specificities. The N-terminus of ER α and ER β is the least conserved region with only 16% amino acid similarity and is shown to contain a ligand independent transactivation site which is important for transcriptional activity (155).

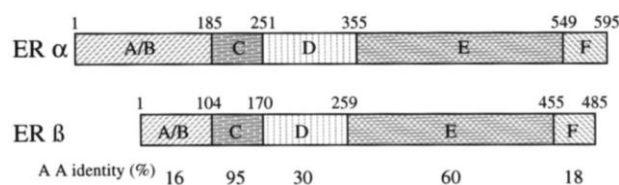


Figure 1.15: Structural comparison of ER α and ER β . The approximate percentage of the amino-acid identity between the structural domains of the two subtypes is given under the ER β figure. The amino acid number is indicated above the figures. Source: Osterlund and Hurd (156).

ERs main function *in vivo* is to act as ligand-inducible transcription factors for genes involved in the regulation of cell growth, proliferation, and differentiation (157). In order for ERs to facilitate transcriptional activation or repression of ER target genes, it must properly recruit co-regulators. Co-regulators are proteins that are tissue-specific and consequently mediate different estrogenic actions in different tissues (158). ER ligands (such as estrogen and ISOs) regulate the recruitment of co-regulators by inducing a distinct formation of the ligand binding and activation domains of ERs so they can bind. Depending on the conformational change induced by the ligand, will determine what co-regulator is recruited by ER α and ER β and whether it will result in transcriptional activation or repression.

Endogenous estrogen, (estradiol) is shown to bind to both ER α and ER β with the same affinity. Consequently estradiol recruits co-regulators non-selectively, triggering both transcriptional activation

and repression pathways for ER α and ER β . Many of the “stimulatory” physiological actions of estradiol are mediated through binding to ER α . Therefore, one of the important roles of ER β when in the presence of ER α , is to modulate this activity by repressing ER α -mediated gene transcription. In its absence, ER β is shown to function as a partial replacement for ER α in estradiol mediated transcription (159). Figure 1.16 describes a study by Lindberg *et al* (159) conducted in wild type (WT) female mice which highlights how ER α behaves as an activator of estradiol mediated gene transcription, whereas ER β is only a modest activator. Furthermore, it illustrates how in the presence of ER α , ER β acts as a dominant repressor of ER α activity.

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This figure is included on page 25 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.16: ER β inhibits ER α mediated gene transcription in the presence of ER α , whereas it can partially replace ER α in the absence of ER α . Source: Lindberg *et al* (159).

The action of ERs can be either genomic or non-genomic in nature. Genomic effects are delayed in onset and prolonged in duration, requiring hours or days to occur. They require transcriptional events which can be mediated directly or indirectly, to modulate protein expression. Non-genomic effects conversely are rapid in onset and short in duration, taking place within minutes through rapid intracellular signalling pathways and do not require transcription to take place.

ERs are primarily localized in the nucleus of target cells and in the absence of stimulation from an appropriate ligand, will form inactive complexes with heat shock proteins (hsp90, hsp70 and hsp56) (137, 138, 160) which then inhibit their ability to initiate transcription. Coupling of a ligand which has diffused through the cell membrane to the unoccupied nuclear ER complex subsequently triggers a cascade of intracellular events, including the phosphorylation of serine and tyrosine residues and dissociation of the heat shock proteins. The receptor is then able to dimerize to form either a homo- or

heterodimer that can bind to a cognate regulatory DNA sequence in the promoter region of the target gene (known as the estrogen response element-ERE) or with other transcription factors in order to regulate transcription (161, 162). This is a classical direct genomic event.

ERs have also been identified on plasma membrane surfaces of cells (163-166). Binding of a ligand to them can regulate gene expression indirectly via coupling to G proteins or generating a second messenger-regulated DNA binding protein, such as a member of the CREB family, adenylyl cyclase (cAMP)/protein kinase C (PKC), cAMP/protein kinase A (PKA), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK). These cascades are capable of regulating transcription of non-ERE containing genes. This is an indirect genomic event. Figure 1.17 outlines potential genomic and non genomic effects of estrogens and ligands for ERs such as ISOs.

NOTE:
This figure is included on page 26 of the print copy of
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Figure 1.17: Genomic and non genomic actions of estrogen and ligands for estrogen receptors.

Source: Lee and McEwen (167).

1.6.2 Isoflavones Action on Estrogen Receptors

The physiological actions of ISOs are often compared with that of estrogen, due to their classification as a phytoestrogen. Whilst they possess the required phenolic B-ring and 4'-hydroxyl group to occupy an ER binding site, ISOs elicit distinct transcriptional actions from that of estrogen (168).

Unlike estradiol which appears non-selective, soy ISOs preferentially activate the binding of ER β to the ERE (145, 168). GEN specifically has a binding affinity which is 20-fold higher for ER β compared to ER α , whilst DAZ expresses a more modest 5-fold higher binding affinity for ER β than ER α (145, 168). The reason why ISOs display a much higher affinity for ER β relates to the structure of ER β 's ligand binding domain (LBD) which is better suited to their structural configuration and ensures stronger dimerisation with the ERE to activate gene transcription. In the case of GEN, its additional hydroxyl group results in stronger binding to the LBD compared to DAZ.

According to trans-activation curves (124), GEN and DAZ are full agonists for both ER α and ER β mediated transcription, giving maximal binding efficacies that are comparable to that of estradiol. However, when the transcription potency of ISOs is compared with estradiol, they are shown to be generally 100-1000 times less effective (169). In the case of GEN, which may bind with the same affinity as estradiol and have a higher transcriptional efficiency, the concentration required to induce transcription was shown, in a study by Muthyala *et al*, to be approximately 60 fold greater compared to estradiol, whereas DAZ was 1000 fold higher (124). Despite their lower estrogenic potency, ISO can still have potential physiological effects due to high circulating levels, which can exceed estradiol by up to 10,000 fold (113).

Studies analysing co regulator protein recruitment by ISOs to ER α and ER β demonstrate they are 1000 fold more potent at triggering transcriptional activity with ER β compared with ER α (170, 171). The reason for this is thought to be due to ISOs being able to induce a functional AF-2 surface in ER β but not ER α which co regulator proteins bind to. ISOs were also shown to be 10-300 fold more potent at triggering transcriptional repression compared to transcriptional activation with ER β . This evidence demonstrates that whilst they may bind to both ER subtypes, ISOs act as weak ER α agonists but potent ER β agonists because they are only effective at triggering transcriptional repression or activation with ER β . This explains why ISOs do not always function as classic estrogen agonists for gene transcription in all tissues. Instead, they act as selective estrogen receptor modulators (SERMs) provoking either an agonistic and/or antagonistic estrogenic effect depending on which ER subtypes are present. Tissues which exclusively express ER β such as the prostate, lung, bone and brain will be responsive to ISOs (5)

and experience estrogenic effects because they are able to stimulate ER β mediated transcriptional repression. In tissues such as the breast, ovary, uterus and vascular epithelium which express both subtypes (5) and are prime sites for estrogen, the estrogenic effects of ISOs will appear as that of an antagonist, as competitive binding of ISOs to ER β will trigger transcriptional repression. Evidence of this nature is best reflected in the observation that supplementation of GEN can exhibit strong estrogenic action in bone and bone marrow to prevent bone loss, without exhibiting estrogenic action in the uterus (172).

1.6.2.1. Isoflavones Action in the Presence of Estrogen

The physiological effects of ISOs also appear to be modulated by an individual's own amount of estradiol. Biological studies looking at effects of ISOs in high and low estradiol environments show that in the presence of high physiologic doses of estradiol, such as that found in peri menopausal women, ISOs act as antagonists, weakening the estrogenic actions of estradiol (168, 173). This inhibitory effect on estradiol action is explained by a competitive reaction for the ER, between ISOs and estradiol. When bound to the receptor, ISOs have an intrinsic lower transcriptional potency which appears to down regulate estradiol's effect on a tissue. In contrast, at low doses of estradiol, close to serum level of postmenopausal women or men, ISOs appear to exert weak estrogen agonist activity (168, 173) through their activation of ER β . Higher concentrations of ISOs are also shown to intensify these agonist and antagonist responses. For example, at concentrations $\geq 5\mu\text{M}$, GEN can antagonise estrogen stimulated cell proliferation similar to the SERM, tamoxifen (174).

1.6.2.2. Other Mechanisms of Action- Anticarcinogenic and Antioxidant Properties of Isoflavones

In conjunction with being able to inhibit estrogen dependent proliferation of cells in certain tissues like the breast, ISOs have other potential anticarcinogenic properties. GEN specifically is shown to inhibit protein tyrosine kinase (PTK) (175) activity which plays a key role in attenuating the growth of cancer cells. (176-178). Furthermore GEN can inhibit epidermal growth factor receptor kinase, cAMP-dependent phosphodiesterase (179), topoisomerase I and II (180), 5 α -reductase (181) and protein histidine kinase(182); which are also involved in pathways that promote cell proliferation and survival.

ISOs simulation of sex hormone binding globulin (SHBG), a circulating transport protein for hormones produced by hepatocytes, is also thought to play a role in preventing hormone dependent cancers as it results in less cellular availability of estrogen (183).

Another physiological property of ISOs that makes them potential anticarcinogens is their ability to function as good antioxidants within cells. The 4'-hydroxyl group, specifically present in their structure, enables them to act as scavenging free radicals (184), preventing the formation of reactive oxygen species that can initiate DNA damage (185). They are also shown to increase the expression of antioxidant proteins such as metallothionein (186), as well as inhibit the expression of stress-response related genes (187) which can contribute to DNA damage and the proliferation of cancerous cells (188).

Antioxidant properties of ISOs can also be anti atherogenic as shown by their ability to inhibit lipid peroxidation, by acting as scavengers of lipid peroxy and metal-ion radicals and suppressing the formation of plasma lipid oxidation products (184, 189). ISOs are also shown to be resistant to oxidative damage (190).

1.7 Equol

1.7.1 Potency of Equol relative to Oestradiol and other Isoflavones on Estrogen Receptors

It has been conclusively demonstrated that when humans consume soy ISOs, those who are equol producers only produce the naturally occurring S-equol enantiomer, not R-equol (191). This is particularly pertinent with respect to equol's potency *in vivo*, as only S-equol is shown to bind to ERs, most specifically ER β (191, 192) with sufficient affinity to be of physiological relevance. In competitive binding affinity assays, S-equol is shown to have a 13-fold preference for ER β compared to ER α (124). R-equol conversely shows a weak preference in favour of ER α . As seen in Table 1.2, the preference of S-equol for ER β is similar to that of GEN and considerably greater than that of DAZ.

In terms of S-equol's binding affinity at equivalent concentrations to estradiol, Setchell *et al* (191) reported approximately 20% for ER β and 2% for ER α . R-equol in comparison was much lower at 1% and 0.47% for ER β and ER α respectively. These values are somewhat higher than those reported earlier by Muthyala *et al* (124) (see Table 1.2). However, they substantiate Setchell *et al*'s observation

that S-equol has a much higher binding affinity for ER β . Muthyala *et al* (124), found the transcriptional potency of equol was greater than that of DAZ when present at equivalent concentrations. Of interest was that equol did not show a significantly higher transcriptional potency for one specific ER subtype (refer Table 1.2). As equol activates the binding of ER β to ERE with slightly more effectiveness than the binding of ER α to ERE, this means DAZ can be changed from a specific activator of ER β to an activator of both ERs in humans who can convert DAZ to equol. As far as its estrogenic potency compared to GEN, equol's transcriptional potency for ER β at equivalent concentrations is less but comparable for ER α .

Ligand	Relative Binding Affinity (RBA ^a %)		Transcriptional Potency (EC ₅₀ ^c)	
	ER α	ER β	ER α	ER β
Estradiol	100	100	0.021	0.11
Daidzein	0.010 \pm 0.006	0.040 \pm 0.001	250	100
Genistein	0.017 \pm 0.003	7.4 \pm 0.5	80	6.6
(\pm) Equol	0.20 \pm 0.02	1.60 \pm 0.04	200	74
R (+) Equol	0.40 \pm 0.04	0.30 \pm 0.02	66	330
S (-) Equol	0.10 \pm 0.01	3.20 \pm 0.06	85	65

Table 1.2: Relative binding affinity expressed as a percentage (RBA^a %) and transcriptional potencies (EC₅₀^c) of isoflavones and equol for ER α and ER β at equivalent concentrations. Table adapted from Muthyala *et al* (192).

1.7.2 Other Pharmacological Activities of Equol.

In addition to its estrogenic effects, equol is also recognised as possessing other potent pharmacological properties. For instance, equol is shown to possess unique anti-androgenic activity (193). Unlike most anti-androgens which block activity directly via the androgen receptor, equol binds to the testosterone metabolite 5 α -dihydrotestosterone (5-DHT), altering its conformation so that it is unable to dimerise and initiate transcription (193). Tissues, such as prostate and epididymis, which are dependent on 5-DHT for cell proliferation are therefore most affected by the production of equol. This can be beneficial for protecting against cancer, as shown by Hedlund *et al* (194) who demonstrated equol's ability to inhibit benign and malignant epithelial cell proliferation of the prostate.

Equol is also shown to possess superior antioxidant activity compared to all the ISOs and respective metabolites (135, 185, 195). The enhanced antioxidant activity of equol (and its 4-hydroxy and 5-hydroxy derivatives), is thought to be directly linked to its reduced, non planar structure which confers greater flexibility to penetrate the interior of cell membranes, particularly lipid particles such as low density lipoproteins (LDL). Once inside the cell, equol inhibits superoxide radical production (O_2^-) by virtue of its ability to enhance NO bioavailability (a potent LDL antioxidant). Furthermore, equol is shown to inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (173) which is involved in cell mediated LDL-oxidation.

1.8 Cardiovascular Health

1.8.1 Cardiovascular Disease

Cardiovascular disease (CVD) is a major health and economic burden throughout the world, especially in developing countries (196) and refers to all clinical disorders that affect the normal functioning of the heart, coronary, cerebral and peripheral blood vessels.

Hypertension, coronary heart disease (CHD), myocardial infarction, angina, peripheral vascular disease, stroke and heart failure are the most common examples of CVD. It is estimated that by 2020, CHD will become the world's single leading public health problem (196).

1.8.1.1 Rates of Incidence

Russia is reported to have the highest death rate from CVD whilst Japan has the lowest. The CVD death rate in Australia is ranked the sixth lowest worldwide, which compared to Japan is around 1.5 times higher.(196). According to the latest figures released by the Australian National Heart Foundation (197), CVD affects more than 3.67 million Australians, killing one Australian every ten minutes. In 2004, CVD was suffered by one in six Australian's and was the leading cause of death, accounting for 35% (48,000) of all fatalities in the country. CHD is reported as the single largest cause of death followed by stroke (197, 198).

1.8.2 Pathogenesis of Cardiovascular Disease

Two major underlying conditions associated with the pathogenesis of CVD are atherosclerosis and arteriosclerosis (199, 200).

Atherosclerosis is the main pathological process leading to coronary artery disease, cerebral artery disease and peripheral artery disease. It is a progressive, degenerative state which begins early in life and progresses gradually through adolescence and early adulthood (200, 201). It is usually asymptomatic for a long period with its rate of progression influenced by CVD risk factors (see section 1.8.4). Atherosclerosis is characterised by the accumulation of excessive amounts of cholesterol-rich lipid plaques beneath the blood vessel endothelium. Plaques result due to inflammatory cells attaching to a dysfunctional endothelium, migrating into the underlying intima and promoting subsequent LDL-C accumulation (202-204). As increasing amounts of LDL-C are deposited in the intima, plaques become more prominent leading to an overall narrowing of arteries. Continued exposure of CVD risk factors, especially regular dietary intake of fat and cholesterol, can cause endothelial cells to retract, rupturing these plaques and exposing them to the circulation, where a blood clot can form (known as a thrombus). The thrombus, which forms as a result of increased platelet aggregation, ultimately leads to occlusion of blood flow through the artery to vital organs such as the heart and the brain (199).

Arteriosclerosis is a disease state that affects all arteries of the CV system and develops with age and in the presence of CVD risk factors; namely hypertension, insulin resistance, and obesity (refer to section 1.8.4 for descriptions). Its characterised by smooth muscle cells (SMC) and calcium (Ca^{+2}) deposits accumulating in the intima and/or media arterial layers to form lesions that lead to increased stiffness and narrowing of the arterial lumen (200). Vascular smooth muscle cell (VSMC) proliferation, which occurs when the endothelium fails to secrete NO, is considered the crucial step in the development of arteriosclerotic lesions as opposed to lipid accumulation in atherosclerotic plaques. The clinical manifestations of atherosclerosis and arteriosclerosis include angina, myocardial infarction, transient cerebral ischemic attacks and strokes.

NOTE:
This figure is included on page 33 of the print copy of
the thesis held in the University of Adelaide Library.

Figure 1.18: Schematic diagram of processes involved in atherosclerosis. Source: Novagen Website (205).

As shown in Figure 1.19, physiological events that precede the development of atherosclerosis and arteriosclerosis include impaired endothelial function (206, 207) and decreased arterial compliance (208-210).

NOTE:
This figure is included on page 33 of the print copy of
the thesis held in the University of Adelaide Library.

Figure 1.19: Physiological events which precede atherosclerosis. Source: HDI/Pulse Wave TM CR-2000 Research Cardiovascular Profiling System: Operators Manual (211).

1.8.3 Biomarkers of Cardiovascular Disease Progression

1.8.3.1 Endothelium

The endothelium is a simple, squamous layer of cells that lines the inner (blood- contacting) surface of all vessels in the CV system. It is the largest active paracrine, endocrine and autocrine organ found in the body and due to the ever- changing haemodynamic environment in which it is located, serves a pivotal role in the normal functioning of arteries (212).

In terms of understanding arterial disease, its role in regulating vascular tone is of particular importance (213, 214). The endothelium controls tone by synthesizing and secreting a variety of dilating

and constricting substances, which act directly and in combination with one another on the blood vessel wall (215). The endothelium also plays an important role in maintaining blood fluidity (216).

Dilating agents secreted directly by the endothelium include nitric oxide (NO), prostacyclin, bradykinin and endothelium derived hyperpolarizing factor (EDHF). Constricting agents that are released include endothelin (ET-1), superoxide anion, endothelium-derived constricting factor, angiotensin II and thromboxane (214). NO is the most physiologically important for controlling vascular tone. Blood fluidity in contrast is maintained by a host of endothelium-derived substances that prevent platelet adhesion and aggregation (212, 216) which are two critically important biological actions against vascular injury, inflammation and thrombosis.

1.8.3.1.1 Nitric Oxide

NO is generated in endothelial cells by the enzyme eNOS which converts the amino acid L-arginine to NO and L-citrulline (217). Synthesised NO diffuses from the endothelium to VSMCs located in the media layer where it elicits relaxation via the cyclic guanosine monophosphate (cGMP) cascade (see Figure 1.20). This pathway activates cGMP-dependent protein kinases (PKG) that increase cGMP formation; reduce intracellular Ca^{+2} concentration and decrease Ca^{+2} -dependent phosphorylation of smooth muscle myosin regulatory light chains (smRLC). These cellular changes open potassium (K^{+}) channels and sequester the release of Ca^{+} from the sarcoplasmic reticulum to initiate the discharge of relaxing factors and cause subsequent dilation of arteries (218).

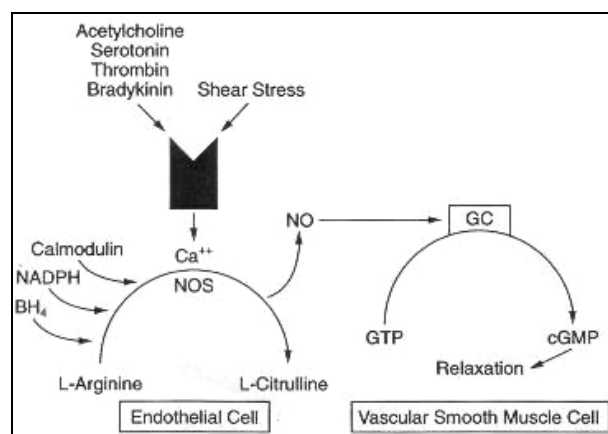


Figure 1.20: Nitric oxide production from the L-Arginine pathway and its activation of smooth muscle relaxation. Source: Kinlay and Ganz (199).

NO released from the endothelium also acts in the lumen and inhibits leukocyte adhesion to the endothelium (219, 220), SMC proliferation (221), platelet aggregation (222) and the release of inflammatory cytokines (219, 221). Physiological factors, such as sheer stress, neurohumoral mediators and pharmacological adrenergic stimulants can all activate receptors on endothelial cells to stimulate NO production (223). Besides stimulating the L-arginine/NO pathway, activation of endothelial receptors can also stimulate the cyclo-oxygenase (COX) pathway to generate prostacyclin (PGI₂) from arachidonic acid. PGI₂ causes SMC relaxation by activating adenylate cyclase (AMP) and increasing cAMP formation (224).

1.8.3.2 Endothelial Dysfunction

Endothelial dysfunction is not confined exclusively to coronary arteries, instead it is a systemic condition that can also affect the peripheral and cerebral vasculature (225, 226). Its existence is best reflected by the failure of the endothelium to elicit NO- mediated vasodilatation due to imbalances in vasodilator and vasoconstrictor secretion (212). Reduced NO bioavailability, accelerated degeneration of NO by oxidative species or increased formation of endothelium- derived contracting factors, like endothelin and angiotensin, are typically responsible for the imbalance (201).

In addition to impaired vasodilatation, endothelial dysfunction is also comprised of a specific state of 'endothelial activation' characterised by enhanced cytokine and adhesion molecule expression. Leukocyte recruitment in the vessel wall occurs as a result (202-204) and triggers inflammation, platelet aggregation, coagulation and production of plasminogen activator inhibitor-1 (recognised inhibitor of fibrinolysis); all milieu that favour atherosclerotic disease progression (227-229).

1.8.3.2.1 Identifying Peripheral Endothelial Dysfunction

To assess the functional integrity of the peripheral endothelium *in vivo*, a test for flow-mediated dilatation (FMD) of the brachial artery is used. The non-invasive technique induces NO- mediated dilatation via BP cuff-induced ischemia (213). Shear stress generated by the release of the cuff exposes the endothelium to a range of tractive forces (230) which in turn triggers the immediate release of NO to initiate vasodilatation via activation of the L-arginine/NO pathway. Shear stress also increases the

expression of enzymes, eNOS (231) and superoxide dismutase, which are involved in NO production and protection against oxidative degeneration respectively (232). Vasodilatation, as measured by the change in diameter of the brachial artery in response to releasing the cuff, is then used as an indicator of whether endothelial function is normal. The correlation between coronary artery endothelium-dependent vasomotor responses and FMD in the brachial artery is well established (233) as is its usefulness as a surrogate in assessing predisposition to atherosclerosis in patients with cardiac risk factors (234-237).

1.8.3.3 Arterial Compliance

Arterial compliance refers to the degree to which the internal diameter of an artery changes in response to a corresponding change in intravascular pressure. It reflects the ability of an artery to expand and recoil in response to ejection of the left ventricular stroke volume (systole) and subsequent relaxation (diastole) (238). The fact that arteries naturally stiffen is not a new phenomenon with several studies demonstrating an age-dependent decline (239). In recent years it has become apparent that a loss in arterial compliance is not solely determined by imbalances in structural elements (collagen and elastin) within the vessel wall and distending pressure (hypertension), but that there is also functional regulation by the sympathetic nervous system (240) and endothelial derived vasoactive substances, specifically NO (241). Such findings indicate that functional abnormalities in the endothelium of arteries may underlie the development of arterial stiffness found in individuals with CVD and its risk factors(241).

As illustrated in Figure 1.21, histological examinations of the intima of stiffened vessels reveal abnormal and disarrayed endothelial cells, reduced NO expression and impaired vascular tone. Infiltration of VSMCs, macrophages and mononuclear cells in the media, increased collagen and frayed elastin molecules in the adventitia and pro- inflammatory milieu in the intima are also present (242).

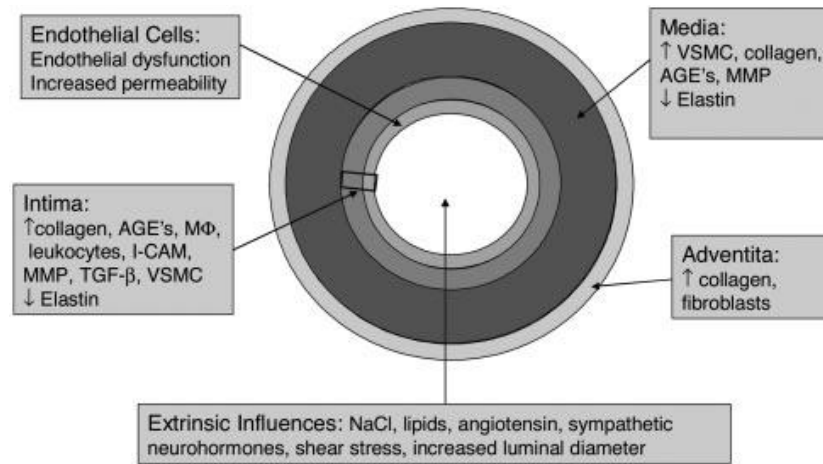


Figure 1.21: Summary of the multiple causes and locations of arterial stiffness. Source: Zieman *et al* (243).

1.8.3.3.1 Identifying Impaired Arterial Compliance

A decrease in arterial compliance has been shown to occur before the appearance of clinically apparent CVD. There is some debate whether reduced arterial compliance is a marker for the development of CVD or is actually involved in its pathogenesis (244). Several studies have investigated this link and found reduced arterial compliance occurs in certain disease states that are themselves associated with increased CV risk, such as hypertension, insulin resistance and hypercholesterolemia (209), implying it is directly involved.

Reduced compliance in large arteries like the aorta has traditionally been used to identify CVD progression (245, 246). However recent studies confirm reduced compliance in small peripheral arteries also denote its existence (209, 239, 247). Measuring compliance in both small and large arteries may therefore prove beneficial in identifying individuals who have, or are at risk of developing CVD.

1.8.4 Modifiable Risk Factors for Cardiovascular Disease

Risk factors can either contribute to the manifestation of CVD by directly promoting those events such as atherosclerosis and arteriosclerosis which lead to CVD or they can identify the underlying conditions that denote their existence such as endothelial dysfunction or arterial stiffness.

It is well documented that people who smoke (248), are obese (249), hypertensive (245, 246), hypercholesterolemic, have raised blood triglycerides (TG) and are insulin resistant (have non-insulin dependent diabetes mellitus) are at increased risk of developing CVD (250-255). Today 90% of

Australian adults have at least one modifiable risk factor for CVD and 25% have three or more risk factors (197). Latest figures released by the National Heart Foundation (197) report that in Australia, 60% of adults are overweight, 51% have elevated blood cholesterol, 30% have high blood pressure (BP) and 8% have diabetes.

It is beyond the scope of this thesis to describe in detail all recognised risk factors that contribute to the physiological aetiology of CVD. However, those which are associated with endothelial dysfunction; a measurable CV biomarker that contributes to the procoagulant, proinflammatory, and proliferative components of atherogenesis are discussed.

1.8.4.1 Hypercholesterolemia

The term hypercholesterolemia refers to excessively high levels of cholesterol in the blood. It is the most recognised risk factor for CVD, specifically CHD, with the National Heart Foundation stating a total cholesterol concentration greater than 5.5 mmol/L reflects increased risk (256). In hypercholesterolemia, it is the proportion of LDL-C to HDL-C that specifically influences the degree to which atherosclerosis is likely to manifest. This is because LDL-C transports cholesterol to cells in arterial walls where it can accumulate to form plaques, whereas HDL-C carries cholesterol from cells to the liver for removal from the body.

Elevated serum levels (> 2.5 mmol/L) of LDL-C and oxidised LDL-C (lipid dense) specifically increase the likelihood of plaque formation beneath the endothelial lining of arteries through enhanced expression of adhesion molecules which recruit inflammatory cells into the vessel intima (199). Several studies demonstrate the close synchrony between elevated serum LDL-C, the expression of cellular adhesion molecules on the endothelium and the development of inflammatory infiltrates in atheromatous lesions (257).

Elevated LDL-C levels also interfere with important signalling pathways (specifically protein kinase C and G proteins) (231) which can manifest endothelial dysfunction. For instance, LDL-C can impair the synthesis of the vasodilating substance NO and promote the generation of superoxide(O_2^-) within vessel walls to reduce NO bioavailability (213). There is also experimental evidence that

Increased LDL-C alters the expression of eNOS receptors on endothelial cells, decreasing NO production (258).

1.8.4.2 Hypertriglyceridemia

Hypertriglyceridemia is a commonly encountered lipid abnormality. It is frequently associated with other lipid and metabolic derangements and according to the National Heart Foundation of Australia can be defined by a triglyceride (TG) level > 1.5 mmol/L (256). Elevated levels in serum can manifest as a result of various genetic defects leading to disordered TG metabolism, high fat diets, obesity, diabetes, hypothyroidism, and certain medications.

Hypertriglyceridemia, in the absence of hypercholesterolemia, has recently been demonstrated to be associated with atherosclerosis (259) and thus can be considered an independent risk factor for CVD, specifically CHD. Since serum TG's do not directly accumulate in the vessel wall to promote atherosclerosis like cholesterol, it's postulated that the disordered metabolism of TG rich lipoprotein species, such as very low density lipoproteins (vLDL) and chylomicrons, are responsible for TG's proatherogenic nature (260). Several studies have demonstrated how vLDL, as well as chylomicron and vLDL remnants, can enter the sub-endothelial space of arterial walls to form plaques as well as be internalised by monocyte macrophages to form foam cells (261, 262).

1.8.4.3 Hypertension

BP is determined by the amount of blood the heart pumps and the degree of resistance to flow through the arteries and other vasculature. Hypertension (high BP) is categorised by the National Heart Foundation as a systolic BP ≥ 140 mmHg and diastolic BP ≥ 90 mmHg and is a common prognostic marker used to identify individuals at greater risk of developing CVD, in particular elevated systolic BP (SBP) (201).

Elevated BP denotes abnormal function or structure of the vasculature, typically at the endothelial interface. Since factors released by an intact endothelium have a significant effect on the microcirculation, particularly the actions of arterioles that regulate systemic vascular resistance and BP,

unexpected alterations in BP infers the endothelium maybe secreting abnormal concentrations of vasodilators or vasoconstrictors.

1.8.4.4 Obesity

Obesity has long been recognised as an independent risk factor for coronary artery atherosclerosis and vascular endothelial dysfunction (263-265). Individuals with a body mass index (BMI) greater than 30 kg/m² are considered to be obese.

Adipose (fat) tissue is a dynamic endocrine organ, which secretes a number of cytokines that contribute to systemic and vascular inflammation (266). Adipocytokines, a specific group of cytokines produced by adipose tissue, have been shown to directly and indirectly regulate processes that contribute to atherosclerosis including hypertension, endothelial dysfunction, insulin resistance and vascular remodelling (see review by Lyon *et al* (266)). Since people who are obese have excessive amounts of adipose tissue, adipocytokine expression will be significantly increased. Moreover adiponectin, a recently described adipocytokine which is shown to improve insulin sensitivity and inhibit vascular inflammation (267, 268) is reduced in obese subjects and only increases with weight loss(269).

Epidemiological studies suggest obesity- induced atherosclerosis may start in early childhood (270) with Tourian *et al* (271) demonstrating the links between severe obesity in children and arterial wall stiffness and endothelial dysfunction.

1.8.4.5 Abdominal Obesity

Selective deposition of body fat in the abdominal region compared to overall obesity is also recognised as an important predictor of CV risk. Recent studies indicate that abdominal fat accumulation, in particular intra-abdominal fat (visceral), is related to impaired endothelial functional (264, 272). The National Heart Foundation states that a waist circumference for males > 94 cm and for females > 80 cm indicates increased risk for CVD, whilst high risk is > 102 cm males; > 88 cm females. Brook *et al* (273) investigated the link between visceral obesity (using waist-to-hip ratio measurements) and vascular endothelial dysfunction. In obese subjects it was found that having a WHR \geq 0.85 was associated with a blunted response in endothelial dependent vasodilatation. Hashimoto *et al* (274)

found similar results in individuals with visceral type obesity compared to subjects with subcutaneous obesity.

1.8.4.6 Insulin Resistance

Insulin resistance is a compensatory response to the diminished ability of muscle, fat and liver cells to respond to the metabolic actions of insulin. It is characterised by increased glucose, insulin and free fatty acids in the bloodstream due to impaired glucose uptake, metabolism or storage by cells (275). Its presence is shown to represent an early biomarker in individuals at high risk for developing CVD, as it frequently coexists with other common proatherogenic disorders such as abdominal obesity, type 2 diabetes, hypertension and dyslipidemia (raised TChol, LDL-C, TG and lowered HDL-C)(276). When these risk factors cluster together they form a condition known as “*metabolic syndrome*” which is four more times likely to manifest CVD than any individual risk factor (277).

Insulin resistance is thought to promote atherosclerosis by altering the endothelium’s capacity to synthesis and release NO (278). Impaired vasodilatation is detected in individuals with insulin resistance as a result of endothelial cells being unable to internalise insulin to stimulate eNOS and increase NO availability. Intracellular defects in insulin- mediated glucose and lipid metabolism also increase levels of reactive oxygen and nitrogen species which further reduces NO availability.

Lipid profiles of people with insulin resistance commonly exhibit hypertriglyceridemia, elevated concentrations of small dense LDL-C and low HDL-C (277). These increased levels of free fatty acids in the bloodstream can further promote atherosclerosis by potentially being deposited into arterial walls.

1.9 Soy and Cardiovascular Disease

1.9.1 Current Strategies to Reduce Cardiovascular Disease

Public health initiatives designed to prevent CVD tend to target its known causes such as excessive fat consumption, smoking, poor physical activity levels and increased stress. Once CVD is established, treatments range from drugs to modify symptoms, to surgical interventions to repair or replace diseased blood vessels. Current pharmaceutical products primarily focus on treatment when

patients are symptomatic and fall into two therapeutic areas - hypertension and lipid reduction.

However none of these therapeutic approaches address the underlying cause of disease.

1.9.2 The Soy Health Claim

Over the last 20 years there has been considerable scientific evidence to support soy's ability to improve the lipid profile following regularly consumption, especially on TChol and LDL-C. Anderson *et al*'s meta-analysis in 1995 was the first publication to validate the widely touted lipid lowering properties of soy (279). The review incorporated results from over 38 controlled clinical trials and reported that significant reductions in plasma TChol (9.3%), LDL-C (12.9%) and TG (10.5%) could occur in individuals with initially high values of plasma lipids following supplementation of SP in the diet, compared to other animal based proteins, such as whey and casein. The meta-analysis also found that in individuals with only mildly elevated plasma TChol (5.2-6.6mmol/L), SP consumption could induce a 4.4% reduction independent of changes in saturated fat, total fat or dietary cholesterol changes.

The hypocholesterolemic effects of soy were officially recognised in 1999 by the US FDA who issued a specific health claim based on the Anderson *et al* meta-analysis for its consumption. It stated "*25 grams a day of SP, as part of a diet low in saturated fat and cholesterol, may reduce the risk of developing heart disease*" (1). The Nutrition Committee of the American Heart Association followed suit and began promoting the consumption of soy to improve elevated lipid profiles (280). In 2001, the U.K Joint Health Claims Initiative (281) released their modified statement specific to soy's beneficial effect on cholesterol.

The claim stated that "*the inclusion of at least 25g soya protein per day as part of a diet low in saturated fat can help reduce blood cholesterol*".

1.9.3 Role of Soy Protein in Cholesterol Reduction.

The exact physiological mechanism behinds soy's hypocholesterolemic effect is yet to be clearly identified. Soy has been shown to alter intestinal absorption of bile acid and dietary cholesterol (282) as well as down regulate critical enzymes involved in fatty acid biosynthesis namely HMG-CoA reductase and cholesterol 7- α -hydroxylase (283). However, the most compelling evidence is an ability to

directly activate LDL-receptors (LDL-R) present in liver cells. Several studies in humans have illustrated this effect with Lovati *et al* (284) reporting an up regulation of LDL-R in monocytes and Baum *et al* (285) reporting increased expression of LDL-R mRNA following SP consumption. LDL-R are cell surface proteins that play a key role in cholesterol homeostasis; thus increased numbers potentially should reduce plasma cholesterol as more LDL-C is being transferred out of the circulation and into tissues like the liver for clearance. Most research has focused on the protein content of whole soy, which accounts for some 40% of its weight, as the direct mediator for this mechanism (283, 286), with the indigestible soy peptides, β -conglycinin (7S globulin) & glycinin (11S globulin) specifically identified as being able to activate LDL-R at the promoter level (287-289). In a recently published study by Cho *et al* (290), several soybean peptides produced from a soybean protein hydrolysate were shown to be able to directly stimulate LDL-R transcription in the human liver cell line.

Soy globulin sub fractions have also been shown to preferentially bind to cell membrane proteins that have recognised antioxidant activity, such as thioredoxin (291). Such binding is thought to promote increased antioxidant capacity in these treated cells, which may further explain the CV benefits exerted by SP, independent of non protein components.

The underlying mechanism by which soy peptides are capable of permeating the gut membrane, entering the bloodstream and exerting an effect on cell receptors in humans is unclear. Research into SP's activation of LDL-R is based primarily on evidence from cell culture studies (289). Rodent studies offer evidence to support soy globulins' postulated ability to permeate the gut wall. For example, rats fed a SP based diet demonstrate an enhanced rate of epithelial cell damage and conversely proliferation of colonic epithelium compared to those receiving a casein protein diet (292). This suggests soy globulins' mechanism of action involves alteration to epithelial junctions prior to the physiological mechanism by which soy delivers cholesterol benefits. Furthermore, it's been demonstrated that some soy peptides derived from the 7S globulin are partially resistant to digestion with endoproteases and consequently are able to enter the bloodstream as functional oligopeptides (293).

1.9.4 Role of Isoflavones in Cholesterol Reduction.

In the past few years, research developments have surfaced that question whether SP *per se* reduces cholesterol. There is compelling scientific evidence to suggest ISOs present in many SP isolates may be equally as important, if not more influential than the protein constituent, for lowering cholesterol when consumed together (4, 5). This is particularly convincing when interpreting the Anderson *et al* meta-analysis which formed the basis of the Soy Health Claim. Lipid reduction was shown to be greatest in subjects with higher baseline cholesterol values. However no dose-response relationship between SP intake and cholesterol reduction, when changes in cholesterol and lipoproteins were adjusted for changes in the control group (animal protein) were detected.

Studies supplementing soybean protein devoid of ISOs have failed to produce a hypocholesterolemic effect (2, 3), instead the full effects of soy on lipid metabolism appears strongest when SP and ISOs are delivered concurrently (6). This is evident by the reported minimal effects of soy ISOs on blood lipid levels alone (7-10).

1.9.5 Molecular Mechanism of Isoflavones on Cholesterol and Triglyceride Reduction

It's well established that ISOs act as phytoestrogens and bind to ERs mediating agonist and antagonist effects. There is now a large body of evidence to show ISOs are able to activate other nuclear receptors which may underlie their abilities to reduce cholesterol. One of the mechanisms ISOs are postulated to effect lipid metabolism is via their activation of nuclear receptors like the peroxisome proliferator activator receptors (PPARs). There are three different isoforms of PPARs (α , γ and δ) each with their own distinctly different tissue distributions which impact on lipid metabolism in the liver, muscle, adipocyte and macrophages. As shown in Figure 1.22, all PPARs heterodimerize with the retinoid X receptor (RXR) and bind to specific regions on the DNA of target genes. These DNA sequences are termed PPREs (peroxisome proliferator hormone response elements; see reviews (294-297)). ISOs *in vitro* have been specifically shown to act as ligands and activate PPAR α and PPAR γ mediated gene expression (298-300). PPAR α is mainly expressed in the liver, heart and skeletal muscle, kidney and brown fat (301, 302) and plays a critical role in lipid metabolism by regulating the expression of key proteins and enzymes involved in the uptake and β -oxidation of free fatty acids.

Transcriptional activation of PPAR α results in increased expression of fatty acid transport protein which is important in the uptake of fats by the liver. Increasing enzymes involved in the β -oxidation pathway in the liver results in increased TG catabolism.

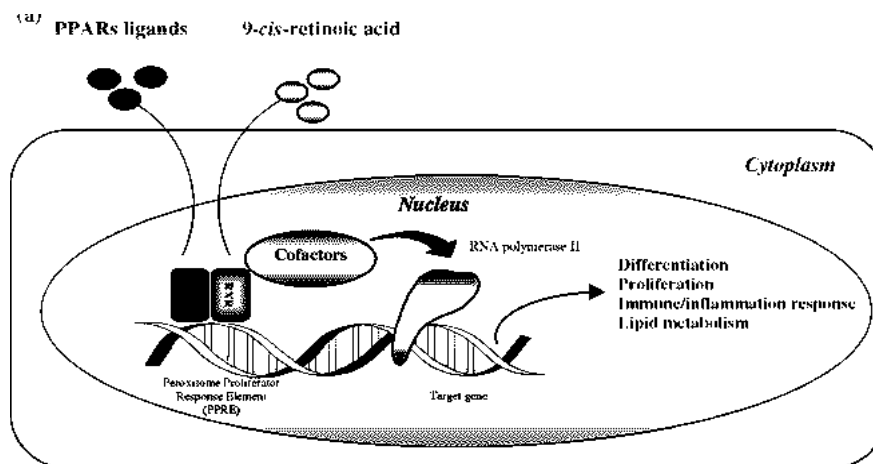


Figure 1.22: General mechanism of PPAR activated transcription. Source: Kuenzli & Saurat (303).

Transcriptional activation of PPAR α is also shown to have other effects on lipoprotein metabolism such as up regulating enzymes involved in HDL-C particle formation, specifically the synthesis of apoA-I and A-II in the liver (304, 305) and increasing bile acid synthesis by upregulating the hepatic expression of nuclear liver X receptor (LXR- α). LXR- α is an important regulator of cholesterol 7 α -hydroxylase, a protein that promotes the conversion of cholesterol to bile acids in the liver (306).

PPAR γ is highly expressed in adipose tissue and is critical in adipogenesis and cholesterol metabolism. Several recent studies (300, 307) highlight GEN's ability to activate PPAR γ and stimulate free fatty acid uptake and TG esterification.

Another postulated mechanism by which ISOs reduce cholesterol is through their ability to up regulate the expression of sterol regulatory element binding proteins (SREBPs), specifically SREBP-2, which binds to promoters of genes involved in cholesterol uptake and biosynthesis such as hydroxymethyl glutaryl-CoA reductase (HMG-CoA reductase) and the LDL-R (308, 309).

1.9.6 Soy and Blood Pressure

A large, population based longitudinal study of Chinese women recently described an inverse relationship between long term soy food intake and BP (310). Evidence from two epidemiological studies (311, 312) suggests ISO consumption and BP are not directly related. To date no observational

studies on ISO intake and BP have been conducted. Evidence for a beneficial effect of ISO on BP has been derived primarily from *in vitro* (313) and animal studies (314) with chronic treatment of GEN shown to decrease arterial BP in rats (315).

In human clinical trials the strongest evidence for a reduction in BP from soy comes from those using SP isolate (316-320), which makes it difficult to ascertain whether the significant BP reductions observed were due primarily to SP or ISO. Furthermore, not one study that has delivered a pure ISO supplement in the range of 50-100 mg/day has reported a significant improvement in BP (9, 321-326), which suggests a putative role of SP in BP reduction. To confound the situation, a recent study by Nestel *et al* reported a significant reduction in SBP in postmenopausal women receiving a synthetic supplement composed of the DAZ metabolite trans tetrahydrodaidzen. After only five weeks, SBP was significantly reduced from 125.6 ± 17.7 mmHg on the placebo treatment to 121.3 ± 12.2 mmHg. Moreover the postmenopausal women in the study were normotensive at the commencement of the study. Dietary interventions using soy foods which incorporate ISOs with SP have failed to report significant reductions in SBP in healthy normotensives (13, 327-332). Only Fuji *et al* (328) reported a significant albeit modest 2mmHg decrease in DBP after 10 weeks of consuming 38g of tofu per day. Only one food study has observed a significant reduction in both SBP and DBP (333). However, subjects who participated in the study were recruited on the basis they were hypertensive (SBP ≥ 155 mmHg and DBP ≥ 100 mmHg). Rivas *et al* (333) observed an unprecedented decrease of 18.4 ± 10.7 mmHg and 15.9 ± 9.8 mmHg for SBP and DBP respectively in individuals consuming 143 mg ISO and 18g of SP/day for 12 weeks. It's important to acknowledge, whilst impressive, the study failed to control other nutritional components in subjects' diets over the three month period such as Ca^{2+} , fibre and Na^{2+} which are known to alter BP and therefore could have exacerbated these reported results.

1.9.7 Soy and Markers of Cardiovascular Disease Progression

1.9.7.1 Soy and Endothelial Function

A higher ISO intake is shown to be associated with better vascular endothelial function in persons who are at a heightened risk of CVD (334). *In vitro* studies demonstrate ISOs and their

metabolites have the ability to enhance endothelial dependent relaxation via increased eNOS activity (335, 336) and expression (313, 337). Enhancement of *in-vivo* endothelial- dependent arterial dilatation is also observed in humans following arterial infusions with pure GEN (338, 339) and dehydroequol (340).

Several human studies have investigated the chronic effects of pure ISO supplementation on endothelial function using the brachial artery vasodilatation test, FMD. Whilst some have seen positive improvements (325, 338, 341) such as Squadrito *et al* (338) who observed a 5.5% increase in FMD after one year treatment of GEN (54mg/day), others report only a trend towards an improvement (342) or no effect (9, 323). Furthermore there are inconsistent findings in the literature (322, 343) for treatment with soy ISO precursors (biochanin A and formononetin) on forearm endothelial function.

Dietary interventions combining SP with ISO in foods are equally as inconsistent. Matthan *et al* (329) who assessed FMD after three diets incorporating products made from either whole soybeans, soy flour, or soymilk that provided 37.5g SP and 50-66 ISO/day found no difference in FMD compared with a control (dairy) diet. This is consistent with a study by Hallund *et al* (330) where 50 mg/day of isolated aglycone ISO delivered in cereal bars to hypercholesterolemic women had no effect on FMD despite significantly increasing plasma nitrate/nitrite levels, which is thought to reflect increased NO production. There are only two dietary interventions that specifically report an improvement in FMD (13, 344). Cuevas *et al* (344) reported a FMD improvement from $5.3 \pm 1.2\%$ at baseline to $9.4 \pm 1.8\%$ after providing 40g SP and 80 mg ISO for four weeks as a protein powder mix. Similarly Clerici *et al* (13) reported a $2.3 \pm 0.8\%$ improvement after just four weeks of consuming only 33mg ISOs in pasta. A decrease in brachial artery blood flow velocity, which is considered a surrogate measure of peripheral vasodilatation, has also be observed after six weeks consuming SP powder drinks containing 107mg ISO and 25g SP (345).

1.9.7.2 Soy and Arterial Compliance

To date only one clinical trial (319) has delivered SP and ISOs concurrently and assessed arterial compliance. The majority of previous studies have focused solely on ISOs as the potential mediator and is confirmed by reports from a cross sectional study in 403 Dutch postmenopausal women

which found a higher dietary intake of ISOs was independently associated with lower aortic stiffness (346).

Nestel *et al* (324) was the first to report improved systemic arterial compliance (SAC) following ISO supplementation. In this earliest study, arterial compliance was measured by ultrasound and determined from a pressure (carotid artery) and volume (outflow into aorta) relationship. After 10 weeks supplementation of 80mg/day ISO, SAC was shown to improve by 23%. The same group later confirmed these observations with an 80mg red clover supplement containing a combination of GEN, DAZ, biochanin A and formononetin. After only 5 weeks, SAC was again shown to improve by 23% (347). The component in the red clover supplement that mediated the effect, was elucidated in an elegant trial by Teede *et al* (322) as formononetin, the precursor of DAZ. 80mg formononetin for six weeks was shown to significantly improved SAC, as well as pulse wave velocity (PWV), another index of arterial stiffness, whilst no effect was seen with an equivalent dose of biochanin A.

Only one trial (330) has assessed the effect of ISOs delivered in a food matrix. In this study by Hallund *et al*, 50 mg of ISOs was incorporated into commercial cereal bars and consumed daily by 30 healthy menopausal women over eight weeks. In contrast to previous observations (322, 324, 347), treatment with the ISO rich cereal bars was actually shown to cause a significant decrease in SAC compared with control cereal bars.

Lastly there is compelling evidence to suggest ISO metabolites may be more potent at improving arterial compliance. The recent study by Nestel *et al* (348) reported 1 g/day of synthetic DAZ metabolite trans tetrahydrodaidzein over five weeks significantly reduced central arterial stiffness as measured by aorta-femoral artery PWV. This is the first study to investigate the effect of ISO metabolites on arterial compliance.

1.9.7.3 Molecular Mechanism of Isoflavones Effects on Vascular Function

1.9.7.3.1 Isoflavones Increase Nitric Oxide Bioavailability

Best known for its activity as a potent vasodilator, NO also plays an important role in inhibiting the proliferation of VSMCs, the aggregation of platelets, and the adherence and infiltration of

inflammatory cells. The pleiotropic effects of NO on the vessel wall therefore means impairment of NO bioactivity or synthesis will have serious implications, not just on endothelial vasodilatation but arterial compliance and BP.

It has been observed that ISO supplementation causes increased plasma nitrites/nitrates levels and decreased plasma endothelin-1 (325), suggesting they have a putative role in NO regulation. The exact mechanism as to how ISOs might regulate NO production and exert a vascular protective effect is still unclear. However, enhanced eNOS activation and expression, as well as up regulation of antioxidant defence enzymes through multiple ER dependent and independent actions on the vascular wall has been demonstrated. As described in section 1.8.3.1.1, eNOS is critical for the production of NO by the endothelium. As seen with estrogen (349), ISOs can bind to ERs in endothelial cells and stimulate eNOS expression via direct and indirect kinase-mediated (350) genomic events (see Figure 1.23).

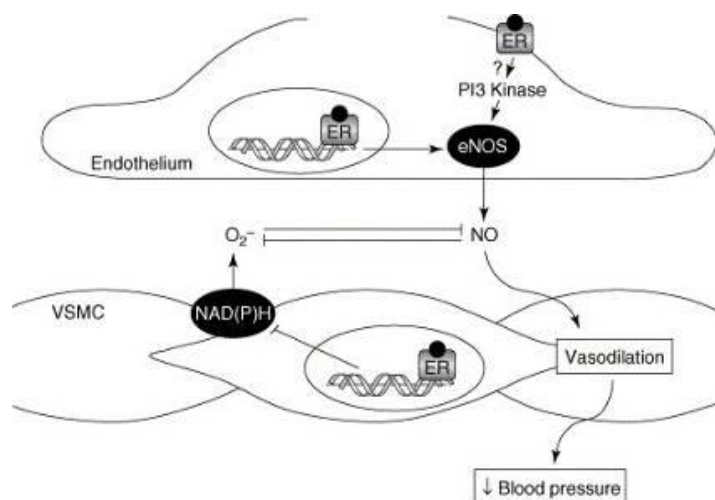


Figure 1.23: Postulated estrogen receptor – dependent genomic and non genomic mechanism by which isoflavones improve vascular function. Source: Dantas and Sandberg (351).

There is also compelling evidence from a recent study by Si and Lui (352) to suggest ISOs can also incite genomic effects on eNOS expression independent of ER and estrogen signalling pathways. Evidence for their direct action on the vascular wall comes from observations that long term exposure of primary human aortic endothelial cells (HAECs) to GEN enhances eNOS gene transcription and protein synthesis in the presence of an ER antagonist. Furthermore, eNOS expression is not mediated by

GEN's ability to inhibit PTK. This is because when DAZ, which is inactive for PTK inhibition, is exposed to HAECs, it is shown to be as potent as GEN in stimulating NO (352).

There is some suggestion ISOs regulate this type of eNOS expression in endothelial cells through binding with the estrogen related receptor $\alpha 1$ (ERR- $\alpha 1$), a nuclear orphan receptor that is able to modulate estrogenic actions, specifically eNOS gene transcription by binding to ERE (353).

PPAR α and PPAR γ are also shown to be present in endothelial cells (354) and their activation have been shown to enhance eNOS expression (355) and NO release (356). As ISOs are agonists of PPAR α and PPAR γ (see review (357)), they may also enhance NO synthesis through activation of these non ER related receptors.

ISOs can also increase NO synthesis through non genomic activation of eNOS. At nutritionally relevant plasma concentrations, direct binding of equol to membrane bound ERs in human endothelial cells activates ERK1/2 and PI3-kinase/AKT signalling pathways which directly phosphorylate eNOS and lead to increased NO production (336). Lui *et al* (342) also demonstrated *in vitro* GEN could phosphorylate eNOS via activation of the cAMP/PKA cascade which is an ER independent intracellular signalling pathway.

With respect to improving endothelial function via enhanced NO bioavailability and reduced oxidative stress, there is evidence to support that ISOs increase the expression of antioxidant defence genes (313, 358). Mahn *et al* (313) demonstrated long term feeding of rats on a diet containing high levels of GEN and DAZ increased synthesis of the antioxidant enzymes manganese superoxide dismutase (MnSOD) and cytochrome c oxidase which inhibit the generation of reactive oxygen species, like superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2), in endothelial cells (359, 360). ISOs induce antioxidant gene expression either via classical nuclear ERs or membrane ER's which signal kinase-mediated modulation of transcription factors. These receptor complexes which are formed then interact with the ERE of antioxidant genes and trigger transcription (see Figure 1.24).

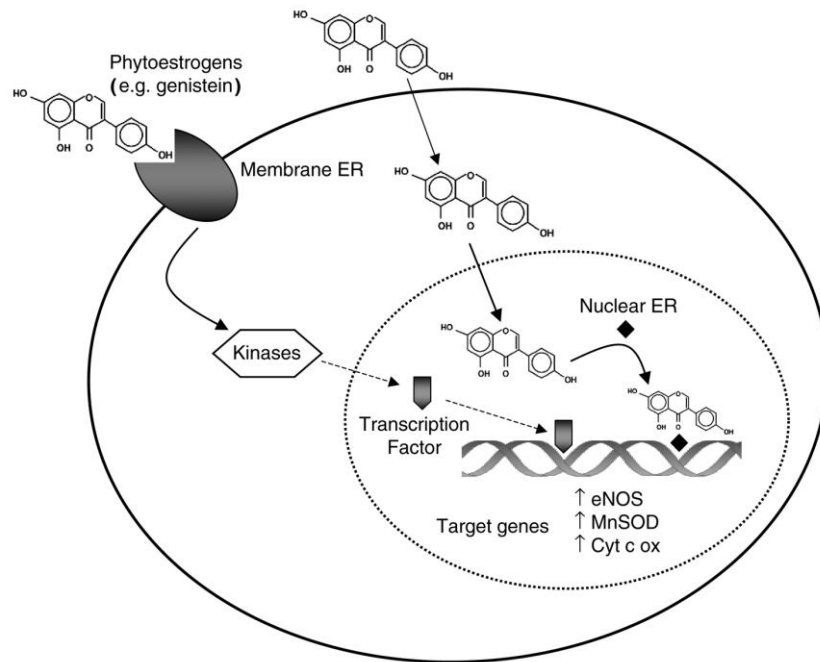


Figure 1.24: Mechanisms by which soy isoflavones may increase antioxidant gene expression (as well as endothelial nitric oxide synthase; eNOS). Source: Valverde and Parker (361).

Evidence from *in vitro* studies (358) demonstrate ISOs can stimulate antioxidant expression for genes that don't have an ERE via activation of transcription factors, like AP-1 and NF- κ B, that can bind directly (362). The MnSOD gene has been specifically shown to have both NF- κ B and AP-1 binding motifs in its promoter region (363). GEN interacts with ERs leading to rapid phosphorylation of the ERK1/2 signalling pathway which activates the NF κ B complex to up regulate MnSOD gene expression (358).

The transcription factor NF- κ B has also been identified in the synthesis NADPH oxidase; a major source of O_2^- in vascular tissue which reduces NO bioavailability (see Figure 1.23)(364, 365). Estrogen has been shown to inhibit NADPH oxidase activity by inhibiting TNF- α -induced NF- κ B activation (366). GEN is also shown to attenuate TNF- α -induced NF- κ B activation in human lymphocytes(367, 368), suggesting it can also inhibit NADPH oxidase production. As shown in Figure 1.25, ISO can modulate NF- κ B activity by either reducing the production of reactive oxygen species (ROS) that phosphorylate the inhibitory subunit I κ B kinase (I κ B) and enables NF- κ B to enter the nucleus, inhibit I κ B directly, reduce NF- κ B's translocation into the nucleus or reduce DNA binding of NF- κ B(369).

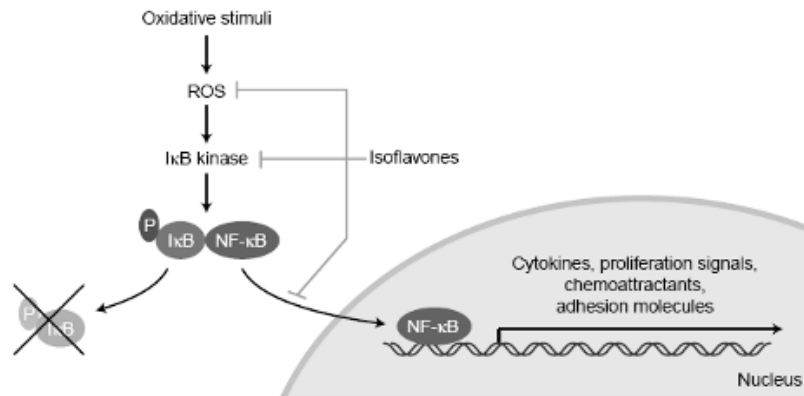


Figure 1.25: The NF-κB pathway as a potential molecular target of isoflavones. Source: Cassidy et al (370).

1.9.7.3.2. Other Mechanisms of Isoflavones in Regulating Vascular Tone and Blood Pressure

ISOs may also improve vascular function by stimulating the synthesis of prostacyclin (PGI₂) (371, 372). PGI₂ a prostaglandin produced in the endothelium from free arachidonic acid through the catalytic activity of two different COX, termed COX-1 and COX-2. PGI₂, chiefly prevents platelet formation and clumping involved in blood clotting. It is also a potent vasodilator able to incite relaxation of VSMCs via opening ATP-dependent K⁺ channels. The exact mechanism by which ISOs increase PGI₂ remains obscure. However, there is strong evidence to suggest the effect is mediated through genomic pathways from classical nuclear ER activation, since treatment of cells with ER antagonists completely abolishes ISO-induced increases in prostacyclin.

Treatment of human endothelial cells with ISOs has also been shown to significantly alter the expression of genes encoding proteins which are centrally involved in regulating vascular tone. GEN specifically, is shown to down regulate genes encoding for endothelin-2 and endothelin-converting enzyme-1 (ECE-1) (373). As previously described, endothelins are potent vasoconstrictors that are also shown to stimulate the production of cytokines and growth factors which promote neutrophil adhesion, platelet aggregation and chemotaxis. Since oxidative stress and inflammation have been implicated in the development of hypertension (374, 375) down-regulation of these genes by ISOs will decrease BP, not only through improved vasodilatation but inhibition of inflammatory responses in the arterial wall.

Soy ISOs may also lower BP through a natriuretic effect. GEN is shown to up-regulate the expression of atrial natriuretic peptide receptor A (ANPA) (373). Activation of this natriuretic peptide

reduces the secretion of inflammatory mediators (i.e. interleukin-1 and TNF α), inhibits the proliferation of VSMCs and along with NO, increases cellular levels of cGMP which decreases endothelin release and promotes decreased BP and arterial tone (376).

GEN's action as a PTK inhibitor (179) can assist in the prevention of VSMC proliferation by antagonising the opening of Ca²⁺ channels to attenuate arterial contractions which can raise BP. Moreover, there is evidence that ISOs and their metabolites can directly block the influx of external Ca²⁺ and release of Ca²⁺ from internal stores to enhance smooth muscle cell relaxation independent of NO, ER related signalling pathways (377, 378).

PPAR α is shown to be expressed in VSMCs (379) as well as the endothelium (354). PPAR α agonists inhibit VSMC growth and proliferation which leads to intimal hyperplasia, by increasing levels of cyclic-dependent kinase inhibitor p27 and decreasing phosphorylation of retinoblastoma protein. They are also shown to decrease inflammatory SMC activation by inhibiting inflammatory response gene expression as a result of PPAR α repression of NF- κ B signalling which produces VCAM-1, IL-6 and endothelin-1 (380). Synthetic PPAR α agonists such as fenofibrate (355) are therefore used to improve BP and arterial compliance. Since ISOs are recognised as agonists for PPAR α (357) as well as modulators of the transcription factor NF- κ B (381) they may induce similar effects.

1.9.8 Clinical Implications of Equol Production in Cardiovascular Disease

In terms of a potential role for equol in the prevention of CVD, Meyer et al (11) conducted a randomized, placebo-controlled crossover trial in 23 mildly hypercholesterolemic subjects and found after retrospective analysis, equol production significantly lowers plasma lipids. In the study, subjects were asked to consume for 5 weeks either dairy or soy-based foods. Despite marked increases in plasma and urinary ISOs after consuming the soy foods, there were no overall differences in subject's plasma lipids, BP or arterial compliance on the diets. Retrospective analysis of a study data however revealed that in eight subjects who produced equol (levels identified in either their plasma or urine were greater than 10 ng/ml), significant reductions were seen for TChol (8.5%), LDL-C (10%), LDL:HDL ratio (13.5%), TG (21%) and lipoprotein(a) (11%) with the soy diet. These reductions were importantly

independent of changes in polyunsaturated fat and other macronutrient intakes which would suggest that the lipid lowering effect of soy may be mediated by equol.

Following on from this study, Krijkamp-Kaspers *et al* (12) conducted a large, parallel, double blind trial investigating the effects of a SP isolate drink containing 25.6g SP and 99mg ISO on vascular function in 202 post menopausal women over one year. At the conclusion of the study no difference in BP or FMD was observed between treatment groups. However, when subjects on the soy treatment diet were categorised based on equol status, those who were producers (n=25) were shown to have a significant decrease in BP (SBP ↓5.6 % and DBP ↓1.6 %). Furthermore, equol producers showed improved FMD responses from baseline (↑1.84%), whereas non producers (n=63) showed a small deterioration in FMD. The small number of subjects in the equol group (n=23) unfortunately meant the difference observed between the two populations for FMD were not statistically significant. However the study provided convincing evidence that equol production is beneficial for significantly lowering BP and improving FMD.

In 2007, Clerici *et al* (13) reported significant improvements in FMD of $2.3 \pm 0.8\%$ for 31 subjects consuming an ISO enriched pasta for four weeks. When these subjects were categorised into groups based on equol status it was actually revealed that only those subjects who produced equol actually exhibited a significant change in FMD from baseline ($P=0.03$), and there was in fact no change from baseline in non equol producers.

These studies clearly highlight the importance of equol in mediating significant improvements in CVD risk factors such as elevated cholesterol, TG and BP. Furthermore they provide convincing evidence that equol is beneficial for improving markers of CVD progression.

1.10. Soy and Cognition

1.10.1 Cognitive Sex Differences

Gender differences in cognitive ability are a widely studied and controversial topic. Women are repeatedly shown to be more superior in language ability (word fluency, spelling, grammatical usage)

(382) and episodic memory tasks (verbal recall of information), whilst males are shown to be more superior at mechanical and visual spatial tasks.

In the past, social scientists have attributed these differences between sexes to be a product of our long evolutionary history as hunters-gathers in which these complimentary roles placed different selection pressures on specific aspects of cognition. Supporters of this schema maintain males have developed brain structures that support cognitive and motor skills needed to ensure successful hunting, such as maintaining orientation while pursuing prey over large unfamiliar territory. Whilst in women, cognition has evolved in ways that support effective gathering, such as the ability to remember the location of potential food sources. The consequence of this evolution is that women are consistently shown to be better at tasks that require memory of object and location and men are better at tasks that require mental transformations of spatial display. As the sexual division of labour between men and women becomes more homogenous in society, it would seem logical that these cognitive sex differences would become less apparent over time. However sex differences, such as that for visual spatial memory, are as large and statistically relevant as when they were first investigated. There is now compelling evidence to suggest it is indeed the role of the sex hormone estrogen which is the major determinant in the organization and maintenance of these cognitive sex differences.

1.10.2 Role of Estrogen Receptors in Cognitive Processes

As described in Table 1.3, ERs are both selectively expressed and co-localised in areas of the brain; predominating in the limbic-related areas (e.g. hypothalamus, pituitary, hippocampus and amygdala) and regions of the cortex.

Table 1.3: Selective and co-expression of estrogen receptors in the brain.

ER- α	ER- β	ER α and ER β
- Hypothalamus; specifically arcuate, periventricular and ventromedial nucleus	- Cerebral cortex	- Pituitary gland
- Temporal cortex	- Prefrontal cortex	- Hypothalamus ; specifically preoptic area
- Pituitary	- Temporal cortex ;specifically layers V and VI	- Cerebral cortex
- Amygdala; specifically hippocampal and cortical nucleus	- Basal forebrain	- Amygdala ;specifically medial nucleus
	- Subiculum	- Entorhinal cortex
	- Midbrain raphe	- Stria terminalis
	- Thalamus	
	- Cerebellum	
	- Hippocampus	
	- Olfactory bulbs	

ER α mRNA is predominately expressed in the hypothalamus and amygdala indicating activation of this receptor subtype modulates neuronal cell populations that are involved in autonomic and reproductive neuroendocrine function; specifically appetite, sexual arousal and thirst in addition to emotional interpretation, information processing and modulation of attention. In contrast, ER β mRNA predominates in the hippocampus, thalamus, entorhinal and prefrontal cortex as well as many widely projecting neural systems such as the basal forebrain cholinergic system, the midbrain serotonin and dopamine systems and the brainstem cholinergic and noradrenergic systems. These areas of the brain are integral in maintaining attention, mood, fine motor skills as well as verbal fluency, memory (declarative and episodic) and visual spatial learning. For that reason it would appear it is the activation of ER β exclusively that plays a putative role in cognition, non-emotional memory and motor functions (156) . High resolution functional magnetic resonance imaging (fMRI) and photo emission tomography (PET) scans reveal increased neuronal activity in the prefrontal cortex (383) and frontal lobe and hippocampal neurocircuitry (384) during verbal fluency tasks . Whereas neuronal activity in the parahippocampal region and parietal lobe is shown to be associated with the performance of visual spatial tasks (384). Table 1.4, describes the location of specific brain regions which are rich in ER β and how their activation is related to specific cognitive processes.

Table 1.4: Areas of the brain where ER β are located and their potential role in cognition.

Area	Location	Role in Cognition
Hippocampus	Part of Limbic System Runs within the inside fold of each temporal lobe	Main component of Memory and Learning Systems <ol style="list-style-type: none"> 1. Declarative 2. Episodic 3. Spatial Learning 4. Visualisation
Entorhinal cortex	Runs between the cortex and hippocampus	Important in Declarative Memory
Cerebral cortex	Largest portion of the brain Convulated outer layer of grey matter	Divided into two hemispheres with each side controlling specific cognitive functions Right Hemisphere <ol style="list-style-type: none"> 1. Spatial Relations 2. Colour Perceptions 3. Visual Interpretation Left Hemisphere <ol style="list-style-type: none"> 1. Analytical tasks- mathematical computation & logical reasoning
Prefrontal cortex		Spatial Working Memory Verbal Fluency
Amygdala	Part of Limbic System Located in anterior part of the temporal lobe Has connections with	Emotional processing of information Attention
Thalamus	Part of Limbic System Sends reciprocal projects to prefrontal cortex	Memory Recall of past information Learning new information

1.10.3 Importance of Cerebral Vasodilatation in Cognitive Performance

There is increasing evidence to suggest improving cerebral vasodilatation can enhance cognition (385). This relationship is thought to arise as a result of improved oxygen and glucose delivery to the blood brain barrier which is utilized by cells to increase growth and survival. The association is most evident in patients who suffer Alzheimer's Disease, as impaired cerebral blood flow is associated with their disease-related declines in cognitive performance (386, 387)

During the execution of mental tasks, blood flow is preferentially distributed to those areas of the brain that are under increased neuronal activity (388). A cross sectional study investigating the effect of estrogen therapy on regional cerebral blood flow using fMRI and PET assessments found a distinct increase in those regions of the brain associated with learning and memory (hippocampus and frontal lobe areas) compared to those individuals who are non-users. Increased blood flow, when

executing verbal memory tasks, was specifically observed in the right inferior frontal region and right parahippocampal gyrus of women on hormone replacement therapy (HRT), whilst for figural memory tasks, increases in the right parahippocampal and right inferior parietal regions were observed. In addition to these significantly different blood flow patterns, the women on HRT exhibited improved scores for memory performance than those women who were non-users. Furthermore, in a longitudinal study assessing the effects of estrogen therapy on cerebral blood flow, women who were users had preserved increases in flow through the right hippocampus, temporal and frontal cortex regions. As ISOs can act as estrogen agonists and these areas of the brain are shown to be rich in ER β , this suggests they may also improve cognitive performance.

1.10.4 Isoflavones and Cognitive Performance

The ability of ISOs to rapidly enter the lipophilic environment of the brain was first reported by Gamache *et al* (389) who demonstrated intra peritoneal injections of GEN and DAZ to adult Sprague-Dawley rats resulted in increased levels in the brain tissue.

Animal studies examining the relative ISO content of specific brain structures, after feeding a phytoestrogen rich, diet have subsequently established that they are most concentrated in regions that are abundant in ER β (390, 391). In a study by Lund *et al*, levels of ISOs in tissue from the frontal cortical region of the brain were found to be approximately 50-fold higher in animals fed a phytoestrogen rich diet compared to those fed a phytoestrogen free diet (391). Moreover, these animals on the ISO diet exhibited improved cognitive performance.

Of the single longitudinal study performed in older males(392), ISO consumption was shown to be detrimental to cognition. Human intervention trials suggest otherwise (393, 394).

File *et al* (393) specifically reported improved non verbal short term memory, mental flexibility and planning ability in healthy adults receiving 60mg ISO equivalents/day for six weeks. Duffy *et al* (394) found similar improvements in cognition with the same dose of ISO over twice as long a period of time as well as enhanced long term episodic memory and attention. Several chronic supplementary trials which provided even greater levels of ISOs have failed to report similar improvements (12, 395, 396).

1.10.5 Molecular Mechanism of Isoflavones Effects for Enhancing Cognition

1.10.5.1 Isoflavones as Neuroprotectants and Neuroenhancers.

As shown in Figure 1.26, ISOs are postulated to improve cognitive function via ER mediated pathways and/or via scavenging antioxidant mechanisms.

In vitro studies demonstrate that at high concentrations, ISOs will act as potent antioxidants, protecting neurons from free radical damage produced from toxic insults (397). Zhan *et al* (398) specifically showed that incorporation of GEN into cortical neuronal cells decreases plasma membrane damage produced by glutamate excitotoxicity and β -amyloid proteins which increase intracellular levels of reactive oxygen species and other milieu that promote cell death.

ISOs at lower concentrations can also exert neuroprotective properties via classical and indirect ER activation, specifically ER β (399), by regulating the expression and activity of proteins involved in apoptotic pathways. GEN is shown to attenuate apoptosis by specifically increasing the expression of anti-apoptotic protein bcl-2 which is normally down regulated in the presence of toxic insults(399-401). Moreover, ISOs increase the expression of β III tubulin, a protein marker of neuronal survival and proliferation and down regulate the expression of BAD, a proapoptotic protein in regions of the brain critical for learning and memory (402).

In addition to protective effects, ISOs improve cognitive performance by altering cholinergic enzyme activity in the cortex, basal forebrain and hippocampus of the brain which leads to increased formation of neurotransmitters and preservation of cholinergic transmissions. Specifically, they are shown to increase the expression and activity of choline acetyltransferase (ChAT), as well as the neurotrophins, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which also assist in neuronal differentiation and synaptic activity (403-405).

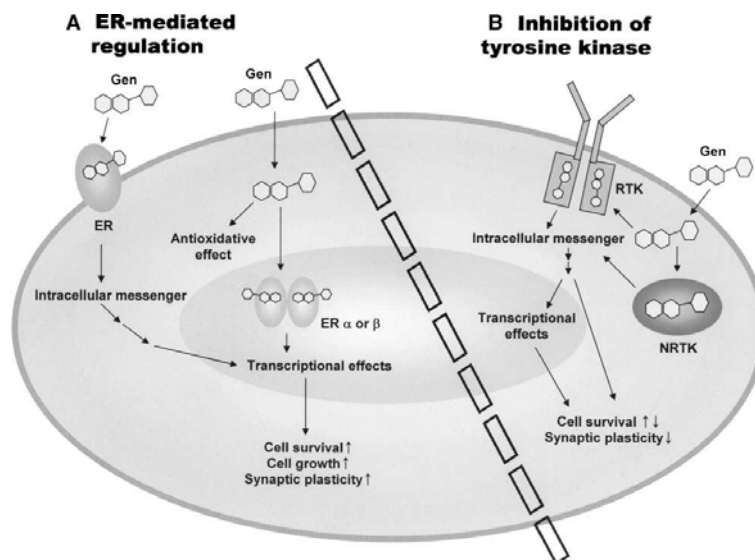


Figure 1.26: Genistein and other isoflavones increase the survival and growth of a neuron, and synaptic plasticity via antioxidant and estrogen receptor-mediated pathways. Source: Lee et al (406).

1.10.5.2 Isoflavone Regulation of Cerebrovascular Blood Flow

NO is a neurotransmitter of the vasodilator nerve in the brain. Activation of ERs in the cerebral vasculature can increase activity and expression of neuronal NOS which produces NO, and via a cyclic cGMP-dependent mechanism, can trigger relaxation of cranial vessels and greater blood flow.

In the brain, ISOs are also shown to increase blood flow via an endothelium ER-independent mechanism. *In vitro* studies demonstrate they specifically block Ca^{2+} entry into VSM to incite relaxation (378) as seen in the periphery. DAZ (407) and equol (408) are also shown to act at the microcirculatory level to increase blood flow in the brain via direct action of the cerebrovasculature. Inhibition of PTK by GEN can also prevent free radical induced vasoconstriction in the brain (409).

1.10.6 Clinical Implications of Equol Production in Cognitive Function.

Only one study to date has elucidated whether equol production provides an additional benefit, beyond that of soy ISOs, in relation to cognitive function (410). Despite no relationship being seen in that study, there are certain properties it possesses that imply it would do so. For instance, equol is shown to have a greater antioxidant capacity than other ISOs and their respective metabolites, which may preserve neurons more advantageously. Furthermore, it has a much higher transcriptional potency on both $ER\beta$ and $ER\alpha$ compared with other ISOs. $ER\beta$ are particularly abundant in the frontal cortex

and hippocampus which control memory and learning. This suggests equol will be able to incite greater ER mediated gene transcription of proteins involved in promoting neural synapses and preventing apoptosis in these areas. There is also evidence to demonstrate equol is able to elicit vasodilatation in both cerebral arteries and the microcirculation which would ensure superior blood supply to regions of the brain during performance of cognitive tasks.

1.11 Thesis Direction and Aims

1.11.1 Direction of Research

There is a plethora of evidence to suggest the physiological properties of ISOs may be beneficial for reducing CVD risk through an effect on lipid reduction, vascular function and several metabolic factors. Moreover, there is increasing molecular and clinical evidence that ISOs can exert a beneficial effect on cognitive performance, through their effects on the vasculature, as well as neurological properties. Opposition remains, nevertheless, to officially acknowledge a CV health benefit from ISOs, as there is a proportionate amount of evidence to support no effect from supplementation.

As ISOs are postulated to enhance CV health and cognition due to their ability to act as estrogen agonists, it is plausible that equol, which has a greater estrogenic potency but is limited in its production by individuals, may mediate these effects. Only a few studies have investigated the importance of equol production in mediating health benefits of ISOs, with the handful that have, albeit in small populations, presenting a convincing argument.

It is evident from the current literature that the role of equol needs to be investigated more robustly using larger populations, in order to resolve the true value of ISOs in the diet. This is not only relevant for the current Soy Health Claim and reducing CVD risk but in the future use of ISOs to enhance cognitive function.

1.11.2 Thesis Aims and Significance

The overall aim of this doctoral thesis was to explore the significance of ISO consumption in the improvement of CVD risk, their potential to enhance cognitive performance and the underlying relevance of equol production in observed physiological improvements. The findings from this thesis are

relevant to understanding the role of ISOs, more specifically equol, in relation to soy's reported health benefits.

To address these aims two intervention trials were conducted. The first was a food-based dietary intervention which was designed to examine the importance of ISO consumption in relation to the current Soy Health Claim and determine the relevance of the recommended quantity of SP when delivered concurrently with ISOs, to lower blood cholesterol. The intervention also explored the effect of ISOs on metabolic risk factors and measures of vascular function, in relation to reducing CVD risk. The second intervention was a supplementation trial designed to investigate the potential of isolated ISOs to enhance cognition and vascular function and whether they were dependent on one another for improvement. The underlying importance of equol in mediating the anticipated health benefits was assessed in each of the interventions.

2.0 Soy Food Intervention

2.1 Rationale for Intervention

Food companies and health practitioners have been promoting the consumption of soy foods to alleviate CVD risk ever since a health claim for SP was released in 1999 stating it could reduce blood cholesterol. Despite knowledge of this benefit, the majority of individuals in Australia find it difficult to consume the FDA's recommendation of 25g SP per day to gain a cholesterol lowering benefit. For most, the quantity is simply unachievable as it requires the consumption per day of at least four serves of soy foods which are traditionally not noted for their palatability.

The current health claim value of 25g/day SP as a definitive threshold for cholesterol reduction can be challenged by dietary interventions which report significant reductions, when consuming a lesser amount. Evidence that ISOs delivered concurrently with SP enhances cholesterol reduction furthermore questions the exclusivity of SP to mediate an effect as the health claim would suggest. Interestingly, Asian countries who are reported to have the lowest incidence of CVD, consume on average half the amount of SP recommended in the health claim. Moreover, they eat traditional soy foods that remain rich in ISOs rather than the myriad of second generation soy foods created by Westerners that are in many cases negligible.

This study will be the first to investigate whether novel foods that combine proportionate amounts of SP and low fat dairy protein (DP), while retaining a full complement of ISOs, can be utilised to lower mildly elevated blood cholesterol, a recognised risk factor for CVD. The design of the novel study foods should provide improved palatability, due to the inclusion of DP, and offer a new marketing strategy to promote the consumption of soy foods in Australia. These novel foods will also allow investigators to ascertain whether a more achievable level of SP, such as 12g, when delivered in combination with a high dose of ISOs will be as effective at lowering cholesterol as 24g SP/day, which is equivalent to the amount that currently supports the health claim.

While the primary outcome of the Soy Food Intervention will be the effect these novel foods have on lowering cholesterol, other indices of risk for CVD will be assessed.

There is strong evidence that ISOs can improve both vascular and metabolic functions via their estrogenic effects on the endothelium and activation of other cellular receptors such as PPARs. The ability of ISOs to improve vasodilator tone can potentially manifest a reduction in BP and improve arterial compliance; two well documented risk factors for CVD. Activation of PPARs by ISOs may also improve anthropometric measures and insulin sensitivity which can prevent the onset of obesity and diabetes.

Data supporting these effects of ISOs is still very limited, but raises some alternate mechanisms by which soy foods can be promoted to offer protection against CVD. Moreover, there is little definitive evidence that following soy consumption, equol production is responsible for mediating improvements to lipid, vascular and metabolic CV risk factors. A large scale study should provide some clarity on this contentious issue.

2.2 General Aims of the Intervention

- a) To develop a range of novel foods enriched with ISOs that combine SP and low-fat DP to enhance the palatability of soy and promote its regular consumption within the community;
- b) investigate whether the postulated hypocholesterolemic benefit of SP could be attained by consuming these novel foods which provide a similar ISO content but either 12g or 24g SP compared with DP consumption;
- c) to determine if supplementing the diet with novel foods enriched with ISOs that combine SP and DP results in other hypothesized CV health benefits of SP only foods, such as improved vascular function and metabolic effects;
- d) To determine the importance of ISOs, especially the potential role of equol, in mediating CV health benefits attributed to SP intake;
- e) Evaluate consumer acceptability and market potential of the novel soy foods incorporating DP.

Aims a) and e) will be addressed exclusively in this chapter. The remaining aims of the intervention will be discussed in the proceeding chapters which assess the effect consuming these novel foods has on plasma lipids, metabolic factors and vascular function.

2.3 Hypotheses of the Intervention

1. Novel foods enriched with ISOs that combine SP with DP will be more palatable/acceptable to consumers compared with novel foods containing SP and ISOs alone;
2. regular consumption of novel foods containing ISOs and SP or a combination of SP and DP in individuals with mildly elevated plasma lipids will produce significant improvements in fasting LDL-C, TChol, TG, HDL-C, glucose and insulin;
3. Increased ISO intake provided by the novel foods will improve anthropometric measures, BP and vascular function; as represented by improved endothelial dependent dilatation and arterial compliance;
4. CV benefits are mediated by equol and will be limited to equol producing individuals.

2.4 Significance and Expected Outcomes

In terms of its significance, this project embodies a highly innovative concept of combining DP and SP with ISOs to potentially improve CVD risk parameters. Evidence supporting the potential health benefits obtained from regular use of soy foods will also further substantiate a health claim for soy foods in Australia which, to date, has only been approved in the US and the UK.

We expect to show from this study that the current health claim recommendation of '25g of SP a day' overestimates the amount of SP necessary to lower blood cholesterol and reduce CVD risk. Instead it is the quantity and subsequent metabolism of ISOs that is more important for reducing blood lipids than SP alone and that novel foods that deliver half the recommended amount of SP (12g) together with 75-90 mg of ISOs will be equally effective.

Unlike previous dietary intervention trials, we expect to show that improvements in endothelial dependent dilatation, arterial compliance, BP and metabolic factors are correlated with the production of equol. This is an important biological relationship that requires further investigation.

2.5 Subjects

2.5.1 Inclusion and Exclusion Criteria

Subjects were eligible for inclusion in the intervention if they had a fasting serum TChol ≥ 5.5 mmol/L, were otherwise healthy, aged between 20-80 years, not on any medication which might influence outcome measures and most importantly were not regular soy consumers. Table 2.1 describes the general exclusion criteria for the intervention.

2.5.2 Justification for Inclusion and General Exclusion Criteria

The decision to recruit individuals between 20-80 years was done to minimise age bias within the intervention. Subjects were excluded from participating if they were taking any form of lipid or BP lowering medication, as they would have affected the primary outcomes of interest in the intervention. Subjects were also ineligible for the intervention if they had a fasting serum TChol ≤ 5.5 mmol/L given that the National Heart Foundation of Australia stipulate a fasting serum TChol ≥ 5.5 mmol/L reflects hypercholesterolemia and subsequent increased risk for CVD. Regular soy consumers were excluded on the basis they would potentially already be benefiting from SP and ISOs in their habitual diets. Additional supplementation with SP and ISOs in these subjects may compromise or mask any effect on the study's outcomes. Subjects with known allergies to soy were obviously excluded as they would have been unable to consume the trial foods. Women who were receiving HRT were excluded as ISOs are reported to mimic some of the physiological actions of estrogen provided exogenously by such medication. Subjects that had been diagnosed with diabetes (411), peripheral vascular disease (412) or were smokers(413, 414) were excluded as these individuals represent a population at risk of having already compromised vascular function. Individuals using phosphodiesterase inhibitors (e.g. Viagra) were also excluded from the study as these types of medication interact with NO dependent pathways and promote vasodilatation. Consumption of >1 g fish oil/day has been shown to positively influence cholesterol levels and blood vessel function (see review by Nestel, P(415)) and therefore regular use could potentially confound study outcomes.

Table 2.1: General exclusion criteria for Soy Food Intervention.

Criteria
<ul style="list-style-type: none">• Consumption of two or more serves of soy products per week• Antihypertensive or hypolipidemic medication• Type I or II diabetes• Smoker• BP: Systolic \geq170 mmHg, Diastolic \geq100 mmHg• Known coronary condition (heart failure, arrhythmia, cardiac valve abnormality, stroke), peripheral vascular, renal or any other CVD• Phosphodiesterase inhibitor medication (eg. Viagra, Levitra)• Known allergies to soy• Fish oil supplementation• Hormone Replacement Therapy (Women Only)• Inability to comply with study protocol in the opinion of study investigators.• Pregnant or lactating• Plant sterol enriched margarines (> 2 serves p/wk)• Known allergies to dairy products (i.e. lactose, cow milk protein intolerant)• Suffer from a blood borne virus (e.g. Active Hepatitis C, Hepatitis B or HIV infection)

2.5.3 Subject Intakes

Subjects were recruited and participated in the intervention in three separate intakes. Two intakes were completed in Adelaide at the Nutritional Physiology Research Centre and one intake of subjects in Perth at the University of Western Australia. The first Adelaide intake was recruited during March- June 2004 and commenced the intervention in July 2004. The second Adelaide intake was recruited from May- July 2005 and commenced the intervention in August 2005. The Perth intake of subjects was recruited during Jan – Feb 2005 and commenced the intervention in March 2005.

2.5.4 Ethical Considerations

Ethical approval for all intakes was obtained from the Human Research Ethics Committees (HREC) of the relevant institutions; University of Adelaide, University of South Adelaide and the University of Western Australia. Refer to Appendix 1 for formal letters of approval sent from each institution's HREC to conduct the intervention. The Therapeutic Goods Administration was also notified of the trial and of the intention to use glyceryl trinitrate (GTN).

2.6 Recruitment

Subjects were recruited by the following means:

- a) Media Press Release, leading to stories in print and electronic media
- b) Paid Volunteer Advertisements and advertorials in metropolitan newspapers
- c) Recruitment flyers
- d) Email and university webpage recruitment advertisements

Refer to Appendix 2 for examples of recruitment material used for the intervention

2.7 Screening of Subjects

2.7.1 Suitability Assessment and Telephone Screening Interview

Individuals who responded to the recruiting campaign were contacted for a brief telephone screening interview to ascertain their potential suitability for participation. The interview involved, asking questions about their general state of health, medical history, and current use of any medications and nutritional supplements. The interview also involved explaining in plain language the key objectives of the study and the different types of assessments that would be undertaken. Time commitments involved with participating in the study was also outlined.

Following the phone interview, individuals who appeared to meet all inclusion criteria were sent a study information pack which included a more detailed diet and lifestyle questionnaire, a volunteer information sheet and a map highlighting the location of the clinic where the screening visit and study assessments would be conducted. After reading the documentation provided in the information pack (see Appendix 2), if individuals were still interested in participating in the study, they were asked to complete the diet and lifestyle questionnaire and return it to the research clinic.

2.7.2 Screening Visit and Information Session

Based on a thorough examination of the returned diet and lifestyle questionnaires of potential study volunteers, those that appeared to meet the entry criteria were contacted by telephone and asked to attend an initial screening visit at the Nutritional Physiology Research Centre Clinic.

At this screening visit, subjects had their height and weight assessed, along with measurements of hip and waist circumference (refer to section 2.9 for protocols for these assessments). Subject's seated BP was also assessed with an Omron Automatic BP monitor (Model T8 with Intellisense). Prior to recordings, subjects were asked to void their bladder and sit quietly for 5-10 minutes. Three recordings were then taken with a minimum interval period of one minute between recordings. A finger prick blood test for plasma TChol concentrations (Reflotron automated analyser, Roche Diagnostics, Basal, Switzerland) was also conducted.

During this visit, it was confirmed that the potential volunteers met the general and specific inclusion and exclusion criteria for the study. Following this screening visit, a letter was sent to subjects notifying them of their acceptance into or exclusion from the study (see Appendix 2). This letter also listed the results of their screening assessments for their personal information.

Following the initial screening visit, all subjects who were recruited for the intervention were asked to attend an evening information session. The main purpose of the session was to provide another opportunity to ask any outstanding questions prior to the commencement of the intervention, meet the other subjects who were also participating and to allow the chief investigators to restate the purpose of the intervention, procedures involved and foods being used. At this session, participants were also provided with the opportunity to taste the food products to be used in the intervention. Time commitments and scheduling of clinic visits was also discussed and confirmed with subjects at the session. Written informed consent was obtained from all subjects at the session (see Appendix 2).

2.8 Methods

2.8.1 Study Design

The invention was supported by an Australian Research Council Linkage Grant (LP0349193) which had So Natural Foods as the industry partner. Furthermore, the study was conducted as a multi-centre collaboration between the Nutritional Physiology Research Centre (which is based in Adelaide South Australia and is jointly affiliated with University of South Australia and University of Adelaide) and the University of Western Australia, School of Medicine and Pharmacology. The intervention used a

three group, Latin Square cross-over design, with each subject undergoing three dietary treatments. Subjects were block-matched into three groups (balanced for age, gender and fasting TChol) and randomised to consume each of the three diets in random order. As shown in Table 2.2, subjects started on either the soy (S), low fat dairy (D) or the soy/dairy (SD) combination food diet for the first six weeks of the intervention. They then crossed over to an alternate dietary treatment for another 6 weeks and again to the final dietary treatment for the remaining 6 weeks of the intervention.

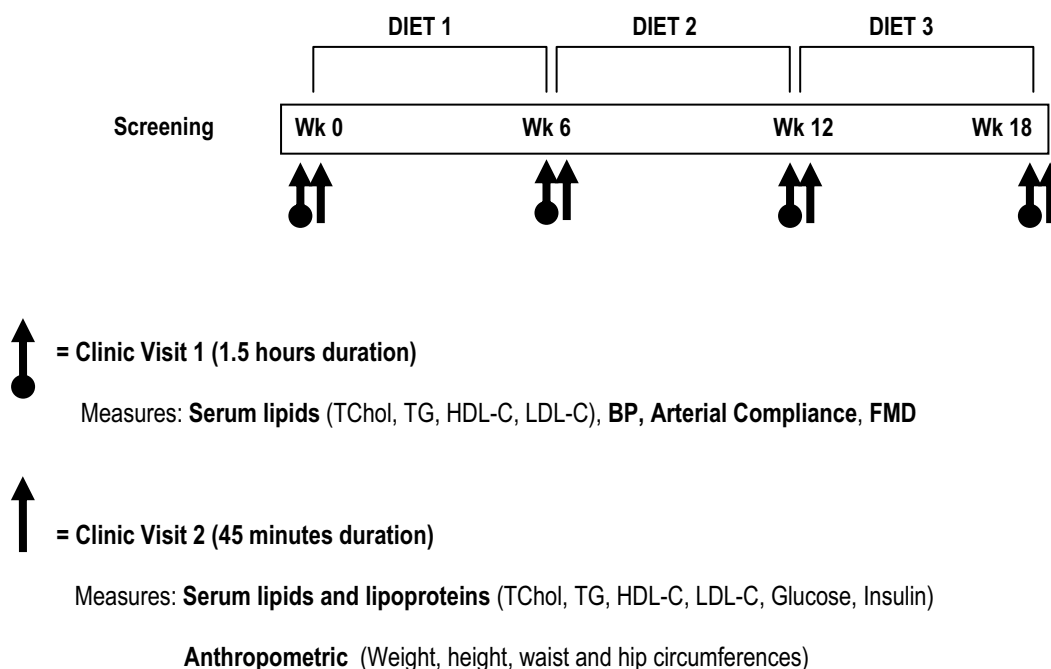
Table 2.2 Randomisation of Treatments on the Soy Food Intervention

Baseline at 0 weeks	0-6 weeks	6-12 weeks	12-18 weeks
Group 1	S	D	SD
	S	SD	D
Group2	D	S	SD
	D	SD	S
Group 3	SD	S	D
	SD	D	S

S=Soy diet, D=Dairy diet (control diet), SD= Soy/Dairy diet

Subjects were required to attend a total of 8 testing sessions over the course of the 18 week intervention, after completing an initial screening visit and attending a volunteer study information evening. As described in Figure 2.1, testing sessions were completed over two consecutive days at the commencement and completion of each of the three 6-week diet phases. The first testing session took approximately 1.5 hours to complete and the second session the following day 45 minutes. A detailed description of outcome measures evaluated at baseline, 6, 12 and 18 weeks of the dietary intervention are described in section 2.9 and the methodology sections of Chapters 3-5.

Figure 2.1: Outline of protocol design for Soy Food Intervention.



2.8.2 Study Protocol

All testing was performed in a quiet, temperature-controlled environment after an overnight fast (minimum 10 hours fasting). As described in the following sections, the study protocol for each intake varied slightly.

2.8.2.1 Intake 1

At the first visit, subjects had their weight recorded and a blood sample collected. Subjects then lay supine for 5-10 minutes to allow their BP to stabilise. BP and arterial compliance was then assessed simultaneously on their right arm using a CR-2000 Research HDI Cardiovascular Profiler (HDI, Egan Minnesota, USA). Assessments were performed in triplicate, with a five minute rest period in between. Subjects then underwent assessments of endothelium-dependent and endothelium-independent vasodilator function using FMD and NTG respectively. Refer to Chapter 5 section 5.4.3 for complete descriptions of these assessment techniques. At the second visit, anthropometric measurements including height, weight, waist and hip measurements were recorded. Refer to section 2.9 for descriptions of individual assessments. Subjects were then seated and a fasting blood sample was collected. Subjects were asked to complete a Food Acceptability Questionnaire (at weeks 6, 12 and 18

only) and return their completed weekly food record sheets and Food Frequency Questionnaires (see Appendix 3). An overnight urine sample was also collected from subjects.

2.8.2.2 Intake 2

Study protocol was the same as for Intake 1. However, no ultrasound assessments for endothelium-dependent and -independent arterial dilatory function were performed.

2.8.2.3 Intake 3

Study protocol was the same as for Intake 1. However no ultrasound assessment for endothelium independent arterial dilatory function was performed.

2.9 Anthropometric Assessments

Prior to anthropometric assessments, subjects were asked to void their bladder (if needed) and wear minimal clothing without shoes.

2.9.1 Weight

Weight was measured to the nearest 0.01 kg using digital scales (Tanita Ultimate Scale™; Model 2000, Tanita Corp., Tokyo, Japan).

2.9.2 Height

Subjects were asked to stand with their feet together, shoulders back and head in a level (Frankfort) plane. Once in this position, height was measured at the highest point of the skull to the nearest 0.1 cm using a wall- fixed stadiometer (SECA, Hamburg, Germany).

2.9.3 Waist and Hip Circumference Measurements

Waist and hip circumference was measured according to International Society for the Advancement of Kinanthropometry protocol (416). Subjects were instructed to stand with their feet shoulder width apart, their gluteal muscles relaxed and arms folded in front of their chest. Hip measurements were taken in a horizontal plane at the level where the buttocks and hips were at their greatest circumference. Waist measurements were also taken in the horizontal plane but at the point where the torso was at its narrowest. For those subjects on whom it was difficult to distinguish a

noticeable waist narrowing, their waist measurement was taken as the smallest horizontal circumference at the midpoint between the lower ribs and iliac crest, as recommended in the National Institute of Health Guidelines (417).

2.9.4 Body Mass Index (BMI)

Body Mass Index (BMI) was calculated using the formula: $BMI = \text{weight (kg)} / \text{height (m}^2\text{)}$.

2.10 Study Foods

2.10.1 Range

Industry partner, So Natural Foods (NSW, Australia) and their affiliate company Freedom Foods (NSW, Australia) provided all soy, dairy and novel soy/dairy combination food products required for the intervention.

Trial foods were based on existing products marketed by these companies that food technologists modified to contain ISOs, SP and/or DP. After performing sensorial tests, the final range selected for use in the intervention was; plain, chocolate and coffee flavoured milks, dry spaghetti, chocolate flavoured cake bars, apricot and bran flavoured cookies and vanilla custard. Milks were provided in ultra heat treated (UHT) tetra packs as 250ml individual servings. Cookies were provided in a packet of nine (net weight 225g) and spaghetti in a 400g packet. Custard was provided as a 200g instant powder mix in a shaker bottle. To prepare custard, 400ml of water or milk was combined with the 200g powder mix. Chocolate cake bars were provided as 60g individual servings.

2.10.2 Nutritional Composition of Study Foods

As previously described in section 2.8.1 foods used in the intervention were specifically designed to provide varying quantities of SP and ISOs. The SP in foods produced for the S and SD phases was achieved by adding SUPRO 760 IP Non GM SP Isolate, containing 88% proteins and 1-3 mg ISO/g SP. In addition to the small quantity of ISOs provided by the SP, the foods were fortified with additional ISOs using Micro IP Non GMO ISOLIFE 2% ISO complex (Soy Health Pty Ltd), which provided 22-30 mg ISO/g. DP in foods was achieved by adding milk protein concentrate MPC70 (M.P.D

Dairy Products), containing 70% DP. Table 2.3 describes the mean SP, DP and ISO content for each serving of the trial foods.

Table 2.3: Protein and isoflavone content per serve of each trial food.

Phase of intervention	Nutrients in Study Food (per serve)		
	SP	DP	ISO
Dairy	0 g	8 g	0 mg
Soy Dairy	4 g	4 g	25- 30 mg
Soy	8 g	0 g	25-30 mg

Subjects were instructed to consume three serves of the trial foods per day from the range available in order to provide a total daily intake of 24g of protein.

Foods formulated for the D diet were specifically designed to be devoid of SP and ISOs as this diet acted as the control phase during the intervention. Novel foods consumed on the SD diet were designed to deliver proportionate amounts of SP and DP (12g each) in conjunction with 75-90 mg of ISOs per day. Whilst foods consumed on the S phase were designed to provide the same ISO intake as the SD foods, but a daily intake of 24 g of SP, which is in accordance with the recommended amount stipulated in the current U.S FDA (1) and U.K JHCI (281) health claims relating to soy intake and cholesterol reduction.

All foods were designed to be low in saturated fat and cholesterol to meet current dietary guidelines (418). The macronutrient profile of each of the trial foods is described in Appendix 4. Due to manufacturing problems, the S diet actually provided subjects with a significantly lower average daily intake of 71.4 ± 1.9 mg ISO/day compared to the SD diet, which provided 76 ± 1.5 mg ISO/day (refer to section 2.16.4).

2.10.3 Availability of Study Foods to Intakes

Not all study foods were available to each of the three intakes. The reason for variations in availability of trial foods was based on results of product acceptability reported by the first intake. Refer to section 2.16.9 for hedonic results of the trial foods from Intake 1. Those products with low palatability ratings were excluded from Intake 2. It was the decision of chief investigators to offer only milk

beverages and custard to subjects from Intake 3 as they were shown to have the greatest rates of consumption in the previous intakes. The availability of foods is reported in Table 2.4 below.

Table 2.4: Individual serving size of trial foods and their availability to the different intakes of subjects during each of the dietary phases.

Food Item	Dietary Phase	Intake 1	Intake 2	Intake 3
Plain Milk	D	X	X	X
	SD	X	X	X
	S	X	X	X
Chocolate Milk	D	X	X	N/A
	SD	X	X	N/A
	S	X	X	X
Coffee Milk	D	N/A	X	X
	SD	N/A	X	X
	S	N/A	X	N/A
Vanilla Custard	D	X	X	X
	SD	X	X	X
	S	X	X	X
Apricot and Bran Cookies	D	X	X	N/A
	SD	X	X	N/A
	S	X	X	N/A
Chocolate Cake Bar	D	X	N/A	N/A
	SD	X	N/A	N/A
	S	X	N/A	N/A
Spaghetti	D	X	N/A	N/A
	SD	X	N/A	N/A
	S	X	N/A	N/A

2.10.4 Assessment of Palatability and Market Potential of Study Foods

To gauge the palatability of the study foods and assess their acceptability in the market place subjects were asked to complete a comprehensive food feedback questionnaire at the end of each dietary phase. The questionnaire addressed issues relating to the hedonics of the study foods by asking subjects to rate their individual appeal on a scale from one to seven, with 1= extremely disliked, 2= quite disliked, 3= slightly disliked, 4= average, 5= slightly liked, 6= quite liked and 7= extremely liked. The questionnaire also asked subjects to comment on the appearance, smell, palatability, mouth feel and texture of the foods as well as whether they would buy this product in the supermarket if it was available and comparable in cost to other brands. Refer to Appendix 3 for a copy of the questionnaire.

2.11 Dietary Assessments of Subjects

During the intervention, subjects were instructed to maintain their normal dietary habits by substituting the trial foods for those they would normally consume. Subjects were also asked to avoid consuming foods known to contain soy for the duration of the study. This was so potential changes in subjects' lipid, metabolic and vascular measures could be attributed solely to the inclusion of SP and ISOs into their diets from the trial foods and not to some other major dietary modification.

2.11.1 Macronutrient Intake of Subjects during Diets

In order to assess whether subject's dietary habits were being maintained over each of the three phases, they were asked to complete a Food Frequency Questionnaire at the end of each six week diet phase. The questionnaire, which was developed by the Victorian Cancer Council for specific use in Australian adults, evaluates both the type and overall frequency of consumption of four categories of foods; cereals (sweets and snacks), dairy products, meat and fish, fruit and vegetables. There is also a separate section related to intake of alcoholic beverages. Based on consumption rates stipulated by subjects in the questionnaire, mean daily energy consumption, dietary macronutrient and micronutrient intake can be derived. The Food Frequency Questionnaire has been used in several large intake studies in Australia and its validity as a reliable measure of dietary intake, specifically in clinical intervention trials, has been established (419-421). Refer to Appendix 3 for a copy of the Food Frequency Questionnaire used.

2.11.2 Isoflavone Intake and Measures of Compliance

Several procedures were implemented during the course of the intervention to ensure subjects maintained their compliance with its protocol. These included the completion of food record forms and urinary analysis of ISO content.

2.11.2.1 Food Record Forms

To ensure subjects were consuming the required three serves of trial foods a day they were asked to record the specific type and amount of each experimental food they consumed on a record form. Food record forms were issued to subjects upon pick up of their trial foods and were returned for

review each fortnight during the three, six week long dietary phases. Refer to Appendix 3 for a copy of food record form. Based on the food record forms completed by subjects, average total and daily ISO intakes were determined for the three arms of the intervention. These values were calculated according to the total ISO content of the individual trial foods.

2.11.2.2 Overnight Urine Collection

At the commencement of each dietary phase, subjects were asked to provide a 50ml sample aliquot of their urine that they had collected overnight for analysis of ISOs. All urine passed by subjects was collected from the time they went to bed, during the night, and till they left the house the next morning. The 50 ml sample aliquot was taken from the total amount collected during this period of time. In order to determine if subjects were compliant with consuming the trial foods during the S and SD phases, and conversely avoiding soy during the D phase of the intervention, the urine samples were analysed for their ISO content using high performance liquid chromatography (HPLC).

2.12 Analysis of Isoflavones in Overnight Urine Samples

2.12.1 Method Development

The method used to quantify ISO's; DAZ, GEN and equol in human urine was adapted from a protocol previously described by King and Bursill (65).

2.12.1.1 HPLC Conditions

A variety of isocratic mobile phases incorporating different ratios of 100% methanol (MeOH), 0.1 M ammonium acetate and 25 mM EDTA or 100% acetonitrile were trialled to ascertain the most accurate separation of ISOs from potential co-elutants. Comparative trials by our lab, found the most efficacious to be an isocratic mobile phase with 45:55:1 of MeOH, 0.1 M ammonium acetate pH 4.6 with glacial acetic acid and 25 mM EDTA disodium salt.

To compensate for the hydrogen reference working electrode in the HPLC system having no inbuilt salt bridge, 50mM potassium chloride (KCl) was added to the mobile phase solution so that 2mM

would pass through the system with each run. It was determined that for 1.1 L of mobile phase produced, 44mls of 50mM KCl need to be added. The working potential of the electrochemical detector (DECADE II E.C.D p.n. 171.0035, Alltech) was trialled at various voltages to maximise the clarity of peaks and minimise background noise. According to Tain *et al* (422) who used HPLC- with ECD to identify ISOs from biological fluids, a voltammograph was established for the ISO's of interest (DAZ, GEN and equol) between +750 and +900 mV. Based on peak area, height and overall delineation of the chromatographs, optimal voltage for analysis was shown to occur at +800 mV.

2.12.1.2 Extraction Protocol

Several solvents were trialled to optimise the recovery of ISOs from samples following enzymatic hydrolysis. Specifically, analytical grade chloroform, dichloromethane, ethyl ether and ethyl acetate were used and their recovery of ISOs, as determined by peak height and concentration from HPLC analysis, were compared. Ethyl acetate was shown to provide the greatest recovery of ISOs.

Optimising the elution of ISOs from the column with either; one wash of 1ml 100% MeOH, one of 800 μ l 80% MeOH or two washes of 800 μ l 80% MeOH was also trialled. It was found that ISOs were optimally recovered after two elution's with 800 μ l 80% MeOH.

2.12.2 Final Method

2.12.2.1 External Standards

External standards for DAZ (Sigma D- 7802), equol (Plantech) and GEN (Sigma G- 664) were prepared based on the method of King and Bursill (1998). Approximately 1 mM stock solutions (equivalent to ~0.25mg/ml) of each pure standard were made in HPLC grade MeOH (Sigma) and stored at -20°C. Solutions were prepared by dissolving 2.54mg of pure DAZ in 10 ml MeOH, 1.21 mg of pure equol in 5ml MeOH and 2.7mg of GEN in MeOH. Stocks were further diluted in MeOH to 40 μ M working stock solutions for DAZ and GEN and 100 μ M for equol. The exact concentrations of each stock solution was calculated by absorbance at the wavelength with maximum absorption (λ_{max}) using molar extinction coefficients (ϵ)(80, 423).

The final concentration of individual standards was adjusted with MeOH to obtain a ~1 mM stock solution and stored at -20°. 50 µM stock solutions of DAZ, GEN and equol were then prepared from their respective 1mM solution with HPLC mobile phase solution and stored at -20°. The 50 µM stock solutions were used in the preparation of a mixed standard containing 2 µM each of DAZ and GEN and 4 µM of equol in mobile phase. To prepare the 2/4/2 µM DAZ/equol/GEN standard mix, 80 µL 50 µM DAZ, 80 µL 50 µM GEN and 160 µL 50 µM equol were added to 1680 µL mobile phase solution.

Serial dilutions of the 2/4/2 µM DAZ/equol/GEN standard mix were then prepared with mobile phase to produce 1/2/1 µM and 0.5/1/0.5 µM standard mixes. The three mixed standard solutions were run at the beginning of each batch of samples to generate a three point calibration curve for which the concentration of ISOs in the unknown samples could be quantified. Regression equations for the standard curves for urinary ISO analysis were rejected if $R^2 < 0.97$ for DAZ and GEN and $R^2 < 0.95$ for equol per run.

2.12.2.2 HPLC Conditions

The HPLC system used for measurement of ISOs in the study consisted of a Shimadzu Prominence auto-sampler (model SIL-20 A) with a dual head pump (model LC-20AD) and degasser (model DGU-20 A₃). ISOs were detected using a VT-03 Electrochemical Flow Cell (INTRO, Antec Leyden) with a hydrogen reference electrochemical working electrode (DECADE II E.C.D p.n. 171.0035, Alltech). The Prominence liquid chromatograph (LC-20AD, Shimadzu) was set to an operational pressure range of 2-220 kfg/cm² and flow rate of 0.8 ml/min. The electrochemical detector was programmed in the DC operating mode, with a working cell potential range of 800 mV at positive amplitude polarity and detection sensitive of 10 nA. The noise filter in the system was set at 1 Hz.

Purified extracted samples were separated for ISOs on a reverse phase C-18 column (Phenomenex Prodigy 5µ ODS (3) 100 Å) that had been fitted with a HPLC guard cartridge (Model KJ0-4282, Phenomenex). Column temperature was programmed at 30° C, approximately 5° above ambient in the laboratory.

An isocratic mobile phase solution of 45:55:1 of 0.1 M ammonium acetate pH 4.6 adjusted with glacial acetic acid, HPLC grade MeOH and 25 mM EDTA disodium salt with 50mM KCl was used for HPLC analysis.

Each sample run was set for 60 minutes with 50 μ L of sample injected onto the column per run. LC solutions analysis (Shimadzu) software was used to collect and interpret sample data.

2.12.2.3 Extraction Protocol

The final extraction protocol for quantifying ISOs from urine was based on a modified version of the methods reported by King and Bursill (1998) (65) but optimised as described in section 2.12.1. Frozen urine samples were thawed, mixed and centrifuged at 4000 rpm at 4 °C for 5 min to remove particulate matter. A 200 μ l aliquot of urine sample was added to a 2 ml glass vial containing 300 μ l Milli-Q water and 100 μ l 1 M ammonium acetate pH 4.6. As previously described (refer to section 1.5.6 of Chapter 1), ISO are present in urine almost entirely as glucuronides and sulphates and must be converted to their aglycone forms for analysis. 10 μ l of glucuronidase/ sulphatase enzyme solution with a minimum enzyme activity of 100,000 units of β - glucuronidase per ml of urine was added to hydrolyse the 200 μ L of urine (Sigma G-1512). The mixture was gently vortexed, capped and incubated for a minimum of 18 hours in a 37 °C water bath overnight with gentle shaking (~20 OPM). Following incubation, 1 ml ethyl acetate was added to each sample which was then vigorously vortex mixed for 1 minute. The sample was centrifuged at 400rpm, 4 °C for 10 min to separate aqueous and ethyl acetate phases. The ethyl acetate phase was removed with a glass pasture pipette into a clean 2 ml glass vial and the remaining organic phase discarded. A second extraction with ethyl acetate was performed on the collected phase to maximise ISO recovery. The ethyl acetate extract was then dried under nitrogen gas at 50°C and stored at -20°C until required for analysis. Stored extracts were re-constituted in 200 μ l of 20% MeOH, and gently vortex mixed for 1 min. After pre-conditioning a 1ml C₁₈ cartridge containing 100mg sorbent (Sep-Pak®, Waters Associates, Inc.) with 2 ml MeOH and 2ml Milli Q H₂O, the reconstituted sample was applied to the cartridge. The cartridge was washed with 1 ml 20% MeOH, and ISOs eluted with two washes of 800 μ l 80 % MeOH. The collected elutant was dried down under nitrogen and reconstituted in 100 μ l mobile phase for HPLC analysis. The sample was centrifuged for 5 min at

4000 rpm to remove any particles prior to injecting 50ul of sample onto the column (Phenomenex Prodigy 5 μ ODS (3) 100 Å). Figure 2.2 is an example of the chromatograph generated by the LC Solution Software program representing the levels of ISOs present in a subject's urine sample.

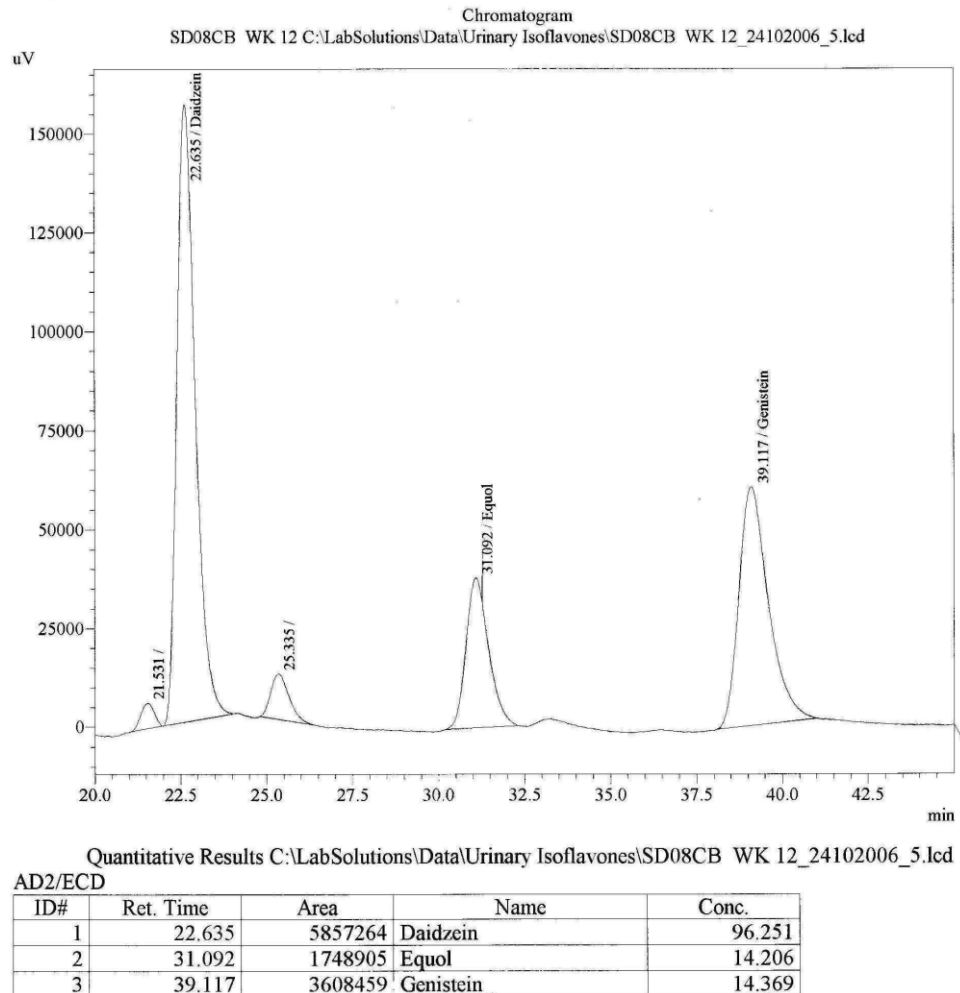


Figure 2.2: Urinary Isoflavone Concentration Chromatograph from HPLC Analysis

2.12.2.4 Recovery of Isoflavones in Urine Sample

To ensure precise and accurate recovery of ISOs in urine an internal standard of known quantity can be added during the extraction process. At present there is no standard protocol for quantifying ISO recovery in urine using an internal standard when performing HPLC. Flavone, has been used as an internal standard by Franke *et al* (62) due to its structural similarity with phytoestrogens and its stability. However, it elutes much later than the last eluting ISO, GEN increasing

the sample run time and is limited to UV detection based analysis. Deuterated standards have also been used in liquid (90) and gas (424) chromatography-Mass Spectrometry.

In order to correct for losses during our hydrolysis and extraction procedure, the recoveries of known amounts of ISO added to urine samples were determined, using the same protocol that was employed for the study samples. All values in this thesis have been corrected for recovery.

2.13 Quantification of Equol Production

As previously described (refer to section 1.5.8 of Chapter 1), equol was first discovered in the urine of pregnant mares in the early 1930's (120). It is an exclusive end product of intestinal bacterial metabolism of the dietary ISO, DAZ and unlike other ISOs, has a chiral centre allowing it to exist as two distinct diastereoisomers (S- and R- enantiomers). S-equol is produced exclusively in humans by intestinal bacteria and is shown to have a higher affinity for ER β in comparison with the relatively inactive R-equol enantiomer (191). For this reason, the specific threshold concentrations of S-equol, in urine and serum have traditionally been used to identify individuals who are producers. These threshold concentrations are generally based upon the distinct demarcation of subjects with low or negligible levels from those with high levels of equol (84, 112). However there are several major limitations with this type of approach for identification. Firstly there is the wide variation in analytical methods currently used to measure S-equol, thus no threshold concentration can be applied universally. Secondly, the level of equol in urine is dependent upon the time after which soy consumption occurred and could lead to underestimations if a 24 hour urine sample is not collected. Lastly the quantity of DAZ consumed will greatly influence the level of equol production.

To eradicate these potential sources of errors, a more robust method developed by Setchell and Cole (125) for identifying equol producers was used in our intervention. The advantage of the Setchell and Cole method is its calculation for equol producer status is determined independently of ISO excretion kinetics and the quantity of DAZ consumed prior to urinary collection.

The absolute urinary concentration of S-equol is expressed as a ratio of subject's absolute DAZ concentration, with any subject who exhibits a log₁₀ ratio value greater than -1.75, deemed to be a producer.

In the Soy Food Intervention, ratios of S-equol: DAZ on both the S and SD phases were averaged for each subject to give an overall concentration ratio. In accordance with the method of Setchell and Cole (125) this averaged ratio was then \log_{10} transformed.

2.14 HPLC Analysis of Isoflavones in Trial Foods

2.14.1 Standards

External standards DAZ (Sigma D- 7802) and GEN (Sigma G- 6649) from Sigma-Aldrich (Castle Hill, NSW, Australia) were prepared to ~ 1 mM stock solutions as described in Section 2.12.2.1 GLY (Indofine GL- 001 >99%) was prepared according to the method of Pettersson and Keissling(425) by dissolving 1 mg of pure GLY in 20ml MeOH to produce a solution equivalent ~ 0.05mg/ml. The exact concentration was calculated by absorbance at the wavelength with maximum absorption (λ_{max}) using GLY's molar extinction coefficient (ϵ) (426).

Stock solutions were used to prepare a top standard mix of 0.5 $\mu\text{g/ml}$ each of DAZ/GEN/GLY in mobile phase. Serial dilutions of the top standard mix were prepared with mobile phase to produce standards of 0.375 $\mu\text{g/ml}$, 0.25 $\mu\text{g/ml}$ and 0.125 $\mu\text{g/ml}$. Mixed standard solutions were run at the beginning of each batch of samples to generate a four point calibration curve. Regression equations for the standard curves for food ISO analysis were rejected if $R^2 < 0.99$ for DAZ, GLY and GEN per run.

2.14.2 HPLC Conditions

Conditions were the same for urinary ISO analysis as described in section 2.12.2.2.

2.14.3 Extraction Method

The extraction protocol used to quantify the concentration of ISOs in food and milk samples was based on the method of Pettersson and Keissling (425) with modifications provided by King *et al* (80). All study foods were extracted in duplicate.

2.14.3.1 Extraction Protocol for Solid Study Foods

Prior to analysis, solid foods from the intervention (e.g. biscuits, custard powder, chocolate bar and spaghetti) were crushed under liquid hydrogen to a fine powder. Five gram of the powder and 50 ml

of analytical grade petroleum spirit (40-60 °C) were then combined in a covered beaker and vigorously mixed for 30 minutes on a magnetic stirrer plate to extract lipids. The petroleum spirit phase was discarded and the food sample allowed to dry in a fume hood. 10 ml Milli-Q H₂O was added to the dried food sample and then mixed with a glass rod until a uniform paste was obtained. ISOs are virtually insoluble in petroleum spirit but in order to recover any traces that were extracted, the petroleum spirit phase was back-extracted with 80% ethanol by vigorous shaking in a sealed tube. Once settled, the upper petroleum spirit phase was aspirated into a waste flask and the remaining ethanol phase made to 70 ml with 80% ethanol. The ethanol phase was added to the food sample paste in the beaker. 10 ml 3M hydrochloric acid was then added to the beaker and the solution mixed well. To extract the ISOs from the food matrix the beaker was placed on a hot plate for 1-2 minutes until the solution reached boiling point. The beaker was then removed and placed in a cold water bath to allow any solid to settle. Once cooled, the supernatant was carefully decanted into a measuring cylinder and made up to a total volume of 100ml with Milli-Q H₂O. The ISOs in the ethanol extract were then hydrolysed as described in Section 2.12.2.3

2.14.3.2 Extraction Protocol for Study Milks

Five ml of study milk and 15 ml of ethanol were combined in a 50 ml screw cap centrifuge tube and mixed thoroughly. 25 ml of petroleum spirit (40-60 °C) was then added, shaken vigorously for one minute and the mixture centrifuged for 10 minutes at 4000 rpm. The upper petroleum spirit phase was then aspirated into a separate 50 ml centrifuge tube using a Pasteur pipette and the bottom ethanolic phase retained. 40 ml 80% ethanol was added to the petroleum spirit phase and vigorously mixed to back-extract any traces of ISOs as described for the solid foods. After the two phases separated, the upper petroleum layer was aspirated and disposed. The remaining ethanolic phase was combined with the first ethanolic phase and made to a volume of 50 ml with Milli-Q H₂O. The ISOs in the ethanol extract were then hydrolysed as described in Section 2.12.2.3.

2.14.4 Hydrolysis Method for Solid and Milk Study Foods.

1.5 ml of the ethanol extract prepared from the study food/milks was added to 1.5 ml 4M HCl in a Kimble tube. The solution was mixed thoroughly and the capped tube placed in a heating block set at 105°C for 30 minutes to hydrolyse glycosidic ISO conjugates into their aglycone form. After 30 minutes, the tubes were cooled in a water bath. If the sample contained suspended matter, the tube was centrifuged at 4000 rpm for 10 min at 4°C. To 2 ml of the hydrolysate, 6 ml Milli-Q H₂O was added to produce an ethanol concentration less than 20% to enable the ISOs in the sample to bind to the C₁₈ Solid Phase Extraction 3 ml cartridge (Sep-Pak®, Waters Associates, Inc). The cartridge was preconditioned with 5 ml MeOH, and 5 ml Milli-Q H₂O using a vacuum filtration manifold (Millipore USA) and the entire volume of diluted hydrolysate washed through slowly. The cartridge was then washed with 5 ml 20% MeOH and the elutant discarded. ISOs were eluted with 5 ml 80% MeOH. Extracts were diluted appropriately in mobile phase and 50 µl injected onto the HPLC column.

2.15. Statistical Analyses

Data were analysed using STATISTICA for WINDOWS software (version 5.1; StatSoft Inc, Tulsa, OK). One-way analysis of variance (ANOVA) with repeated measures using baseline scores as fixed covariates was used to examine the overall effects of the different diet treatments on the dependent variables. To determine the effects of the diet treatments on dependent variables, and their interaction with gender and/or equol production, three-way ANOVA with repeated measures was used. Where ANOVA showed significant main effects, differences between means were determined post-hoc using Tukey's HSD test. To optimise the analysis of differences between treatments, where appropriate, a nested ANOVA design was used to examine changes in dependent variables. In the instance of SP's interactive effect, SD and S were nested in time to examine changes in dependent variables. When examining the interactive effect of equol production on dependent variables, equol status and diet treatments were nested in time. A test for difference between two proportions was used to determine if there was a statistical difference in the proportion of males and females who were or weren't equol producers in the trial. Relationships between variables were determined using linear regression. Statistical significance was set at a level of $P < 0.05$. All data in tables and graphs are shown as mean \pm

SEM unless stated otherwise. The participation of 90 subjects in the intervention was calculated to give 80% power to detect a 5% reduction of TChol at $\alpha=0.05$ (11).

2.16 Results

2.16.1 Profile of Subjects in the Intervention

One hundred and sixty seven individuals were screened for participation in the intervention. After the screening visit, 126 individuals were eligible for inclusion and chose to take part in the study. After being randomised in three successive intakes (n=52, 50 and 24), 91 completed the intervention. Figure 2.3 describes subject recruitment for each intake. Lack of compliance to the protocol (n=24), poor tolerance to study foods (n=4), change in work commitments (n=4), interstate/overseas travel (n=2) and use of banned medication (n=1) were the primary reasons for the withdrawal of 35 subjects. Baseline characteristics of subjects are described in Table 2.5. For the n=91 individuals who completed the intervention, diets were undertaken in the following orders; D→SD→S (n=25), D→S→SD (n=5), SD→S→D (n=25), SD→D→S (n=4), S→D→SD (n=27), S→SD→D (n=5).

Table 2.5: Baseline characteristics of all subjects in Soy Food Intervention.

	Mean	Range	Recommended Range
Gender (M / F)	34 / 57		-
Age (y)	52.7 ± 1.0	21-78	-
Weight (kg)	77.1 ± 14.5	44.2-128.6	-
BMI (kg/m²)	27.3 ± 4.5	17.6-36.6	18.5-24.9
Waist Circumference (cm)	88.9 ± 12.6	58-125.5	M ≤ 94 cm, W ≤ 80 cm
Hip Circumference (cm)	104.4 ± 9.1	88.5-128	-
Waist:Hip Ratio	0.85 ± 0.09	0.66-1.03	M ≤ 1, W ≤ 0.85
T Chol (mmol/L)	5.63 ± 0.81	4.16-8.2	≤ 5.5
LDL-C (mmol/L)	3.60 ± 0.68	1.64-5.27	≤ 2.0
HDL-C (mmol/L)	1.27 ± 0.47	0.36-2.75	≥ 1.0
TG (mmol/L)	1.72 ± 1.01	0.48-6.59	≤ 1.6
Glucose (mmol/L)	4.95 ± 0.13	3.0 – 7.8	≤ 6.1
Insulin (mmol/L)	12.40 ± 0.60	4.8 – 26.1	-
SBP (mmHg)	123.2 ± 11.1	106 – 153	≤ 140
DBP (mmHg)	71.7 ± 8.1	57 - 98	≤ 90

Values= Mean ± SD

2.16.2 Gender Variation in Soy Food Intervention

Of the 91 subjects who completed the intervention, 34 were males and 57 were females. Gender distribution over the three intakes was; Adelaide 1st intake (M=13/F=22), Adelaide 2nd Intake (M=9/F=11) and Perth Intake (M=12/F=24).

2.16.3 Compliance of Subjects

In terms of the proportion of experimental foods consumed, compliance according to food record forms during the intervention for all intakes was 96.6 ± 0.4 %. For all subjects (n=91), compliance was similar on the diets (D = 97 ± 0.6 %; SD = 97 ± 0.5 %; S= 96 ± 0.7 %). Mean days spent on each diet was also similar (D= 41.4 ± 0.3 days; SD= 41.4 ± 0.2 days; S= 41.3 ± 0.2 days).

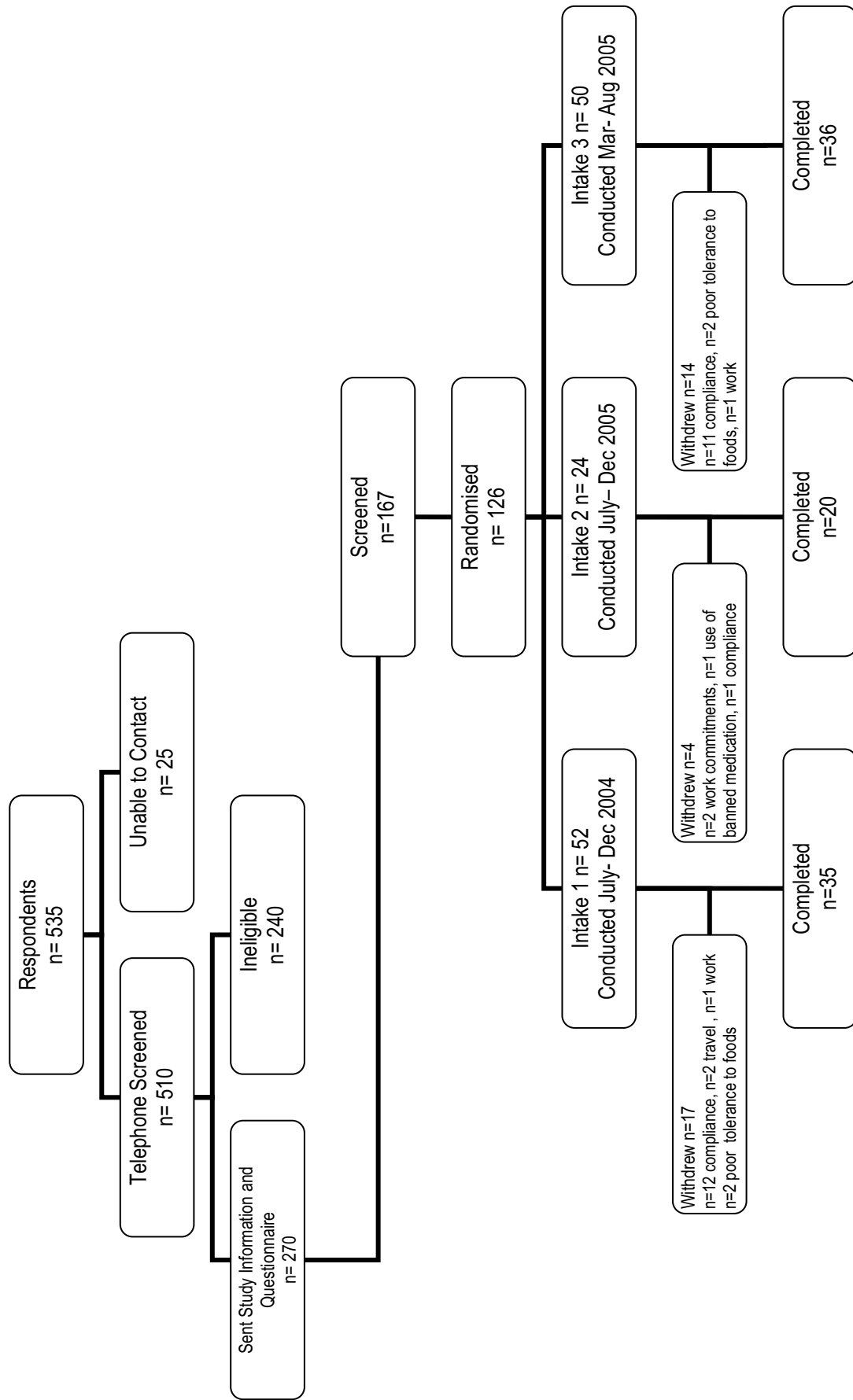


Figure 2.3: Subject recruitment, randomisation and completion rates during the Soy Food Intervention.

2.16.4 Isoflavone Content of Study Foods

HPLC analysis revealed differing quantities of ISOs present in the range of trial foods manufactured for the SD and S treatments of the intervention. Listed in Table 2.6 are the total aglycone amounts of DAZ and GEN present in one serving of each trial food used in each intake.

Table 2.6: Total isoflavone content (in mg) of trial foods per serving.

Food Item	Serving Size	Diet Phase	Aglycone DAZ + GEN Concentration (mg/serve)		
			Intake 1 (n=35)	Intake 2 (n=36)	Intake 3 (n=20)
Plain Milk	250 ml	D	0	0	0
		SD	19.4	29.6	28.7
		S	20.8	32	31.7
Chocolate Milk	250 ml	D	0	0	N/A
		SD	43.7	30.9	N/A
		S	30.9	18.6	39.4
Coffee Milk	250 ml	D	N/A	0	0
		SD	32.0	25.8	25.0
		S	25.8	25.4	N/A
Vanilla Custard	200g (1/3 bottle)	D	2.9	0.2	0
		SD	14.1	16.2	23.1
		S	14.5	15.7	15.4
Apricot and Bran Cookies	3 biscuits	D	1.8	0	N/A
		SD	14.2	25.4	N/A
		S	13.2	29.9	N/A
Chocolate Cake Bar	1 bar (60 g)	D	1.2	N/A	N/A
		SD	19	N/A	N/A
		S	29.8	N/A	N/A
Spaghetti	63 g dry (1/4 packet)	D	0.2	N/A	N/A
		SD	16.4	N/A	N/A
		S	15.6	N/A	N/A

Table 2.7 describes the mean concentrations of individual ISOs found in the trial foods manufactured for use in all intakes of the intervention. On average, the chocolate milk provided the highest intake of DAZ, GEN and GLY. Whilst, the instant vanilla custard mix provided the least amount of DAZ and the dry spaghetti the least amount of GEN and GLY.

Table 2.7: Mean daidzein, genistein and glycitein concentrations in trial foods.

Food Item	Serving Size	Diet Phase	Concentration (mg/serve)			Total
			DAZ	GEN	GLY	
Plain Milk	250 ml	D	0	0	0	0
		SD	11.9	7.4	7.1	26.4
		S	12.2	9.1	6.4	27.7
Chocolate Milk	250 ml	D	0	0	0	0
		SD	18.8	10.9	7.6	37.3
		S	14.5	8.1	7.1	29.7
Coffee Milk	250 ml	D	0	0	0	0
		SD	14.2	6.7	6.7	27.6
		S	13.1	6.5	6.0	25.6
Vanilla Custard	200g	D	0.3	0.1	0.5	0.9
		SD	6.9	4.2	4.1	15.2
		S	7.0	4.0	4.1	15.1
Apricot and Bran Cookies	3 biscuits (100 g)	D	0.1	0.1	0.2	0.4
		SD	11.8	3.7	6.1	21.6
		S	10.9	3.2	5.7	19.8
Chocolate Cake Bar	1 bar (60 g)	D	0.5	0.1	0.1	0.7
		SD	12.6	5.3	6.9	24.8
		S	9.8	4.3	5.6	19.7
Spaghetti	63 g dry (1/4 packet)	D	0	0.2	0.1	0.3
		SD	8.5	3.0	4.1	15.6
		S	8.3	4.3	3.8	16.4

2.16.5 Macronutrient and Energy Intakes of Subjects during the Intervention

Analysis of Food Frequency Questionnaires showed a significant difference in subject's macronutrient intake between treatments, specifically in relation to saturated and unsaturated fat intake. As shown in Table 2.8, consumption of saturated fat was significantly higher on the D diet compared with the SD and S diets ($P < 0.01$). However intakes did not differ between SD and S ($P = 0.19$). Unsaturated fat intake was significantly greater on the S diet compared to SD ($P = 0.04$) and D ($P = 0.01$). When unsaturated fat intake was expressed as a ratio to saturated fat, there was a significant difference across all treatments ($P < 0.01$), with ratios becoming progressively lower with consumption of D (0.65), SD (0.51) and S (0.41) respectively. When the consumption of intervention foods was removed from the Food Frequency Questionnaire data for each treatment, saturated fat intake was consistent across the diets ($P = 0.60$) indicating the elevation in saturated fat intake during the D diet was a direct result of consuming the intervention foods rather than a change in background diet. Sugar intake was also significantly lower on the S diet compared with D and SD ($P < 0.01$).

Table 2.8: Macronutrient intake of subjects during intervention based on Food Frequency Questionnaire data analysis.

Macronutrient	Mean Macronutrient Intakes		
	D	SD	S
Energy KJ/d	7407 ± 284	7107 ± 266	7326 ± 349
Protein g/d	88 ± 4	83 ± 3	88 ± 4
Carbohydrate g/d	194 ± 8	194 ± 8	192 ± 8
Total Fat g/d	61 ± 3	58 ± 3	60 ± 3
Saturated Fat g/d	22 ± 1 ^a	19 ± 1	17 ± 1
Unsaturated Fat g/d	34 ± 2	34 ± 2	38 ± 2 ^b
Saturated: Unsaturated Fat	0.65 ± 0.02 ^c	0.51 ± 0.03 ^c	0.42 ± 0.02 ^c
Sodium mg/d	2354 ± 108	2352 ± 103	2302 ± 101
Fibre g/d	24 ± 1	24 ± 1	25 ± 1
Total Sugars g/d	75 ± 4	80 ± 4	68 ± 4 ^d
Alcohol g/d	12 ± 2	13 ± 2	11 ± 2

^a Significantly different to SD and S (P<0.01) , ^b Significantly different to SD and D (P<0.04),

^c Significantly different from each other (P< 0.01), ^d Significantly different to SD and D (P<0.01)

As shown in Table 2.9, when different macronutrients in subjects diets was expressed as the percentage of energy they contributed, all were within the American Health Association's (AHA) recommendations (427). The only exception was that of saturated fat intake on the diets, which was slightly higher than the recommended <7% daily requirement.

Table 2.9: Percent energy derived from macronutrients on diet treatments for all subjects.

	AHA CVD Dietary Recommendation	Mean % Energy breakdown over diet treatments		
		D	SD	S
Protein %	15-25	20.11 ± 0.42	20.01 ± 0.96	20.76 ± 0.39
Carbohydrate %	45-65	44.23 ± 0.97	47.06 ± 2.26	45.12 ± 0.81
Total Fat %	20-35	30.89 ± 0.71	31.38 ± 2.06	30.88 ± 0.63
Saturated Fat %	<7	10.82 ± 0.37	9.49 ± 1.08	8.06 ± 0.40
Unsaturated Fat %	N/A	1.91 ± 0.05	2.11 ± 0.11	2.21 ± 0.05
Polyunsaturated Fat %	N/A	5.33 ± 0.22	5.38 ± 0.31	6.89 ± 0.26
Monounsaturated Fat %	N/A	9.32 ± 0.30	9.07 ± 0.88	12.41 ± 0.28

2.16.6 Intake of Study Foods by intakes

All intakes derived the majority of their required daily intake of experimental foods from plain and flavoured milks as described in Table 2.10.

Table 2.10: Summary of trial food consumption rates (as a percentage of total serves) for intakes.

Diet Phase	Intake			Mean % for all intakes	
	Intake 1 (n=35)	Intake 2 (n=36)	Intake 3 (n=20)		
D	Plain Milk	32.0	37.0	46.9	38.6
	Chocolate Milk	29.6	27.7	-	28.7
	Coffee Milk	-	20.2	41.2	30.7
	Vanilla Custard	6.3	6.1	11.9	8.1
	Apricot Bran Cookies	18.2	9.0	-	13.6
	Chocolate Cake Bar	10.2	-	-	10.2
	Spaghetti	3.7	-	-	3.7
SD	Plain Milk	27.9	33.0	48.0	36.3
	Chocolate Milk	30.0	29.2	-	29.6
	Coffee Milk	2.7	22.3	40.2	21.7
	Vanilla Custard	6.1	6.4	11.8	8.1
	Apricot Bran Cookies	20.5	9.1	-	14.8
	Chocolate Cake Bar	8.6	-	-	8.6
	Spaghetti	4.3	-	-	4.3
S	Plain Milk	29.2	31.9	45.0	35.4
	Chocolate Milk	33.9	31.3	41.3	35.5
	Coffee Milk	1.3	22.3	-	11.8
	Vanilla Custard	5.4	5.3	13.7	8.1
	Apricot Bran Cookies	19.1	9.2	-	14.2
	Chocolate Cake Bar	7.4	-	-	7.4
	Spaghetti	3.6	-	-	3.6

2.16.7 Isoflavone Intake of Subjects during Diet Treatments

One-way repeated measures ANOVA revealed all diet treatments provided subjects with significantly different intakes of ISOs ($P < 0.05$) (D vs SD and S : $P < 0.01$, SD vs S : $P < 0.05$). As shown in Table 2.11, mean ISO consumption/day over all intakes was greatest on the SD treatment.

Table 2.11: Mean daily and treatment isoflavone consumption rates for all subjects and across intakes.

Diet Phase	Mean Intake (mg)	Intake 1 (n=35)	Intake 2 (n=36)	Intake 3 (n=20)	All subjects (n=91)
D	Daily	1.3 ± 0.2	0.04 ± 0	0 ± 0	0.5 ± 0.1
	Phase	51.8 ± 6.4	1.49 ± 0.2	0 ± 0	20.5 ± 3.6
SD	Daily	70 ± 3.5	81.1 ± 1.0	77.2 ± 1.1	76 ± 1.5
	Phase	2941 ± 147	3305 ± 47	3210 ± 66	3144 ± 63
S	Daily	57 ± 2.3	72.3 ± 1.3	95.3 ± 3.0	71.4 ± 1.9
	Phase	2386 ± 99	2921 ± 63	3987 ± 128	2945.7 ± 82

2.16.8 Equol Producers in Trial

Results from overnight urinary ISO analysis revealed 30/91 subjects were “equol producers”, which represents 33% of the entire study population.

Of the 30 identified equol producing individuals, 11 were males and 19 were females. A test for difference between two proportions revealed no difference ($P=0.93$) between the proportion of male or female producers within the study population; males (32.4%), females (33.3%). Figure 2.4, illustrates the log₁₀ ratio values for concentrations of equol:DAZ of all subjects. As shown, a log₁₀ ratio values ≥ 1.75 indicates the subject is a producer of equol.

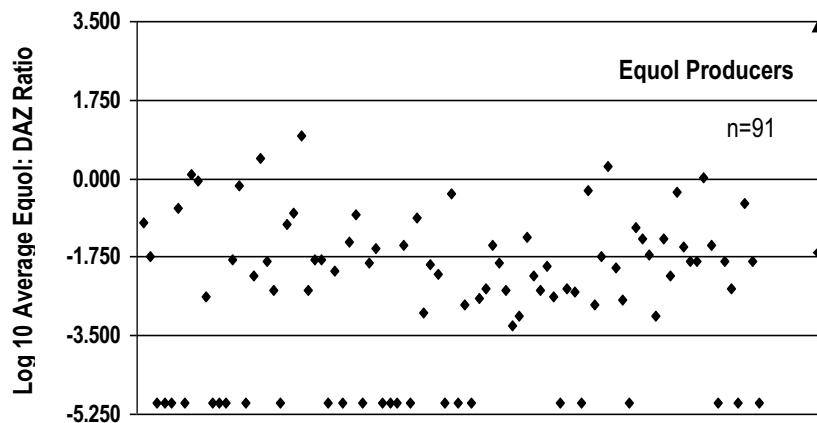


Figure 2.4: Log 10 of mean equol:daidzein concentration ratio of all subjects.

2.16.9 Palatability of Study Foods

Table 2.12 describes the mean hedonic scores given by subjects who consumed the trial foods during the diet treatments (refer to section 2.9.4 for description of scores). Chocolate milk rated the highest for hedonics across all diet treatments, with an average rating of 5.3, indicating they were

“slightly liked” by subjects who consumed it. In contrast, the dry spaghetti rated the lowest for hedonics across all diet treatments, with an average rating of 2.7, indicating it was “slightly disliked”. All D trial foods rated the highest in hedonics compared to SD and S diet versions, except for spaghetti. SD and S trial foods were received equally.

Table 2.12: Mean hedonic scores for trial foods used during the Soy Food Intervention.

Food Item	Treatment					
	D		SD		S	
	Intake	Av Rating	Intake	Av Rating	Intake	Av Rating
Plain Milk	1 n=27		1 n=27		1 n=29	
	2 n=36	5.2 ± 0.2 (n=78)	2 n=35	4.3 ± 0.2 (n=76)	2 n=36	4.1 ± 0.2 (n=80)
	3 n=15		3 n=14		3 n=15	
Chocolate Milk	1 n=28		1 n=29		1 n=28	
	2 n=34	5.6 ± 0.2 (n=62)	2 n=32	5.0 ± 0.2 (n=61)	2 n=34	5.1 ± 0.2 (n=78)
	3 n=0		3 n=0		3 n=16	
Coffee Milk	1 n=0		1 n=8		1 n=2	
	2 n=32	5.1 ± 0.2 (n=46)	2 n=30	4.6 ± 0.2 (n=53)	2 n=33	4.2 ± 0.3 (n=35)
	3 n=14		3 n=15		3 n=16	
Vanilla Custard	1 n=22		1 n=18		1 n=19	
	2 n=25	5.0 ± 0.2 (n=57)	2 n=26	4.5 ± 0.2 (n=53)	2 n=27	4.4 ± 0.1 (n=56)
	3 n=9		3 n=9		3 n=10	
Apricot and Bran Cookies	1 n=28		1 n=30		1 n=28	
	2 n=32	4.5 ± 0.2 (n=60)	2 n=32	4.3 ± 0.2 (n=53)	2 n=32	4.3 ± 0.23 (n=60)
	3 n=0		3 n=0		3 n=0	
Chocolate Cake Bar	1 n=23		1 n=19		1 n=20	
	2 n=0	4.1 ± 0.3 (n=23)	2 n=0	3.3 ± 0.4 (n=19)	2 n=0	3.3 ± 1.7 (n=20)
	3 n=0		3 n=0		3 n=0	
Spaghetti	1 =16		1 n=17		1 n=19	
	2 n=0	2.8 ± 0.5 (n=16)	2 n=0	3.2 ± 0.4 (n=17)	2 n=0	2.1 ± 0.3 (n=19)
	3 n=0		3 n=0		3 n=0	

2.16.10 Market Potential of Trial Foods

Subjects were asked whether they would purchase the trial foods if available to them at comparable prices to other similar products in the supermarket. These results are listed in Table 2.13. On average, all D trial foods, except spaghetti and the chocolate cake bar, rated the highest in terms of subject's intent to purchase if available when compared to equivalent SD or S versions. Of all the trial foods manufactured for the SD and S diets, chocolate milk had the highest market potential with 54.1% and 55.1% respectively of consumers in the intervention indicating they would purchase if available. The only product manufactured for the SD diet which rated the highest in terms of market potential compared to D and S diet versions was the spaghetti. 41.7% of those subjects who consumed the spaghetti on the SD diet indicated they would purchase if available, compared to only 33.3% and 5.6% on the D and S diets respectively.

Table 2.13: Percentage of subjects willing to purchase the trial foods used in the Soy Food Intervention.

% of Individuals who would purchase the trial foods			
Food Item	D	SD	S
Plain Milk	69.2 % (n=78)	46.1 % (n=76)	45 % (n= 80)
Chocolate Milk	64.5 % (n=62)	54.1 % (n=61)	55.1 % (n=78)
Coffee Milk	52.2 % (n=46)	47.2% (n=53)	31.7 % (n= 35)
Vanilla Custard	56.1% (n=57)	43.4% (n=53)	48.2 % (n= 56)
Apricot Bran Cookies	50 % (n=60)	35.5 % (n= 62)	38.3 % (n= 60)
Chocolate Cake Bar	21.7 % (n=23)	26.7 % (n= 19)	11.1 % (n= 20)
Spaghetti	33.3% (n=16)	41.7 % (n= 17)	5.6 % (n= 19)

2.17 Discussion

2.17.1 Characteristics of Subjects in the Intervention

Subject's baseline TChol levels ranged considerably from 4.16 - 8.2 mmol/L. Although the inclusion criterion for this measure was set at ≥ 5.5 mmol/L, it appears that the automated analyser (Reflotron Plus, Roche Diagnostics, Basal, Switzerland) used at screening overestimated subjects true values; up to 24% in some instances. The group's collective baseline TChol level nevertheless reflects they were mildly hypercholesterolemic. Elevated baseline LDL-C and TG values for the group also indicates mild hyperlipidemia (428).

Subjects in the intervention were shown to be overweight according to the group's BMI value and had higher than recommended waist and hip circumference measurements (429). Such characteristics are known to potentiate the development of other CVD risk factors such as hypertension and insulin resistance. However this does not appear to be the case in these subjects as their baseline values for SBP, DBP, glucose and insulin were within normal ranges.

There was a non- deliberate, disproportionate number of females compared to males involved in the study ($P < 0.001$). However this did not affect the ratio of male to female equol producers in the group which was exactly 1:1. The number of equol producers in the trial (30/91) furthermore parallels what might be expected in a typical western population (75).

2.17.2 Attrition and Compliance of Subjects in the Intervention

Ninety-one subjects completed the intervention out of a possible 126, indicating a drop out rate of 28%. This level of attrition is much higher than other soy food interventions (11, 330, 430) which report only 10% on average. The primary reason given by subjects who withdrew was an inability to comply with the study protocol ($n=24$). The palatability of the trial foods most likely caused this failure as suggested by the low hedonic scores given by those subjects who did consume them.

In spite of the unanticipated high drop out rate, those individuals who did complete the intervention did so with a very high level of compliance, averaging 96.6 ± 0.4 % across all diet

treatments. Such a level of adherence mirrors other interventions (>90%) which have investigated effects on soy foods on lipids and vascular function (8, 11, 330, 430).

2.17.3 Strength of Intervention to Detect Outcome Measures.

The intervention used a double blinded, placebo controlled, cross over Latin square study design. One of the reasons for selecting this type of design was to ensure no carry-over effect from the diets on outcome measures occurred. It also minimised the overall duration of the intervention as the need for an extended wash out period between the treatments was eliminated.

The other advantage of using a Latin square design as opposed to a parallel was all subjects underwent the three treatment diets so the number of subjects required to participate could be reduced since a control group was not required. By also using a stratified randomisation approach to assign orders that the subjects undertook the diets, variability in subject's response to the diets was removed.

Double blinding the study investigators and subjects as to the order of the treatments during the intervention also helped remove any bias in analysing results and assisted with subject's compliance to the protocol.

2.17.4 Variability of Isoflavone Content in Trial Foods

A daily ISO intake range of 75-90 mg/day had been proposed for the SD and S diets as such quantities are reported to benefit cholesterol reduction (398, 431) when delivered with SP. Each trial food, manufactured for consumption during the SD and S diets, should have provided between 25-30 mg of aglycone ISOs. HPLC analysis subsequently revealed that the actual ISO content of the foods ranged considerably from 15.2-37.3 mg on the SD diet and 15.1-29.7mg on the S Diet. Whilst a small amount of ISO was provided from the SP isolate which was added to the foods in order to increase their SP content (refer to section 2.10.2), the majority came from an ISO complex. Since whole soybeans can vary in their ISO content due to genetic and environmental factors (refer to 1.4.4) it's quite possible that the batches of ISO complex that were incorporated into the foods actually provided an amount of ISO than was different from the 22-30mg /g it was reported to contain.

It also appeared from HPLC results that certain food matrices retained the ISOs far better than others. For all intakes, it was shown that the plain and flavoured milks provided the highest amounts of ISOs, whereas the solid matrix of the spaghetti provided the least.

2.17.5 Macronutrient and Isoflavone Intakes of Subjects during the Intervention

It was made very clear to subjects that trial foods should not be “additions” in their diets but rather should be substituted in for similar products they would normally consume in order to maintain their typical diet. Since subject’s protein intake was similar on all the diet treatments and their percentage of energy derived from protein within the AHA dietary recommendations, it could be argued that they were correctly following this instruction.

Despite designing all trial foods to be low in fat it does appear that those consumed on the SD and S diets provided subjects with significantly less saturated fat. A higher intake of unsaturated fat was also observed from the foods on the S diet. These differences in fat from the trial foods can be directly attributed to the inclusion of SP.

Sugar intake, which was defined as simple carbohydrates, was found to be lower on the S diet compared to the other diets. This may be due to subjects consuming predominately plain and chocolate milk during the phase rather than the other snack/dessert type foods that provided more sugar (refer to Appendix 4 for macronutrient profiles of the individual trial foods manufactured for each diet phase).

Variability in ISO content of the trial foods meant subjects mean intakes ranged from 34.5-118.2 mg/day over both SD and S treatments depending upon their favoured food choices (refer to Table 2.7). Disparity in foods chosen by subjects for the different diet phases, meant mean daily ISO intake was marginally higher on the SD diet (76 ± 1.5 mg/day) compared to S (71.4 ± 1.9 mg/day) ($P < 0.05$).

2.17.6 Hedonics of Trial foods and their Potential Marketability

The sensory appeal of soy has long been a major determinant for mass consumer acceptability. The current study attempted to address the issue of soy’s poor palatability by combing it with an already highly favoured taste (low fat dairy). It was hoped by doing this; it would produce a novel food matrix able to deliver soy’s key nutrients thought to optimise CV health with a distinctly greater market appeal

than soy products currently available. Foods used in the study were based on the formulation of existing So Natural and Freedom Foods products known to have consumer appeal and were modified to include additional ISOs, SP and/or DP.

Results from the food acceptability questionnaire which subjects were asked to complete after each blinded diet treatment, indicate all trial foods (excluding spaghetti) manufactured for the D treatment had the greatest market and sensory appeal in comparison to SD and S diet versions. Foods manufactured for consumption on the SD and S diets appeared to be equally tolerated with plain and flavoured milks rating the highest for hedonics and market appeal. This would suggest milk would be the most marketable of all the food matrices developed to deliver SP and ISOs to the wider community.

2.18 Summary

With respect to marketing the novel foods used in the intervention, it would appear plain and flavoured soy dairy combination milks would be the best matrix with which to promote the incorporation of SP and ISOs into consumer's current diets.

Outcomes from the intervention relating to effects of the novel and soy based foods on lipids, metabolic factors and vascular function are discussed in the proceeding chapters 3-5.

3.0 Effect of Soy Foods on Blood Lipids

3.1 Introduction

CVD is the leading cause of death in western societies (432). Elevated blood cholesterol is a well established modifiable risk factor for CVD, with every 10% reduction in TChol reflective of a 15% decrease in CVD mortality risk and 11% decline in total mortality (433). LDL-C levels have been shown to be an even stronger predictor for CVD risk and remain the most significant risk factor in the development of atherosclerosis, a condition which denotes the early progression of CVD (434, 435).

Antihyperlipidemic medications such as hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors or “statins” as they are commonly referred to, are the current first-line drugs for controlling cholesterol in patients with risk factors for CVD (436). However, contraindications from statins in certain individuals coupled with their limited effectiveness when taken sporadically, have resulted in a need for alternative treatments. Dietary modification and the incorporation of functional foods (e.g. plant sterol enriched margarine spreads) into the diet have become the leading non-pharmacological therapies for positively altering cholesterol levels. Low saturated fat diets have long been recommended as the first step for reducing LDL-C and subsequent risk for CVD (437). More recently, regular consumption of dietary fibre has been demonstrated to reduce CVD risk (438). Interestingly, epidemiological studies repeatedly report, Asian countries for which soy based foods are a staple component of their diet, as having the lowest incidence of CVD, implying soy may offer a CV benefit (45, 439, 440).

In the last twenty years, a large body of scientific evidence has emerged to support these epidemiological findings and to associate soy consumption with reductions in blood cholesterol. Anderson *et al's* (279) meta analysis in 1995 was the first comprehensive review to confirm soy's lipid lowering properties and later provided the basis of a health claim released in 1999 by the U.S FDA (1) which stated that “25g of SP per day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease”. In 2002 the U.K Joint Health Claims Initiative (281) released their own more

specific health claim for SP which stated, “The inclusion of at least 25g of soya protein per day, as part of a diet low in saturated fat, can help reduce blood cholesterol levels”.

In Anderson *et al*'s meta- analysis of 38 controlled clinical studies, there was no dose-response relationship between SP intake and cholesterol reduction, when changes in cholesterol and lipoproteins were adjusted for changes in the control group (animal protein). This raised the contentious issue as to whether an additional component in soy, such as its ISO content, may contribute to cholesterol reduction- a hypothesis Anderson *et al*'s meta-analysis failed to address. With respect to the health claim, the FDA made a specific point of stipulating, “the evidence did not support a significant role for ISOs in cholesterol- lowering effects of SP” (1). Controversially, the Nutrition and Metabolism Committee of the American Heart Association released their own scientific advisory Statement for Healthcare professionals and concluded; findings were inconclusive for SP being the sole attributing factor. However, a more recent statement released by the American Heart Associate questions ISOs ability to independently reduce CVD risk (441).

Another aspect of the health claim is whether individuals with only mildly elevated cholesterol levels would benefit from soy consumption, given that no significant reduction occurred in subjects with baseline cholesterol values (between 5.2-6.6 mmol/L) in the Anderson *et al* meta-analysis when receiving on average 47g/day SP.

Despite several recent meta-analyses reporting a maximum hypocholesterolemic benefit, particularly on LDL-C when SP and ISO are delivered concurrently(398, 431, 442, 443) others show no dose response effect between ISO consumption and LDL- C reduction (7, 324, 347, 444-447).

Setchell *et al* (123) hypothesised that the inconsistency in results from trials investigating effects of SP delivered in combination with ISO may be related to the ability of individuals to convert DAZ into equol through bacterial fermentation in the large intestine (448). Only one third of the adult population in western society harbours the specific gut micro flora necessary to produce equol (95) compared with 50-55% of the Asian population(70, 449, 450). This may explain why Asian countries in epidemiological studies who consume soy foods in their daily diet have a lower incidence of CVD than those who consume a typical Western diet (451).

As is evident from several *in vitro* studies, equol mediates potent agonist effects on ER β compared with DAZ or GEN (123, 452). Its presence may therefore prove critical in assisting in the many intricate mechanisms by which ISOs are hypothesised to reduce lipids.

The influence of equol production in eliciting significant reductions in cholesterol is most evident in the recent study by Meyer *et al* (11), where retrospective analysis of results from 23 individuals receiving 30g of SP and 80mg of ISO/day for five weeks, revealed that only those individuals (n=8) identified as “equol-producers” experienced significant reductions in TC, LDL-C , TG and HDL-C. Although only a small study, it raises the question as to whether or not an individual, if able to produce equol, would need to consume the large quantities of SP suggested by the current health claim if taken in combination with ISOs. This may explain why some cross-sectional and clinical studies administering lower amounts of SP (<20g /day) are still able to demonstrate a significant reduction in cholesterol (439, 440, 453-455).

3.2 Aims of the Intervention in Relation to Plasma Lipids

- a) Investigate whether hypocholesterolemic benefits can be attained by consuming novel foods which provide 12g SP per day with 75-90 mg ISOs;
- b) Compare effects of the novel foods on lipids against soy foods that provide 24g SP (i.e. similar to that in health claims) and 75-90 mg ISOs to ascertain the importance of SP for mediating a potential hypocholesterolemic benefit;
- c) Investigate whether hypocholesterolemic benefits from the novel and soy foods would be limited to equol producers;
- d) Ascertain to what extent regular soy consumption benefits mild hypercholesterolemics at CVD risk.

3.3 Methods

3.3.1 Subjects and Recruitment

Data were collected from all subjects who were recruited as part of the Soy Food Intervention. Refer to Chapter 2 section 2.5.1 for subject inclusion and exclusion criteria. Methods of recruitment are also described in section 2.6.

3.3.2 Study Design and Protocol

Refer to Chapter 2 section 2.8.1 and 2.8.2 for a description of the study design and protocol.

3.3.2.1 Protocol for Collecting Blood Samples

Blood samples were collected from subjects on each morning visit after an overnight fast (> 10 hours fasting) using venipuncture. 16ml of blood was drawn from the antecubital vein into EDTA-containing vacutainer tubes on the first morning. 8mls of blood was also collected into an EDTA-tube as well as 6mls of blood into a sodium fluoride vacutainer tube the following morning. The collection of blood samples at each visit allowed lipids to be assessed over two days (i.e. duplicates) and the average of the two samples was taken to be the measured value.

3.3.3 Blood Plasma Analysis

All tubes containing fasting blood samples were immediately placed on ice and later centrifuged (Hettich Zentrifugen Universal 32 R, Tuttlingen, Germany) at 3000 g for 10 minutes at 4°C within 2 hours of collection. Plasma was then drawn off, aliquot in epindoffs and stored at -80°C. Samples were analysed in a single batch once subjects completed all three diet treatments to minimize laboratory variability

The concentration of subject's plasma TChol, HDL-C and TG were determined on a Konelab 20XTi auto- analyser (Thermo Electron, Victoria, AUS) with appropriate standards and reagents. A modified Friedewald equation was used to calculate LDL-C from TG and HDL-C concentrations (456).

3.3.4 Study Foods, Intake and Compliance

Refer to Chapter 2 section 2.10 for detailed description of the range, composition and availability of trial foods consumed in the study. For protocols used to measure study food compliance and macronutrient intake on the diets see section 2.11.

3.3.5 Identification of Equol Producers in the Intervention

Overnight urine samples were collected after the completion of each treatment (refer to Chapter 2 section 2.11.2.2 for protocol). The established method of HPLC using ECD was employed for identifying equol producers based on the samples collected within the study cohort. Subjects were subsequently identified based on the method established by Setchell and Cole (125) as described in section 2.13.

3.4 Statistics

Refer to Chapter 2 section 2.15 for statistical procedures used to analyse data.

3.5 Results

3.5.1 Plasma Lipids during Dietary Phases of the Intervention

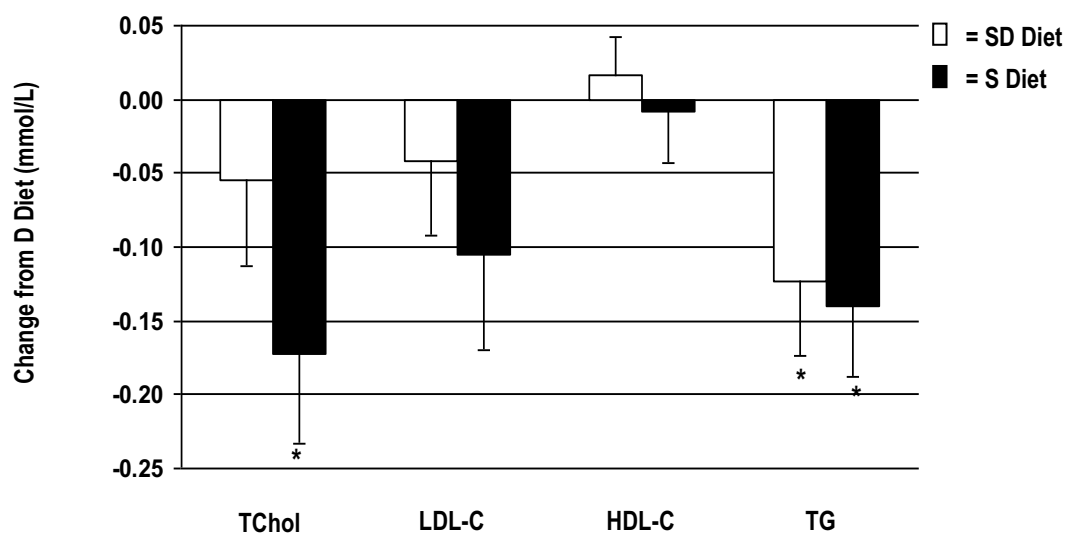
Blood lipid concentrations at the end of each diet treatment are presented in Table 3.1. One-way repeated measures ANOVA showed significant main effect of the diet treatments for TChol ($P=0.014$) and TG ($P<0.001$), but not for LDL-C ($P=0.22$) or HDL-C ($P=0.72$). Post hoc analysis revealed that compared with D, TChol decreased by 3% during the S diet ($P=0.01$), and TG was reduced by 3.5% and 4% on the SD ($P=0.03$) and S ($P=0.009$) diets respectively, with no difference between SD and S in the magnitude of this effect on TG ($P=0.94$). The ratio of TChol:HDL-C on the D, SD and S treatments was 5.05, 5.01 and 4.76 respectively. Despite a trend for the ratio of TChol:HDL-C to be lower on the S treatment compared with SD and D, this difference did not reach statistical significance ($P=0.06$). Figure 3.1 illustrates changes in lipid values on both the SD and S diets from D.

Table 3.1: Lipid concentrations and changes across diets for all subjects

Lipids	Absolute Concentration (mmol/L)			P-value
	D	SD	S	
TChol	5.65 ± 0.07	5.60 ± 0.08	5.48 ± 0.09	0.01
LDL-C	3.53 ± 0.07	3.49 ± 0.07	3.43 ± 0.07	0.22
HDL-C	1.33 ± 0.05	1.35 ± 0.06	1.32 ± 0.05	0.72
TG	1.81 ± 0.12	1.68 ± 0.11	1.67 ± 0.11	<0.001
TChol:HDL-C	5.05 ± 0.29	5.01 ± 0.32	4.76 ± 0.23	0.06

All units are presented as mmol/L

Figure 3.1: Change in lipids on SD and S Diets from D (Control) for all subjects.



* Significantly different to Dairy ($P \leq 0.05$)

3.5.2 Influence of Macronutrient Intake on Plasma Lipids

Linear regression analysis found a significant correlation between saturated fat intake and TChol ($P=0.01$). There was no significant correlation although between TChol and ratio of saturated to unsaturated fat within subjects across the diets ($P=0.12$). Similarly, when sugar intake which was determined as a component of total carbohydrates, was correlated with TG values of subjects on the treatment diets, linear regression showed no significant relationship ($P=0.95$).

3.5.3 Influence of Equol Production on Plasma Lipids

There was no significant interactive effect between equol production and diet in terms of changes in TChol (P=0.94), TG (P=0.68), LDL-C (P=0.75) or HDL-C (P=0.74). When equol production status was nested into the effect of the SD and S diets on blood lipids, no significant interactions between diet treatment and equol production status on changes in TChol (P=0.90), LDL-C (P=0.79), HDL-C (P=0.70) and TG (P=0.96) were observed.

As shown in Table 3.2, a dietary effect was observed for non equol producers with plasma TG shown to be significantly lower in these individuals after completing the S diet compared to D (P=0.03)

Table 3.2: Lipid concentrations and changes on diets based on equol status.

	Lipids	Absolute Concentration (mmol/L)			P-value	% Difference from D	
		D	SD	S		(SD-D/D*100)	(S-D/D*100)
Equol Producers n=30	TChol	5.49 ± 0.11	5.43 ± 0.14	5.27 ± 0.12	0.15	-0.64 ± 1.97	-3.31 ± 2.12
	LDL-C	3.44 ± 0.12	3.40 ± 0.12	3.28 ± 0.10	0.34	0.52 ± 3.33	-2.00 ± 4.02
	HDL-C	1.26 ± 0.09	1.30 ± 0.09	1.28 ± 0.09	0.84	2.22 ± 3.46	6.09 ± 6.90
	TG	1.77 ± 0.20	1.67 ± 0.21	1.64 ± 0.16	0.25	-5.34 ± 4.70	-1.94 ± 4.59
Non-Equol Producers n=61	TChol	5.73 ± 0.09	5.68 ± 0.10	5.58 ± 0.11	0.10	-0.68 ± 1.20	-2.61 ± 1.17
	LDL-C	3.58 ± 0.09	3.54 ± 0.10	3.50 ± 0.10	0.57	-0.55 ± 1.61	-0.77 ± 2.35
	HDL-C	1.36 ± 0.07	1.37 ± 0.07	1.34 ± 0.06	0.67	1.91 ± 1.99	0.96 ± 2.25
	TG	1.82 ± 0.14	1.69 ± 0.12	1.68 ± 0.14	0.03	-2.52 ± 3.78	-5.02 ± 3.05

All units are presented as mmol/L

Figure 3.2 and 3.3 illustrate the absolute change in lipids for equol and non-equol producers on the SD and S diets compared to D respectively.

Figure 3.2: Change in lipids on the SD Diet compared to D (control) for equol and non-equol producers.

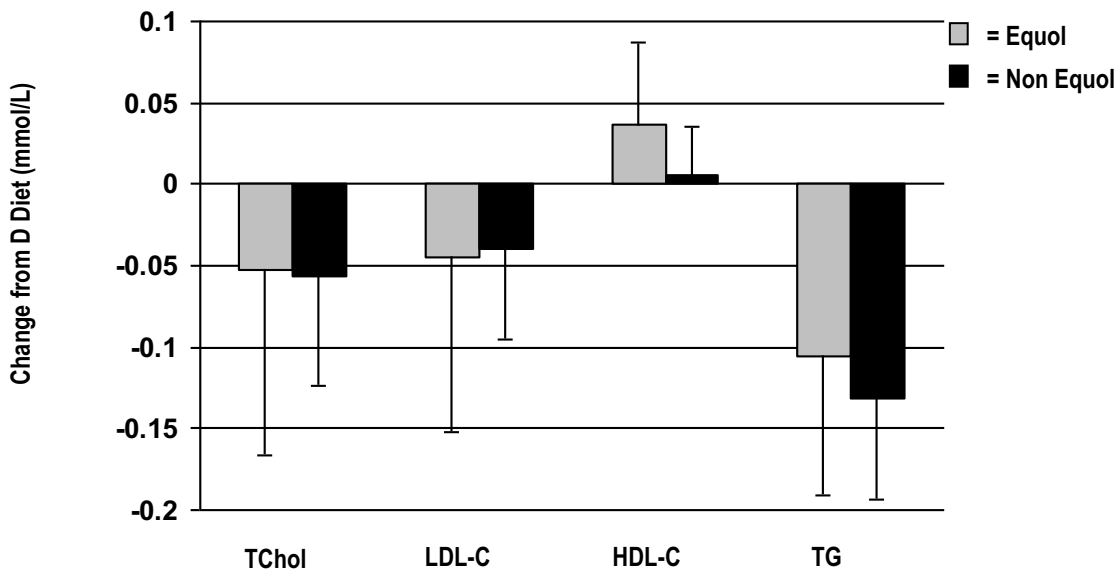
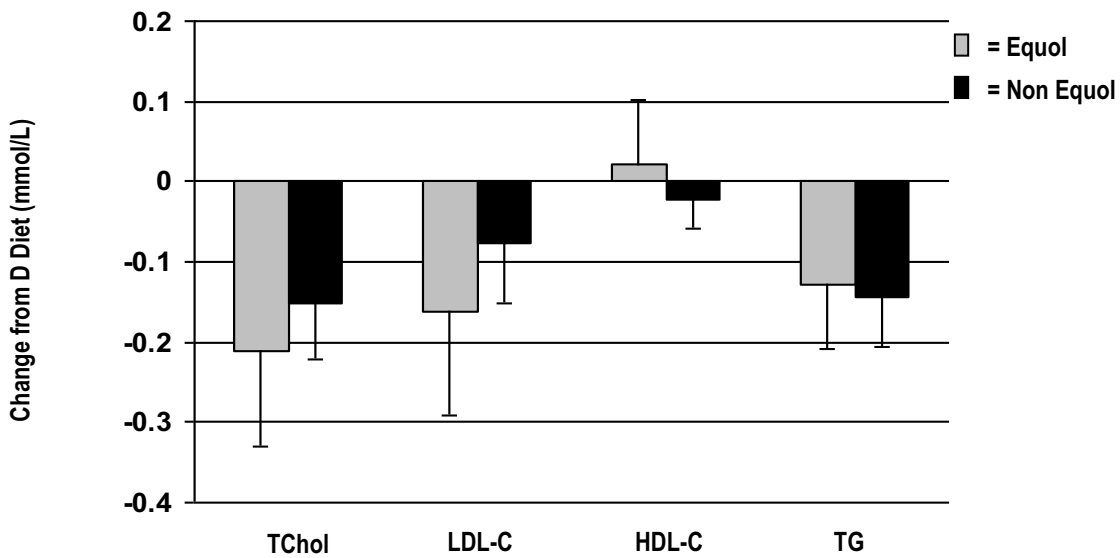


Figure 3.3 Change in lipids on the S Diet compared to D (control) for equol and non-equol producers.



3.5.4 Effect of Gender on Lipids

There were significant differences in lipids values for males and females at baseline with HDL-C significantly higher ($P=0.009$) and TG significantly lower ($P=0.01$) in females compared to males. Two ways repeated measures ANOVA found during the diet treatments there was no effect of gender on TChol or LDL-C ($P= 0.47$ for both) . There was a significantly lower HDL-C ($P=0.004$) and higher TG ($P=0.002$) concentration for males compared to females over the diet treatments as shown in Table 3.3.

Table 3.3 Influence of gender on lipid concentrations on the diets.

	Lipid	D	SD	S
Males n=34	TChol	5.64 \pm 0.11	5.55 \pm 0.14	5.34 \pm 0.14
	LDL-C	3.53 \pm 0.14	3.45 \pm 0.16	3.30 \pm 0.13
	HDL-C	1.13 \pm 0.09	1.17 \pm 0.09	1.14 \pm 0.08
	TG	2.22 \pm 0.21	2.12 \pm 0.20	2.08 \pm 0.21
Females n=57	TChol	5.66 \pm 0.09	5.63 \pm 0.10	5.56 \pm 0.11
	LDL-C	3.54 \pm 0.09	3.52 \pm 0.08	3.51 \pm 0.08
	HDL-C	1.45 \pm 0.06*	1.45 \pm 0.07*	1.43 \pm 0.06*
	TG	1.56 \pm 0.12*	1.42 \pm 0.11*	1.42 \pm 0.10*

All units are presented as mmol/L, *Significantly different from Males ($P \leq 0.05$)

When subject's lipids were expressed as a percent difference from D on both SD and S, as seen in Figures 3.4 and 3.5 there was no significant difference between genders ($P>0.36$). There was a trend albeit non significant on the S diet for males to experience a greater reduction in TChol and LDL-C in conjunction with an improvement in HDL-C compared with females ($P>0.08$).

Figure 3.4: Percent change in lipids on SD Diet from D for genders.

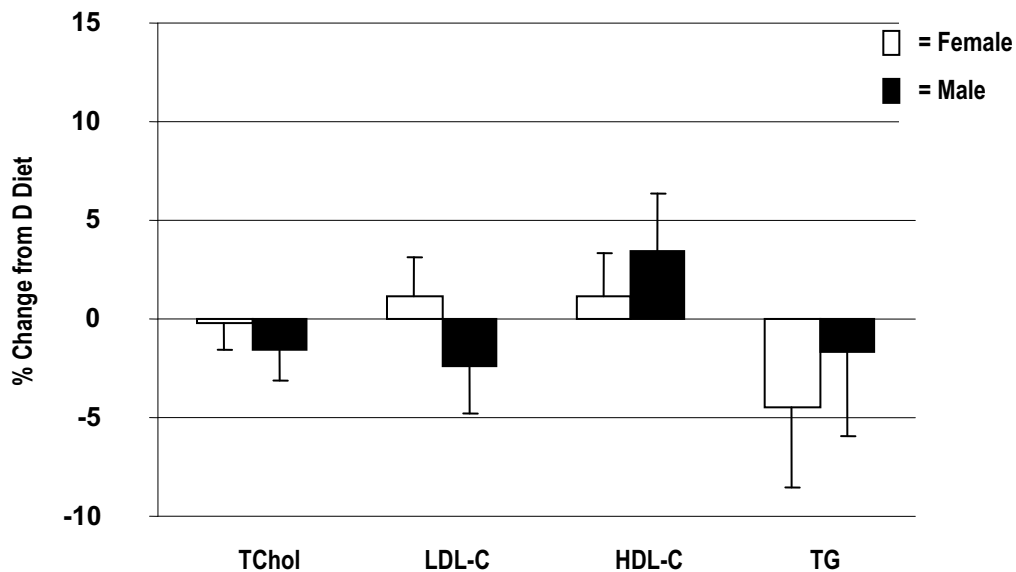
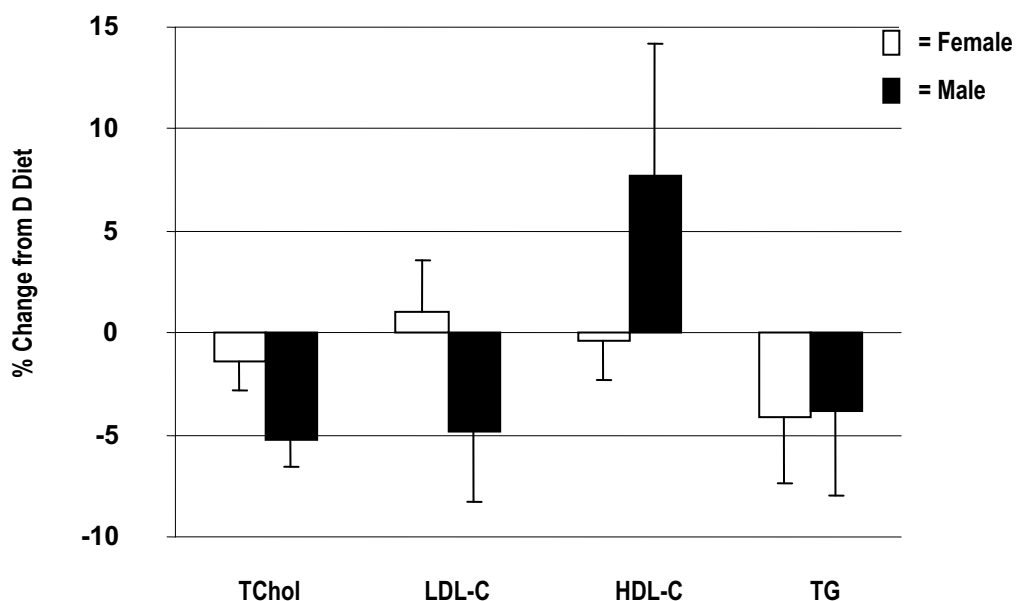


Figure 3.5: Percent Change in Lipids on S Diet from D for genders.



3.5.5 Correlation between Isoflavone Intake and Changes in Lipids

The different ISO contents of the trial foods resulted in a small, but significantly different intake of ISOs during the different dietary phases ($D < S < SD$; $P < 0.05$) (refer to Chapter 2 section 2.16.7). Linear regression analysis found no correlation between mean ISO intake/day and changes in lipid parameters (for absolute or % change compared with D) for all subjects or equol- producers on the SD ($P > 0.37$) and S ($P > 0.08$) diets.

Table 3.4: Correlation between changes in isoflavone intake/day and changes in lipid parameters.

	Lipid	SD Diet		S Diet	
		Absolute ▲		Absolute ▲	
All subjects n=91	TChol	r=0.05	P=0.65	R=0.12	P=0.24
	LDL-C	r=0.09	P=0.39	R=0.18	P=0.08
	HDL-C	r=0.01	P=0.90	R=0.03	P=0.78
	TG	r=0.02	P=0.82	R=0.12	P=0.27
Equol Producers n=31	TChol	r=0.08	P=0.67	R=0.22	P=0.25
	LDL-C	r=0.04	P=0.83	R=0.29	P=0.13
	HDL-C	r=0.06	P=0.78	R=0.05	P=0.81
	TG	r=0.07	P=0.73	R=0.18	P=0.35

3.5.6 Nested Analysis of Soy vs Dairy Consumption on Lipids

A significant effect of SP intake (either 12 or 24 g/day) on plasma TChol (P=0.03) and TG (P=0.004) was observed compared to DP. However this was not observed for LDL-C (P=0.14) or HDL-C (P=0.88). When equol production was nested in the analysis, no effect was observed (P>0.08).

3.6 Discussion

3.6.1 Effect of Trial Foods on Blood Cholesterol

Several trials have used differently processed forms of soybean (whole, fermented or unfermented) or products derived from soy flour (textured SP) or soymilk (tofu and yoghurt) with varying quantities of ISOs and compared the effects of these foods on blood cholesterol against foods containing animal protein (8, 11, 329, 447, 457). However none to date has looked at the impact of soy foods in which SP has been partially replaced with more palatable DP whilst retaining the full complement of ISOs. A major objective of the Soy Food Intervention was to therefore assess what effect consuming these novel foods would have on plasma cholesterol, specifically LDL-C, as its reduction in such trials is what prompted the FDA to declare regular SP consumption reduces a person's risk for heart disease.

Both LDL-C and TChol levels of mild hypercholesterolemics in the current study failed to be significantly reduced when consuming the novel foods on the SD diet that provided only 12g SP and a statistically higher daily intake of ISOs.

Soy foods providing 24g SP on the S diet also failed to induce a significant change in LDL-C which was unexpected considering recent reviews and meta-analyses (441, 458, 459) stipulate this amount of SP each day should produce at least a 3% reduction. In spite of the trivial 1% reduction in LDL-C, a significant 3% improvement in subject's TChol was identified on the diet. The reduction of 0.17 ± 0.06 mmol/L may have been lower than expected for mild hypercholesterolemics (398) consuming this amount of SP and ISOs, yet sufficient power within our subjects cohort (n=91) recognised its statistical significance.

The failure of both diets to achieve clinically significant reductions in LDL-C cannot be attributed to poor compliance by the subjects which was >96 % on all diets. Instead, it may have been because their initial baseline levels were only mildly elevated (3.60 ± 0.7 mmol/L) and that higher levels that would qualify for statin treatment such as that of subjects in a similar, smaller scale study (430) which reported a 5 % LDL-C reduction from 25g/day SP (4.8 ± 0.5 mmol/L) is necessary.

Given that there was no significant decrease in LDL-C levels, it's likely the modest 3% reduction in TChol seen on the S diet is in fact due to both a reduced saturated fat and increased unsaturated fat intake which occurred as a result of consuming the trial foods. This outcome would confirm the study of Gardner *et al* (430, 460) who reported that up to one half of cholesterol reduction from soy milk occurs due to its decreased saturated fat and cholesterol levels and increased polyunsaturated fat content.

Due to the SD diet providing a similar intake of saturated fat to the S diet, nesting results from these diets maintained the significant reduction in subjects TChol (P=0.03).

No significant change was observed in HDL-C on the diets. The inability to see a benefit in HDL-C on the treatments may have been related to the length of the diets with evidence to demonstrate ISO supplementation greater than six weeks is necessary to mediate significant improvements (461) . When HDL-C was expressed as a proportion of TChol there was a trend for the ratio to be lower in subjects after the S treatment compared to SD and D. Some argue the ratio of TChol:HDL-C is more

precise than TChol or LDL-C in estimating the risk of CVD (462-464) as it takes into account the amount of cholesterol in the TG-rich very low density lipoprotein (vLDL) fraction, which is positively correlated with CHD risk (465).

3.6.2 Influence of Isoflavones on Lipids

The ISOs provided by trial foods on the diets were not shown to directly influence subjects' lipids. This finding supports a previous study by Hall *et al* (447) where 50 mg/day of isolated ISOs delivered in cereal bars to hypercholesterolemic women had no effect on blood lipids. A similar study by Hodgson *et al* (446) also observed no improvement on lipids or lipoproteins of healthy adults when consuming 55 mg ISOs/day.

Clerici *et al* recently showed significant improvements in TChol and LDL-C could be obtained without SP when directly delivering 33mg/day of ISOs in aglycone form in a pasta food matrix over an eight week period. If the aglycone form of ISOs is required to mediate a cholesterol benefit, studies such as the present using glycoside conjugates which require digestion by gastro-intestinal bacteria to become biologically active, may be expected to have more variable outcomes.

In the study, plasma TGs was shown to significantly improve after both diets. Although increased dietary sugar can increase TGs (466, 467) and consumption was shown to be lower on the SD and S diets, it did not account for the decrease in TG when controlled for across the diets. As ISO intake was relatively comparable on these two treatments and SP was halved in the SD diet, the mediator for reduction could be due to the inclusion of ISOs. A review by Ricketts *et al* (357) clearly demonstrates ISO's ability to activate PPARs which regulate cellular lipid metabolism. Activation of PPAR- α specifically by ISOs, improves blood and hepatic TG concentrations via increased fatty acid oxidation(468). GEN, has also been shown to activate PPAR γ resulting in an up regulation of adipogenesis which increases the uptake of fatty acids from plasma and subsequent redistribution into adipocytes (307).

3.6.3 Influence of Equol Production on Lipids

A key objective of the present study was to establish whether equol production resulted in a hypocholesterolemic effect when consuming the novel soy foods used in the SD treatment and soy foods delivering the recommended daily amount of SP in the S treatment.

The study of Meyer *et al* (11) was the first human intervention to investigate retrospectively this correlation between equol production and lipid lowering benefits from soy foods. Despite only a relatively small number of equol producers (n=8) in the study, the authors demonstrated significant reductions in the lipid profiles of equol producers compared to non producers. Of the 91 subjects who completed the present study, 30 were identified as equol producers which is the largest known cohort identified in a dietary intervention investigating the effect of soy on cholesterol reduction. This is also the first study to look at the current health claim in relation to equol producers and investigated whether or not this unique sub-population require the recommended daily dose of SP to elicit positive changes in their lipid profile or whether a reduced intake, given in combination with ISOs offers equivalent benefits.

When subjects were categorised into equol or non-equol producers, there was no significant difference in effects on TChol, LDL-C, HDL-C or TG between groups. Despite equol producers having on average a greater LDL-C reduction on the S diet and greater increases in HDL-C on both SD and S treatments, the large variance in their responses on the diets failed to make the trend significant. Attempting to optimise the analysis by nesting equol production and diet treatments also failed to demonstrate an effect on blood lipids. Our finding subsequently confirm that of another recent, but smaller study, which also showed no difference in the effect of SP on blood lipids of equol producers and non producers (430).

3.6.4 Effect of Soy Foods in Relation to Reducing Cardiovascular Disease Risk

Since the release of a health claim for SP over 10 years ago, the CVD effects of soy have become a topic of intense scientific and public scrutiny. The notable lack of effect on LDL-C, even when consuming the FDA and JHCI recommended amount of SP indeed questions the appropriateness of the current Soy Health Claims(1, 281) as a preventative measure for reducing heart disease. This is

because LDL-C is shown to have the strongest correlation with CVD morbidity and mortality(434, 435) and is the principal criterion on which the National Cholesterol Education Program estimates risk and recommends therapy for CVD (437).

The modest 3% reduction we did observe in TChol for subjects on the S diet is not only clinically insignificant if a 10% reduction in TChol is necessary to mediate a 15% decrease in CVD mortality risk and 11% decline in total mortality (433), but it may be accounted for by a lower saturated fat intake on the diet as a result of the test foods. Results from the current study demonstrate soy consumption alone is not the answer to reduce heart disease risk in mild hypercholesterolemics and reiterates the position currently held by the American Heart Association Science Advisory Committee than SP and ISOs offer minimal CV benefits (441). Other FDA health claims which state to similarly reduce risk for heart disease such as viscous soluble fiber from psyllium, highlight the inconsequential nature of a 1 % reduction in LDL-C with 24g SP/day when a standard daily psyllium dose of 10.2 g is shown to reduce LDL-C by 7% (438). Consumption of margarines containing plant sterols and stanols in doses of 0.7g/day and 2.5g/day have also been shown to mediate LDL-C reductions in the range of 6.7%-11.3% (469) . Even if the novel soy foods were consumed by mild hypercholesterolemics in combination with a low saturated (and *trans*) fatty acid and cholesterol diet, which can potentially evoke a 10-20% reduction in LDL-C(470-472), the reduction in LDL-C would not compare to the effects expected with a starting dose of statins, the current first line drug therapy (minimum 40% reduction in LDL-C)(436).

3.7 Summary

When regularly consumed by mild hypercholesterolemic subjects, the current study found that foods delivering 24g SP/day or, alternatively, 12g/day but with an equal amount of ISOs did not significantly reduce LDL-C. This outcome was unrelated to the ability of individuals to produce equol. A 3% reduction in TChol was observed on the S diet compared with the D diet, but could be accounted for by a reduced saturated fat intake on the diet as a result of the trial foods. The results of this study call

into question the ability of SP to significantly reduce cholesterol and the risk of heart disease as stated in current health claims for soy foods.

Consumption of soy foods was shown to improve plasma TG, which when elevated is recognised as a CVD risk factor. It's likely the benefit in TG is attributable to ISOs rather than SP provided by the diets.

4.0 Effect of Soy Foods on Metabolic Risk Factors

4.1 Introduction

Obesity and insulin resistance are two closely related metabolic disorders that are known to increase a person's risk of CHD. They are both characterised by impaired glucose tolerance and hyperinsulinemia and have a tendency to manifest in each others presence (473) . Importantly, they play a cardinal role in the development of other CVD risk factors such as diabetes, hyperlipidemia and hypertension, which when clustered together (metabolic syndrome), pose considerable CVD risk.

While much focus still remains on its ability to lower cholesterol, evidence is emerging to suggest soy may also reduce CVD by preventing the development of obesity and insulin resistance (307, 474-477, 478). Several intervention studies in animals (476-478) and humans (474, 475) have provided evidence that compared with casein, consumption of soy improves glucose control, insulin sensitivity and body composition. However conjecture remains as there are still some studies that report no effect (479-481).

The physiological actions of ISOs on PPARs have been identified as mediating these metabolic improvements. As demonstrated by Mezei *et al* (307), a SP diet high in ISOs increases PPAR- γ mediated expression of insulin sensitizing genes in adipocytes, which inturn improves glucose tolerance. The activation of PPAR α in skeletal muscle cells is also shown to increase levels of enzymes necessary for fatty acid oxidation which prevents the accumulation of free fatty acids in adipocytes (468). Besides their action on PPAR's, ISOs are also shown to favourably affect insulin sensitivity and glucose control by acting as α -glycosidase inhibitors which can limit intestinal glucose absorption and postprandial hyperglycaemia (482).

In terms of the potential of equol to prevent insulin resistance and obesity, no human study has reported a significant effect. One study in ovariectomized rats has demonstrated that dietary

administration of equol can attenuate body weight gain and intra-abdominal fat accumulation which may transpire in humans as well (483).

4.2 Aims

- a) To investigate whether SP and ISOs provided by novel foods improve metabolic risk factors (ie. insulin resistance, glucose sensitivity and anthropometric measurements for obesity) in mild hypercholesterolemics;
- b) to determine if the inclusion of varying quantities of SP (12g or 24g/day) in novel soy foods enhances the effect of ISOs on improving metabolic risk factors;
- c) To determine the importance, if any, of equol in mediating metabolic improvements.

4.3 Methods

4.3.1 Subjects and Recruitment

Data were collected from all subjects who were recruited as part of the Soy Food Intervention. Refer to Chapter 2 section 2.5 for subject inclusion and exclusion criteria. Methods of recruitment are also described in section 2.6.

4.3.2 Study Design and Protocol

Refer to Chapter 2 section 2.8.1 and 2.8.2 for a description of the design and protocol.

4.3.3 Anthropometric Assessments

Refer to Chapter 2 section 2.9 for protocols used to assess subjects weight, height and waist to hip measurements.

4.3.4 Metabolic Assessments

4.3.4.1 Plasma Glucose and Insulin

Plasma glucose and insulin were measured from blood samples collected in sodium fluoride vacutainer tubes. Refer to Chapter 3 section 3.3.2.1 for protocol used for blood collection. Plasma

glucose concentrations were determined using a Konelab 20XTi auto- analyser (Thermo Electron, Victoria, AUS) with appropriate standards and reagents. Plasma insulin concentrations were determined using a highly specific and sensitive double-antibody commercial Human Insulin Specific RIA Kit (LINCO Research Inc, Missouri, USA) with an inter-assay coefficient of variation of 3.4% and an intra-assay variability of 2.5% (Linco Research, Inc., St. Charles, MO).

4.3.4.2 HOMA 2 Calculation

The updated HOMA2 (or computer) model with nonlinear solutions, which uses paired fasting glucose and insulin values was calculated (HOMA Calculator version 2.2) according to Wallace *et al* (484) and yielded the following assessments: HOMA2-IR and HOMA2-%B-cell function (HOMA2%B), where 100% is normal B-cell function.

4.3.5 Study Foods, Intake and Compliance

Refer to Chapter 2 section 2.10 for detailed description of the range, composition and availability of trial foods consumed in the study. Refer to section 2.11 for protocols used to measure study food, macronutrient intake and compliance.

4.3.6 Identification of Equol Producers in the Intervention

Overnight urine samples were collected after the completion of each treatment and analysed for the presence of ISOs (refer to Chapter 2 section 2.11.2.2 and 2.12.2 for protocols used). Subjects were subsequently identified as equol producers based on the method established by Setchell and Cole (125) described in section 2.13.

4.4 Statistics

Refer to Chapter 2 section 2.15 for statistical procedures used to analyse data.

4.5 Results

4.5.1 Effect of Diets on Anthropometric and Metabolic Factors

As shown in Table 4.1, one way ANOVA with repeated measures found no difference in anthropometric or metabolic measurements of all subjects during the treatment diets.

Table 4.1: Anthropometric and metabolic measurements of all subjects on the diets.

	Absolute Concentration (mmol/L)			P-value
	D	SD	S	
Weight (kg)	77.9 ±1.5	78.0 ±1.5	77.9 ±1.5	0.55
BMI (kg/(m²))	27.6 ± 0.5	27.6 ± 0.5	27.5 ± 0.5	0.67
Waist Circumference (cm)	88.3 ± 1.3	88.5 ± 1.4	88.5 ±1.3	0.69
Hip Circumference (cm)	103.9 ± 1.0	104.2 ± 1.0	103.9 ± 1.0	0.37
Waist:Hip Ratio	0.8 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.60
Glucose (mmol/L)	4.98 ± 0.13	5.00 ± 0.14	4.95 ± 0.14	0.26
Insulin (mmol/L)	11.96 ± 0.41	12.4 1 ± 0.45	12.29 ± 0.43	0.62
HOMA	1.53 ± 0.05	1.58 ± 0.06	1.57 ± 0.06	0.59

When subjects were categorised based on their baseline fasting glucose values, 57 subjects were found to be glucose sensitive (fasting glucose < 5.6 mmol/L) and 35 subjects glucose intolerant (fasting glucose >5.6 mmol/L). As shown in Table 4.2, there was no difference in anthropometric or metabolic measurements across the diets for either glucose sensitive or intolerant subjects. There was also no interactive effect observed between the glucose status of subjects and diet treatments for insulin (P=0.94), HOMA (P=0.48) and all anthropometric measurements (P>0.50).

Table 4.2: Anthropometric and metabolic measures on the diets based on glucose tolerance.

		D	SD	S	P-value
Glucose	Glucose (mmol/L)	4.29 ± 0.14	4.28 ± 0.14	4.21 ± 0.13	0.06
Sensitive	Weight (kg)	78.7 ± 1.8	78.91 ± 1.77	78.68 ± 1.79	0.49
N=57	BMI (kg/m²)	27.5 ± 0.6	27.55 ± 0.58	27.48 ± 0.59	0.68
	Waist Circumference (cm)	88.3 ± 1.6	88.56 ± 1.58	88.63 ± 1.58	0.71
	Hip Circumference (cm)	103.5 ± 1.1	103.85 ± 1.07	103.32 ± 1.13	0.30
	Waist:Hip Ratio	0.85 ± 0.01	0.85 ± 0.01	0.86 ± 0.01	0.42
	Insulin (mmol/L)	11.68 ± 0.54	12.04 ± 0.55	11.91 ± 0.56	0.82
	HOMA	1.47 ± 0.06	1.56 ± 0.06	1.54 ± 0.06	0.35
Glucose	Glucose (mmol/L)	6.18 ± 0.09	6.24 ± 0.09	6.23 ± 0.11	0.68
Intolerant	Weight (kg)	76.5 ± 2.8	76.5 ± 2.9	76.5 ± 2.9	0.93
N=35	BMI (kg/m²)	27.7 ± 0.8	27.6 ± 0.9	27.6 ± 0.9	0.83
	Waist Circumference (cm)	88.2 ± 2.3	88.5 ± 2.6	88.3 ± 2.5	0.90
	Hip Circumference (cm)	104.6 ± 1.7	104.8 ± 1.8	104.8 ± 1.7	0.79
	Waist:Hip Ratio	0.84 ± 0.02	0.84 ± 0.02	0.84 ± 0.02	0.97
	Insulin (mmol/L)	12.43 ± 0.63	13.03 ± 0.75	12.93 ± 0.68	0.42
	HOMA	1.64 ± 0.10	1.62 ± 0.11	1.60 ± 0.11	0.87

4.5.2 Influence of Equol Production on Anthropometric and Metabolic Measures

As shown in Table 4.3, equol producers has significantly lower waist and hip circumference measurements ($P < 0.02$) and waist:hip ratio values ($P = 0.03$) at baseline than non-equol producers.

Table 4.3: Baseline anthropometric and metabolic measures for equol and non equol producers.

	Equol (n=30)	Non- Equol (n=61)	P-value
Gender (M / F)	11/19	26/35	-
Age (y)	55.4 \pm 8.5	51.4 \pm 10.4	0.12
Weight (kg)	73.6 \pm 11.5	78.8 \pm 15.6	0.11
BMI (kg/(m ²))	26.6 \pm 5.0	27.6 \pm 4.3	0.36
Waist Circumference (cm)	83.0 \pm 10.0	91.7 \pm 12.9	< 0.01
Hip Circumference (cm)	99.9 \pm 8.7	105.9 \pm 8.7	0.02
Waist:Hip Ratio	0.82 \pm 0.1	0.86 \pm 0.1	0.03
Glucose (mmol/L)	4.83 \pm 1.31	5.01 \pm 1.24	0.53
Insulin (mmol/L)	13.28 \pm 7.41	11.97 \pm 4.77	0.31
HOMA	1.53 \pm 0.10	1.54 \pm 0.08	0.96

Two-way repeated measures ANOVA found no significant interactive effect between equol production and diet treatments for any anthropometric ($P > 0.15$) and metabolic ($P > 0.31$) measurements during the intervention. Equol producer's significantly lower waist and hip circumference measurements ($P < 0.01$) and waist:hip ratio values ($P = 0.05$) was maintained over the three treatment periods compared with non-equol producers (see Table 4.4).

Table 4.4: Anthropometric and metabolic measures on diets for equol and non equol producers

		D	SD	S	P- value
Non- Equol Producers n=61	Weight (Kg)	79.5 ± 2.0	79.6 ± 2.0	79.4 ± 2.0	0.63
	BMI (kg/m ²)	27.9 ± 0.56	27.8 ± 0.6	27.8 ± 0.6	0.29
	Waist Circumference (cm)	91.2 ± 1.6	91.4 ± 1.7	91.5 ± 1.7	0.67
	Hip Circumference (cm)	105.9 ± 1.1	106.3 ± 1.1	105.8 ± 1.2	0.26
	Waist:Hip Ratio	0.86 ± 0.01	0.86 ± 0.01	0.86 ± 0.01	0.33
	Glucose (mmol/L)	5.04 ± 0.16	5.07 ± 0.17	5.03 ± 0.17	0.69
	Insulin (mmol/L)	11.85 ± 0.54	12.22 ± 0.57	12.18 ± 0.50	0.67
	HOMA	1.52 ± 0.07	1.56 ± 0.07	1.57 ± 0.07	0.66
Equol Producers n=30	Weight (Kg)	74.5 ± 2.1	74.8 ± 2.2	74.8 ± 2.2	0.35
	BMI (kg/m ²)	27.0 ± 0.9	27.1 ± 1.0	27.1 ± 0.9	0.29
	Waist Circumference (cm)	82.4 ± 1.9	82.6 ± 2.0	82.4 ± 1.7	0.89
	Hip Circumference (cm)	99.9 ± 1.6	100.0 ± 1.6	100.1 ± 1.5	0.87
	Waist:Hip Ratio	0.82 ± 0.01	0.82 ± 0.01	0.82 ± 0.01	0.96
	Glucose (mmol/L)	4.88 ± 0.24	4.86 ± 0.25	4.79 ± 0.24	0.30
	Insulin (mmol/L)	12.18 ± 0.62	12.80 ± 0.68	12.52 ± 0.84	0.74
	HOMA	1.56 ± 0.08	1.62 ± 0.10	1.56 ± 0.11	0.75

4.5.3 Correlation between Isoflavone Intake and Changes in Anthropometric and Metabolic

Markers

Linear regression analysis found no correlation between mean ISO intake/day and changes in anthropometric measures for all subjects or equol producers on the SD ($P>0.37$, $r<0.01$) or S ($P>0.08$, $r<0.01$) diets. Furthermore there was no correlation observed for changes in glucose or insulin on either SD ($P>0.32$, $r>0.01$) or S ($P>0.48$, $r>0.01$) for all subjects or equol producers.

4.5.4 Influence of Gender and Age on Anthropometric and Metabolic Measures

Females had significantly lower weight, waist circumference and waist:hip ratio values ($P<0.01$) compared to males at baseline and across all diet treatments. There was no gender effect on any other anthropometric ($P>0.63$) or metabolic measures ($P>0.45$). Furthermore there was no relationship between age of subjects and changes on the diets for all anthropometric ($P>0.11$) or measures ($P>0.09$).

Two way repeated measures ANOVA found no interactive effect between gender and the effect of the diet treatments on any anthropometric or metabolic measures.

4.6 Discussion

4.6.1 Effect of Diets on Metabolic Measures

Insulin resistance and impaired glucose tolerance (fasting glucose ≥ 5.6 mmol/L) are common features in individuals who are either pre diabetic or have diabetes.

Animal studies were the first to report that soy improves insulin resistance (476, 485) and can decrease the onset (486) or occurrence of diabetes (487). HOMA is a common model used to estimate an individual's level of insulin resistance from fasting plasma insulin and glucose concentrations (488). Despite other human clinical studies showing insulin sensitising effects, when SP with ISO is consumed (311, 489), in the present intervention, subject's metabolic biomarkers were unaffected by the diets. This finding supports Hall *et al* (447) who also found no improvement in HOMA, fasting insulin and glucose concentrations in healthy postmenopausal women receiving 50mg ISO/day for 8 weeks. It's likely that there was no scope within our study population to detect a change in these measures, as all subjects had normal insulin sensitivity and glucose tolerance according to their baseline fasting glucose (4.95 ± 0.13 mmol/L) and HOMA scores (1.53 ± 0.06).

When subjects were categorised on the basis of whether they were initially glucose intolerant, those 34 subjects who did have baseline plasma glucose levels > 5.6 mmol/L were not shown to have improved glucose tolerance or insulin sensitivity on any of the diets. The fact that these subjects had a mean HOMA score of 1.54 ± 0.11 at baseline and a value > 3.8 indicates insulin resistance (490), implies their degree of glucose impairment was not clinically significant.

4.6.2 Effects of Diets on Anthropometric Measures

Obesity and abdominal fat distribution are independent risk factors for CVD and are frequently associated with other CVD risk factors and markers of disease progression such as dyslipidemia, atherosclerosis, diabetes and CHD. Several human and animal (491-496) studies report the inclusion of

soy in the diet can induce significant anti-obesity effects and lead to an improved body composition. Whilst the SP fraction β -conglycinin, has been linked with reducing weight gain and suppressing appetite in animals (497) Naaz *et al* (498) demonstrated ISO supplementation can also attenuate body fat gain by specifically decreasing adipose weight and adipocyte circumference.

In the present study, none of the diet treatments were shown to significantly improve anthropometric measures although this is possibly due to the short intervention periods. According to Australia's National Heart Foundation Guidelines for reducing CVD risk, subjects recruited for participation in the intervention were overweight (BMI: 27.3 ± 4.5) and had higher than recommended waist circumference measurements (males 95.6 ± 10.1 , recommended ≤ 94 cm; females 84.8 ± 12.3 , recommended ≤ 80 cm) and thus were susceptible to possible body composition changes with the inclusion of both SP and ISOs in their diets. As might be expected, females in the intervention had significantly lower baseline body weight, waist circumference and waist:hip ratio values compared to males ($P < 0.01$) and this trend carried across all the diet treatments. However, these differences in gender were not shown to influence the effect of the diet treatments on anthropometric measurements. As reported in Chapter 2 section 2.16.5, despite subjects deriving a higher proportion of their energy from protein and less from carbohydrates and unsaturated fat, weight loss was not reported across the diet treatments as might be expected with such a diet (499-501).

Subject's intake of ISOs on the diets was not shown to directly influence their anthropometric measurements. Whilst the lack of a direct effect on BMI and waist-to-hip circumference may parallel other studies (480, 481), it does not support the findings of Goodman-Gruen and Kritz-Silverstein (502) who specifically found dietary ISO intake to be inversely correlated with body weight, BMI and waist circumference in healthy post-menopausal women.

4.6.3 Effect of Equol on Anthropometric and Metabolic Measures

A recent animal study (483) showed the administration of dietary equol could attenuate body weight gain and intra-abdominal fat accumulation in ovariectomized rats. In the current study however, equol production failed to affect anthropometric measures of subjects. Equol producers were found to

have smaller waist and hip circumference measurements as well as lower waist:hip ratio values at baseline and across the diet treatments compared to non-producers, implying producers in the study had a more advantageous body composition than non-producers. Estrogen is shown to have anti-obesity effects via activation of ER α in the hypothalamus; which play an important role in controlling energy balance, body fat distribution and weight gain (503, 504). As equol is a potent ER α agonist compared to, DAZ and GEN (124), its production may account for the observation that producers in the present study had lower waist: hip ratios. As seen with other studies (447), equol production did not improve effects of the diets on glucose and insulin. This could be due to the fact that all subjects in the study did not have impaired glucose tolerance or insulin resistance.

4.7 Summary

Despite evidence to suggest SP and ISOs can beneficially influence metabolic factors, the novel foods consumed as part of the Soy Food Intervention did not significantly improve glucose, insulin, HOMA or anthropometric measures in mild hypercholesterolemics. Furthermore, equol production did not influence the effect of the diets on these measures, but it was of interest that in this self-selected population, equol producers had significantly slimmer body shapes than non-producers.

5.0 Effect of Soy Foods on Markers of Circulatory Function

5.1 Introduction

A healthy endothelium is quintessential for maintaining vascular homeostasis. While it's most recognised for regulating vascular tone by synthesising substances that undergo complex interactions with cells in the vessel wall and lumen, the endothelium also serves to control blood fluidity and inflammation processes (see review by Gordon *et al* (505)).

Recent studies have demonstrated that dysfunction of the endothelium, specifically a loss of control over vascular tone, correlates with risk for an initial or recurrent CV event (506). Endothelial dysfunction typically manifests in the presence of CVD risk factors and is characterised by imbalances in production of vasodilator and vasoconstrictor substances. Loss of NO bioavailability due to reduced synthesis and increased scavenging by reactive oxygen species, is a cardinal feature in dysfunction (507). Consequently, its existence is best conferred by the failure of the endothelium to elicit NO-mediated vasodilatation, which can be assessed using a test for FMD of the brachial artery (234).

In addition to abnormal vasoreactivity, dysfunction of the endothelium is characterised by enhanced cytokine and adhesion molecule expression. Leukocytes are recruited into the vessel wall as a result, triggering inflammation, platelet aggregation and coagulation, as well as the proliferation of VSMCs and deposition of calcium into the underlying vascular tissue (202-204, 508)(refer to Chapter 1 section 1.8.3.2). Loss of tone and the milieu that accompanies endothelial dysfunction has discernible effects on arterial compliance and BP, as it promotes intimal thickening and restructuring of the vasculature. Improving endothelial function, via increased expression of NO is therefore highly desirable in order to prevent arterial stiffening and elevated BP which overt the manifestation of atherosclerosis.

Observational studies in postmenopausal women demonstrate HRT prevents the develop of atherosclerosis (509, 510) with its cardio protective benefit attributed to estrogen's ability to improve vascular function via its action on ERs in tissue. ER β is specifically located along the plasma membrane

of human vascular endothelial cells (511, 512) and has been shown *in vitro* to play a crucial role in regulating vascular homeostasis (513) by inducing genomic and non genomic pathways which increase the expression and/or activity of eNOS and subsequent synthesis of NO (350, 514). As ISOs contain the same phenolic ring structure as that present in estradiol and are known to bind preferentially to ER β (512, 515), albeit with a lower affinity (516), soy supplementation has been investigated for its potential role in improving endothelial function and manifestations of its dysfunction such as arterial compliance and BP.

In vitro and animal studies (313, 322, 517, 518) conclusively demonstrate ISOs can stimulate eNOS activity and expression, via genomic and non genomic events, with evidence they can also regulate NO expression independent of ER mediated pathways (refer to Chapter 1 section 1.9.7.3.1 for detailed description of molecular mechanisms). Furthermore, ISOs are shown to up regulate the expression of antioxidant defence genes and act directly as antioxidants ((519) which can increase NO bioavailability by decreasing degradation from free radicals.

In addition to NO production, ISO mediate an array of ER independent, non NO mediated events which may positively impact on vascular tone to promote increased arterial compliance and reductions in BP. As described in Chapter 1 section 1.9.7.3.2, ISOs *in vivo* can down regulate genes encoding for potent vasoconstrictors, alter transcription factors (e.g. NF- κ B) that induce reactive oxygen specie formation, stimulate receptors (e.g. PPAR, ANPA) that inhibit VSMC proliferation and inflammatory cytokine production and directly incite smooth muscle relaxation.

Evidence from human interventions remains inconclusive as to whether the benefits of ISOs *in vivo* transpire into improved endothelial function when measured by NO- dependent vasodilatation. Several studies using isolated ISOs (13, 325, 338) and ISOs with SP (344, 345, 520) have reported significant improvements to FMD. Whilst studies using soy ISO precursors (322, 343), glycoside conjugates delivered in a supplement (323, 342), food matrix (329, 330) or in combination with SP (12) report only trends or in some cases no effect at all on FMD. Furthermore, evidence for an effect on arterial compliance and BP with ISOs remains inconclusive with some studies reporting an improvement with supplementation (316, 320, 322, 324, 333, 347) and others no effect (9, 321, 327, 330).

There is evidence to demonstrate that brachial artery infusion of the DAZ metabolite dehydroequol increases forearm blood flow (340). Yet only two studies have looked at the influence of equol production on improvements in FMD and BP, with no study investigating effects on arterial compliance. The first study by Kreijkamp-Kaspers *et al* (12) involved 202 post menopausal women who were assigned either a soy treatment (25.6g of SP with 99 mg of ISO/day) or placebo treatment (milk protein) for one year. At the conclusion of the study, there was no difference between treatment groups. However, when those on the soy treatment were categorised based on equol status, producers were found to have displayed improvements, albeit non significant, in FMD compared to those women who were not. The authors also reported significant reductions in BP for equol producers (SBP -5.6 %, DBP-1.6%). The other study by Clerici *et al* (13) reported increased FMD in equol subjects compared to non producers advocating equol's role in enhancing vascular function.

5.2 Aims of the Intervention in Relation to Vascular Function

- a) to ascertain whether chronic ISO supplementation delivered in novel food matrices improves vascular function as measured by FMD, arterial compliance and BP in individuals with mild hypercholesterolemia;
- b) To determine if the inclusion of varying quantities of SP (12g or 24g/day) in novel soy foods enhances the effect of ISOs on vascular function;
- c) To determine the importance, if any, of equol in mediating vascular improvements.

5.3 Methods

5.3.1 Subjects and Recruitment

Data were collected from those subjects who were recruited as part of the Soy Food Intervention. Refer to Chapter 1 section 2.5.1 for subject inclusion and exclusion criteria. Methods of recruitment are also described in section 2.6.

5.3.2 Study Design and Protocol

For a description of the study design and protocol refer to Chapter 2 section 2.8.1 and 2.8.2. All subjects underwent assessments for BP and arterial compliance. Only subjects who were recruited from

Intake 1 and 3 (based in Adelaide) underwent assessments for peripheral endothelial function (viz. FMD).

5.4 Protocols for Vascular Assessments

5.4.1 Measuring Blood Pressure

During study visits, subject's BP was assessed with the CR- 2000 Research HDI CV Profiler (HDI, Egan Minnesota, USA). Subjects were instructed to lie supine on a plinth with their legs straight, uncrossed and left arm resting flat to the side for five minutes in a temperature controlled room ($24 \pm 2^{\circ}\text{C}$). During this time, subjects were encouraged to relax, lie quietly and breathe normally. After allowing subject's BP to normalise, a sphygmomanometer BP cuff attached to the CV Profiler, was securely positioned around the subject's left upper arm so the lead was aligned over the brachial artery in the front of the inner elbow (antecubital fossa). Subjects were instructed not to talk during periods of cuff inflation. Three recordings of BP were taken with a minimum five minute interval between repeat measurements. Values for BP provided by the CV Profiler included SBP, BAP, mean arterial BP (MAP) and pulse pressure (PP).

5.4.2 Measuring Arterial Compliance

Arterial compliance was also assessed using the CR-2000 Research HDI CV Profiler which uses the pulse signal from a subject's radial artery to derive a BP waveform. In addition to providing approximate values for BP and pulse rate, the contour of the waveform provides information related to the structure and physiological function of the arterial system. As shown in Figure 5.1, the waveform is a function of the volume and velocity of blood ejected by the heart during left ventricular contraction as well as the structural and functional aspects of the large and small arteries. As the aortic valve closes after ejection of the stroke volume, the resulting diastolic decay curve, describes the distensibility of the succeeding arterial tree (211, 521).

NOTE:

This figure is included on page 131 of the print copy of the thesis held in the University of Adelaide Library.

Figure 5.1: Interpretation of blood pressure waveform derived from CV Profiler. Source: HDI Diagnostics Inc (522).

Two values from the CV Profiler that are of most benefit in predicting CVD risk are the large artery elasticity index (LAEI) and small artery elasticity index (SAEI), also known as C1 and C2 respectively. They are derived from a 30- second collection of radial artery BP waveforms that are analysed according to a modified Windkessel model (523, 524) (see Figure 5.2). Reductions in SAEI manifest in the presence of endothelial dysfunction in the micro vascular circulation and continue to fall as vascular disease progresses. Furthermore, its reduction precedes significant decreases in LAEI (which reflects the elastic behaviour of the aorta and large arteries).

Other values provided by the CV Profiler are for systemic vascular resistance (SVR) which is calculated from MAP/cardiac output during the diastolic decay portion of the wave form and for total vascular impedance (TVI) which is calculated from LAEI (C1), SAEI (C2) and SVR values. SVR is a measure of the resistance to blood flow offered by all of the systemic vasculature, whilst TVI provides a relative value for the forces impeding steady blood flow throughout the peripheral vasculature only.

NOTE:

This figure is included on page 132 of the print copy of the thesis held in the University of Adelaide Library.

Figure 5.2: Model of CV Profiler rationale for analysis and determination of LAEI (C1) and SAEI (C2).

Source: HDI Diagnostics Inc (522).

To measure LAEI and SAEI, an appropriately sized BP cuff was placed around subjects upper left arm to allow for repeatable measurements and their right wrist (palm facing upwards) secured in a comfortable support. Attention was made to avoid over extending the wrist in the support, and care was also taken to avoid strapping the wrist stabiliser too tight on the forearm region to prevent pooling of blood and inaccurate readings. A pressure transducer attached to the CV Profiler machine was then positioned on the skin of the wrist where there was maximum pulsation (located using index finger). After acquiring a suitable waveform and signal strength (above 20%), a recording was taken. The measurement (which took approximately five minutes) was repeated a further two times with a five minute interval period between successive readings. The average of these three readings was taken to represent LAEI ,SAEI, SVR and TVI (see Appendix 3 for example of a print out assessment from the CV Profiler).

5.4.3 Measuring Peripheral Vascular Function

Endothelial dependent and independent peripheral vasodilator function was assessed using images taken from the brachial artery using a 12Hz transducer under upper vascular extremities exam mode. All images were recorded as raw-data, in the form of three second video loops, so that image

quality could be improved and measurements for arterial diameter could be made post-examination. The 12 Hz transducer was selected as it provided optimal axial resolution of the vascular wall (525).

5.4.3.1 Endothelial Dependent Vasodilatation

To assess endothelial-dependent peripheral arterial dilatory function, a test for FMD as described by Celermajer *et al* (234) was performed. In the assessment, a BP cuff was securely positioned around the subject's right forearm immediately distal to the humeral epicondyle. Two-dimensional, pulse wave mode ultrasound images of the lumen/arterial wall interface of the brachial artery were then obtained in the distal third of the upper right arm using a high resolution 12MHz linear array transducer (LOGIQ 5, GE Medical Systems, Wisconsin, USA). After optimizing the longitudinal brachial artery image with the depth and gain settings, the transducer was fixed into position with an adjustable clamp and three baseline images were recorded in order to calculate the average vessel diameter in diastole. The sphygmomanometer BP cuff was then inflated to 200 mmHg for five minutes before being deflated gradually over a ten second period to induce FMD. Arterial images were recorded ten seconds prior to initial cuff inflation, ten seconds prior to cuff deflation and then every 30 seconds for a total of three minutes post cuff release.

5.4.3.2 Endothelial Independent Vasodilatation

Once subjects had sufficiently recovered from the FMD assessment, the high-resolution 12MHz linear array transducer was once again positioned over the brachial artery and three baseline images recorded to re-establish average vessel diameter. To assess endothelial independent peripheral arterial function, the non-prescription drug nitroglycerate (NTG) was administered sublingually. Immediately after subjects placed the tablet containing 300µg of NTG underneath their tongue, ultrasound recordings were taken every minute for ten minutes.

NTG is predominately used to prevent or relieve angina and dilates both arteries and veins, so blood flow and oxygen supply to the heart is increased. It initiates vasodilatation by providing an exogenous source of NO that is capable of acting directly on smooth muscle surrounding arteries. Like

any drug, there are potential side effects that may occur from taking NTG. TGA approval was therefore acquired to use NTG in the intervention.

5.4.3.3 Analysis of Peripheral Vascular Function Assessments

As shown in Figure 5.3, the arterial diameter of the recorded ultrasound clips was measured using digital callipers (LOGIQ 5 1.1X software GE Medical Systems, Wisconsin, USA) between the anterior and posterior intima- lumen interfaces (distance between I-lines).

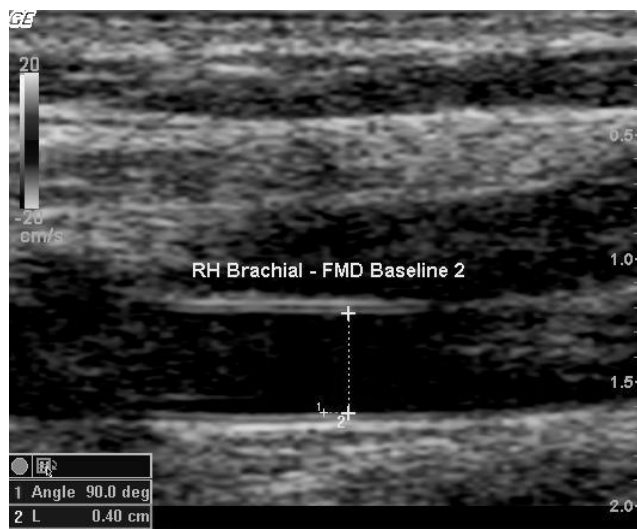


Figure 5.3: Ultrasound analysis of brachial arterial diameter using digital callipers.

If both I- lines were not visible, the distance between the anterior and posterior media lumen interfaces (distance between M-lines) was measured (see Figure 5.4). To retain consistency while performing image analysis, the arterial diameter of subjects was measured in end diastole phase and at $90 \pm 0.1^\circ$ to the posterior intima. All measurements were taken from the same landmark along the arterial wall. Rationale for measuring intima to intima as opposed to the more traditional protocol of anterior to posterior M-line (which represents the media-adventitia interface) is that its assessment avoids any minute compression of the intima-media complex that inevitably occurs with the thinning of smooth muscle cell fibres during arterial vasodilatation (526). Several studies have also reported diffuse atherosclerotic thickening in brachial artery walls in post-mortem samples from people with known atherosclerosis (527, 528). This would imply measuring from the inner luminal diameter gives a more accurate estimation of endothelial function compared to the assessment of the luminal diameter M-line

to M-line, which takes into account in its measurement any intima-media thickness (529). All ultrasound images were transferred onto rewritable CDs once assessed.

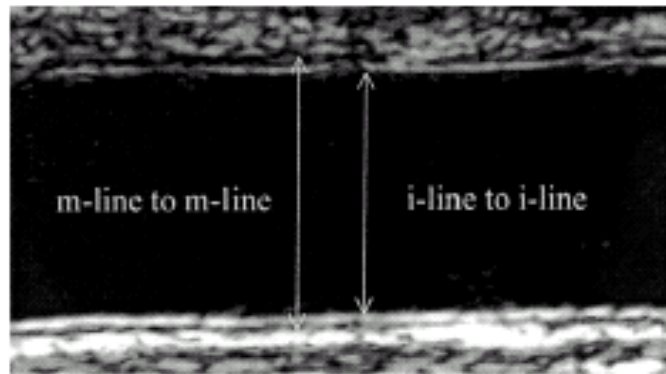


Figure 5.4: Analysis of brachial arterial diameter using either the posterior and anterior M-lines or I-lines. Source: Jarvisalo *et al* (529).

5.5 Study Foods, Intake and Compliance

A detailed description of the range, composition and availability of trial foods consumed in the study is described in Chapter 2 section 2.10. The protocols used to measure subject compliance as well as trial food and macronutrient intake are described in section 2.11.

5.6 Statistics

In addition to statistical procedures stated in the Chapter 2 section 2.15, one way analysis of variance with repeated measures using brachial artery diameter pre occlusion as a changing covariate was used to examine the different effect of the treatments on FMD. To compare the effects of DP vs SP with ISOs *per sec* on FMD, a contrast analysis was performed. Absolute values for FMD of subjects on the SD and S diets were combined to form an average “soy” value and then subtracted from double the absolute value when subjects were on the D diet ($2D - [SD+S]$). The difference was then compared using a one sample T-test (SPSS, version 10, Chicago, USA).

5.7 Results

5.7.1 Subjects

Table 5.1 describes baseline vascular characteristics of subjects (n=91) in the intervention. As previously stated, only endothelial function was assessed in Adelaide based subjects (intake1 and 3) (n=55) . Seventeen of the 55 subjects, who underwent assessments for FMD, were identified as equal producers. Of this 17, 11 were female and 6 were male. A test for difference between two proportions revealed a significant difference ($P<0.01$) between the ratio of male to female equal producers within the study population; males (35.3%), females (64.7 %).

Table 5.1: Baseline vascular characteristics of subjects.

	All subjects (n=91)
SBP (mmHg)	123.2 ± 11.1
DBP (mmHg)	71.7 ± 8.1
MAP (mmHg)	89.7 ± 8.9
PP (mmHg)	51.2 ± 7.0
SAEI (ml/mmHg x 100)	6.7 ± 3.4
LAEI (ml/mmHg x 10)	17.1 ± 5.9
SVR (dyne•sec•cm ⁻⁵)	1397 ± 209
TVI (dyne•sec•cm ⁻⁵)	130.8 ± 41.4

Mean + SD

5.7.2 Effects of Diets on Vascular Function

5.7.2.1 Arterial Compliance and Blood Pressure for All Subjects (n=91)

As shown in Table 5.2, subject's arterial compliance did not differ between diet treatments. However an effect on BP was observed (see Table 5.3), with all subjects exhibiting a higher DBP on the SD diet compared to D ($P=0.03$). When subjects were categorised according to equal producing status, no effect was observed on arterial compliance or BP variables.

Table 5.2: Effect of diet treatments on arterial compliance for all subjects.

		Treatment			P-value
		D	SD	S	
SAEI (ml/mmHg x 100)	All	6.8 ± 0.3	6.7 ± 0.3	7.0 ± 0.4	0.71
	Equol	7.4 ± 0.7	7.5 ± 0.7	7.7 ± 0.7	0.82
	Non- Equol	6.5 ± 0.4	6.3 ± 0.4	6.6 ± 0.4	0.80
LAEI (ml/mmHg x 10)	All	16.7 ± 0.5	16.6 ± 0.5	16.3 ± 0.4	0.71
	Equol	16.7 ± 0.8	16.8 ± 0.8	16.5 ± 0.7	0.85
	Non- Equol	16.7 ± 0.6	16.5 ± 0.6	16.5 ± 0.6	0.65
SVR (dyne•sec•cm⁻⁵)	All	1361 ± 21	1363 ± 22	1365 ± 23	0.97
	Equol	1330 ± 32	1333 ± 33	1343 ± 37	0.87
	Non- Equol	1376 ± 27	1378 ± 28	1377 ± 29	0.94
TVI (dyne•sec•cm⁻⁵)	All	127.5 ± 3.5	127.1 ± 3.3	128.4 ± 3.3	0.80
	Equol	124.5 ± 5.9	125.0 ± 5.7	124.2 ± 5.6	0.92
	Non- Equol	129.1 ± 4.3	128.2 ± 4.2	130.4 ± 4.0	0.81

Table 5.3: Effect of diet treatments on blood pressure for all subjects.

		Treatment			P-value
		D	SD	S	
SBP (mmHg)	All	121.2 ± 1.0	122.7 ± 1.1	122.1 ± 1.1	0.22
	Equol	119.5 ± 1.5	122.1 ± 1.4	120.7 ± 1.4	0.25
	Non- Equol	122.1 ± 1.3	122.9 ± 1.5	122.7 ± 1.5	0.66
DBP (mmHg)	All	70.0 ± 0.8	71.4 ± 0.8	70.8 ± 0.8	0.03 *
	Equol	69.2 ± 1.5	70.9 ± 1.2	70.4 ± 1.3	0.16
	Non- Equol	70.4 ± 0.9	71.6 ± 1.0	71.0 ± 1.1	0.15
MAP (mmHg)	All	88.1 ± 1.0	89.4 ± 0.9	88.8 ± 0.9	0.12
	Equol	86.7 ± 1.5	88.8 ± 1.1	87.9 ± 1.3	0.16
	Non- Equol	88.7 ± 1.2	89.8 ± 1.3	89.3 ± 1.2	0.47
PP (mmHg)	All	51.2 ± 0.6	51.3 ± 0.7	51.3 ± 0.7	0.99
	Equol	50.2 ± 1.0	51.2 ± 1.1	50.4 ± 1.2	0.58
	Non- Equol	51.6 ± 0.8	51.3 ± 0.9	51.8 ± 0.9	0.81

* SD significantly different to D

5.7.2.2 Peripheral Vascular Function for Adelaide Based Subjects (n=55)

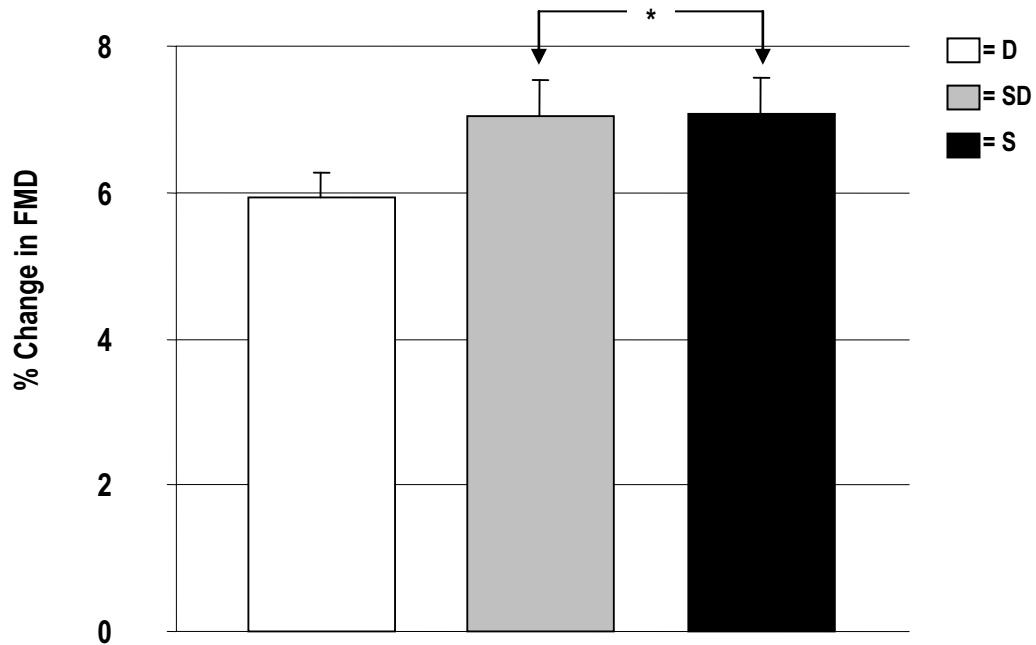
Endothelial dependent and independent vasodilator function in the peripheral circulation was assessed using FMD and NTG mediated dilatation respectively. As shown in Table 5.4, one way ANOVA with repeated measures using baseline arterial diameter as a fixed covariate revealed no significant diet effect on changes in FMD and NTG vasodilatation for all, equol and non-equol subjects.

Table 5.4: Effect of diets on peripheral endothelial dependent and independent vasodilatation.

			Treatment			P-value
			D	SD	S	
FMD	All Subjects (n=55)	Ab	0.026 ± 0.002	0.030 ± 0.002	0.030 ± 0.002	0.19
		%	5.9 ± 0.4	7.1 ± 0.5	7.1 ± 0.5	0.12
	Equol Producers (n=17)	Ab	0.027 ± 0.002	0.034 ± 0.004	0.031 ± 0.003	0.43
		%	6.6 ± 0.5	8.4 ± 1.1	7.6 ± 0.8	0.37
	Non- Equol Producers (n=38)	Ab	0.025 ± 0.002	0.028 ± 0.002	0.029 ± 0.003	0.33
		%	5.6 ± 0.5	6.5 ± 0.5	6.8 ± 0.6	0.24
NTG	All Subjects (n=34)	Ab	0.085 ± 0.004	0.085 ± 0.004	0.086 ± 0.003	0.95
		%	20.3 ± 1.0	20.2 ± 1.1	20.3 ± 0.9	0.99
	Equol Producers (n=13)	Ab	0.085 ± 0.006	0.091 ± 0.006	0.092 ± 0.007	0.62
		%	21.0 ± 1.7	21.8 ± 1.5	21.5 ± 1.6	0.90
	Non- Equol Producers (n=21)	Ab	0.085 ± 0.005	0.083 ± 0.005	0.031 ± 0.003	0.90
		%	20.0 ± 1.3	19.5 ± 1.4	19.7 ± 1.0	0.93

Results from a one sample t-test contrast analysis nesting changes in FMD on both SD and S diets (see Figure 5.5) did reveal a significant improvement in subjects FMD response when they consumed a “soy” diet (enriched with SP \geq 12g/day and ISO) compared to DP only (P=0.03).

Figure 5.5 Nested analysis for effect of SD and S Diets on FMD compared to D for n=55 subjects.



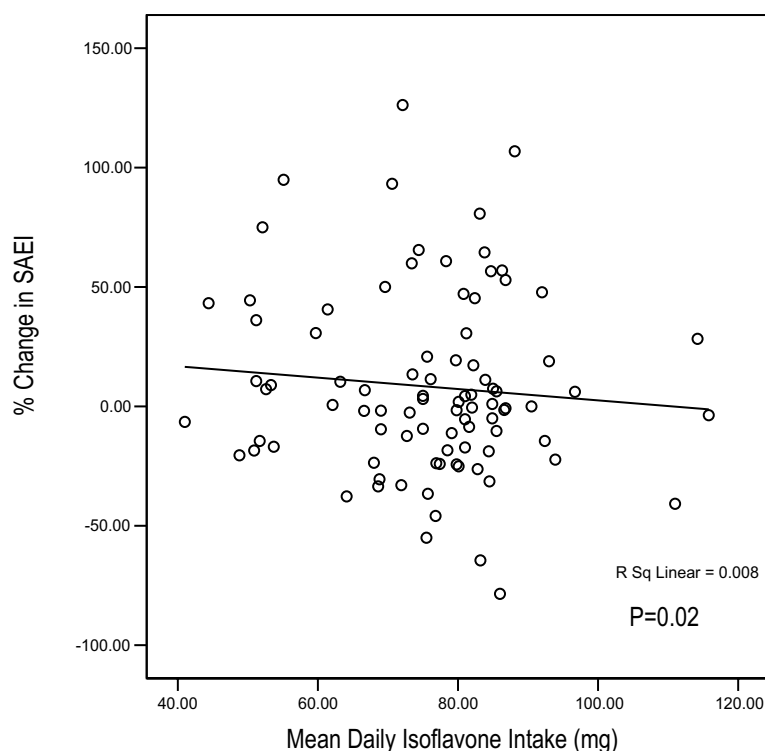
* Significantly different to D (P= 0.03)

5.7.3 Influence of Isoflavone Intake on Vascular Function

Linear regression analysis found no correlation between mean ISO intake/day and absolute or % changes in FMD (compared with D) for all, equol and non-equol producing subjects when on the SD and S diets (P>0.25). There was however a trend (r=0.470, P=0.06) for equol producer's % change in FMD on the SD diet to correlated with their ISO intake.

For arterial compliance measurements, linear regression found a significant inverse correlation between all subjects mean ISO intake/day and % change in SAEI (r=0.25, P=0.02) on the SD diet. Specifically, when subjects had increased ISO intake they were shown to have decreased peripheral arterial compliance (see Figure 5.6). With respect to BP, there was no significant correlations between subjects (all, equol and non equol producers) ISO intake/day and changes in BP variables.

Figure 5.6: Correlation between mean isoflavone intake/day and % change in SAEI for all subjects (n=91) on the SD diet.



5.7.4 Influence of Equol Production on Vascular Function

Two way ANOVA with repeated measures revealed no difference in absolute or % change in FMD for equol and non equol producers on the SD(P=0.07) and S(P=0.49) diets (compared to D). Moreover, there was no difference in changes to BP and arterial compliance variables between these individuals on either diet (P>0.27 for all).

When FMD values for subjects on both the SD and S diets were grouped together, there was a trend for the equol producers to experience a greater improvement in FMD than non-equol producers (P=0.08). There was no significant interactive effect between equol production and diet in terms of changes in dependent variables of BP (P>0.45) and arterial compliance (P>0.79).

5.7.5 Influence of Plasma Lipids on Flow Mediated Dilatation

Linear regression revealed no significant correlations between changes in subject's lipids (TChol, LDL-C, HDL-C and TG) and FMD responses on the SD and S diet compared to D. Grouping

subjects into equol or non equol producers similarly revealed no relationship. A positive inverse trend was seen for equol producers (n=17) between absolute change in LDL-C and FMD on the SD diet (r=0.465, P=0.06).

As for any dietary influence on FMD, linear regression revealed no significant correlation between subject's absolute saturated fat intake and changes in FMD on the SD diet (absolute FMD change; r= 0.18, P= 0.20 and % FMD change r=0.18, P=0.19) or S diet (absolute FMD change; r= 0.02, P=0.91, % FMD change; r.< 0.01, P=0.99) compared to D.

5.7.6 Influence of Age and Gender on Vascular Function

Subject's age did not influence their FMD response on either the SD diet (absolute FMD change; r= 0.04, P= 0.76 and % FMD change; r=0.05, P=0.74) or S diet (absolute FMD change; r= 0.07, P=0.60, % FMD change; r= 0.04, P=0.78) compared to D. Two-way ANOVA furthermore revealed no difference in absolute (P=0.50) and % (P=0.85) change in FMD between males and females in the study. There was also no interaction between gender and the effect of the diets on FMD (P>0.22).

For arterial compliance, two way ANOVA revealed a significant difference between genders with men shown to have significantly higher LAEI and SAEI values compared to females on the diets(P<0.01 for both). There was also a significant interactive effect between gender and the effects of the diets on SVR (P=0.04).

For BP, there was no difference in responses between genders (P>0.31) on the diets nor was there an interactive effect between gender and effects of diet on BP variables (P>0.24).

5.8 Discussion

5.8.1 Effects of Trial Foods on Peripheral Endothelial Function

FMD reflects the ability of the vascular endothelium to release NO. Despite *in vivo* evidence to indicate ISOs can up regulate the activity and expression of eNOS to increase NO production, the FMD responses of subjects did not improve when consuming the soy foods in the intervention. This finding reflects that of another study (329) whereby hypercholesterolemic individuals, receiving comparable amounts of ISOs and SP to that of the SD and S diets, observed no benefit to FMD.

Endothelial dysfunction is common in individuals who have risk factors for CVD and can be identified by a blunted FMD response of 1-4% (414, 530-532). It was postulated that subjects recruited in the Soy Food Intervention may have compromised endothelial function as they had elevated blood cholesterol levels (5.63 ± 0.81 mmol/L) and a mean WHR ≥ 0.85 which have both been shown to manifest a blunted FMD response (273). However, results on the D diet (which acted as the control phase) indicated subjects had normal endothelial function reflected by their FMD response of 5.93 ± 0.35 % which is comparable to that seen by healthy individuals in other studies (533).

When analysing subjects' FMD responses on the diets, their baseline diameter was deliberately controlled for, as individuals with naturally large arterial diameters (>5 mm) are not shown to respond to the FMD test as effectively as those with a smaller diameter (525). Controlling for baseline arterial diameter did not alter the effects of the diets on FMD ($P>0.12$). There was a trend for subjects to have a higher % change (of approximately 1%) on both SD and S diets compared with D. The change in subject's FMD response on the SD and S diets was virtually identical at $7.05 \pm 0.47\%$ and $7.06 \pm 0.49\%$ respectively. Since the SP content of the two diets varied considerably (12g vs 24g/day) unlike their ISO content (SD: 76 ± 1.5 mg/day vs S: 71.4 ± 1.9 mg/day), it's possible the ISOs provided by foods during these diets mediated the 1% improvement in FMD. Several studies have reported that it is the ISOs in soy, not SP, that is responsible for improving endothelial dependent vasodilatation (13, 325, 338, 341). It has been suggested that an increase of at least 2% reflects a clinically significant change in FMD (235). If all subjects in the intervention had undergone assessments for FMD instead of only $n=55$, then there may have been enough power to detect the 1% change on SD and S diets as being statistically significant. When the two diets were nested together, a significant improvement in FMD ($P=0.03$) was seen compared with D, suggesting consumption of a "soy enriched diet" in individuals with normal endothelial function is beneficial. On both the SD and S diets, subjects consumed the ISOs in the form of glycoside conjugates. A recent study by Clerici *et al* (13) eluded that the composition, rather than the dose of ISOs consumed by an individual, is more influential for improving FMD. The authors of the study found consumption of only 33mg of aglycone equivalents (ISO) per day in a pasta food matrix for four weeks could significantly improve FMD by 2.3 ± 0.8 %. It was not stated whether these

individuals had impaired endothelial function prior to the intervention although they were shown to have much higher blood cholesterol levels (TChol 6.76 ± 0.08 mmol/L) than subjects in the present study. It's postulated that delivering ISOs as aglycone equivalents results in greater interact with ER β s as they are directly taken up into the circulation from the lumen. In comparison, glucoside conjugates need to initially be hydrolysed by gut bacteria before they can mediate effects on ER β s. This ability to metabolise ISOs can vary between individuals and subsequently influence their bioavailability in plasma. Evidence from a study that delivered pure GEN (as aglycones) via arterial infusion (339) and reported improvements of 65 % in forearm blood flow compared to delivering GEN as a glycoside supplement with reported improvements of 11% (325) would support this theory.

There is evidence to show that a reduced FMD response can be related to plasma lipid disturbances (534). However, in the current study no significant associations were found when changes in FMD were correlated with changes in subject's lipids. There was a trend for equol producers to exhibit a more favourable FMD response ($P=0.06$) when their LDL-C levels decreased on the SD diet. This type of relationship is also observed when taking statin medication (535). Increased saturated fat in the diet has been reported to impair endothelial vasodilatation (536). Although subject's saturated fat intake on the SD and S diets was not shown to directly improve their FMD response, the fact that it was significantly lower than on the D diet may have contributed partially to the improvement observed.

In the present study, equol production was not shown to influence endothelial function although there was a trend, as seen in a previous study (12) for producers to have greater FMD improvements. Only one trial (13) has shown equol production directly improves FMD. The inability of the present study to detect a similar effect of statistical significance may be due to the small number of producers (only $n=17$) within the group.

To ensure that the conclusions drawn from subjects' FMD results pertained specifically to endothelial function, subjects' response to a non- endothelium dependent vasodilator was measured on each of the diets. NTG was used in the study to perform this assessment, as it provided an external source of NO (independent of that produced by the endothelium). As observed, there was no difference in NTG – mediated vasodilatation of subjects. Moreover the approximate 20% increase seen is

comparable with responses observed in other studies involving soy supplementation (330, 344, 345). Changes in FMD on the diets could therefore be exclusively attributed to improvements in endothelial function.

5.8.2 Effects of Diet Treatments on Arterial Compliance and Blood Pressure

It's well established that arteries naturally stiffen with age which can result in a simultaneous rise in SBP (537, 538). Age related changes aside, a loss of compliance and elevation in BP can also serve as reliable predictors of CVD risk. This is because they are shown to manifest in the presence of a dysfunctional endothelium which promotes vascular remodelling and loss of tone.

Arterial compliance and BP were both assessed using a HDI CV profiler. With respect to arterial compliance, the system provided elastic indices for small (SAEI) and large (LAEI) arteries as well as measures of systemic (SVR) and whole body (TVI) resistance. The reliability and repeatability of this technique for measuring arterial compliance has been well established (539), along with a range of normal indices, based on age and gender which can be used to identify at risk populations. In the present study, diet treatments were not shown to effect arterial compliance, irrespective of whether subjects produced equol or not. The lack of improvement in the study maybe related to the subjects already having normal levels of compliance (baseline SAEI: 6.7 ± 3.4 ml/mmHg x100, LAEI: 17 ± 5.9 ml/mmHg x10). Generally a LAEI value between 15-17 ml/mmHg x10 and SAEI value between 6-8 ml/mmHg x100 indicate normal arterial compliance, irrespective of an individual's age or gender (540). If the mean age of the group (52.7 ± 1.0 years) is taken into consideration, then standardised levels indicate our subjects had approximately 10% and 35% higher SAEI and LAEI values respectively prior to commencing the intervention (539).

The results from the current study contradict previous studies (322, 324, 347) which have reported that ISO supplementation can improve compliance in healthy individuals. The duration of the diets (only six weeks) may have contributed to why no significant improvement was observed, as the majority of studies reporting beneficial effects have been conducted over periods greater than eight weeks. Only one other study which is comparable in duration and ISO levels has reported an

improvement. Teede *et al* (322) demonstrated in only five weeks with 80mg of formononetin (the ISO precursor of DAZ) that a significant improvement in systemic arterial compliance of 23% was possible.

When subject's ISO intake was correlated with changes in their arterial compliance, a significant inverse relationship was observed on the SD diet. As ISO consumption increased, subject's peripheral arterial compliance decreased. Although this relationship wasn't particularly strong ($r=0.25$), it was still unexpected in view of the current literature which reports ISOs are beneficial for improving compliance and that SP (which was lower on the SD diet) has no effect on compliance. The only other study to report a similar negative effect was that by Hallund *et al* (330) when delivering 50mg ISOs in cereal bars. After eight weeks, a significant decrease in systemic arterial compliance (SAC) compared with control cereal bars was observed.

With respect to BP, all subjects recruited were normotensive prior to commencing the treatments. A significant diet effect on DBP for all subjects was detected, with levels significantly elevated on the SD diet ($71.4 + 0.8$ mmHg) compared to D ($70.0 + 0.8$ mmHg). This was not anticipated given the similarity in subjects DBP values on the different diets. Despite a trend for equol producers to have lower BP values across the diets (see Table 5.3), there was no significant difference in how the diets affected these individuals.

5.9 Summary

The current study found that foods delivering ISOs and varying quantities of SP, when regularly consumed by mild hypercholesterolemics, could significantly improve peripheral vasodilatation compared with foods containing only DP.

Equol production was not shown to influence the effects of ISO supplementation on endothelial function, arterial compliance or BP. These observations are in contrast to evidence which suggests equol has favourable vascular benefits (12, 13, 340). Despite the small number of equol producers in the studies, positive correlations were observed for changes in vascular parameters within these individuals which warrant further investigation.

6.0 Effect of Isoflavone Supplementation on Cognitive Function

Results from the Soy Food Intervention demonstrate ISOs consumed in novel soy foods has favourable effects on peripheral vascular function. This observation, in conjunction with evidence that ISOs may provide mental health benefits through enhanced cerebral vasodilatation and neurotrophic effects, provided the rationale for conducting a chronic supplement intervention examining effects of isolated ISOs on cognition. It was hypothesised an improvement in cognition by the ISOs may be related to their ability to improve vascular function.

6.1 Introduction

The performance of cognitive tasks is often dependent on a person's level of intelligence. Research has also shown that factors such as age, gender, past experiences as well as genetics, diet and health status are also highly influential. Whilst cognitive impairment occurs naturally with aging, several factors are shown to contribute to its decline through their acceleration of cerebral degenerative changes. Alcohol consumption(541), illicit (542) and prescribed medications (543), head trauma (544) and sexually transmitted diseases (i.e. AIDS and syphilis)(545) all impair cognitive performance by reducing synaptic density, neuronal growth and promoting brain atrophy. Furthermore, metabolic and CV conditions like atherosclerosis (546), hyperlipidemia (547), high BP (548), obesity (549), and diabetes (550) can also exacerbate cognitive decline . There is now suggestion that cerebral vascular dysfunction is also involved in the development of mild cognitive impairment (see review by O'Brien *et al* (551)). Poor local and global cerebral perfusion has been implicated in its underlying aetiology which is supported by evidence that increased blood flow mediated by Ca²⁺ antagonists and vasodilators have positive effects on cognition (552, 553).

The localisation of ERs in the brain and revelation that estrogen is involved in the organisation and maintenance of some cognitive tasks, led to the discovery that estrogen protects against cognitive decline and aids in the maintenance of cognitive function. Observational studies in non-demented, post-

menopausal women undergoing HRT are consistently reported to have less age-associated cognitive decline (554-556). Furthermore longitudinal studies of women on HRT report improved learning, memory recall and verbal recognition of information compared with women who are not (557). Recently, HRT has been shown to enhance cognition by decreasing cerebral vascular tone and increasing blood flow in those areas of the brain that are stimulated during learning of visual and memory tasks (558) (see Review by Krause *et al* (559)).

In the brain, the most critical sites for cognitive development and processing are the hippocampus, frontal cortex and thalamus (151). Moreover, these areas control cognitive processes which are specifically shown to differ between genders. Whilst ERs are located throughout the entire brain, ER β is shown to be highly prevalent in each of these areas (146, 156, 560) suggesting the activation of this specific receptor subtype mediates the beneficial effects seen in cognition with estrogen. As ISOs, particularly GEN, display a high binding affinity for ER β , there has been considerable interest in whether ISOs can improve and preserve cognition by mimicking the effects of estrogen. *In vitro* studies have demonstrated that ISOs can improve cognitive performance via ER mediated events that enhance brain cell survival, growth and neuroplasticity (see Chapter 1 section 1.10.5.1). Lee *et al* (561) verified this ability to improve cholinergic enzyme activity in the brain and increase neuron density in rat models fed soy diets enriched with ISOs. Furthermore, the authors illustrated how these physiological events transpired into improved cognitive performance (404, 561, 562).

Another postulated mechanism by which ISOs may improve cognition is via enhanced cranial vasodilatation (see Chapter 1 section 1.10.5.2). Animal studies, such as that by Sobey *et al* (563), demonstrate short term treatment with DAZ can enhance vasodilatation in cerebral arteries by increasing the expression of key proteins which modulate eNOS activity. Torregrosa *et al* (378) confirms ISOs ability to induce relaxation of isolated cerebral arteries, although the mechanism was not shown to be related to increased NO production but an inhibition of extra cellular Ca²⁺ influx to VSM. *In vivo* studies also demonstrate ISOs can directly act on the micro cerebral vasculature to enhance perfusion (407, 408).

Although no human interventions have specifically assessed the effect of ISOs on cerebral vascular function, a recent study by Lavi *et al* (564) suggests measuring peripheral endothelial function can provide a surrogate measure of cerebral blood flow. The authors observed patients with reduced cerebral blood flow also demonstrated impaired FMD, suggesting the deficiency of the endothelium in the peripheral circulation to release NO emulates impaired NO production in the cerebral vasculature.

To date, evidence from human studies in relation to effects of ISOs on cognitive function have been inconclusive. Some studies in post-menopausal women (393, 394, 396) and healthy adults (565-567) demonstrate ISO supplementation is beneficial for cognitive performance, whilst several chronic supplementary trials which provide even greater levels of ISOs have failed to reproduce the same effects (12, 395, 396). It has been suggested that inconsistencies in health benefits from ISOs may be due to the influence of equol. This is most evident in studies investigating effects of ISOs on lipids (11) and vascular function (12) in which an effect was only evident in participants who demonstrated elevated levels of equol. Only one study has investigated whether equol production influences cognition, with no effect found (410). The lack of previous studies investigating the effect of equol may explain discrepancies seen to date in improved cognitive performance from ISOs.

As for effects in males, there appears to be some debate within the literature as to whether ISOs are detrimental or beneficial for cognition. Evidence for an effect of soy on cognition in males, first came from an epidemiological study conducted in healthy elderly Japanese-American men living in Oahu (392). The study investigated whether tofu consumption during mid-life affected their cognitive performance and brain weight several decades later. The authors observed a correlation between atrophy and deterioration in brain function, when more than two servings of tofu per week were consumed. While the study provided no direct evidence that the brain atrophy and deterioration in cognitive function observed was due to ISOs present in tofu, it was indirectly implied to be a contributing factor. Subsequent animal studies support the claim that ISOs may be detrimental to cognition, with observations that cognitive performance in male rats can be significantly impaired after ISO supplementation (391, 562). Furthermore, ISOs supplementation in rats has been observed to worsen

specific cognitive traits like visual conceptualisation which males are predisposed to perform better at than females.

Only a handful of studies have evaluated the effect of soy consumption on cognition in human males. All have been conducted using young, healthy populations (566-568). Furthermore, only one of these has directly correlated effects on cognition with ISO consumption. This particular study by File *et al* (567) found consumption of a high soy diet enriched with 100mg ISO/day, for 10 weeks, improved immediate and long term episodic memory, mental flexibility and planning ability in males compared with a control diet. Whilst authors did observe a beneficial effect in the males, this study also included females and improvements in cognition were not shown to be consistent across genders, with females improving more at certain cognitive tasks after ISO supplementation and males showing impaired performance in others.

As previously discussed (see Chapter 1.10.1), men and women differ in their ability to perform cognitive tasks and estrogen is shown to mediate and maintain these differences. Evidence that it may be possible to enhance the execution of tasks in males that are usually performed better by females came from a study involving genetically male transsexuals who had been undergoing estrogen treatment for several months whilst awaiting gender reassignment surgery (569). The authors of the study reported that males receiving estrogen compared to those not on hormone therapies had improved verbal memory performance; a task which females are superior at performing. Furthermore, visual spatial function which males naturally perform better at, was preserved in those undergoing estrogen therapy, indicating estrogen can provide in males similar benefits for cognitive performance, as seen in females, without detrimental effects on other cognitive processes.

6.2 Aims

Due to the limited number of studies undertaken to assess the impact of ISO supplementation on cognition in males and the lack of any well-controlled trials in older healthy males, we aimed in our supplement intervention to investigate;

1. Whether healthy elderly males exhibited improved cognitive performance, specifically in relation to learning and memory following chronic ISO supplementation;
2. Determine if ISO supplementation in males could enhance specific cognitive traits at which women excel;
3. Determine if ISO supplementation was detrimental to the performance of tasks at which males excel;
4. Investigate the effect of ISO supplementation on peripheral vascular function viz. FMD
5. Determine whether any benefit observed in cognition from ISO supplementation is related to improved peripheral vascular function;
6. Determine the importance of equol in mediating benefits to cognition or peripheral vascular function.

6.3 Hypotheses

- a) ISO supplementation will improve cognitive function and assessments of FMD in males.
- b) Improvements in cognition, which is known to be influenced by improved cerebral circulation, will correlate with improvements in peripheral endothelial function.
- c) Peripheral endothelial function is a surrogate measure for cerebral circulation.

6.4 Significance and Expected Outcomes

There is some evidence that alterations in cognitive function may be in part mediated by changes in cerebral perfusion, which might be secondary to alterations in vascular dilatory and/or endothelial function. Whilst it is still questionable whether peripheral endothelial function is a surrogate for cerebral circulation, we hope our study will be the first to correlate improvements in cognition, which is known to be influenced by improved cerebral circulation, with improvements in peripheral endothelial function. Moreover, this will also be the first prospective study to look at the effect ISO supplementation has on cognition and vascular function exclusively in older healthy males. To date, there have been limited studies investigating these effects in a male population. We expect to show that ISO supplementation has beneficial effects on both cognition and vascular function.

6.5 Subjects

6.5.1 Inclusion and Exclusion Criteria

Subjects were eligible for inclusion in the intervention if they were healthy males, aged 30-70 years, not on any medication which might confound outcome measures and most importantly were not regular soy consumers. Table 6.1 describes the general exclusion criteria for the intervention.

Table 6.1 General exclusion criteria for Isoflavone Supplement Intervention.

Criteria
<ul style="list-style-type: none">• Consumption of two or more serves of soy products per week• Regular use of medications deemed likely to confound the study outcomes (antihypertensives , antidepressants, sedatives, stimulants)• Type I or II diabetes• Smoker• BP: Systolic \geq170 mmHg, Diastolic \geq100 mmHg• Known coronary condition (heart failure, arrhythmia, cardiac valve abnormality, stroke), peripheral vascular, renal or any other CVD• Phosphodiesterase inhibitor medication (eg. Viagra, Levitra)• Fish oil capsules (>1g p/day)• Inability to comply with study protocol in the opinion of study investigators.• Participant in any other current

6.5.2 Justification of Exclusion Criteria

Subjects were excluded for participation in the study if they were habitual soy consumers (>2 serves of soy foods per week), were taking >1g/day of fish oil and were taking any prescribed medications or over the counter supplements that may influence cognition, mood, depression or anxiety. Habitual soy consumers were excluded to avoid confounding due to additional ISO intake. Fish oil has been shown to positively affect cognition, specifically memory and learning functions which were being investigated in the current trial (570). Antidepressant, sedatives, stimulants and mood altering medications/supplements were excluded because they stimulate chemical changes within the brain to increase levels of the three main neurotransmitters; serotonin, noradrenalin and dopamine. ISO are

postulated to act on these cholinergic systems within the brain and also stimulate neurotransmitter release so these drugs may confound the effects of ISOs.

Subjects were excluded for participation if that they had been diagnosed with diabetes (411), peripheral vascular disease (412) or were smokers (414) as these individuals are known to have endothelial dysfunction (see Review by Drexler,H) (571). Individuals using phosphodiesterase inhibitors (e.g. Viagra) were also excluded from the study as these types of medication interact with NO dependent pathways and promote vasodilatation. Use of antihypertensive and lipid lowering medication (534) and fish oil (415) have also been shown to positively influence vascular function and therefore would confound potential effects of ISOs.

6.6 Recruitment of Subjects

Subjects were recruited by the following means:

- a) Paid volunteer advertisements in metropolitan newspaper
- b) Local radio and television broadcasts
- c) Recruitment flyers
- d) Email and university webpage recruitment advertisements

Refer to Appendix 5 for examples of recruitment material used for the intervention

6.7 Screening of Subjects

6.7.1 Suitability Assessment and Telephone Screening Interview

Refer to Chapter 2 section 2.7.1 for description of protocols used. See Appendix 5 for documentation provided in the information pack to potential participants.

6.7.2 Screening Visit and Information Session

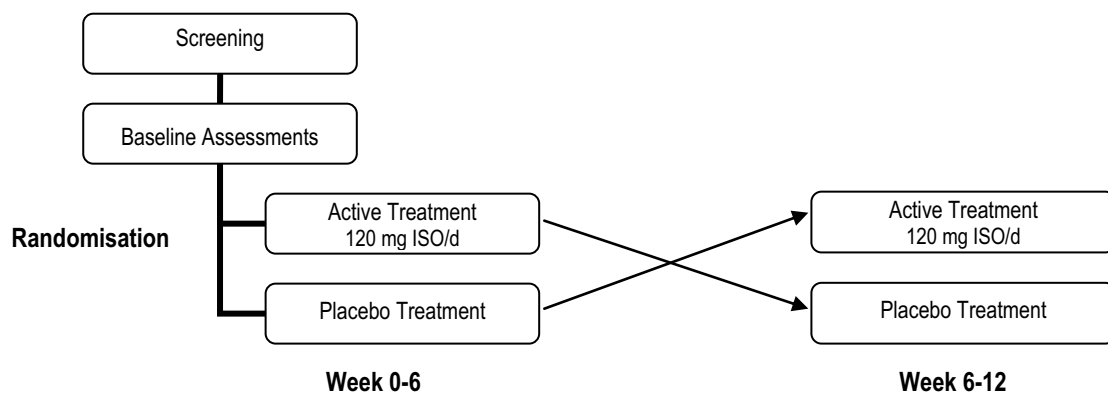
Based on a thorough examination of the returned diet and lifestyle questionnaire from potential study volunteers, those that appeared to meet the entry criteria were contacted by telephone and asked to attend an initial screening visit at the Nutritional Physiology Research Centre.

At the screening visit, subjects had their height and weight assessed (refer to Chapter 2 sections 2.9.1 and 2.9.2 for protocols for these assessments). Subject's BP was also assessed with an Omron Automatic BP monitor (Model T8 with Intellisense). Prior to recordings, subjects were asked to void their bladder. BP was recorded after 5-10 minutes of subjects sitting quietly. Three recordings were then taken with a minimum interval period of one minute between recordings.

6.8 Study Design

The intervention used a placebo-controlled, cross-over design of 12 weeks duration. The 40 subjects recruited underwent an initial battery of standardised cognitive tests designed to assess several aspects of executive mental function (i.e. planning, memory recall, information processing, and attention/alertness) as well as an assessment of peripheral vascular function viz. FMD. Once baseline parameters had been established, subjects were then randomised to commence either an active treatment (120 mg ISO/day) or placebo (120 mg raitilose + fibre/day) phase during the first six weeks of the intervention. After the first six weeks, subjects crossed over to the alternate treatment. A schematic outline of the protocol is provided in Figure 6.1.

Figure 6.1: Outline of protocol for Isoflavone Supplement Intervention.



Subjects on the active treatment received 120mg/day of ISOs prepared from Soy Life 40% (see attached product specification sheet in Appendix 6). The ISOs were consumed as two x 30mg ISO capsules twice daily, whilst those subjects in the placebo group took two matching placebo capsules which each contained 30 mg of raitilose and fibre. All ISO and placebo supplements used in the

intervention were provided as an in-kind donation by Soy Health Pty Ltd (NSW, Australia) and were manufactured by Frutarom Pty Ltd (Amsterdam, Netherlands). Refer to section 6.13 for detailed description of supplements used.

Subjects were required to attend three testing sessions (baseline, 6 and 12 weeks) over the course of the intervention, with each session taking approximately 1.5 hours to complete. Outcome measures evaluated after each treatment included repeat assessments of standardised cognitive tests and peripheral vascular function viz. FMD, as performed at baseline.

An overnight urine specimen was also collected at each time-point to determine urinary ISO content, including equol content to identify subjects who were equol producers. Throughout the intervention, subjects were asked to maintain their normal eating patterns and exercise routines as well as cease consumption of any products containing soy.

6.9 Study Protocol

Clinic visits were conducted after a minimum six hour fast from food and minimum two hour fast from fluids (except water) and stimulants (caffeine, alcohol). Upon arrival, subjects had their weight measured as described in Chapter 2 section 2.9.1. FMD was then performed to assess endothelium-dependent arterial dilatory function as described in Chapter 5 section 5.4.3.1. Following the FMD assessment, subjects were given a small snack consisting of a 250ml glass of orange juice and a plain sweet biscuit. They then completed a battery of cognitive assessments which took approximately one hour to complete. These tests were conducted in a quiet room with plain white walls to limit distractions. The tests included in the battery were:

- Rey Auditory Verbal Learning Test (RAVLT)
- Paired Associates Learning Task.
- Backwards Digit Span
- Letter Number Sequencing Task.
- Novel Spatial Working Memory Task
- Mental Rotation Task

- Initial Letter Fluency Task
- Trail Making Test

A detailed description of each of the tests can be found in section 6.11

6.10 Ethical Considerations

Ethics approval was obtained from the HREC of the University of Adelaide and the University of South Australia. Refer to Appendix 1 for formal letters of approval sent by each institution.

6.11 Cognitive Assessments

As there is strong evidence from the current literature to suggest ISOs influence those regions of the brain that control executive function, learning and memory (such as the hippocampus and prefrontal cortex) a battery of eight cognitive tests which specifically measure these functions was used in the intervention. Listed in Table 6.2 are the eight cognitive tests which were performed, a brief description of the specific cognitive function being assessed and the specific region of the brain that pertains to that function. All assessments which composed the battery of cognitive tests used in the intervention were performed in a quiet environment by an experienced study investigator who had been trained by a professional psychologist on how to administer the tests proficiently.

Table 6.2: Battery of cognitive tests used in the Isoflavone Supplement Intervention.

Cognitive Test	Area of Brain Affected by Assessment	Cognitive Function	Duration of Test
Spatial Working Memory	Prefrontal cortex	Object and spatial location recall Working Memory	10 minutes
Paired Associates Learning	Hippocampus	Declarative Memory, Learning Auditory memory recall	5-10 minutes
Backwards Digit Span	Prefrontal cortex	Working Memory	3-5 minutes
Letter Number Sequencing	Prefrontal cortex	Working Memory	5-10 minutes
Mental Rotation	Hippocampus Parietal lobe	Visual-Spatial processing	5 minutes
RAVLT	Hippocampus	Learning Auditory memory recall	10-15 minutes
Trail Making	Prefrontal cortex Parietal lobe	Planning ability	3-5 minutes
Initial Letter Fluency	Frontal Lobe	Spontaneous mental flexibility Strategic retrieval of verbal info	3 minutes

In the intervention specific aspects of memory, learning, visual spatial processing, executive mental function and planning ability were assessed. The following sections define these aspects of cognition and outline the protocol and design of the tests performed in order to evaluate them.

6.11.1 Memory and Learning

Memory can be defined as the retention of, and ability to recall, information, personal experiences, and procedures (skills and habits). Depending on the length of time information is stored for, categorises memory as sensory, short or long term (572).

Sensory memory corresponds with the initial 200 - 500 ms after an item is perceived. An example of sensory memory would be looking at an item, and remembering what it looked like with just a second of observation, or memorization. The capacity of sensory memory is approximately 12 items, but this degrades very quickly (within a few hundred milliseconds). Therefore this type of memory cannot be prolonged via rehearsal. Some of the information in sensory memory is then transferred to short-term memory. Short-term memory involves the temporary storage and management of information required to carry out complex cognitive tasks such as learning, reasoning, and comprehension. It is

characterised by the ability to recall information collected from several seconds to a minute without rehearsal. Like sensory memory, its capacity is also very limited.

Unlike sensory and short term memory, long-term memory has an unlimited capacity to store information that can last for as little as 30 seconds to as long as decades. It is typically divided into two types: procedural and declarative. Procedural memory holds information concerning action and sequences of actions and is encoded and stored by the cerebellum and the striatum. Declarative memory holds knowledge, facts, information, ideas, or anything that can be recalled and described in words, pictures, or symbols from specific events or general knowledge.

In sensory and short-term memory, there is a temporary potentiation of neural connections in regions of the frontal lobe and the parietal lobe. Through the process of rehearsal and meaningful association, short term memory can be converted into long term memory through a physical change in the structure of neurons which result in more stable, permanent potentiation throughout the brain (Milner *et al* 1998). The hippocampus is essential to the consolidation of information from short-term to long-term memory.

Compared to memory which is knowledge encoded, stored and later retrieved; learning can be defined as the development of a change in behaviour or acquisition of knowledge as a result of experience. It is usually adaptive or useful (573).

6.11.2 Auditory Memory Recall

Auditory memory recall refers to the ability to recall information that was given verbally usually as units, words, numbers, sentences, phonemes. Information can also be verbalised in paired associates. This type of memory recall engages two separate mental processes. The first is the learning of the information (i.e. words); the second is the formation of a bond between the information (i.e. two words). Cognitive tests which employ both learning and auditory memory recall of single and paired information are the Rey Auditory Verbal Learning Test (574) and Paired Associates Learning Task(575).

6.11.2.1 Rey Auditory Verbal Learning Test

Immediate and delayed memory recall function in subjects was assessed using the widely validated and standardised Rey Auditory Verbal Learning Test (RAVLT) (574). The RAVLT test required subjects to verbally recall a list of 15 words (List A) read aloud to them at a speed of one word per second; immediately over five trials (A1-A5); after a distracter list of 15 words (list B) was read aloud (immediate delayed recall; A6); and after a 20- minute delay (A7).

From the RAVLT test, subjects received an overall score based on the total number of words they remembered from list A over the first five trials, the number of words remembered following the distracter list and after the number of words after the 20 minute delay (A1+ A2+ A3, A4, A5 + A6 +A7= overall test rating)

Subjects immediate memory recall function was scored according to the total number of words remembered from List A following the distracter list (A6). In general, 1-2 words are lost compared to the total number recalled for trial 5 (A5) after hearing the distracter list. Delayed memory recall was scored according to the total number of words subjects could recite from List A after the 20 minute delay (A7).

Rate of learning was calculated for subjects according to the difference in number of words recalled from trial A1 and A5. A high score (A5- A1) represents a fast rate of learning. For instance most young healthy adults (aged 20-39 years) can recall 6-7 words on trial 1 (A1) and 12-13 words by trial 5 (A5) indicating a sound degree of learning. See Appendix 7 for an example of the RAVLT test.

6.11.2.2 Paired-Associated Learning Task

Verbal Paired Associates Learning Task was used from the Wechsler Memory Scale III (WMS III) (575) and tests the ability to remember novel word pair associations. First a list of word pairs is read out, then the first word from each pair is given and the examinee is asked to provide the associated word pair. This is done in 4 consecutive trials to test immediate recall memory, and then once more later to test delayed recall. See Appendix 7 for example of the test.

6.11.3 Working Memory

Working memory is defined as the ability to temporarily store and hold information in a readily assessable form while other cognitive decisions or operations are taking place and to manipulate that information or use it to guide action (576, 577). It is considered to play a crucial role in a wide variety of complex cognitive activities, including mental calculations, reasoning and language comprehension.

In all working memory tasks the information held accessible in memory is continually changing or being updated as the task proceeds. In both humans and animals, the activation of the prefrontal cortex is linked to the performance of tasks with a working memory component (577-579).

Working memory tasks typically contain an auditory and/or spatial component.

Two auditory working memory tasks used in the battery were the Backwards Digit Span and Letter Number Sequencing Task. Both tests were derived from the WAIS III (575). Spatial working memory was assessed using a novel multi-trial task developed by Duff and Hampson (580).

6.11.3.1 Backwards Digit Span Task

The Backwards Digit Span Task involves reading a sequence of numbers aloud at the rate of one number per second to a subject. At the end of the sequence, the person being tested was asked to recall the numbers in reverse order they were spoken. A new sequence was then read aloud to the subject and again they are asked to recall the numbers said in reverse order. The test began with 2 to 3 numbers, and the sequence became progressively longer until the person committed errors two times in a row. Recognisable patterns (for example 2, 4, 6, 8) were avoided. See Appendix 7 for example of the Backwards Digit Span Task.

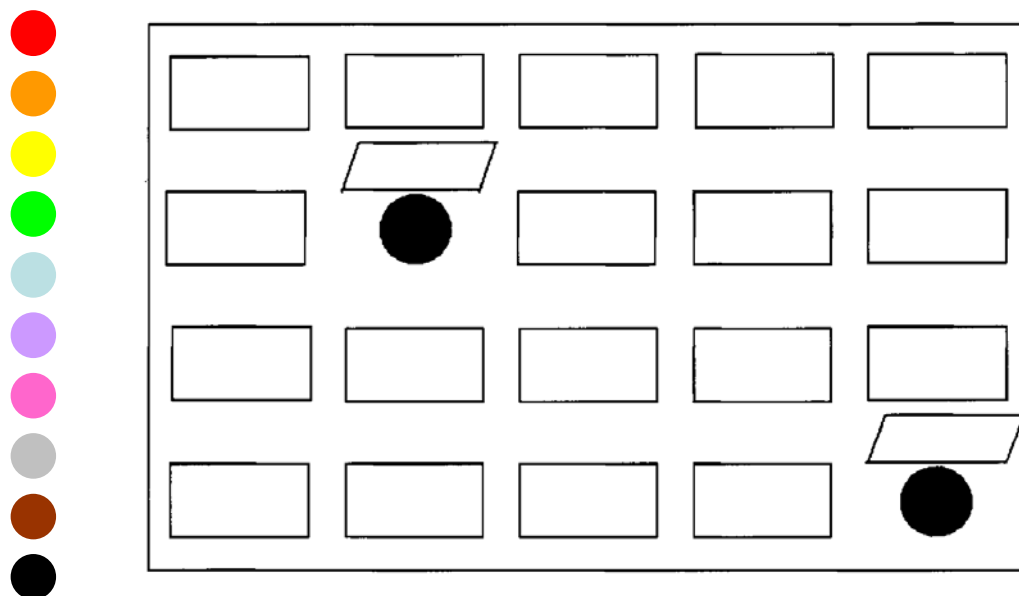
6.11.3.2 Letter Number Sequencing Task

In the Letter-Number Sequencing Task, letters and numbers are provided verbally in random order, and examinees were required to recall them while rearranging them so that they give the numbers first and then the letters, each in ascending order. See Appendix 7 for an example of the Letter Number Sequencing Task.

6.11.4 Spatial Working Memory Task

In the Novel Spatial Working Memory Task, subjects had to search through and progressively discover the location of ten pairs of coloured dots concealed in an array of twenty cards (5 x 4) (see Figure 6.2). The objective of the task was for subjects to locate the matching pairs in as few choices as possible. Time taken to find all pairs was also recorded. Proficient performance in the task was heavily dependent on subjects maintaining and continually updating representations of the spatial locations of where they had already found and matched a pair of coloured dots. This is because they have access to all the cards during the test and require this information to avoid re-searching those locations.

Figure 6.2: Schematic diagram of Novel Spatial Working Memory Task.



6.11.5 Visual- Spatial Processing

Visual-Spatial processing is the ability to perceive, analyse, synthesize, and think with non-linguistic visual patterns and spatial configurations. A number of distinct cognitive tasks fall under this broad category such as the ability to manipulate objects or patterns mentally, the ability to identify visual representations that appear in obscure or vague circumstances, and the ability to store and recall visual representations. The Mental Rotation Task is a common test used to evaluate an individual's ability to mentally rotate visual forms.

6.11.5.1 Mental Rotation Task

The Mental Rotation Task is frequently shown to be performed better by men than women (581). The task requires the examinee to mentally rotate an object in space. For the present study, John Chay's online Mental Rotation Task was used (582). Participants are shown two different rotations of a multi-faceted object and asked to identify whether the second object is a correct rotation of the first object (see Figure 6.3). Number of correct responses and speed of responding (in milliseconds) are recorded.

Figure 6.3: Schematic diagram of question from the Mental Rotation Task. Source (582).

NOTE:
This figure is included on page 162 of the print copy of
the thesis held in the University of Adelaide Library.

6.11.6 Executive Mental Function

Executive mental function relates to the brain's ability to differentiate between conflicting thoughts and predict outcomes to assist with planning and development of strategies.

Mental flexibility and the ability to ignore distractions from task- irrelevant information was assessed using the Initial Letter Fluency Task (583, 584).

6.11.6.1 Initial Letter Fluency Task

The Initial Letter Fluency Task is widely used in clinical neuropsychology due to its sensitivity in detecting executive cognitive dysfunction associated with frontal lobe damage. In contrast to semantic fluency tasks that require subjects to list as many words as they can think of belonging to a particular category, the initial- letter fluency task requires a novel strategy of word retrieval based on a single letter of the alphabet. This method employs a heavier degree of monitoring by subjects to eliminate the production of illegitimate, semantically related words.

The test used in the intervention was a shortened version of the Controlled Oral Word Association Test (585, 586). It required subjects to state as many words as possible that began with a specific letter of the alphabet in one minute. Subjects were instructed that the words they stated must be composed of four or more letters, may not begin with a capital letter (which meant excluding words such as people's names, places, brand names etc), must not be a number and were not allowed to be a word they may have previously stated but with a different ending. Subjects were scored based on the number of words they recited over the minute that complied with the test rules.

6.11.7 Planning Ability

Planning involves the identification and organisation of steps and elements required to carry out an intention or achieve a goal. In order to plan effectively, an individual must be able to conceptualise change and deal objectively with choices and ideas. Good impulse control and intact memory function is also required. To assess subjects' planning ability *per se* in the intervention the Trail Making Test (587, 588) was used.

6.11.7.1 Trail Making Test

The test was administered as two parts; A and B. In part A, subjects were given a piece of paper containing randomly placed numbered (1-25) circles. They were asked to draw as quickly as possible and without taking their pens off the sheet of paper a line connecting the circled numbers in their consecutive order i.e. 1 to 2, 2 to 3 etc. In part B, subjects were given a piece of paper that had both randomly placed numbered (1-25) and lettered (A-Y) circles. They were again asked to as quickly as possible draw a continuous line linking the numbered and lettered circles in consecutive order but were asked to alternate between the two sequences i.e. link A to 1, 1 to B, B to 2 etc. The time taken to successfully complete Part A and Part B with no errors was recorded in seconds. A difference score between Part B and Part A (B-A) was then calculated to remove the element of speed from the evaluation. The reported reliability coefficient for this test varies considerably with most quoting above 0.6 and several reporting >0.8. Both Part A and Part B have been shown to correlate very highly with one another and the difference score B-A correlates highly with scores from other mental ability tests and

with ratings for severity of cognitive impairment. See Appendix 7 for Trail Making Test (Part A and Part B) used in the intervention.

6.12 Assessment of Peripheral Vascular Function

Subject's peripheral endothelial function was assessed using a test for FMD of the brachial artery. Refer to Chapter 5 section 5.4.3.1 for a description of protocol used and section 5.4.3.3 for method used when analysing results from the test.

6.13 Composition of Isoflavone and Matching Placebo Supplements

ISO supplements used in the intervention were provided as an in-kind donation from Soy Health Pty Ltd (NSW, Australia). Supplements were prepared using the product Soy Life 40% (see Appendix 6 for product specifications) by affiliate company Frutarom (Nutrilab B.V, Giessen, The Netherlands). The ISO profile of Soy Life 40% has a typical soy germ ratio of GEN:DAZ:GLY which is 15:50:35 and provides 400mg of total ISOs (in aglycone, glucoside, malonyl glucoside and acetyl glucoside forms) per gram. The ISO supplements were manufactured as 500mg capsules and designed to contain a total of 45.6 mg ISO, with 28.7 mg being aglycone equivalents. Table 6.3 describes the breakdown of aglycone, glucoside, malonyl glucoside, acetyl glucoside and total aglycone equivalents of DAZ, GEN and GLY present in each 500mg capsule

Table 6.3: Composition of isoflavone supplements used in the intervention.

	DAZ	GEN	GLY	Total ISO
Aglycone	0.49 mg	0.09 mg	0.35 mg	0.93 mg
Glycoside	26.44 mg	3.98 mg	13.10 mg	43.52 mg
Acetyl- Glycoside	0.59 mg	0.09 mg	0.28 mg	0.96 mg
Malonyl- Glucoside	0.05 mg	0.01 mg	0.01 mg	0.07 mg
Total aglycone equivalents	17.08 mg	2.69 mg	8.89 mg	28.66 mg

Consumption of 4 x 500 mg ISO supplements therefore provided subjects with a daily total aglycone ISO intake of 114.2 mg. Of the 114.2 mg ISOs, 68.3 mg was DAZ and 10.8 mg was GEN,

providing a ratio of DAZ:GEN in each supplement of approximately 6:1. The equivalent 500mg placebo capsules used in the interventions were carbohydrate based and contained a combination of standard raffilose and fibre.

6.14 Measures of Compliance

Each subject was administered a vial containing 180 capsules (either placebo or ISO supplements) for each treatment period. A maximum of 168 capsules were meant to be taken by subjects over the six weeks, with an additional 12 provided to allow for any extra days subjects were on the treatment. Compliance was determined from the number of capsules subjects returned after each treatment period from the maximum number that should have consumed.

6.15 Overnight Urine Collection

Refer to Chapter 2 section 2.11.2.2 for protocol used to obtain an overnight urine sample.

6.16 Identification of Equol Producers in the Intervention

The established method of HPLC using ECD was employed for identifying equol producers within the study cohort from overnight urine samples that were collected after the active treatment phase. Refer to Chapter 2 section 2.12.2 for the final method used to quantify ISOs present in subject's urine samples. Subjects were identified as an equol producer according to the method established by Setchell and Cole (125), described in section 2.13.

6.17 Statistics

Data were analysed using STATISTICA for WINDOWS software (version 5.1; StatSoft Inc, Tulsa, OK) and *SPSS for Windows* (version 12.0, SPSS Inc, Illinois, USA). One way analysis of variance with repeated measures was used to examine the effect of treatments on dependent variables. Baseline cognitive test scores and baseline arterial diameter were used as fixed covariates to analyse the different effect of the treatments on cognitive test and FMD scores respectively. To determine if there was a treatment interaction with equol production and/or age, two-way ANOVA with repeated measures was used. Where ANOVA showed significant main effects, differences between means were

determined post-hoc using Tukey's HSD test. Relationships between independent variables (age and daily ISO intake) on dependent variables were determined using linear regression (SPSS, version 10, Chicago, USA). Where ANOVA showed significant main effects, differences between means were determined post-hoc using Tukey's HSD test. For the Spatial Working Memory Task with multiple components in its assessment, a one way MANOVA with repeated measures was performed to determine an overall effect of treatments. Relationships between variables were determined using linear regression (SPSS, version 10, Chicago, USA). Statistical significance was set at $P < 0.05$. All data are shown as mean \pm SEM unless otherwise stated. The participation of 40 males in the intervention was calculated to give 80% power to detect a significant improvement in working memory performance at $\alpha = 0.05$. The calculation was based on the results of a pilot study performed in 20 subjects who participated in the second Adelaide based intake of the chronic dietary intervention.

6.18 Results

6.18.1 Subjects

Forty- two males were screened for participation in the intervention. Of this, 40 were eligible for inclusion and chose to take part in the study. After 12 weeks, 34 completed the intervention. Inability to comply with the study protocol ($n=4$), change in banned study medication ($n=1$) and surgery for a medical condition unrelated to the study ($n=1$) were the reasons cited by the six subjects who withdrew. Figure 6.4 described recruitment, withdrawals and final completion of subjects in the intervention. Baseline characteristics of the $n=34$ subjects who completed the intervention are shown in Table 6.4.

Table 6.4: Screening characteristics of males in the Isoflavone Supplement Intervention.

	Subjects (n=34)
Age (y)	48.7 ± 10.3
Weight (kg)	85.1 ± 13.3
Height (cm)	175.4 ± 7.1
BMI (kg/(m²))	27.6 ± 3.6
SBP (mmHg)	125.9 ± 11.8
DBP (mmHg)	77.8 ± 9.9

Mean + SD

6.18.2 Equol Producers in Intervention

Eight of the 34 males who completed the study, were identified as equol producers which represents 23.5% of the study population.

6.18.3 Compliance during Intervention

Subject compliance during the active and placebo treatments was 91.9 ± 1.4% and 91.5 ± 1.4% respectively.

6.18.4 Isoflavone Intake of Subjects during Active Treatment of Intervention

Mean daily ISO intake for subjects during the active treatment was 105.0 ± 9.3 mg.

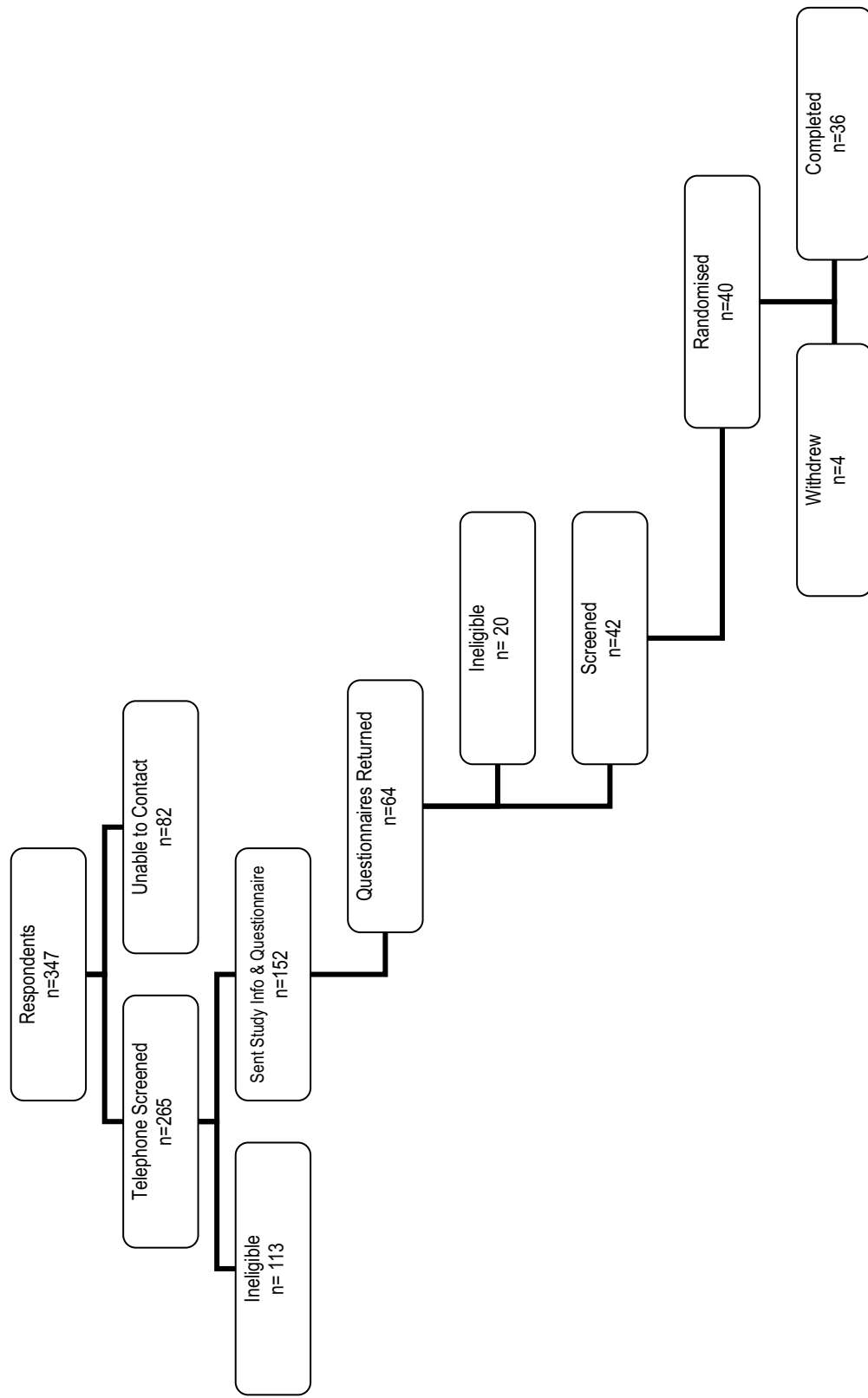


Figure 6.4: Subject recruitment, randomisation and completion rates for the Isoflavone Supplement Intervention.

6.18.5 Effect of Isoflavone Supplementation on Cognitive Performance

Table 6.5 describes the absolute scores and changes for each cognitive test performed on the two treatments. One way MANOVA with repeated measures revealed a significant overall improvement in subjects performance of the Novel Spatial Working Task on the active treatment compared to the placebo (P=0.01) with each of the three test components individually shown to significantly improve; total number of pairs viewed (P=0.01); number of memory errors made throughout the test (P=0.02) and time in seconds to complete task (P=0.03). One way ANOVA with repeat measures revealed no significant treatment effect with respect to any of the other cognitive tests.

6.18.6 Effect of Isoflavone Supplementation on Peripheral Endothelial Function

Table 6.6 shows the absolute and % changes in FMD of subjects on the different treatments. A paired T-test revealed no difference in subjects (all, equol or non equol producer) FMD response on the active treatment compared with the placebo. When subjects pre-occlusion (baseline) arterial diameter was controlled for, the % change in FMD across treatments was still non- significant (P=0.40).

Table 6.6: Effect of treatments on FMD response of subjects in the Isoflavone Supplement Intervention.

		Treatment		
		Active	Placebo	P-value
All subjects (n=34)	Absolute Change FMD	0.027 ± 0.002	0.026 ± 0.002	0.78
	% Change FMD	5.53 ± 0.48	5.40 ± 0.52	0.37
Equol Producers (n=8)	Absolute Change FMD	0.025 ± 0.005	0.029 ± 0.005	0.56
	% Change FMD	5.04 ± 1.01	5.12 ± 0.81	0.56
Non Equol Producers (n=26)	Absolute Change FMD	0.028 ± 0.002	0.026 ± 0.003	0.41
	% Change FMD	5.68 ± 0.56	5.20 ± 0.58	0.40

COGNITIVE TEST	All subjects (n=34)			Equol Subjects (n=8)			Non Equol Subjects (n=26)		
	Active	Placebo	Absolute ▲	Active	Placebo	Absolute ▲	Active	Placebo	Absolute ▲
Paired Associate Learning Task (nos of words)									
-First Recall	2.88 ± 0.40	2.56 ± 0.35	0.32 ± 0.24	3.25 ± 1.13	2.63 ± 0.80	0.63 ± 0.50	2.77 ± 0.41	2.54 ± 0.39	0.23 ± 0.27
-Total Recall	19.41 ± 1.46	18.06 ± 1.46	1.35 ± 0.94	18.00 ± 3.73	17.50 ± 3.47	0.50 ± 1.74	19.85 ± 1.57	18.23 ± 1.62	1.62 ± 1.12
-Delayed Recall	5.50 ± 0.37	5.18 ± 0.39	0.32 ± 0.27	5.13 ± 0.95	5.00 ± 0.85	0.13 ± 0.44	5.62 ± 0.39	5.23 ± 0.45	0.38 ± 0.33
Spatial Working Memory Task									
-Pairs Viewed	56.44 ± 2.75	68.44 ± 5.08	12.00 ± 4.62*	58.25 ± 4.56	78.13 ± 15.40	19.88 ± 13.77	55.88 ± 3.35	65.46 ± 4.76	9.58 ± 4.41*
-Memory Errors	73.15 ± 5.37	95.09 ± 9.43	21.94 ± 8.54*	75.88 ± 8.93	109.88 ± 26.48	34.00 ± 23.24	72.31 ± 6.54	90.54 ± 9.46	18.23 ± 8.77*
-Time to complete task (sec)	344.38 ± 23.72	413.49 ± 36.95	69.11 ± 31.06*	392.27 ± 45.57	505.25 ± 127.91	112.98 ± 113.69	329.65 ± 27.48	385.26 ± 28.48	55.61 ± 22.82*
RAVLT (nos of words)									
-First Recall	7.62 ± 0.43	7.29 ± 0.38	0.32 ± 0.38	8.25 ± 0.88	7.13 ± 0.55	1.13 ± 0.61	7.42 ± 0.50	7.35 ± 0.47	0.08 ± 0.45
-Total Recall	55.12 ± 1.49	54.29 ± 1.60	0.82 ± 1.24	53.75 ± 3.57	53.00 ± 2.29	0.75 ± 2.39	55.54 ± 1.64	54.69 ± 1.99	0.85 ± 1.47†
-Immediate Recall after distraction	10.82 ± 0.57	10.97 ± 0.51	-0.15 ± 0.39	10.13 ± 1.11	10.75 ± 0.84	-0.63 ± 1.10	11.04 ± 0.66	11.04 ± 0.62	0 ± 0.40
-Delayed Recall (20 mins)	10.62 ± 0.65	10.29 ± 0.58	0.32 ± 0.45	8.38 ± 1.36	9.38 ± 1.03	-1.00 ± 1.56	11.31 ± 0.69	10.58 ± 0.69	0.73 ± 0.34*
Initial Letter Fluency Task (nos of words)	26.12 ± 1.30	25.65 ± 1.20	0.47 ± 0.96	29.38 ± 3.53	28.00 ± 3.13	1.38 ± 2.07†	25.12 ± 1.30	24.92 ± 1.25	0.19 ± 1.10
Digit Backwards Task (nos of words)	7.35 ± 0.42	6.97 ± 0.38	0.38 ± 0.39	8.25 ± 0.75	7.38 ± 0.68	0.88 ± 0.40	7.08 ± 0.50	6.85 ± 0.45	0.23 ± 0.50
Trail Making Test (seconds)									
-Trial A	28.91 ± 1.67	28.35 ± 1.17	-0.48 ± 1.40	27.17 ± 2.38	27.65 ± 1.52	0.48 ± 1.69	29.47 ± 2.08	28.56 ± 1.47	-0.78 ± 1.77
-Trial B	67.50 ± 4.07	69.77 ± 4.37	1.80 ± 3.83	58.28 ± 3.16	62.17 ± 3.71	3.89 ± 5.01	70.45 ± 5.17	72.11 ± 5.55	1.16 ± 4.81
-Total (B-A)	39.14 ± 3.58	41.43 ± 4.22	2.29 ± 4.19	31.12 ± 2.42	34.52 ± 4.67	3.41 ± 5.79	41.61 ± 4.53	43.55 ± 5.35	1.94 ± 5.23
Letter Number Sequencing Task (nos correct)	10.38 ± 0.36	10.71 ± 0.40	-0.32 ± 0.35	11.63 ± 0.65	11.38 ± 0.63	0.25 ± 0.75	10.00 ± 0.40	10.50 ± 0.48	-0.50 ± 0.40
Mental Rotation Task									
-Number Correct	24.65 ± 0.60	24.62 ± 0.64	0.03 ± 0.40	25.63 ± 1.25	25.38 ± 1.15	0.25 ± 0.67	24.35 ± 0.68	24.38 ± 0.77	-0.04 ± 0.48
-Time to complete task (sec)	110.21 ± 8.88	113.14 ± 9.84	2.93 ± 5.76	92.00 ± 7.14	102.16 ± 10.34	10.16 ± 7.50	115.81 ± 11.24	116.51 ± 12.49	0.70 ± 7.19

Table 6.5: Absolute and change scores of cognitive tests in Isoflavone Supplement Intervention for all subjects, equol and non equol producers.

*Active treatment score significant improvement from Placebo Score P<0.05, † significantly different to non equol producers, ‡ significantly different to equol producers

6.18.7 Influence of Peripheral Endothelial Function on Cognition

Subject's FMD response was correlated against their cognitive scores for components of the Spatial Working Memory Task (pairs view, mental errors, time to complete task) that were all shown to significantly improve with ISO supplementation. Linear regression found no significant relationship between absolute and % change in FMD and scores for components of the test across treatments for all subjects ($P < 0.35$). When subjects were categorised based on equol status, a significant inverse relationship between non equol subjects' absolute change in time to complete the Spatial Working Memory Task and absolute change in FMD was observed ($r = 0.40, P = 0.045$). Furthermore trends for an inverse relationship between absolute change in pairs viewed ($r = 0.39, P = 0.052$) and mental errors ($r = 0.38, P = 0.057$) compared to absolute change in FMD were also observed within these individuals. No significant correlation between changes in FMD and components of Spatial Working Memory Task was observed for equol producers, despite a trend for a negative inverse relationship between % change in FMD and time to complete the task ($r = 0.68, P = 0.064$).

6.18.8 Correlation between Isoflavone Intake and Changes in Cognitive and Vascular Function

6.18.8.1 Isoflavone Consumption and Cognitive Performance

There was no significant correlation between subjects mean daily ISO intake and changes in their cognitive test scores. When subjects were categorised based on equol status, a significantly positive correlation between ISO intake and total recall of words for the RAVLT test was observed for non equol producers ($P = 0.026, R = 0.437$) (see Figure 6.5) indicating that a higher ISO intake resulted in subjects being able to recall a greater number of words after five attempts of learning the list. An inverse relationship was observed for equol producers in the Initial Letter Fluency Task ($P = 0.006, R = 0.861$) as shown in Figure 6.6 indicating those subjects who consumed a greater amount of ISOs were able to list fewer words in one minute.

Figure 6.5: Correlation between mean daily isoflavone intake and total recall component of the RAVLT for non equol producers (n=26) only.

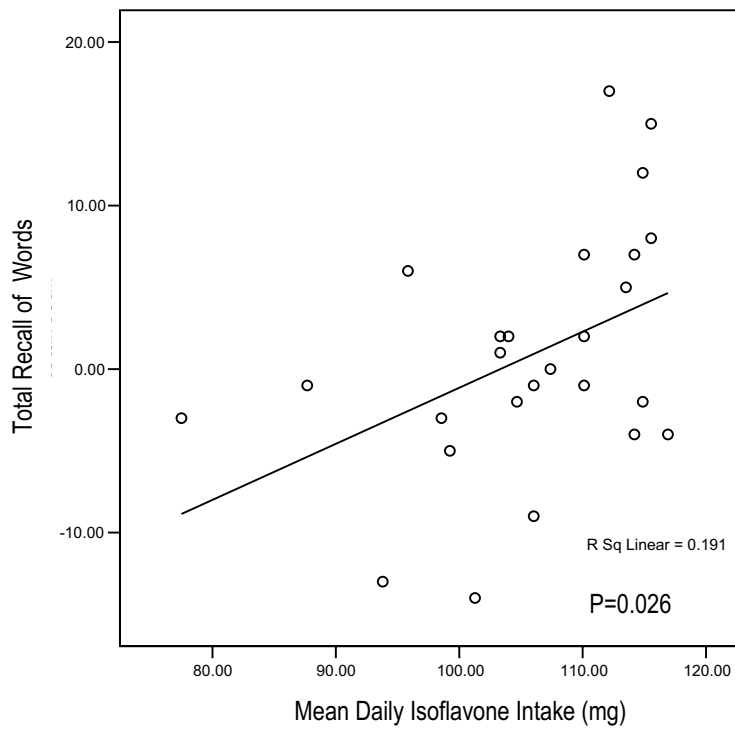
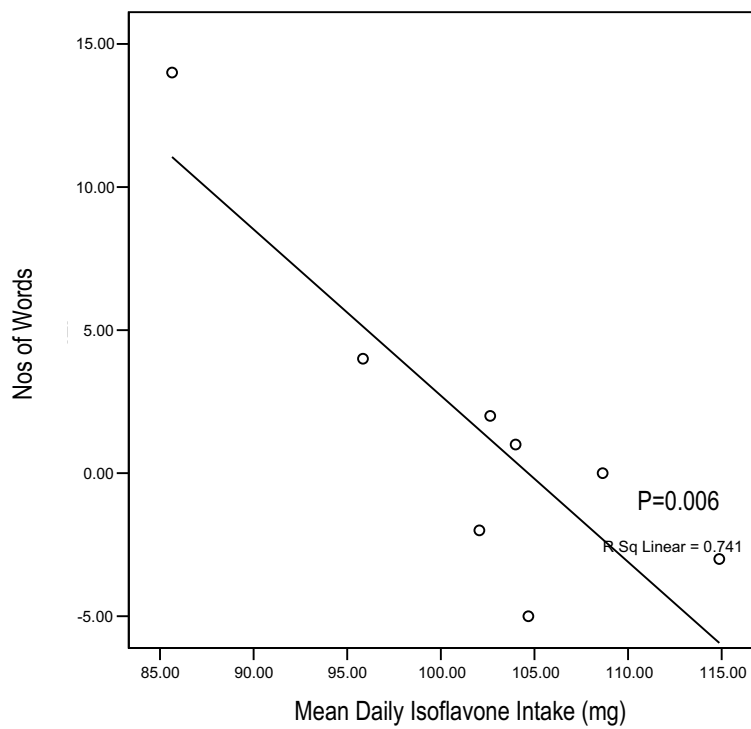


Figure 6.6: Correlation between mean daily isoflavone intake and Initial Letter Fluency Task for equol producers (n=8) only.



6.18.8.2 Isoflavone Consumption and Peripheral Endothelial Function

For all subjects, changes in FMD on the active treatment compared with placebo was significantly correlated with mean daily ISO intake (absolute change; $r = 0.38$, $P = 0.03$ and % change $r = 0.34$, $P = 0.05$) indicating an increased ISO intake improved subjects FMD response (see Figure 6.7). When subjects were categorised based on equol status (as shown in Figure 6.8) there was a significant positive correlation between changes in FMD and mean daily ISO intake for non equol producers (absolute change; $r = 0.50$, $P = 0.01$, and % change; $r = 0.46$, $P = 0.02$). No correlation was observed for equol producers (absolute change; $r = 0.001$, $P = 0.99$ and % change $r = 0.006$, $P = 0.99$).

Figure 6.7: Correlation between mean daily isoflavone intake on the active treatment and % change in FMD for all subjects ($n = 34$).

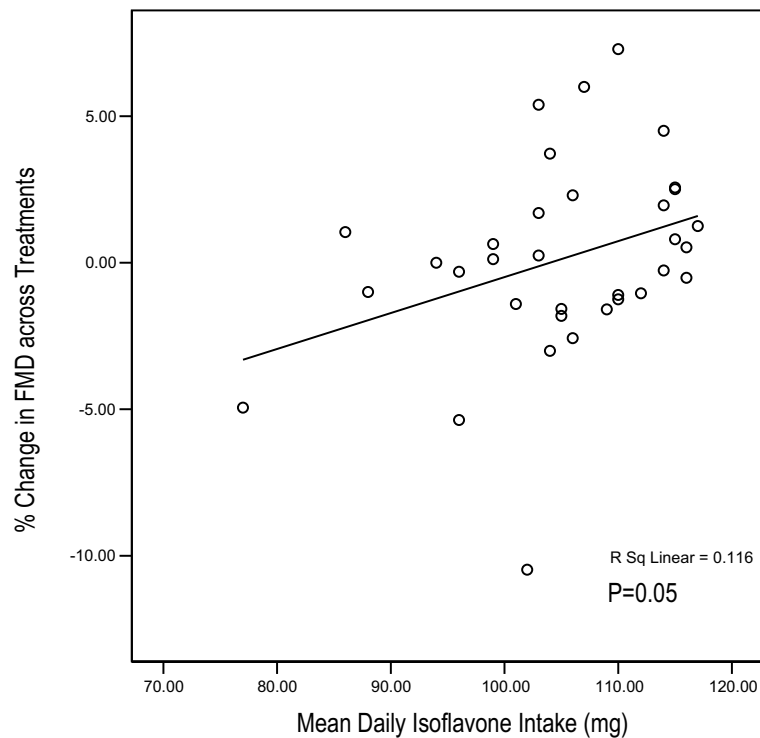
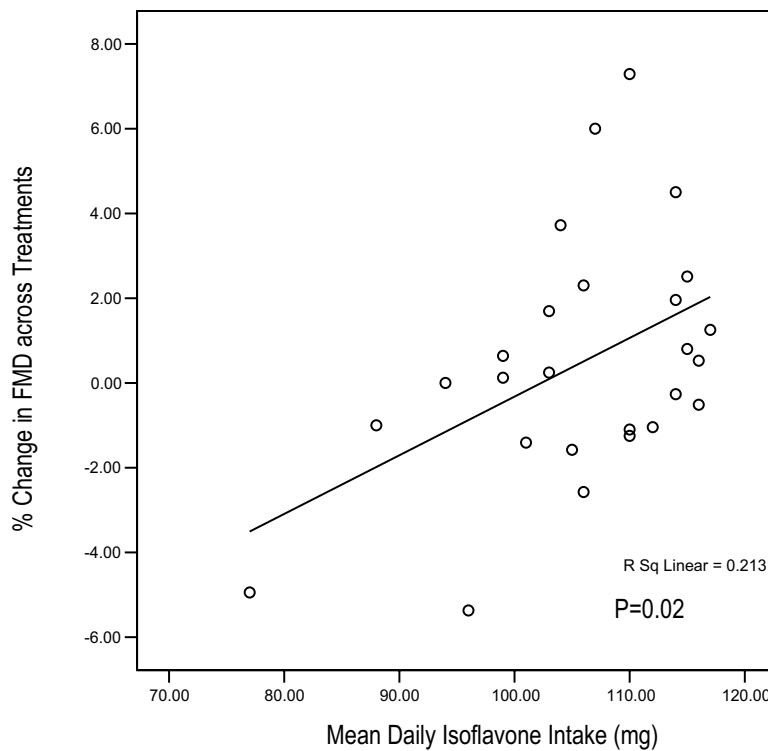


Figure 6.8: Correlation between mean daily isoflavone intake on the active treatment and % change in FMD for non equol producers (n=24) only.



6.18.9 Influence of Equol Production on Cognitive and Vascular Function

6.18.9.1 Influence of Equol on Cognition

When subjects were categorised based on their equol producer status, the significant improvements seen in all subjects for the Novel Spatial Working Memory Task was preserved only for non equol producers. Non equol producers were also shown to perform significantly better after the active treatment phase on the delayed recall component of the RAVLT test (P=0.044) compared with the placebo treatment. With respect to treatment effects between equol and non equol producers there was no significant difference between their performance of the cognitive tests except for the Letter Number Sequencing Task, which equol producers performed significantly better on (P=0.036) and the delayed recall component of the RAVLT test which non equol producers performed better on (P=0.041).

6.18.9.2 Influence of Equol on Flow Mediated Dilatation

Two way ANOVA with repeated measures revealed no difference in the absolute ($P=0.97$) or percent ($P=0.91$) change in FMD of equol and non equol producers, nor was there a significant interactive effect between equol status and the change in FMD across the treatments ($P>0.27$).

6.18.10 Influence of Age on Cognitive and Vascular Function

Linear regression showed no correlation between the age of subjects and their absolute change in all cognitive test scores ($r>0.003$, $P>0.12$) and measures of FMD across the treatments (absolute FMD change; $r=0.09$, $P=0.60$ and % FMD change; $r=0.19$, $P=0.70$)

6.19 Discussion

6.19.1 Cognitive Performance during Intervention and the Effect of Equol Production

Our ISO supplementation intervention was the first to assess the direct effect of ISO on cognitive function in an older healthy male population. The only evidence for the effect of soy on cognition in an elderly male population (aged 71 to 93 years) comes from an epidemiological study which reported a negative correlation between mid life tofu consumption and cognitive performance (392). Another study has looked at ISO consumption on cognitive performance in males and found beneficial effects (567). However the intervention did not consist exclusively of male participants. Instead it was a healthy mixed gender population (15 men and 12 women) and the participants were all relatively young (all under 40 years of age). Furthermore only one other study (410) has correlated improvements in cognitive performance with equol production. Although no effect was observed, the study population was exclusively female.

Of the eight cognitive assessments performed in the intervention, only the Novel Spatial Working Memory Task was shown to significantly improve after ISO supplementation.

In this multi- component test, ISO supplementation resulted in 17.5% less pairs being viewed, 23.1% fewer memory errors and a 16.7% reduction in time to complete the task, compared to subjects who undertook the placebo treatment. The fact that only one test was shown to significantly improve over the

six week period was somewhat unexpected, as the battery that was used, contained tests specifically reported in previous trials to improve with ISO supplementation (393, 394, 396).

In terms of equol production, only eight subjects were identified as equol producers, representing 23.5% of the study population which was lower than anticipated. Equol producers outperformed non equol producers on only one test out of the eight undertaken. This particular test was the Letter Number Sequencing Task ($P=0.036$) which, as described in section 6.11.3.2, assesses auditory working memory. Equol producers were shown to have a 2 % improvement overall compared with a 4% decline in non equol. Conspicuously equol producers were actually shown to perform poorer on the delayed recall component of the RAVLT ($P=0.041$), with non equol producers exhibiting a 7% improvement compared with an 11% decline in equol producers. This result indicates that equol producers in the study had inferior learning and delayed verbal recall skills than non equol producers.

Although compliance was good during the intervention, and no correlation was observed between ISO intake and improvements in test scores for all subjects, when subjects were categorised, based on equol status, linear regression found a significant negative correlation between mean daily ISO intake of equol producers and performance on the Initial Letter Fluency Task. This finding indicates that higher intakes of ISOs were actually detrimental to equol producers' ability to perform the task. The reason for this finding, along with equol producers' poorer performance on the delayed recall component of the RAVLT is unclear, considering the multitude of evidence supporting equol as having superior estrogenic properties and thus a potentially greater ability to improve cognitive performance (refer to Chapter 1 section 1.7.1). The smaller than anticipated (95) sample size of equol producers in the study ($n=8$) may have contributed to this finding by inflating the potential for Type 1 error. What is indicative of our results is that the improvement observed in spatial working memory is not related at all to equol production.

A plausible explanation as to why no improvements were observed for the other tests could be associated with the composition of the supplement used in the intervention. As previously described, equol is a more potent agonist at ER β than its precursor DAZ. Consequently the study capsules were deliberately manufactured to be composed predominately of DAZ in order to favour those who were

potential equol producers in the study. The four capsules subjects consumed daily for six weeks provided a daily total aglycone equivalent intake of 114.2 mg. Of this total, 68.3 mg was DAZ and 10.8 mg GEN, representing a ratio of DAZ:GEN of approximately 6:1.

Considering the small number of equol producers in the present study (n=8) and GEN's higher binding affinity and transcriptional potency for ER β compared to DAZ, these ISO supplements in retrospect may not have been conducive for mediating genomic events that increase the formation of neurotransmitters to preserve cholinergic transmissions or the expression neurotropic factors (403, 404) to attenuate neuronal apoptosis. Furthermore GEN is shown to be more potent at inciting cerebral relaxation via non ER mediated actions than DAZ (378) despite evidence that DAZ can improve cerebral perfusion of the microcirculation whereas GEN does not (407).

In the study by File *et al* (567) which observed improvements in memory and mental flexibility in healthy males, subjects received a high ISO diet for 10 weeks which was designed to deliver 100mg ISO/day. The authors, like in other studies (393, 394, 589), did not disclose the specific quantities of DAZ to GEN that constituted the 100 mg given to these subjects. It is therefore plausible that File *et al*'s study, as well as others that have reported beneficial effects, the ratio of GEN to DAZ has been far greater than that used in the present study. In the study by Kreijkamp-Kaspers *et al* which investigated over a year the effect of consuming 25.6g SP and 99mg ISO/day on cognition in 202 healthy post menopausal women (410), the ISO supplement used consisted of 52 mg GEN, 41 mg DAZ and 6mg GLY. The authors used similar tests to those in the present intervention (RAVLT, Initial Letter Fluency Task and Trail Making Test) and found no significant improvements, only trends when subjects were on the soy treatment. This would suggest, in conjunction with the present study's findings, that a greater proportion to DAZ (ratio >1) and higher dose (>52mg) of GEN is required to see an improvement in cognition.

6.19.2 Effect of Isoflavones on Cognitive Tests with Known Sexual Differences

One of the key objectives of the intervention was to ascertain whether ISO supplementation in males could improve those cognitive abilities in which females generally perform better. This is due to

evidence that estrogen therapy in males can be beneficial (569). It was also an intention to investigate whether ISOs were detrimental to the performance of those tasks which males generally perform well, as supplementation has been observed to be in some animal studies (391, 562).

In terms of sex differences in cognition, women have been repeatedly shown to outperform males in certain tests of verbal ability (word fluency, spelling, grammatical usage, verbal recollection) (590, 591), whilst males appear to excel at specific mathematical and visual spatial tasks (382, 592, 593). Gender differences in specific types of memory performance have also been established, with females more dominant at spatial working memory tasks (580) which require both object and location recall and males more dominant at recalling spatial information (594).

Estrogen and the location of ER β s in brain areas such as the hippocampus, frontal lobe and cortex which control memory and learning function have been implicated in the differentiation and maintenance of these dimorphic cognitive sex differences (595-597). As a result, certain cognitive tests in the battery used in the intervention were known to be performed better by certain genders. For instance, women are shown to perform better at the Novel Spatial Working Memory Task (580), Paired Associate Learning Task (598) and RAVLT (599, 600). These tests appear to be directly influenced by estrogen, for which women have naturally higher circulating levels, rather than a difference in the integrity of the prefrontal cortex and hippocampal formation which is responsible for executing these tasks (601). Since ISOs can mimic the action of the main endogenous estrogen, estradiol, and activate estrogen receptors, especially ER β , we predicted that the males in our intervention would show improved scores for these tests whilst undertaking the ISO treatment.

The Novel Spatial Working Memory Task was the only cognitive task shown to significantly improve during the intervention. This result reflects an enhancement of the male's spatial working memory. The other three tests which women are shown to perform better at only showed trends for improved performance following the ISO treatment. The improvement observed in the Novel Spatial Working Memory Task is suspected to be due to increased binding of ISOs to ER β s in the prefrontal cortex, which plays a critical role in working memory. Neuroendocrine research shows the prefrontal cortex is highly susceptible to the organizational and activation effects of sex steroids. For example,

both androgen and estrogen binding neurons have been identified in the prefrontal cortex of juvenile and adult rhesus monkeys (602-604), as has the enzyme aromatase which converts testosterone to estrogen (603-605). In the human female brain, the prefrontal cortex is shown to be one of the highest binding sites for estrogen(606) and in a recent neuroimaging study, it was demonstrated that changes in task-induced prefrontal activity were associated with variations in female hormonal status (607).

The Novel Spatial Working Memory Task used in the intervention was heavily modelled on past tests measuring working memory in which prefrontal involvement has been determined unequivocally (608-611). When completing the task, women are normally shown to view less pairs, make fewer mental errors and require less time to complete the task compared with males. Furthermore, there is no evidence to suggest this sex difference in performance is attributable to differences in overall ability, attention, perceptual speed or speed of verbal access. In the intervention, all of our male subjects viewed significantly less pairs (17.5%), made fewer mental errors (23.1%) and performed the task in a shorter amount of time (16.7%) when undergoing the ISO treatment. This observation provides the first known evidence that ISO supplementation in healthy older males can significantly improve spatial working memory which is a female dominant cognitive process.

There were three tests in our cognitive battery which were known to have no gender difference and they were the Letter Number Sequencing Task, Initial Letter Fluency Task and the Backward Digit Span Task (612-615). In the intervention, ISO treatment was not shown to enhance males' performance for these tests, which is similar to the lack of effect seen by Kreijkamp-Kaspers *et al* (410) for postmenopausal women consuming 99mg ISO /day for 12 months. Overall the Trail Making Test is not considered to be subject to gender bias. However, there is evidence that women may perform somewhat slower than men on Part B (616). Part B of the Trail Making Test is shown to be more sensitive to cognitive flexibility than Part A and places increased demands on subjects motor speed and visual search (617, 618). In the intervention, the time taken to complete Part B of the Trail Making Test did not differ for subjects during each of the treatments, suggesting ISOs were not detrimental to male's performance of this task.

In tasks requiring the use of spatial cues, researchers have consistently found males exhibit facilitated spatial learning and better spatial memory compared with females (594, 619-621). The underlying mechanism for this difference is not fully understood though there is strong evidence to suggest that gonadal steroids modulate this sexually dimorphic ability, specifically testosterone through interneuronal conversion to estradiol in the brain (622). For instance, the castration of male rats is shown to inhibit visual spatial performance, whilst treatment of intact and ovariectomized female rats with testosterone improves their spatial ability (623, 624). Several animal studies have investigated the effect of ISOs on cognitive tasks that favour males, like visual spatial function, using the Mental Rotation Task (625). Two studies found consumption of ISOs was detrimental to performance of the Mental Rotation Task in male rats compared to females (391, 562). In contrast, elderly male rats consuming a high ISO diet have been reported to exhibit improved spatial memory in a delayed matching-to-place, water maze, behavioural task (561).

In the intervention, performance of the Mental Rotation Task was consistent across the treatments. A lack of difference indicates our males' visual spatial performance was not adversely affected by ISO supplementation and disproves previous evidence that ISOs are harmful to this specific cognitive function in males; albeit in animal models.

6.19.3 Effect of Isoflavones on Peripheral Endothelial Function

The number of human intervention studies that have investigated the effect of pure ISO supplementation on endothelial function is remarkably low (9, 322, 323, 325, 338, 342). Furthermore, no study to date has looked exclusively at ISOs' effect on FMD in a male population. An earlier study by Yildiri *et al* (520) did look at the effect of a soy diet in hypercholesterolemic males (in which 60% of the males source of dietary proteins were substituted by soy) and found a significant improvement of endothelial dependent dilatation. Although in the present study no fasting plasma sample was collected to verify that subjects were not hypercholesterolemic, they were screened for the use of any lipid lowering medication. As no subjects were using these types of medications or any that lower BP or improve mental state, we could assume this is the first intervention to assess ISOs' effects on

endothelial function exclusively in a healthy, older male population. Despite providing one of the highest doses of ISOs (114mg/day over six weeks), no difference was observed in subjects' FMD response. Only a 0.5% increase in FMD was reported in subjects after undertaking the active treatment compared with placebo. This finding is in stark contrast to studies that delivered less ISOs and reported significant improvements (13). Specifically, supplementation with GEN has been shown to improve FMD by 5% (338). Furthermore, no effect of equol was identified (all $P > 0.37$) with respect to subjects' FMD response although this may be related to the small number of producers identified within the study ($n=8$).

When % change in FMD was correlated against subjects' intake of ISOs on the active treatment, a significant positive relationship was observed ($r=0.38$, $P=0.03$), indicating a greater consumption of ISOs by the healthy males, improved their vascular function. This finding supports evidence from epidemiological studies that similarly show ISO intake is correlated with improved endothelial function (334).

6.19.4 Correlation between Cognitive Performance and Flow Mediated Dilatation Response of Subjects in the Intervention

The significant improvement in subjects' performance of the Novel Spatial Working Memory Task does not appear to be mediated by an improvement in blood flow, as suggested by the lack of positive correlation between subjects' FMD response and performance during the tests. In fact, there was a tendency for a negative inverse relationship to occur, significantly in the case of non equol producers, inferring subjects' performance during the test declined as peripheral blood flow improved. This apparent negative association between FMD and performance of the Novel Spatial Working Memory Task does not entirely preclude increased cerebral blood flow as the potential mediator of the improvement. This is due to evidence that demonstrates blood flow in the peripheral and cerebral circulations are not closely related (626, 627).

6.20 Summary

When older healthy males consumed ISOs, an improvement in spatial working memory was observed. This type of cognitive process is traditionally performed better by females because of the sex hormone estrogen. The test used to assess this cognitive function was the Novel Spatial Working Memory Task. An improvement in all aspects of this multi-component test denotes an enhanced ability by the males to retain and continually update information based on visual cues. An increase in blood flow is unlikely to have mediated this cognitive improvement as peripheral endothelial function was not shown to improve nor correlate with subject's performance of the task. Instead the ability of ISOs to bind to ER β and mediate transcriptional events that enhance neuronal growth, survival and synaptogenesis in the prefrontal cortex is most likely to have instigated the effect. Importantly, ISOs were not shown to be detrimental to the performance of cognitive tasks that males naturally excel at, such as visual spatial memory (as measured by the Mental Rotation Task).

Equol was not shown to be responsible for the improvements seen in the males' spatial working memory. Furthermore equol and non equol producers were shown to differ in performance in only two of the administered tests.

7.0 General Discussion

This thesis comprises two dietary intervention trials that investigated whether ISOs present in soy promote CV and mental health benefits. The relevance of the ISO metabolite equol was also investigated as there is limited, but compelling evidence, to suggest that producers may exhibit greater health benefits.

7.1 Key Outcomes from the Interventions

When novel foods containing varying amounts of SP and ISO were consumed by mild hypercholesterolemics, they were shown to improve both plasma lipids and vascular function. Specifically an improvement in TG, TChol and endothelial mediated vasodilatation was observed.

Whilst these findings indicate that soy consumption promotes a more favourable lipid profile, in the case of TChol, the benefit appears unrelated to the inclusion of either SP or ISOs but may be attributable to the consequent decrease in saturated fat consumption. On the other hand, the reduction in TG and improvement in FMD may be attributable to ISOs. In healthy males, ISO consumption also appears to enhance cognitive function, which is most likely due to their estrogenic properties.

The observed benefits in the interventions were not shown to be mediated to any significant extent by the production of equol, indicating any individual who consumes ISOs, in a soy food or as a supplement, is capable of achieving these health benefits.

7.1.1 Benefits of Novel Soy Foods in Relation to Cardiovascular Risk Reduction

At present, a daily intake of 25g of SP, potentially consumed as four individual 6.25g servings, is recommended to reduce blood cholesterol and inturn CVD risk. We explored the concept that novel foods delivering ISO with a reduced amount of SP (only 12g/day) could reduce blood cholesterol as effectively in mild hypercholesterolemics as the recommended amount of SP. The motivation for investigating these novel foods is that the current health claim is difficult for most individuals to adhere to on a daily basis due to soy's poor palatability and that a lower amount of SP, in the vicinity of

12g/day, is far more achievable. There is also support that ISOs, and more importantly the production of equol, can enhance cholesterol reduction which may reduce the amount of SP that is needed in order to achieve a benefit. The ability of these novel soy foods to improve metabolic factors and vascular function was also investigated as there is compelling evidence to show ISOs and equol can independently improve these risk factors/biomarkers of CVD.

The results of the Soy Food Intervention revealed that consuming novel soy foods enriched with ISOs and only 12g SP/day reduced CVD risk in mild hypercholesterolemics by significantly improving plasma TG, TChol and endothelial vasodilatation. As hypercholesterolemia, hypertriglyceridemia and endothelial dysfunction are all associated with the development of atherosclerosis, the pathological condition underling all CVD (259), these novel foods do appear to provide substantial CV benefits.

TG reduction was undeniably due to consuming the novel foods as opposed to some metabolic or dietary improvement since subject's weight, insulin, glucose and carbohydrate intake did not change during the intervention.

The ISOs present in these novel foods also appeared to mediate the reduction in TG as there was no dose response relationship between TG and SP consumption. For instance, on the S diet, which provided twice the content of SP, a comparative reduction in TG was observed.

When the effect of the novel foods was nested with those providing the recommended amount of SP/day, a significant reduction in TChol was observed. The low saturated fat content of SP is thought to have manifested the improvement in cholesterol, as opposed to some direct physiological effect of SP or ISOs, which supports the stance of the American Heart Association Science Advisory Committee (441).

The consumption of novel soy foods also led to a significant improvement in normal endothelial function. Impaired endothelial dilatation is recognised as one of the principle pathological processes of atherosclerosis and is associated with CVD risk (234, 628). Moreover, FMD is seen as a predictor of future CV events, albeit it has a modest prognostic accuracy compared with blood cholesterol (629). As suspected, the improvement in subjects' vascular function appears related to the ISO content of the novel foods rather than SP. This is supported by the significant linear relationship between ISO intake

and change in FMD, when subjects were supplemented with ISO alone (during the ISO Supplementation Trial). Improvements in FMD are most likely mediated by ISOs ability to bind to ER β and PPARs, which are shown to up regulate eNOS activity and increase NO bioavailability.

It has been suggested that inconsistencies in soy's reported health benefits are due to the ISO metabolite equol. Previous studies support this, with its production shown to directly correlate with improvements in lipids (11) and vascular function (13). The Soy Food Intervention was specifically devised to be one of the largest studies to investigate the impact of equol production on lipids and vascular function. Moreover, it was to be the first to investigate the current Soy Health Claim in regards to its legitimacy for equol producers. Despite the hypothesis that equol producers would experience significant improvements in plasma lipids at a potentially reduced SP intake (12g /day), no influence of equol was observed on either the SD or S Diet. Equol production was also not shown to enhance metabolic risk factors or vascular function despite a trend for producers to have a greater FMD response (P=0.08) when consuming a soy diet enriched with ISO. This finding implies that equol production is not necessary to reduce CVD risk in individuals, and that consumption of novel soy food that provide less than the recommended amount of SP but are rich in ISOs, can be beneficial for all individuals.

7.1.2 Benefits of Isoflavones in Relation to Cognition in Healthy Males

In recent years, soy consumption has been shown to manifest improvements in cognitive performance. This phenomenon has been specifically attributed to neurological properties of ISOs. We explored the hypothesis that isolated ISOs can improve vascular and cognitive function by looking at effects of supplementation in a healthy, older male population. ISOs have previously been reported to be detrimental to cognitive performance in these individuals. Moreover, there are limited studies which have exclusively assessed the impact of ISO supplementation on FMD in an exclusively male population. Another objective was to ascertain whether improvements in cognition and vascular function were interrelated. In the Supplement Intervention, ISO treatment was shown to significantly improve cognitive performance in healthy males. Specifically, the Novel Spatial Working Task (women are shown to out perform males) was improved following supplementation. This is the first known study to demonstrate that ISO supplementation can improve a cognitive process that is naturally regulated by

estrogen, most likely through its ability to activate ER β . Results from the intervention furthermore refute earlier evidence (392) that soy is detrimental to cognition in males, as ISO supplementation was not shown to diminish their performance in tasks they naturally perform better than women, such as the Mental Rotation Task.

Although there is evidence to suggest increasing cerebral blood flow leads to improvements in cognition, it is unlikely that males' performance of the Novel Spatial Working Memory Task was mediated by such an effect. This was because no improvement in FMD, a measure of peripheral blood flow which has been previously shown to reflect changes in cerebral blood flow (564) was observed in subjects. Furthermore, subjects' FMD response did not correlate with their performance of the task.

Only one other study is known to have correlated improvements in cognition with equol production (630). The ISO Supplement Intervention was designed to be the first to investigate the effect of equol on cognition and vascular function in males. Only one cognitive test out of the eight performed was shown to be positively influenced by equol production (Letter Number Sequencing Task). Unlike previous observations (13), equol was not shown to influence vascular function. Although there was only a small number of equol producers in the intervention (n=8), results demonstrate equol production is not critical for improving cognition performance.

Whilst the ISO Supplement Intervention acts as a promising pilot study and presents a good argument that ISOs can improve certain cognitive traits in males, its major outcome on visual spatial function warrants confirmation in a larger scale study, perhaps utilising a supplement that has a higher content of GEN.

7.2 Significance of Key Outcomes from Interventions

The ability of ISOs to improve CVD risk is an important area of research, especially since the FDA is reviewing past and current research to determine the ongoing validity of the Soy Health Claim and the American Health Association Science Advisory Committee have recently released a statement denouncing they offer any significant CV benefit (441).

Although ISO consumption was not found to directly affect blood cholesterol in the Soy Food Intervention, it was found to significantly improve TG and vascular function, irrespective of whether the recommended amount of SP was also consumed. This has potential public health implications as it demonstrates ISOs can, along with SP, play a potential role in reducing CVD risk

The risk for atherosclerosis-related events is significantly increased when TG levels are elevated (above 1.6 mmol/L). For clinicians, prescribing ISO supplementation or a diet incorporating traditional non-fermented soy foods (which are typically rich in ISOs) could be a useful alternative treatment option when TGs begin approaching this level. Unlike fibrates, the most common medication used to lower TGs, ISOs do not cause any adverse side effects.

The fact that we were able to demonstrate ISOs from soy foods can improve a normal FMD response is of clinical significance as this is not only recognised as a predictive marker for CVD (629) but may prevent the development of other CVD risk factors, such as insulin resistance that can manifest in the presence of impaired vasodilatation (see review by Cersosimo and DeFonzo(631)). The amount of ISOs required to achieve this benefit (approx 70-75 mg/day) is also highly achievable and within internationally recognised limits of safety (41, 42).

Collectively, results from the Soy Food Intervention would support that the current Soy Health Claim be amended to not only acknowledge a role for ISOs but to state that regular soy consumption can reduce heart disease through the improvement of vascular function.

Although the physiological actions of ISOs and SP did not appear to mediate the observed improvement in TChol, the outcome does emphasise that soy foods are a good source of high quality protein that is low in saturated fat. By developing these novel study foods that incorporate soy into a high palatable food matrix, it offers a potential avenue in which to promote greater consumption by the general public. It also illustrates how soy consumption, at as little as 12g/day, can lower cholesterol when substituted for DP foods which are typically high in saturated fat and cholesterol.

Cognitive impairment without dementia affects a very large segment of the elderly population, with over one fifth of elderly Americans estimated to have measurable cognitive deficiencies (632). Outcomes from the ISO Supplement Intervention advocate that ISOs can beneficially affect cognitive

function which may be important for this specific population. With further research, the implication could be that ISO supplements and foods fortified with ISOs are promoted for improving mental health in the elderly community.

7.3 Study Limitations of Interventions

7.3.1 Soy Food Intervention

In retrospect, there were several limitations associated with the Soy Food Intervention. The first was that not all subjects actually had mildly elevated TChol at the start of the intervention (≥ 5.5 mmol/L). The decision to screen and recruit subjects based on results from a capillary blood sample (finger prick) could explain this. A cholesterol test of blood taken from a vein is shown to be far more accurate than blood taken from a capillary (in a finger tip), with several studies (633, 634) reporting capillary TChol as 3-4% higher than a venous source measured with the same analyzer. The fact that some subjects had normal blood cholesterol levels in the intervention may also explain why only a modest 3% reduction on the S diet for TChol was observed, as there is evidence to support a strong gradient relationship between baseline cholesterol levels and reductions in cholesterol (279).

In addition to elevated TChol, some subjects were also clinically obese, glucose intolerant and/or had central adiposity. Since the subject population was not homogenous but rather had varying CVD risk profiles, it made it difficult to ascertain effects of diet treatments on metabolic factors. Moreover, the intervention was only adequately powered to detect a significant change in blood cholesterol.

The design of the intervention made it difficult to attribute the beneficial effects of lipids and vascular function solely to ISOs provided by the soy foods because both treatment diets contained a portion of SP. Whilst correlations were performed between the subjects mean daily ISO intake and changes in these measures, the tight ranges in ISO intake reported made it difficult to draw any strong linear relationships. In hindsight it would have been advantageous to have a fourth treatment period which provided subjects with only ISOs (e.g. 75-90 mg) and no SP or DP each day.

Another potential limitation in the design of the intervention was the length in duration of the diets. Whilst it is well recognised that LDL-C and TChol can significantly change over a period of a few weeks, there is evidence to suggest HDL-C takes much longer to improve (410). The duration of the diets may have also been too brief to mediate structural changes that transpired into significant improvements in vascular function, specifically when assessing arterial compliance (322, 324, 347).

The time at which clinic visits were conducted may also have presented a limitation in detecting significant improvements in FMD. If ISOs mediate vasodilatation via non-genomic mechanisms (336, 342), performing the assessment the morning after an overnight fast would not have been the time when ISO bioavailability in the circulation was optimal.

The decision to collect only an overnight urine sample from subjects, as opposed to a 24 hour collection, meant that we were unable to use urinary concentrations of GEN and DAZ as an additional biomarker of subjects' adherence to the study protocol. The pharmacokinetics of ISOs in soy foods, after their initial digestion, typically ranges from 6-8 hours (65). As subjects were asked to fast for a minimum of 10 hours prior to their clinic visits, and thus were not consuming any soy foods that could be metabolised overnight, levels of ISOs present in their urine would not reflect their true compliance.

In the study, compliance was determined from food record forms only, which were completed by the subjects themselves. As subjects recruited in the trial were initially non soy consumers and most trial foods on the treatment diets were not well received (as reflected by subjects' poor hedonic ratings of the foods), it's highly possible they may have overstated their actual trial food intake. As results in the intervention were dependent on the optimal adherence of subjects to the study protocol, it's possible the lack of a significant effect on measures such as LDL-C, particularly seen on the S diet, were due to subjects actually consuming lower than expected intakes of SP and ISOs. The use of a Food Frequency Questionnaires to ascertain subjects' macronutrient intakes on the diets, may have incorrectly estimated fat and sugar consumption which was shown to differ significantly on the diets. Instead, a three day weighed food record, completed by subjects at the end of each diet might have provided a more accurate detail of their macronutrient intake enabling us to speculate more thoroughly whether changes in subjects' diets accounted for the changes in lipids.

7.3.2 Isoflavone Supplement Intervention

A potential limitation of the ISO Supplement Intervention was the small number of male participants who completed the trial (n=34). Despite predicting sufficient power with 40 male subjects, only one of the eight tests showed a significant improvement following ISO supplementation. An explanation for this could be that the males in the intervention had no apparent impairment in their cognitive function and a greater number of subjects would be needed to detect significant improvements in a healthy population. It is also possible the tests we used were not sensitive enough to detect significant improvements in a healthy population. The Novel Spatial Working Memory Task has specifically been devised to be sensitive enough to detect significant improvements in a healthy population and interestingly this was the only task that we found significant improvements in. The other tests used were developed to detect differences in populations who have known/severe cognitive impairments.

Despite testing subjects at approximately the same time of day before and after each treatment phase, there is evidence that levels of testosterone can influence cognitive performance of males (635-637). Blood samples were not collected at the beginning of the cognitive testing sessions. Therefore subjects' testosterone level could not be determined and used as a covariate in our analysis of their performance. There is also no evidence that testosterone levels in males are altered by dietary ISOs (638, 639).

7.4 Future Directions of Research

The ISO Supplement Intervention is the first to demonstrate ISOs can improve a female dominant cognitive process whilst maintaining normal function in older healthy males, irrespective of whether they produce equol. Future studies looking at the effects of equol on cognitive performance are still warranted, as the small number in the present study does not determine conclusively whether equol production is beneficial or not.

A dietary intervention delivering equol as opposed to ISOs in soy foods could be performed in the future to further ascertain the effect of this metabolite on lipids, vascular function and metabolic

factors. This is due to recent evidence that suggests the direct delivery of the metabolite beneficially affects these measures (483, 640). Consuming equol would furthermore remove individual variability in its production and reduce the number of subjects required to ascertain its effects.



To summarise, results from this thesis support the hypothesis that ISOs consumed with SP can protect against CVD by providing a beneficial effect on plasma lipids and vascular function. Furthermore the results identify a novel effect of ISOs on the performance of cognitive tasks that demonstrate an estrogenic dependence in males. Interestingly, these CV and mental health benefits were not shown to be mediated to any significant extent by the production of equol which is shown to possess superior estrogenic properties compared to ISOs.

Our definitive dietary intervention also calls into question the ability of SP to significantly reduce cholesterol and the risk of CVD as stated in current Soy Health Claim. When regularly consumed by mild hypercholesterolemic subjects, soy foods delivering SP equivalent to the recommended amount did not significantly reduce LDL-C. Moreover the reduction in TChol observed could be accounted for by a reduced saturated fat intake.

