The effect of soy isoflavone intake on cardiovascular disease risk factors - potential role of equol

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By

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Abstract

Isoflavones, the subclass of flavonoids found in soybeans, may reduce the risk of cardiovascular disease (CVD). However, the current evidence is inconsistent. The capability to produce the isoflavone metabolite equol in 20-60 % of the population might underlie the benefits of isoflavone intake on vascular health. In a retrospective analysis of a 12-month intervention containing isoflavones (100 mg isoflavones, as aglycones equivalents), postmenopausal women with Type 2 diabetes who were equol producers (EPs) had significantly lower diastolic blood pressure (BP), mean arterial BP and arterial stiffness (assessed by pulse wave velocity (PWV)) (mean change from baseline (Δ)$\pm$SEM; -2.2$\pm$1.3 mmHg, -1.2$\pm$1.3 mmHg, -0.7$\pm$0.4 m/s, respectively, P<0.01) than non-EPs. Subsequently, an acute crossover double-blind study was conducted where EP and non-EP males at elevated heart disease risk were prospectively recruited (n=14 per group) to determine whether EP phenotype was associated with differential vascular responses. The effects of an isoflavone supplement (80mg; single-dose) on endothelial function, PWV and BP were assessed at baseline, and at the anticipated T_{max} for plasma isoflavones (6h) and equol (24h). After isoflavone intake, EPs had significantly decreased PWV at 24h ($\Delta$$\pm$SEM: isoflavone -0.2$\pm$0.2, placebo +0.6$\pm$0.2; P<0.01). However, isoflavone intake had no effect on the vascular measures at the 6h and 24h timepoints. To further investigate the acute vascular effects of S-equol *per se,* synthetic *S*-equol supplements (40 mg) were consumed by non-EPs at the anticipated T_{max} for plasma S-equol (2h) and this showed no significant vascular benefits.

In conclusion, acute isoflavone intake improved arterial stiffness in male EPs at increased CVD risk, with the magnitude of change potentially equating to a 10-12% risk reduction in risk of CVD if sustained. Synthetic equol intake had no acute benefit on vascular health in non-EPs. These data suggest that the EP phenotype may partly predict the effectiveness of soy isoflavones on arterial stiffness. Further long term RCTs with prospectively recruited EPs are required to confirm the importance of equol production for cardiovascular health.

Table of Contents

List of figures

List of tables

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Chapter 1   Literature review: the effect of soy isoflavone intake on cardiovascular disease risk factors - potential role of equol

1.1   Introduction

Isoflavones are heterocyclic compounds found in soybeans and belong to the flavonoid family that have been studied extensively for their health effects. In particular, soy isoflavones may offer benefits on cardiovascular disease risk factors such as blood pressure [1], endothelial function [2, 3] and arterial stiffness [4].

However, the evidence for health benefits is inconsistent [5-16] and the metabolic ability to produce the unique isoflavone metabolite equol might lead to differential health effects following isoflavone intake [17]. Between 20-60% of populations can produce equol from the major soy isoflavone daidzein, with the highest prevalence occurring in Asian populations [18]. Equol is of interest because it has higher bioavailability *in vivo* [19] and *in vitro* it has been shown to have greater antioxidant [20-22] and vascular benefits [23, 24] compared to its parent compound. In addition, recent observational and retrospective analyses of intervention trials support the hypothesis that people who have the ability to produce equol may have better cardiovascular health [25-28] and may benefit more from isoflavone intakes that target cardiovascular disease risk factors [6].

This chapter reviews cardiovascular disease development and risk factors, soy isoflavones and their effects on cardiovascular disease risk factors, the isoflavone metabolite equol and its role in explaining any potential benefits of isoflavones on vascular function, and briefly current knowledge on the bioavailability of isoflavone and the gut metabolite equol.

1.2 Cardiovascular diseases and atherosclerosis

Cardiovascular disease (CVD) is the main cause of death worldwide; 17.1 million deaths from CVD are estimated each year [29]. CVDs consist of coronary heart disease, cerebro-vascular disease (i.e. stroke and ischemic heart attack) and peripheral vascular disease, all of which develop from atherosclerosis and thrombosis in the arteries [30]. Atherosclerosis is a thickening of the arterial lining, which is a consequence of the development of atheromatous plaques. Accumulated lipids within the arterial wall form foam cells that can progress into mature plaque with proliferation of connective tissue, adhesion of platelets, proliferation and migration of smooth muscle cells and infiltration of immune cells (Figure 1.1). Plaques can grow and significantly reduce the blood flow through an artery and when the plaques rupture, thrombus (blood clot) occurs that can occlude the artery [31, 32]. CVD risk factors include elevated plasma lipid levels [33], endothelium dysfunction [34], arterial stiffness [35] and hypertension [36]. Of importance, endothelial dysfunction, arterial stiffness and hypertension are demonstrated strong predictors of CVD risk [34, 37-39] and are described further in subsequent sections.
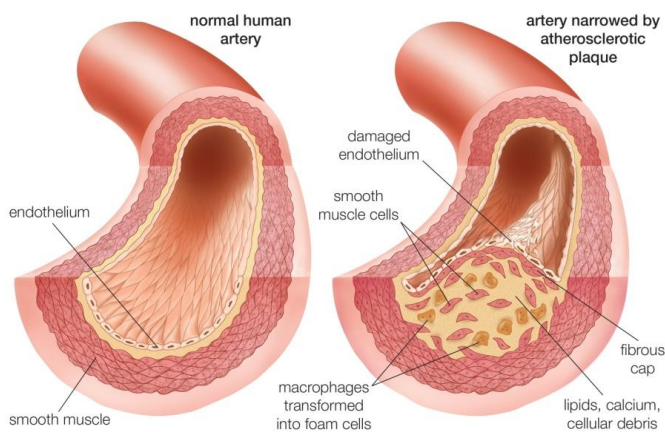
Figure 1.1 Development of atherosclerosis
(Figure reproduced from Encyclopaedia Britannica, 2010 [40]).

1.2.1 Endothelial dysfunction

Endothelial dysfunction is implicated by the loss of sufficient dilation of an artery in reaction to endothelial-derived stimulus [41] and is a major early marker of the development of atherosclerosis and CVD [41]. Additionally, endothelial dysfunction is positively associated with CVD co-morbidities, such as hypercholesterolemia, hypertension, and type 2 diabetes mellitus [42]. Human studies have shown that dietary and lifestyle interventions such as diet rich in flavonoids could reverse endothelial dysfunction and in turn may prevent development of CVDs [43].

The mechanism by which the dysfunctional endothelium-dependent vasodilation occurs has been suggested through an imbalance of endothelium-derived vasodilators and contracting factors; particularly a decreased bioavailability of vasodilators and antithrombogenic factors such as nitric oxide (NO), and/or increased presence of vasoconstrictors and prothrombotic factors [43]. During endothelial dysfunction additional impaired activations in the endothelium take place that further lead to atherosclerosis progression, in particular decreased anticoagulation, increased levels of pro-inflammatory factors (adhesion molecules and cytokines) and elevated smooth muscle proliferation [44].

Endothelial function can be measured at the coronary or peripheral circulation by a variety of techniques such as coronary angiography, flow-mediated dilation (FMD) of the brachial artery [41, 45], plethysmography of the forearm circulation, and finger plethysmography (using EndoPAT) [46]. The main principle for most of these techniques is the measurement of the dilatation of the coronary or brachial arteries, in response to a stimulus, either after intra-arterial infusion of exogenous vasodilators (acetylcholine or bradykinin), or post the occlusion of the artery (when the blood flow increases and creates an amplified tangential force or shear stress on the endothelium causing the dilatation of the vessel [47]). The shear stress derived hyperaemia reaction and the initiated endothelium-dependent changes in vascular tone [48] are most likely the result of the release of the most potent vasodilator in the endothelium, NO [34, 41].

FMD measurement, using high-resolution ultrasound of the artery, is the most commonly used method for assessment of endothelium function. However, the limitation of using this

method is that it is operator dependent, which requires extensive training, and the technique does not control for potential tone alterations in the systemic nervous system [49].

As an operator independent alternative, finger plethysmography (using EndoPAT device) is widely used to assess endothelial function by measuring pulse wave profiles, following brachial artery occlusion, at a finger artery. The EndoPAT records changes in plethysmographic pressure caused by the arterial pulse in the finger and interprets it into a peripheral arterial tone (PAT). When using EndoPAT, measurements on the other arm are used to correct for any potential simultaneous endothelium-independent changes (for details, see Figure 3.5, page 97) [48].

1.2.2 Arterial stiffness

Increased arterial stiffness is positively associated with risk for fatal cardiovascular events including myocardial infarction and stroke [35] and it is also correlated with endothelial dysfunction and hypertension [38].

Arterial stiffness is considered an important prognostic factor that has been targeted by human studies for protection against CVD development and in terms of the clinical importance, it has been shown in meta-analyses that reductions in PWV equal to 1 m/s might lead to 13-14% reduction in CVD risk [50]. A recent review reported that dietary components could improve arterial stiffness, in particular the flavonoid subclasses: isoflavones, anthocyanins and to a lesser extent flavan-3-ols [51].

During aging and/or development of CVD, the arteries become more stiff and in return less compliant, leading to an increase in cardiac muscle contraction to accommodate the resistant arteries and this might result in left ventricular hypertrophy (overstretch of cardiac walls) and cardiac failure over time [52].

Arterial stiffness originates from two different components, structural and dynamic alterations in the arterial wall [53] (Figure 1.2). The structural changes occurs in the arterial media layer as the collagen to elastin ratio increases, which might be due to elevated collagen, elastin fragmentation, arterial calcification, glycation of both elastin and collagen, and cross-linking of collagen by advanced glycation end- products (AGE) [53]. The cross-linking between collagen and elastin is essential for the strength and elasticity

to the arteries. However, during ageing, cross-linking of collagen might increase due to cross-linking amino acids histidinoalanine and pentosidine in collagen itself, or due to an increase in the formation of AGE [54]. AGE are produced by the non-enzymatic glycation between the amino groups and sugars in proteins that build bridges between collagen fibres and thus decreases the elasticity of the arterial wall [53, 54]. The dynamic changes also occur in the arterial media layer and it is related to the tone of smooth muscle cells which depends on the release of vasodilators and vasoconstrictors from the endothelium such as Nitric Oxide (NO) [53]. This emphasizes the connection between arterial stiffness and endothelial dysfunction.
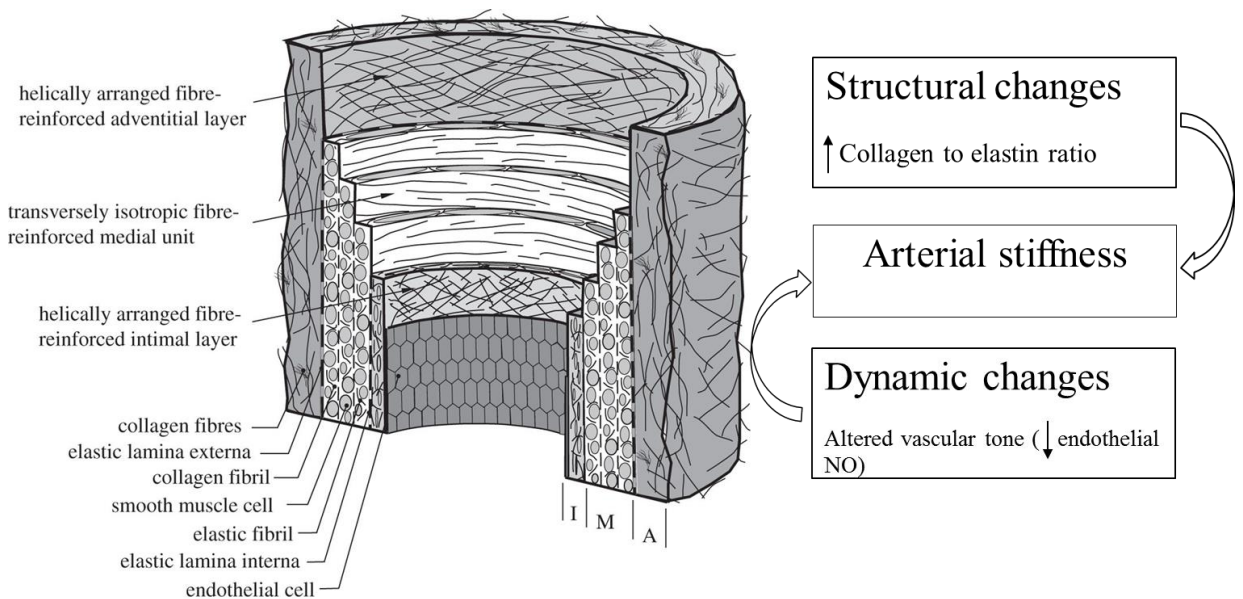
Figure 1.2 The structure of an artery and arterial stiffness causes
The arterial stiffness could be related structural (increased collagen to elastin ratio) and/or dynamic alterations (altered vascular tone induced by vasoactive compounds) in the arterial media layer. The media is the middle layer of the artery that is made up of elastic and collagen fibrils, smooth muscle cells, and elastic laminae. There are two other layers that compose the artery: the inner layer (intima) consisting of collagen fibrils, a single layer of endothelial cells, and a thin basal membrane, and the outer layer (adventitia) which is surrounded by loose connective tissue and has helically arranged collagen fibrils (figure is adapted from Tsamis et al., 2013 [54]).

Arterial stiffness influences pulse wave reflections [52, 55]. Laurent et al. reviewed the most accepted model of the arterial tree: a propagative model that resembles a visco-elastic tube that has different elastic properties allowing the generation of a pressure wave which travels forward [55]. When the pressure wave travels down through a tube of numerous

branches of decreased diameter, it is amplified as a result of going through the central arteries branching into peripheral arteries that are more muscular and less elastic where the resistance increases and creates reflected waves. In healthy arteries, the reflected waves arrive during late systole to diastole and subsequently contribute to the magnitude of diastole through constructive wave interference [55].

During CVD development, central arteries such as the carotid become stiffer and have a decreased diameter that repositions points of pulse wave reflections to earlier segments along the arterial tree. Thus, reflected waves will arrive to the heart closer to systole, augmenting it and delaying the diastole and in turn, this may result in overstretching of the cardiac walls and decreased coronary perfusion [38, 55].

Arterial stiffness can be evaluated by different methods including regional stiffness (pulse wave velocity (PWV)), local stiffness (magnetic resonance imaging (MRI)), and arterial compliance and wave reflections (pulse wave analysis (PWA)) [38, 55]. The gold standard method is the central/aortic PWV that can be estimated between the carotid and femoral arteries [38, 55].

1.2.3 Hypertension

Hypertension is a major risk factor for CVD development and mortality [56]. It is highly associated with stroke, coronary and peripheral arterial diseases [56].

Hypertension is diagnosed in subjects who have persistently high blood pressure; with a systolic blood pressure equal or more than 140 mm Hg and/ or diastolic blood pressure equal or more than 90 mm Hg [56].

Hypertension is likely to be reduced by fruit and vegetable-rich diets, particularly due to their flavonoid content [57]. A reduction of 2-3 mmHg in diastolic blood pressure in adults (50–69 years) could lead to 10-20% reductions in risk of stroke and coronary artery disease [58].

Two types of hypertension are frequently described: primary hypertension, which is due to increased total peripheral resistance, and isolated systolic hypertension, which is caused by increased pulse pressure [59]. In both instances, uncontrolled hypertension can cause cardiac perfusion and may result in cardiac arrest. High blood pressure can also cause aneurysms to develop in arteries that are associated with blood clots and their rupture [56]. The cause of hypertension is likely to include dysfunction of either the sympathetic

nervous system [60], the renal renin-angiotensin aldosterone system [61], endothelial dysfunction, and/ or vascular inflammation [61, 62].

As mentioned previously, all the discussed CVD risk factors could be modified by dietary factors, which could prevent CVD disease. In particular, intakes of flavonoids might provide protection against CVD development [13]. Recent meta-analyses suggest that soy isoflavone intake, a subclass of flavonoids, may improve endothelial function [2, 3, 13], arterial stiffness [4] and blood pressure [1].

1.3   Isoflavones and cardiovascular disease (CVD)

1.3.1 Soy isoflavones- Biological properties, chemical structure and soy content

During the past 20 years, extensive research has investigated the cardio-protective effects of soy isoflavones [63, 64]. Isoflavones are natural heterocyclic phenols, present predominantly in soybeans. Research has identified around 370 isoflavones, with genistein, daidzein and glycitein as the major ones in soybeans [65]. The basic chemical structure of isoflavones consists of two benzene rings (A and B) bound to a heterocyclic pyrone ring (C) (Figure 1.3).

 Isoflavones are classified as a subgroup of phytoestrogens since they have the potential to exert both oestrogen agonist and antagonist effects. This might be due to similarity in their chemical structure with the 17β oestradiol [66]. As potential selective oestrogen receptor modulators (SERMs) [67, 68], it has been suggested that isoflavones might have more favourable cardio-protective effects compared to oestrogen.

R1	R2	Compound
H	H	Daidzein
OH	H	Genistein
H	OCH_3	Glycitein

R3	R4	R5	Compound
H	H	H	Daidzin
OH	H	H	Genistin
H	OCH_3	H	Glycitin
H	H	$COCH_3$	Acetyldaidzin
OH	H	$COCH_3$	Acetylgenistin
H	OCH_3	$COCH_3$	Acetylglycitin
H	H	$COCH_2COOH$	Malonyldaidzin
OH	H	$COCH_2COOH$	Malonylgenistin
H	OCH_3	$COCH_2COOH$	Malonylglycitin
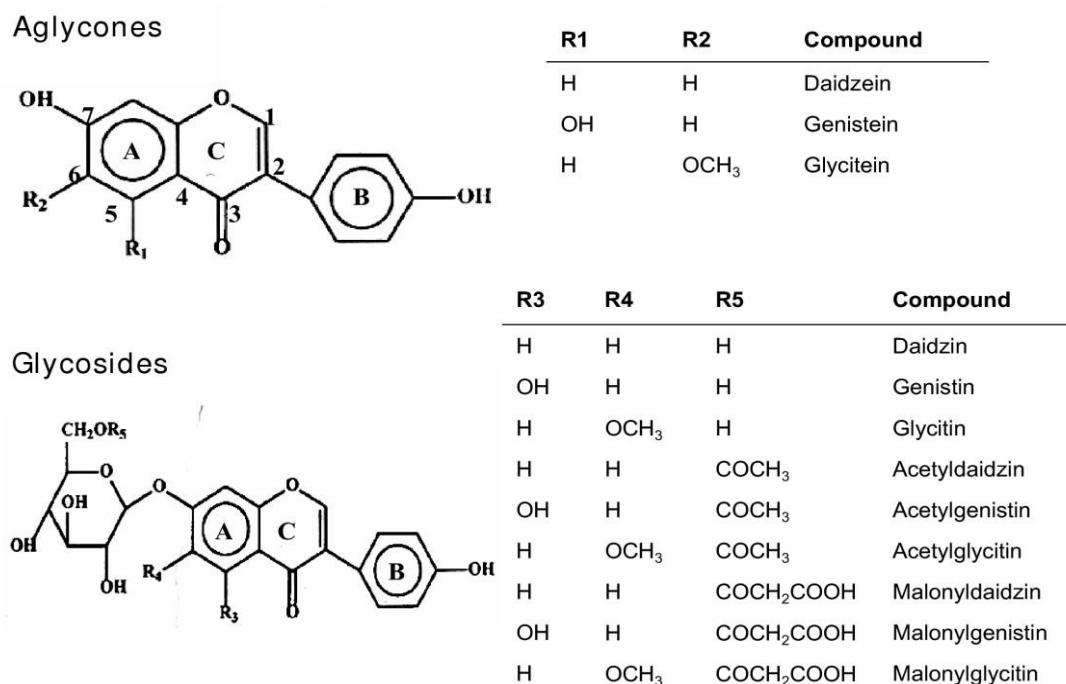
Figure 1.3 Chemical structures of soy isoflavones

Chemical structures are shown for major isoflavones in food: daidzein, genistein and glycitein existing as aglycones or glycosides that are bound to acetyl or malonyl groups (figure adapted from Anupongsanugool *et al.*, 2005 [65]).

Daidzein, genistein and glycitein are the aglycone forms, while daidzin, genistin, and glycitin are the glycoside forms that are found mainly in soy food and products, which are often bound to acetyl or malonyl groups.

The soy isoflavone content and composition vary in different soy foods according to the cultivation, environmental conditions and processing methods [69]. For instance, isoflavone levels may range from 2.66 isoflavones mg/g protein in roasted soybeans to 0.73 mg/g protein in soy protein concentrate (Table1.1). This loss of isoflavone is due to alcohol extraction methods that wash out the isoflavones [69].

Table1.1 Isoflavone content of soy foods and products

Soy food and products	Mean total isoflavone content (mg/g of food)
Soybeans (green, raw)	0.49
Soybeans (mature seeds, sprouted, raw)	0.34
Soybeans (roasted)	2.66
Soy flour (textured)	1.72
Soy protein isolate	0.91
Soy protein concentrate	0.73
Miso soup (mix, dry)	0.07
Tempeh	0.61
Tofu (silken)	0.18
Tofu yogurt	0.16
Soy hot dog (frozen, unprepared)	0.01
Soy milk (original, vanilla)	0.18
Soy sauce	0.01

Table adapted from Bhathena *et al*., 2002 & USDA., 2008 [70, 71].

1.3.2 Epidemiology - Soy isoflavone intake and CVD

In Asian countries the intake of isoflavones has been estimated to range from 25–50 mg per day [72, 73], whereas the population in the UK is thought to consume just 1 mg per day [74, 75]. This greater habitual intake of isoflavones in Asian countries may be one contributor to the low incidence of CVD in this region.

Some epidemiological research suggests that dietary intake of soy isoflavones may protect against CVD initiation and progression [73, 76-79]. A large cohort with 18 years follow up has shown that nuts and soy intakes were two of most important independent contributors to decreased overall and CVD mortality [76]. It was also reported that higher isoflavone intake was positively associated with enhanced endothelial function and better clinical atherosclerotic symptoms in patients with coronary artery disease (CAD) or stroke [78].

1.3.3 Randomised controlled trials (RCTs) - the effects of soy isoflavones on CVD risk factors

Cardio-protective effects of soy isoflavones have been investigated in human RCTs such as improvements in the plasma lipid profile, hemodynamic and endothelial function [5-16]. In particular, benefits of soy isoflavones on lipid lowering cholesterol levels have been reported extensively [64]. Recent meta-analyses suggest that the cardio-protective effects of soy intake are related mainly to its constituent isoflavones [3] and they extend beyond enhanced lipids to benefits on endothelial function [2, 3, 13], arterial stiffness [4] and blood pressure [1]. It has been shown that infusion of 10 to 300 nmol/min of genistein caused NO-dependent vasodilation in the brachial artery with comparable efficiency to 17β-oestradiol in male and female subjects [80]. Subsequently, a meta-analysis of 9 RCTs has also shown that isoflavone supplement intake significantly improved endothelial function (assessed by FMD) only in postmenopausal women at increased CVD risk having low FMD levels, but not in those with high FMD levels at baseline [2].

Furthermore, a more recent meta-analysis of 17 RCTs providing 33-120 mg/d (as isoflavone aglycones) for 4-52 weeks, has shown a potentially clinical relevant increase of 1.98 % in FMD in women and men by isolated isoflavone intake alone compared to an FMD increase of 0.72 % by isoflavone containing soy protein [3].

Another meta-analysis of 14 RCTs has shown that intakes of 25-375 mg soy isoflavones (as aglycone units) for 2-24 weeks significantly improve hemodynamic function (estimated decrease of 1.92 mmHg in systolic blood pressure) in subjects with normal blood pressure or pre-hypertension [1]. Regarding effects on arterial stiffness, few studies have shown improvements on PWV and/or systemic artery compliance (SAC) in response to isoflavone supplementation [81-83]. Nestel et al. [82] showed enhanced SAC in 21 peri- and post-menopausal women following intake of 80 mg per day isoflavone (1.3:1:0.1, genistein, daidzein, glycitein respectively) for 5 weeks. Similarly, a 6 week intake of 80 mg per day (red clover isoflavones, precursors for daidzein and genistein) significantly increased SAC and lowered PWV in 80 healthy men and postmenopausal women [81]. Interestingly, few trials investigated effects of isoflavone metabolites, and one of them showed that consumption of the isoflavone metabolite trans-tetrahydrodaidzein (THD) (1g per day for 5 weeks) significantly decreased central PWV and systolic BP in 25 postmenopausal women and overweight men [84].

In contrast, some studies have reported no effects or contradictory findings [15, 16]. A 6 month intake of dietary soy protein (118 mg as isoflavones aglycone) observed no improvements on endothelial function, arterial stiffness or ambulatory blood pressure in 41 hypertensive men and women [16]. Similarly, a large RCT [15] reported no significant change in endothelial function in 202 postmenopausal women after an intervention of soy protein (99 mg isoflavones/d for 12 months) and systolic BP increased in the soy group compared to the placebo group.

These equivocal data might be explained by different reasons such as study populations, isoflavone dose and source, consumption duration, collection time of endpoints and inter-individual variability in metabolism in particular the ability to produce a unique isoflavone called "equol". For instance, the time of collection of study endpoints might be a crucial determinant for findings and it is suggested that the vascular benefits of isoflavones could be positively related to the bioavailable levels of isoflavones and their metabolites [9]. A single dose of 80 mg isoflavones has showed significant increases in FMD and plasma Nitric Oxide concentrations (NO) in postmenopausal women at the anticipated peak time for the major isoflavones daidzein and genistein [9].

Additionally, it is believed that the matrix of the consumed isoflavones affects their bioavailability and thus isoflavones consumed as pure compounds, or dietary source may generate differential health effects [85]. It has been previously reported that isoflavone was more bioavailable from a supplement source than from soy based cheese (food source) [86]. This might be because the dietary matrix contains lipids, proteins and carbohydrates that bind to isoflavones and limit or delay their absorption, or due to isoflavone losses or structure conversions through multiple cooking steps like heating and grinding steps [87]. Therefore, the source of isoflavone intake may induce significant changes to its bioavailability and in turn vascular bioactivity. From the Beavers et al. meta-analysis, it is suggested that consumption of isolated isoflavone supplements could improve endothelial dysfunction more than isoflavone containing soy protein [3]. However, one recent study has shown that whole soy intake significantly reduced plasma levels of lipid and inflammation markers among prehypertensive postmenopausal women and these effects were not showed after consumption of purified daidzein [88].

1.3.4 The vascular protective effects of soy isoflavones: mechanism of actions and potential biomarkers

From the available evidence, the most beneficial effects of isoflavone interventions tend to be improvements in the endothelial dysfunction, which is an early step in the progression of atherosclerosis that results from lipid deposition, inflammation and oxidative stress in the arteries [34].

In vivo and in vitro studies have been undertaken to examine how isoflavones exert their vasodilation effect, in particular focusing on mechanisms that underlie endothelial vasodilation and constriction [39]. The endothelium has crucial functions in blood vessels: it modulates immune responses, regulates coagulation and clotting, and controls the vascular tone through vasodilation and vasoconstriction [89]. NO is an essential endothelial-derived vasodilator that is produced from the conversion of L-citrulline to L-arginine by endothelial nitric oxide synthase (eNOS) [90]. The maintenance of an adequate level of endothelial NO can protect the vessels against atherosclerosis because it promotes vaso-relaxation, inhibits migration of adjacent vascular smooth muscle cells (VSMC), prevents platelet aggregation and expression of monocyte adhesion and adhesion molecules [89]. However, under abnormal physiological conditions, NO is thought to be lost through reaction with reactive oxygen species (ROS) to form peroxynitrites ($ONOO^-$) [89, 91].

It has been proposed that the positive effects of chronic and acute exposure to isoflavones on endothelial function are explained by elevated bioavailable levels of NO [9, 92] and this could be a result of increased expression of eNOS [93, 94] or due to reduced activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH normally generates superoxide ($O_2^{.-}$) that binds to NO and forms peroxynitrite ($ONOO^-$) and thus reduces the bioavailable NO [95].

Furthermore, some *in vitro* work has reported that daidzein and genistein induce increased prostacyclin (PGI2) production (i.e. a vasodilator and inhibitor of platelet aggregation) [96]. Two *in vitro* and *in vivo* studies have also shown a significant increase in PGI2 production in human umbilical vein cells (HUVEC) treated with serum obtained from post-menopausal women consuming isoflavones (55 mg/ day, for 6 months) compared to treatment with baseline serum [97]. An additional proposed mechanism underlying the

vascular actions of isoflavones might involve inhibition of endothelial-derived vasoconstrictors such as Endothelin-1 [11, 98] and angiotensin converting enzyme (ACE) [39, 99]; both are part of the Ang II-induced hypertension that occurs in endothelial dysfunction and vascular inflammation (Figure 1.4) [61, 100].
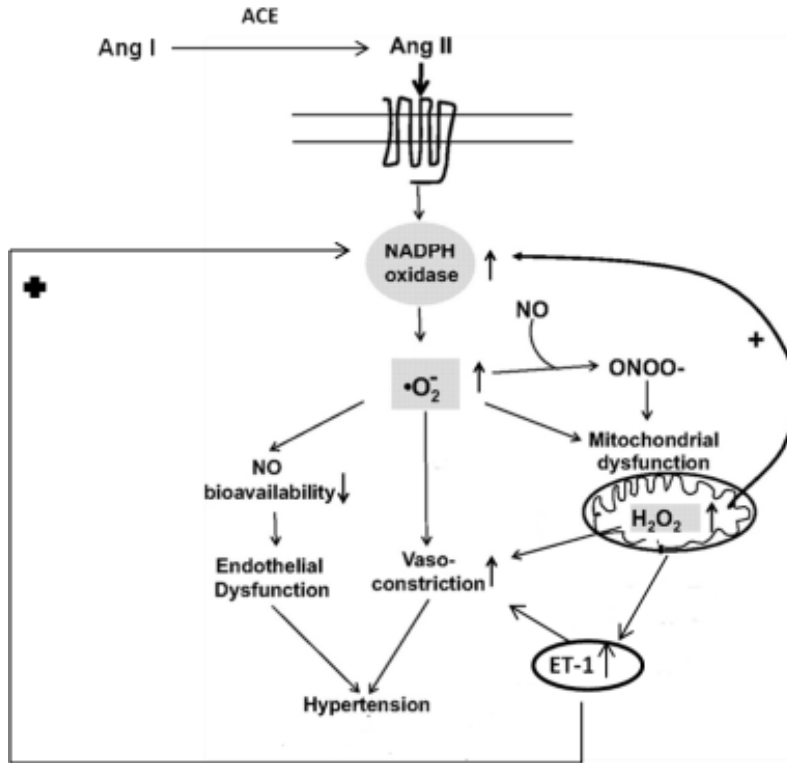
Figure 1.4 Pathway of Ang II-induced hypertension Ang I is converted to Ang II by ACE and then Ang II binds to the Ang I receptor that lead to ROS generation through activation of NADPH oxidase. NADPH oxidase-derived superoxide (O2$^{.-}$) reacts with NO to form peroxynitrite (ONOO$^-$). Both O2$^{.-}$ and ONOO$^-$ increase production of mitochondrial ROS leading to mitochondrial dysfunction. The mitochondria release H_2O_2 that induces ET-1 and further activate NADPH oxidase. Collectively, these lead to elevated ROS production and reduced NO bioavailability, which contributes to increased endothelial dysfunction and vasoconstriction and may result in hypertension. *ACE, Angiotensin converting enzyme; **Ang,** Angiotensin; **ET-1**, Endothelin-1; **NADPH**, Nicotinamide adenine dinucleotide phosphate hydrogen; **NO,** Nitric oxide, **ROS**, reactive oxygen species* (figure adapted from Fukai., 2009 [100]).

Potential effects of isoflavones on insulin resistance

Results from RCTs suggest that consumption of soy isoflavones may have positive effects on insulin resistance [14, 101-104]. In such studies insulin resistance is generally estimated using the homeostatic model assessment-insulin resistance (HOMA-IR) [105, 106], which is considered as an independent predictor of CVD [107, 108]. It is likely that part of the insulin sensitising benefits on CVD are related to the role of insulin in vascular function [109]. Insulin stimulates vaso-relaxation by NO production via phosphoinositide 3-kinase/ protein kinase B (PI3K/Akt) pathway, and in contrast, it induces vasoconstriction by ET-1 secretion through the mitogen-activated protein kinase (MAPK) pathway (Figure 1.5). Insulin resistance creates endothelial imbalance by selectively defect PI3K/Akt pathway [110] and/or up-regulation of Ang II-induced pathway [111].

It has also been shown that soy isoflavone reduces insulin resistance in rats, which was accompanied by reduced levels of inflammatory markers such as resistin [112].
Resistin is a cytokine that is secreted by adipose and circulating mononuclear cells [113]. It is suggested as an important marker of vascular inflammation and atherosclerosis [114-117]. Human studies have also reported that plasma levels of resistin are positively correlated with severity of coronary heart disease, hypertension [118, 119] and development of heart failure even after adjusting for insulin resistance [120].
In vitro work has shown that resistin down regulated eNOS expression via increased production of mitochondrial ROS and activation of MAPK proteins in human coronary endothelial cells. This down-regulation was reversed by treatment with antioxidants [121]. Additionally, resistin has been shown to selectively impair the insulin-induced IRS-1 (insulin receptor substrate-1) pathway that activates eNOS and vaso-relaxation in endothelial cells [122].

Insulin

Insulin receptor

IRS-1/2

Ras

PI3K

Raf

AKT

MEK

eNOS

MAPK

L-citrulline

L-arginine → NO

ET-1
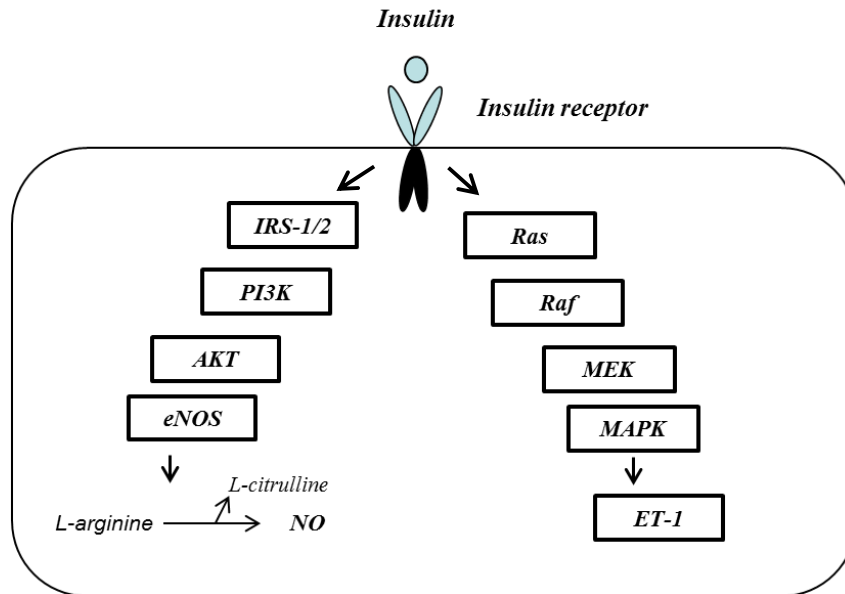
Figure 1.5 Antagonistic insulin-evoked pathways in endothelium
Binding of insulin to its own tyrosine kinase receptor; results in activation of IRS-1/PI3K, with subsequent phosphorylation of Akt and activation of eNOS leading to increase NO levels. In contrast, insulin can activate the Ras/Raf/mitogen-activated protein kinase (MAPK) branch that increases the secretion of ET-1. *Akt, protein kinase B; eNOS, endothelial nitric oxide synthase; IRS-1, Insulin receptor substrate-1 (IRS-1); MAPK, mitogen-activated protein kinase; NO, Nitric oxide; PI3K, phosphatidylinositol 3-kinase (*figure adapted from Potenza *et al.,* 2009 [110]).

Analyses of vascular-related biomarkers in RCTs and differential effects of isoflavones and metabolites

Few human studies have been conducted to investigate the effect of isoflavone interventions on the previously discussed vascular-related biomarkers such as NO, ET-1 and resistin (Table 1.2). In two studies, levels of NO have been shown to significantly increase following isoflavone consumption, while data were not conclusive on ET-1 and resistin. Yet further human studies to investigate related biomarkers that might unravel the mechanism of bioactivity of isoflavones in vascular health.

Different bioactivity of different isoflavones and their metabolites might explain the inconsistent health benefits reported in isoflavone supplementing RCTs [123-125]. It has also been reported that genistein increases eNOS expression more than that of daidzein *in vitro* [126]. These differential effects might be due to different produced metabolites that are suggested to be more physiologically active; such as 3'-OH-genistein, 3'-, 6-, and 8-OH-daidzein and in particular the gut daidzein metabolite equol [127].

Table 1.2 Effects of isoflavones intake on vascular-related biomarkers in published RCTs

Biomarker	Isoflavones RCTs	Outcome
ET- 1	• 117 healthy postmenopausal women, 50 mg/d soy isoflavone (genistein-to-daidzein, 2:1), 8 weeks [10]	no effect
	• 30 healthy postmenopausal women, 50 mg/d soy isoflavones (genistein-to-daidzein, 2:1), 8 weeks [11]	no effect
	• 79 healthy postmenopausal women, 54 mg/d genistein, 1 year [98]	reduced
NO	• 79 healthy postmenopausal women, 54 mg/d genistein, 1 year [98]	increased
	• 22 healthy postmenopausal women, 80 mg isoflavones of soy bean extract, acute phase [9]	increased
Resistin	• 33 men with prostate cancer, 20 g soy protein (160 mg isoflavones), 12 week [128]	no effect
	• 75 healthy postmenopausal women, 20 g soy protein (160 mg total isoflavones: genistein 64 mg, daidzein 63 mg, and glycitein 34 mg), 12 weeks [5]	no effect

ET-1, Endothelin-1; NO, Nitric Oxide; RCT, Randomised controlled trials.

1.4 Potential role of equol on the vascular effects of soy isoflavones

1.4.1 Equol - biological importance, and discovery

Equol is an isoflavone metabolite that has been proposed to be more potent than its parent compound daidzein, exhibiting higher binding affinity for the oestrogen receptor [129], antioxidant activity [130] and more vascular bioactivity [24, 131]. Equol has shown similar oestrogenic activity to genistein and activated transcription of oestrogen receptors more strongly than other isoflavones [132]. Furthermore, it has been reported that equol and its metabolites (4-hydroxy- and 5-hydroxy- equol) are more potent antioxidants relative to daidzein, genistein and their glycosylated forms [133].

Equol was discovered in the urine of pregnant mares in 1932 by Marrian and Haslewood during experiments to isolate oestrone [134]. This compound was thought to be a contaminant of the hormone hydroxyestrin. Crystallization of the ether-soluble phenolic fraction of toluene extracts of the urine by chloroform led to extracting appreciable quantities of this compound from the urine of both pregnant and non-pregnant mares. Therefore, it was concluded that this compound was not specifically linked to pregnancy and not associated with high levels of oestrogenic hormones. Further, multiple analytical applications allowed its chemical structuring, and it was called "equol" due to its equine source [18, 135]. Further research showed that sheep grazing on clover in South Western Australia developed a reproductive disease called clover disease and subsequent analytical studies showed that those infertility symptoms were due to significantly high circulating levels of equol metabolised from the isoflavone formononetin in the clover plant [18]. Formononetin is demethylated by human hepatic enzymes to yield the major soy isoflavones genistein and daidzein [136]. In 1982, Axelson et al. was the first to find equol in human blood and urine after consuming soy by comparing it to the sample isolated in 1932 and this discovery led to the valuable scientific finding of soy as a food source rich in isoflavones [137, 138]. Afterwards, it was defined that equol is exclusively metabolised by the intestinal microflora from the soy isoflavone daidzein. However, there is a high

inter-subject variability in the levels of excretion of equol in humans. Research has shown that only 25%-30 % of Western individuals are equol producers, i.e., can metabolise daidzein to equol after soy consumption [6, 139, 140], whereas 50–60% of Asian and western vegetarian subjects are equol producers [141]. To accurately assess the equol production status it is essential to challenge with sufficient amounts of daidzein and allow for a sufficient transit time until the substrate reaches the distal colon and obtains optimal intra-luminal redox conditions activating the microbiological reaction [18]. Multiple methods have been developed with variable soy challenge periods and cutoffs [17, 142, 143] such as an urinary detection limit of more than 0.68 nmol/mL [144]. However, this is criticized, since low levels of equol appear in the urine of all humans consuming animal-derived foods like Cow's milk [145]. Setchell et al. developed a standard method [141] that is independent of the amount of daidzein ingested and of different analytical methods measuring isoflavones [26, 144].

1.4.2 Equol - Chemical structure and biological properties

Equol is chemically known as 7-hydroxy-3-(4′-hydroxyphenyl)-chroman, 4′.7-isoflavandiol, or 4′,7-dihydroxyisoflavan, and its formal name is 3,4-dihydro-3-(4-hydroxyphenyl)-2H-1-benzopyran-7-ol. It has the molecular composition of $C_{15}H_{14}O_3$ and a molecular weight of 242.27 g/mol [18]. Equol is a non-polar, insoluble and chiral molecule with an asymmetric centre at position C-3. Thus, it can exist as two distinct optically active isomers R- (+) and S- (-) equol [18] (Figure1.5). It has been shown that both equol isomers have higher binding affinity for oestrogen receptor (ER) α and ER β than that of the parent compound daidzein [146]. However, these enantiomers have shown different biological and chemical effects. S-equol has a more planar shape, which makes it structurally more similar to oestradiol, and hence it possesses higher binding affinity to oestrogen receptors compared to R-equol and daidzein [147]. An early *in vitro* study showed that *S*-equol had four times the molar binding affinity of daidzein to oestrogen receptors [147]. S-equol also shows higher binding affinity to ERβ than to ERα, while the R-equol shows higher binding affinity to ERα than to ERβ. It was reported that the relative binding affinities of the S and R-enantiomers were at around 20 % and 1 % of that of 17 β-oestradiol for ERβ and 0.47 % and 2 % of that of 17 β-oestradiol for ERα, respectively

[129]. The oestrogenic binding affinities of the S-equol were also similar to that of the most oestrogenic isoflavone genistein. In conclusion, the order of binding affinities to ERs is reported to be S-equol≈Genistein > R-equol > Daidzein [146]. S-equol is considered a selective oestrogen receptor modulator (SERM) because of its selective affinity to bind ERβ. This is of particular importance for postmenopausal health because isoflavones and S-equol as SERMs may offer an alternative for synthetic oestrogen replacement therapies that have been associated with an increased risk of breast and endometrial cancers [64].

On the other hand, R-equol showed chemo-preventative effects in an animal model which was not apparent with S-equol [148]. Despite the different biological actions of S-equol and R-equol, both were shown to be antagonists for dihydrotesterone which suggest a potential role for equol in androgen-related diseases such as prostate cancer or skin disease [125, 149].
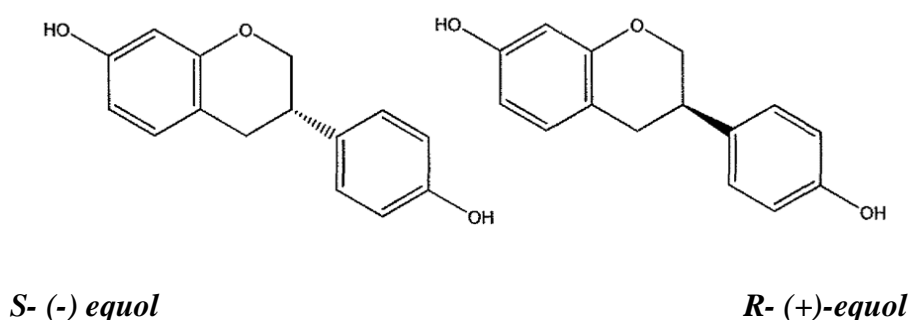
S- (-) equol   **R- (+)-equol**

Figure 1.6 The two enantiomers of equol, S- (-) equol and R- (+) equol.

1.4.3 Methods for quantification of equol concentrations in biological samples

Equol concentration can be quantified in blood and urine by electrospray ionisation liquid chromatography-mass spectrometry (LCMS) or gas chromatography-mass spectrometry (GC-MS) techniques. First, biological samples are hydrolysed by glucuronidase and sulfatase enzymes, extracted, and undergo a preparation method of volatile derivatives in the case of GC-MS analysis [127] or injected into LCMS [150].

S- and R-equol are quantified separately by chiral-phase high performance liquid chromatography (HPLC) method; the identification of S- and R-equol depends on the retention time of the eluting peak relative to the mass chromatograms of pure standards of S- and R-equol analysed under identical conditions [129]. Alternatively, other analytical methods have been developed to analyse racemic equol in the urine such as time-resolved fluoroimmunoassay [151] and enzyme-linked immunoassay (ELISA) [152].

Most studies have used the racemic form of equol while each isomer should be studied individually, since they possess different biological actions [129]. The form S-equol is of more interest because it is more structurally similar to oestrogen and posseses higher binding affinity to oestrogen receptors than daidzein and R-equol (as mentioned earlier in section 1.4.2, page 27). In addition, S-equol is exclusively found in the urine and plasma of equol producer mammals after consuming soy, and it is the only form produced by the human intestinal bacteria [129].

1.4.4 Synthesis of S-equol (SE5OH) supplements

Equol can be synthesised from daidzein by catalytic hydrogenation and because of the different properties of the two equol isomers experimental methods have been developed for the selective synthesis of S-equol and R-equol [153]. S-equol has been found to be produced by microbiological reactions. For example, Otsuka Pharmaceutical Co., Ltd has developed a natural S-equol supplement, called SE5-OH, which is the standardized soy-based product of fermentation, sterilisation and drying of a soy germ solution with the lactic acid bacterium *Lactococcus garvieae* (20-92 strain). In evaluation of toxicity and safety of SE5-OH, it has been reported to be not genotoxic and has a no-observed-adverse-effect-level (NOAEL) of 13 mg equol/kg/day [154]. Therefore, SE5-0H supplements would be a source of natural S-isomer equol that could be utilised in human interventions to assess the potential effects of equol proposed by *in vitro* and animal research.

1.4.5 Vascular effects of equol and potential mechanisms of action

In cell culture and animal studies, treatment with equol has been shown to induce beneficial effects on endothelial function (Table 1.3) and antioxidant status or defence system (Table 1.4). Moreover, equol showed higher bioactivity, in particular greater vasodilation [23, 24] (Table 1.3) and antioxidant effects [20-22], than its parent compound daidzein and other major isoflavones (Table 1.4).

An *in vitro* study supports evidence of vasodilation effects of equol using endothelial cell models e.g. human umbilical endothelial cells (HUVECs) and human aortic endothelial cells (HAECs). Treatments of 1-100 nmol/L equol for 2 minutes caused rapid relaxation of endothelium intact aortic rings in a dose-dependent manner which was inhibited by pre-treatment with 100 µmol/L of the NOS inhibitor L-NAME (N-nitro-L-arginine methyl ester) [93]. Equol caused a rapid increase in cGMP accumulation which was inhibited by L-NAME and subsequently it was proposed that equol stimulated the endothelium to release NO at resting cytosolic Ca+2 levels by activation of extracellular signal-regulated kinase (ERK) 1/2 and protein kinase B (AKt) [93]. Additionally, equol induced vaso-relaxation in normotensive rats independent of an intact endothelium, K^+ channels, NOS activity and gender. Similar findings were obtained for daidzein. This study also compared equol and daidzein, showing that equol induced vaso-relaxation during angiotensin II-induced hypertension, while daidzein did not. Equol also displayed a weak antioxidant ability assessed as reduced NADPH-induced superoxide levels in the basilar artery compared to negligible ability by daidzein. It was concluded that equol has stronger vaso-relaxant effect during hypertension compared to daidzein and thus it could be more potent therapeutic tool against cerebral vascular disease [23].

In addition, in porcine and human pulmonary arterial endothelial cells equol has been shown to reverse detrimental effects induced by HIV protease inhibitor ritonavir (HIV PI RTV) such as increased oxidative stress (expressed by superoxide production), eNOS down-regulation and vasomotor dysfunction in arterial rings [155]. Treating fresh porcine pulmonary artery rings with 15 µmol/L of RTV for 24 hr significantly reduced the endothelium-dependent relaxation induced by bradykinin and this effect was regressed by treatment with 0.1, 1, 10 µmol/L of equol in a dose-dependent manner. Using two cultures

of human pulmonary artery endothelial cells (HPAEC) and the porcine pulmonary arteries, it was shown that equol reversed the detrimental effects of RTV including reduction of eNOS expression, and production of superoxide anion [155].

More recently, a putative pathway was proposed to explain the acute vasodilation effects of equol [94]. It suggested that equol stimulated the endothelium to release NO by activation of ERK 1/2 and AKt, which leads to phosphorylation of eNOS (at serine1177). The suggested pathway relates the kinase activation to transactivation of G-protein coupled receptor 30 (GPR30)/epidermal growth factor receptor (EGFR). Subsequently, EGFR activation leads to ERK 1/2 activation via proto-oncogene c-Src, whilst mitochondrial reactive oxygen species (ROS) facilitate AKt activation via phosphoinositide 3-kinase (PI-3K) (Figure 1.7). Furthermore, brachial artery infusion of dehydroequol (a potential precursor of equol) metabolite at 3 μmol/min in forearm resistance arteries in six healthy men, resulted in a dose-dependent increase in forearm blood flow and these responses were diminished by inhibition of eNOS [156].
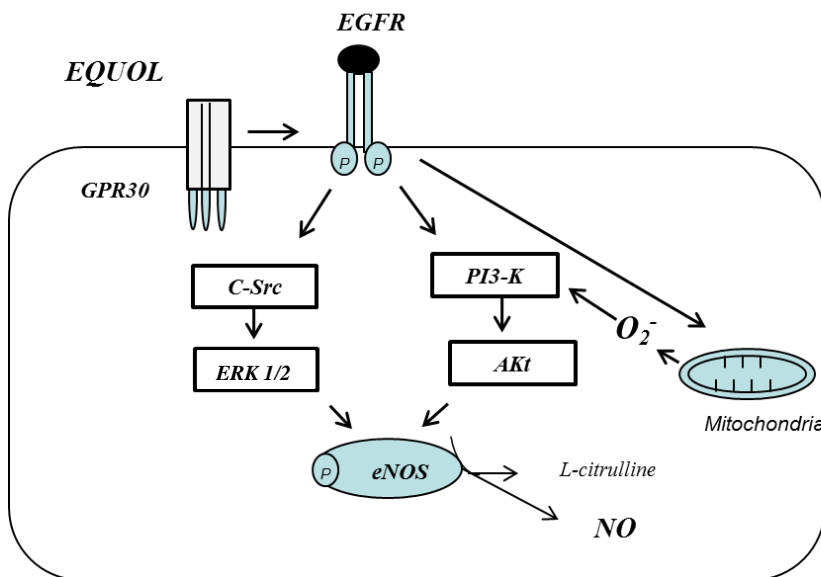
Figure 1.7 Proposed mechanism underlying vasodilation effects of equol
Equol induces endothelial vasodilation through kinase activation, and subsequently NO production with the involvement of mitochondrial derived ROS. Kinase activation might be related to the transactivation of GPR30/ EGFR by equol. Activated EGFR stimulates ERK 1/2 activation via activation of proto-oncogene c-Src. While mitochondrial reactive ROS activates Akt via activation of PI-3K. The two activated proteins ERK 1/2 and Akt then induce phosphorylation of eNOS to release NO. *AKt, protein kinase B; c-Src, proto-oncogene; EGFR, epidermal growth factor receptor; ERK 1/2, extracellular signal-regulated kinase; GPR30, G-protein coupled receptor 30; ROS, reactive oxygen species* (figure adapted from Rowlands *et al*., 2011 [94]).

Equol has also shown antioxidants effects on LDL modification and its implication in endothelial cell injury and atherosclerosis development (Table 1.4). Equol has been shown to inhibit H_2O_2-induced apoptosis via reduced levels of intracellular ROS in bovine aortic endothelial cells and in human umbilical vein endothelial cells (HUVECs) [157]. Another study examining macrophages similarly reported that equol inhibited LDL oxidation and modification through reduced superoxide production and increased intracellular NO levels. However, this study suggested that these effects may be due to inhibition of NADPH oxidase activity instead of an upregulation of eNOS [20].

One important aspect to note is that almost all of the reviewed *in vitro* studies have used a racemic mixture of equol (50:50) for their investigations and it is worthwhile to investigate any differential effects between the two enantiomers S-equol and R-equol.

Table 1.3 Effects of equol on vascular function in cell culture and animal studies

Reference	Study model	Findings
[24]	*Ex vivo* platelet study	Equol had antagonistic effects on the thromboxane A2 (TXA2) receptor (membrane surface receptor that is involved in platelet aggregation and vascular smooth muscle contraction). Out of all major isoflavones tested, including genistein, daidzein and glycitein, equol was found to have the highest binding affinity to the TXA2 receptor.
[93]	HUVECs and HAECs	Treatments of 1-100 nmol/L equol for 2 minutes caused rapid relaxation of endothelium intact aortic rings in a dose-dependent manner, which was inhibited by pre-treatment with 100 μmol/L of the NOS inhibitor L-NAME.
[23]	Carotid and basilar arteries isolated from normotensive and hypertensive rats	Equol induced vaso-relaxation independent of an intact endothelium, K^+ channels, NOS activity and gender and similar findings were obtained for daidzein in normotensive rats. However, in hypertension, equol has been shown to induce vasodilation while there was no response following daidzein treatement

*AKt, protein kinase B; **ERK**, extracellular signal-regulated kinase; **HAECs**, Human aortic endothelial cells; **HUVECs**, Human umbilical vein endothelial cells; **L-NAME**, N-nitro-L-arginine methyl ester; **NOS**, Nitric oxide synthase; **TXA2**, Thromboxane A2.*

Table 1.4 Antioxidant effects of equol in cell culture and *in vitro* studies

Reference	*Study model*	*Findings*
[155]	Porcine and human pulmonary arterial endothelial cells	Equol has been shown to reverse detrimental effects induced by HIV PI RTV such as increased oxidative stress, eNOS down-regulation and vasomotor dysfunction
[157]	Bovine aortic endothelial cells	Equol has been shown to inhibit H_2O_2-induced apoptosis via reduced levels of intracellular ROS
[20]	Macrophages	Equol inhibited LDL oxidation and modification through reduced superoxide production and increased intracellular NO levels
[21]	Liver cancer cell model (HepG2 human hepatocellular carcinoma cells)	Equol induced activity and protein expression of catalase and total superoxide dismutase (which are essential in the antioxidant defence system) to a greater extent than daidzein
[22]	*In vitro* oxidation of lipoproteins in serum	Equol inhibited lipoproteins oxidation more efficiently than genistein and approximately 10 times better than daidzein

*eNOS, endothelial Nitric oxide synthase; **HIV PI RTV**, HIV protease inhibitor ritonavir; **HUVECs**, Human umbilical vein endothelial cells; **LDL**, Low density lipoprotein; **NADPH**, Nicotinamide adenine dinucleotide phosphate.*

Potential beneficial effect of equol on insulin sensitivity have been also proposed and studied recently using C3H10T1/2 and 3T3-L1 cell lines (i.e. mouse cell models to study adipocyte differentiation [158]). It was demonstrated that equol at 1 μmol/L significantly increased adipocyte differentiation and insulin-stimulated glucose uptake through activation of peroxisome proliferator-activated receptor-gamma (PPARγ) [159]. These data may explain the potential insulin-sensitizing effects of soy isoflavones because PPARγ is a nuclear transcription factor that regulates responsiveness to insulin in adipose cells and thus it is a target for anti-diabetic drugs that enhance insulin resistance [160].

Collectively, these *in vitro* data support further investigations to examine cardio-protective effects of equol *in vivo*.

1.4.6 Cross-sectional studies: equol producer phenotype and CVD factors

The previous *in vitro* studies support the potential role of the isoflavone metabolite equol on vascular health and suggest that the capability to produce the bioactive metabolite equol might explain benefits of isoflavone intake on vascular function.

Cross-sectional studies have investigated possible beneficial associations between equol producer status and CVD risk factors. Four out of five recent observational studies have shown that the equol producer phenotype is associated with reduced levels of various CVD risk factors in mostly Asian populations (Table 1.5). When equol producers were compared to non-equol producers, they had better cholesterol profiles, lower triglycerides (TG) [25, 26], free fatty acids (FFA) [25], leptin, leptin/BMI [27], waist to hip ratios [26], free fat mass [25] in a variety of populations including healthy subjects, postmenopausal prehypertensive, pre-diabetic, or diabetic individuals. Few of these studies investigated blood pressure [25, 26, 28] and inflammatory biomarkers such as uric acid [26] and hs-CRP [25]. Equol producers were reported to have lower blood pressure than non-equol producers in two studies [25, 28] while one study showed no difference between the two groups [26]. Overall, the available data from observational studies on Asian populations suggests positive association between equol producer phenotype and better cardio-metabolic health and this might be partly related to their high daily intake of isoflavone (up to 50 mg [63]).

1.4.7 Isoflavone interventions: equol producer phenotype and CVD factors

A variety of isoflavone RCTs investigated if equol producer phenotype predicts the efficiency of soy isoflavones interventions on circulating lipid profile, blood pressure, inflammation and metabolic markers; 5 out of 7 studies have reported more profound benefits in equol producers than non-equol producers mainly on plasma lipids (Table 1.6). All of these studies have followed a retrospective analysis quantifying urinary or plasma equol levels to stratify between equol and non-equol producers. Two isoflavone RCTs have shown greater benefits on vascular function (FMD, and blood pressure) in equol producers compared to non-equol producers and the reported mean concentrations of plasma equol were 105 nmol/L [6], and 190 nmol/L [15] in equol producers.

Kreijkamp-Kaspers et al. [15] showed that 12 month consumption of soy protein (99 mg isoflavones aglycone equivalents/day) in postmenopausal women resulted in trends towards a decreased systolic and diastolic blood pressure and an enhanced endothelial function (assessed by FMD) in the equol producer compared to non-equol producer. Additionally, a 4 week intervention of soy germ-rich pasta (33 mg isoflavone aglycones/day) induced significant improvements in endothelial function (assessed by FMD) in hypercholesterolemia equol producer men and women [6]. Soy germ pasta was selected because it is rich in aglycones like fermented soy products that contribute the most to the Asian diet and thus it might exert more beneficial health effects based on the supporting evidence from observational data (Table 1.6).

Meyer et al. did not show effects on vascular function including blood pressure and arterial compliance. However, only equol producing subjects (women and men, mildly hyper-cholesterolemic and/or hypertensive) had significantly enhanced lipid profiles, including significantly reduced total cholesterol, LDL cholesterol, LDL: HDL-cholesterol ratio, TG and lipoprotein (a) after consuming the soy diet (80mg isoflavone aglycones units per day for 5 weeks) [161]. Likewise, Wong et al. has also shown significant reductions of LDL-cholesterol levels after 4 week consumption of soy food (10-73 mg isoflavones aglycones) in 43 postmenopausal females and 42 males with high cholesterol profile. Moreover, these effects were more profound in equol producers that maintained significantly higher levels of HDL-cholesterol compared to non-equol producers [162]. In contrast, two studies

reported no differential effects of isoflavone interventions on plasma glucose, insulin, lipid metabolism and inflammatory biomarkers between equol producers and non-equol producers [10, 163].

The current literature on RCTs that retrospectively analysed equol producer phenotype supports a potential role of equol producer phenotype on the effectiveness of soy isoflavones interventions on lipid profile and inflammatory markers. However further investigations are required on the vascular function in particular endothelial function, arterial stiffness and blood pressure. In addition, there is a need for trials which prospectively recruit based on equol producer phenotype to investigate further if equol producers benefit more from isoflavone interventions than non-equol producers.

To date, only one study has been conducted which had prospectively recruited equol producers and non-equol producers to study their differential vascular responses after isoflavone intake. This clinical study prospectively screened postmenopausal women for equol production ability and found that equol producers had significantly lower systolic and diastolic blood pressure and mean arterial pressure, and better endothelial function than non-equol producers did at enrolment to the study independent of isoflavone intake [164-166]. The non-significant responses to isoflavone supplementation in the latter trial might (in part) contribute to the study limitations, i.e. use of oestrogen replacement therapy and non-matching of equol and non-equol producers for vascular function.

This variability in previous data can be explained by different study designs such as isoflavone dose, source, study populations, consumption duration, equol production assessment method, outcome measurement time and inter-individual variability depending on their health status. Additionally, the limitations of retrospective analysis that does not provide causality between equol producer phenotype and CVD risk, nor answers whether there are subtle differences in CVD risk in equol and non-equol producers.

Consequently, robust RCTs are required to examine if the equol producer phenotype explains the potential benefits of isoflavones interventions on vascular function, particularly endothelial function and arterial stiffness. Additionally, in order to confirm the bioefficacy of equol, it is of interest to investigate its direct health effects *per se in vivo* and the possibility of achieving benefits on vascular function by equol supplements in people who lack the endogenous ability to produce equol.

To date, there have been only few RCTs that reported potential benefits of equol supplements. These focused mainly on improving postmenopausal symptoms such as

intensity and frequency of hot flashes, muscle pain, and bone density loss [167-170]. Only one study considered metabolic-related markers and one secondary vascular outcome (Cardio-Ankle Vascular Index (CAVI)). It has been shown that daily consumption of 10 mg, 20 mg or 40 mg S-(-)equol (SE5OH) supplements for 8 weeks significantly reduces the frequency of hot flushes, intensity of muscle and joint pain more than isoflavone intake (48 mg isoflavone aglycones units) in postmenopausal women [170]. Notably, the dose of 40 mg/day was more beneficial in decrease the frequency of hot flashes than isoflavones in women who had more than 8 hot flashes per day. Similarly, a trial showed that daily consumption of 10 mg S-(-)equol (SE5OH) supplements for 12 weeks significantly reduces the intensity of hot flashes, neck or shoulder muscle stiffness in non-equol producer postmenopausal women [169]. Another RCT of 54 overweight/obese equol and non-equol producers showed that the same dose of SE5OH supplements for 12 weeks improved metabolic-related biomarkers, including reduced circulating levels of glycated haemoglobin (HbA1c), and LDL- cholesterol. In addition, there were decreased levels of CAVI, which is a marker of the arterial stiffness of the artery from the aortic valve to the peripheries (in the ankle) independent of blood pressure [168]. However, the latter study showed greater effects in the female non-equol producer group, but it had a limitation in the method used to identify equol producer phenotype that might have been implicated by their analysis method and limit of detection of equol [168].

It is also notable that many of the previously reviewed RCTs have limited their sample population to postmenopausal women, and there is a gap in studying other population subgroups and in particular the response in males.

Table 1.5 Summary of cross-sectional studies on associations between equol producer phenotype and CVD risk factors

Ref	Study population	Method to identify EP	EP %	Confounding factors adjusted for	Outcome
[26]	100 women and 102 men, Chinese, healthy, 26-69 y	3 day isoflavone challenge	50%	None	EPs showed lower serum uric acid, TG, and waist/hip, higher HDL cholesterol than non-EPs, and no difference on BP
[28]	633 women and 758 men, Korean, healthy, average 53 y	habitual intake	70%	Area, sex, age, smoking and alcohol consumption	EPs showed lower diastolic BP than non-EPs
[144]	100 women and 100 men, Chinese, healthy, 20-75 y	3 day isoflavone challenge	60%	BMI	No association between plasma lipids or uric acid and EP phenotype
[25]	595 postmenopausal women, Chinese, pre-hypertensive, 48-70 y	7 day daidzein challenge	53%	Age, menopausal year, physical activity, dietary energy, isoflavone and fat intake (for BP and body composition) and BMI (for TG, FFA and hs-CRP)	EPs had lower systolic and diastolic BP, serum TG, FFA, hs-CRP and higher fat free mass than non-EP
[27]	34 men and 45 women, Japanese, pre-diabetic or diabetic patients, NA for age	NA	NA	NA	EPs showed lower leptin and leptin/BMI than non-EPs in women only.

*BP, Blood pressure; **BMI**, Body mass index, **EP**, Equol producer; **FFA**, Free fatty acids; **HDL**, High density lipoprotein, **hs-CRP**; high sensitivity-C-reactive protein; **NA**, No available data (abstract access only); **TG**, Triglycerides.*

Table 1.6 Summary of interventional studies that investigated beneficial effects of isoflavone and equol producer phenotype on CVD risk factors

Ref	Study population	Method to identify EP	EP %	Intervention (aglycones), duration	Outcome	Plasma/urine equol (mean, nmol/L)
[171]	97 postmenopausal women, healthy, >60 y	Serum equol, retrospective analysis, no challenge, >20 nmol/l	49%	105 mg, soy protein and/ isoflavone aglycones units, 12 mo	EPs showed significantly reduced total Cholesterol/HDL-C, LDL/HDL-C from baseline at 12 mo but data were not significantly different from non-EPs.	Plasma: 190
[15]	202 women, postmenopausal, healthy, 60–75 y	plasma equol, retrospective analysis, no challenge, >83 nmol/L	30%	99 mg, soy protein powder, 12 mo	EPs showed trends towards decreased systolic and diastolic BP, and increased FMD, and these data were in the opposite direction in non-EPs	NA
[161]	10 women and 13 men, mildly hyper-cholesterolemic and/or hypertensive, 54 y (mean)	plasma equol, retrospective analysis, no challenge, if detected in plasma/urine	35%	80 mg, soy milk/yogurt, 5 wk	EPs had significantly reduced total Cholesterol, LDL-C, LDL/HDL-C, TG and lipoprotein (a) after soy intervention compared to placebo, and there was no effects on BP/ arterial compliance	NA
[162]	42 hyper-cholesterolemic men and 43 postmenopausal women, 60 y (mean)	Urine equol, retrospective analysis, ≥1000 nmol/d and log equol: daidzein ratio of > -1.75	35%	10-73 mg, soy food, 4 wk	EPs and Non-EPs had similarly reduced LDL-C, and EPs had maintained high HDL-C compared to significantly reduced levels in non-EPs	Urine: 1334 creatinine
[6]	29 women and men, hyper-cholesterolemic, 58 y	Serum equol, retrospective analysis, log equol: daidzein ratio of > -1.75	69%	33 mg, soy germ pasta, 4 wk	EPs showed significantly reduced plasma LDL-C, total cholesterol, and hsCRP; and greater FMD than Non-EPs	Plasma: 105

Ref	Study population	Method to identify EP	EP %	Intervention (aglycones), duration	Outcome	Plasma/urine equol (mean, nmol/L)
[163]	33 men, 58 women, mildly hyper-cholesterlemic, 18-80 y	Urine equol, retrospective analysis, log equol: daidzein ratio of > -1.75	33%	70-80 mg, isoflavones and soy protein, 6 wk	EPs did not show significant differences in response from non-EPs on plasma lipids	NA
[10],	117 postmenopausal women, healthy,45-70 y	Urinary equol, retrospective, >936 nmol/L or serum equol >39 nmol/L	28%	50 mg, soy enriched cereal bar, 8 wk	EPs did not show significant difference in response from non-EPs on inflammatory biomarkers, blood pressure; plasma TC, LDL-C, HDL-C, total:HDL-C, TG, NEFA, glucose, insulin, or HOMA-IR. Only differences in Lp(a) and % small dense LDL concentrations were shown in equol producers.	Urine: 2.61 mg/ d

BP, Blood pressure; EP, Equol producer; FMD, Flow mediated dilatation; HDL-C, High density lipoprotein-Cholesterol; HOMA, Homeostatic model assessment of insulin resistance; hs-CRP; high sensitivity-C-reactive protein; LDL-C, Low density lipoprotein-Cholesterol; Lp(a), Lipoprotein (a); NA, No available data; NEFA, Non- esterified fatty acids; TG, Triglycerides.

1.4.8 Potential factors affecting equol producer phenotype

Differences in equol producer phenotype frequency

In contrast to animals, the excretion of equol is highly variable in humans. As mentioned earlier, not all healthy humans possess the ability to convert daidzein to equol. It was reported that daily ingestion of 500 ml of soy milk for 3 days resulted in mean concentration of 139 nmol/L and 12574 nmol/L, of equol in serum and urine respectively [141]. Generally, it is estimated that around 30-50 % of humans can produce equol from daidzein [18, 172]. However, there is evidence that the prevalence of equol producers is higher among Asian populations than western populations [173]. One study has found that 38 % and 58% of Japanese women and men had serum equol higher than 20 nmol/L, in comparison with 2.2% and 0% of British women and men [140]. However, this result might be in part due to the habitual intake of soy in Asian populations. In addition, the chemical form of isoflavone intake was considered as a possible factor. The Asian diet is rich in fermented soy products, which is rich in isoflavone aglycones that are suggested to be more rapidly and easier to absorb than glycosides and thus attain higher bio-availability than the non-fermented soy and in turn yield more equol [6]. However, it has also been suggested that equol might be produced more from isoflavone glycosides as they possess longer transit time in the intestine, allowing for more bacterial metabolic reactions to take place [174, 175]. Another study by Song et al. found that the prevalence of equol production status was 51% in Korean American females compared to 36% in Caucasian American females and reported no dietary association between regular soy intake and the equol production phenotype [139]. Collectively, there is a research gap regarding the reasons behind the high prevalence of equol producer phenotype in Asian populations and research has been conducted to investigate factors that might influence the equol producer phenotype.

Gut microbiota and equol producer phenotype

In the 1980s, it was found that equol production depends on the intestine microflora from studies on germ-free animals and antibiotic use [137, 138]. Further research suggested that the ability to produce equol is determined by the intestinal microflora profile (Table 1.5). *In vitro* work has suggested that the equol production capability is most probably accredited to specific strains of the intestinal microflora; in support of this, it has been shown that faecal flora extracted from equol producers, had the capacity to metabolise incubated daidzein to equol whereas faecal flora of non-equol producer did not [176].

Research has been conducted to identify the intestinal bacteria species that are able to metabolise equol and develop probiotics that might induce this ability in non-equol producers. *In vitro* culture of human faecal flora after soy intake identified three bacteria species which showed ability to convert daidzein to equol; the gram-negative *Bacteroides ovatus* spp, the gram-positive *Ruminococcus productus* spp and *Strepotococcus intermedius* spp [17]. In addition, a mixture of the faecal bacteria *Lactobacillus* sp. Niu-O16 and *Eggerthella* sp. Julong 732 have been found to transform daidzein to dihydrodaidzein and dihydrodaidzein to S-equol respectively [177]. Another nine strains of equol-producing bacteria (*Adlercreutzia equolifaciens* gen. nov, sp. nov) were isolated from human faecal microflora and classified as obligately anaerobic, asaccharolytic and gram-positive coccobacilli. Seven of these new strains could produce equol from daidzein via dihydrodaidzein and the left two (FJC-A10 and FJC-A161) are able to metabolize only dihydrodaidzein to equol similarly to the previously identified Julong732 [178]. Recently, another daidzein-metabolising bacterium was isolated; the NATTS strain which found to be a Gram-positive, non-spore-forming rod bacterium and belong to the genus *Slackia* sp. Interestingly, 40% of the Japanese population have a mean of 10^6 cells of *Slackia* sp per gram [179].

Table 1.7 Microflora species associated with equol producer phenotype

Bacteria	*Ref*
Slackia isoflavoniconvertens	[180]
Slackia equolifaciens	[181]
Adlercreutzia equolifaciens	[178]
Lactococcus garvieae	[182]
Lactococcus sp. strain 20-92	[183]
Lactobacillus sp. Niu-O16 and Eggerthella sp. Julong 732	[177]
FJC-A10 and FJC-A161	[178]
NATTS strain (Slackia)	[179]

An alternative hypothesis proposed that a mixture of bacterial strains could be responsible for equol production because of the complex composition of intestinal microflora. Decroos et al. (2005) prepared a mixed culture of intestinal bacteria (EPC4; consisting of *Lactobacillus mucosae* EPI2, *Enterococcus faecium* EPI1 and *Finegoldia magna* EPI3 and *Veillonella* sp strain EP) that efficiently metabolises daidzein into equol [184]. EPC4 was also supplemented with propionate and butyrate, which are major short chain fatty acids produced by microflora fermentation of complex carbohydrates such as non-starch polysaccharides in dietary fibers and resistant starch [185]. This supplementation increased the equol production, suggesting a positive influence of a diet rich in complex carbohydrates. In contrast, treatment with fructo-oligosaccharides inhibited equol production. Interestingly, the equol-producing ability of EPC4 was preserved when added to a faecal bacterial culture of non-equol producer [184]. Further experiments reported that EPC4 was also successful in converting the faecal profile of non-equol producers to equol producers, after being administered to stimulator of the human intestinal microbial ecosystem (SHIME, a model for a gastrointestinal tract of an adult human) which was fed soy germ powder and inoculated with faecal samples of non-equol producers [186]. In contrast, studies have failed to change equol producer status by probiotic consumption (including *Lactobacillus acidophilus* and *Bifidobacterium bifidus* which are not specifically equol producing bacteria but found to be highly associated with equol producer phenotype) [187, 188].

Overall, it seems that the literature includes some data about specific colonic bacterial species that are capable of producing equol from daidzein and these information are useful for future research. In particular, there is still a lack of human RCTs providing probiotics

that are specifically designed for equol-producing strains or alternatively faecal transplant studies to investigate the possibility to change a non-equol producer to equol producer.

Effects of antibiotic administration on equol producer phenotype

Studies since the 1980s administering antibiotics have shown that equol production depends on the intestine microflora [18, 137, 138]. More recent studies have suggested using antibiotics to aid in identifying the specific strains of equol-producing bacteria. Blair et al. [189] performed a 1 month antibiotic treatment to female monkeys and showed variable effects of antibiotics on metabolism of equol, isoflavones and their metabolites. It has been shown that vancomycin, kanamycin, and a mixture of vanomycin and kanamycin induced significant reduction in plasma equol levels whereas doxycycline treatment did not influence plasma equol [189]. In accordance, plasma concentrations of daidzein were increased by kanamycin, and a mixture of vancomycin and kanamycin but also, surprisingly, by deoxycycline and this might be attributed to variable influence of different antibiotics like effects on metabolism of isoflavones or on their absorption [189]. Similar *in vitro* results were found for metronidazole and kanamycin in faecal inoculates from equol producers and non-producers incubated with daidzein [190]. It is suggested that equol production is a permanent capability except during administration of antibiotic – "once an equol producer, always an equol producer" [17, 172]. In contrast, some studies reported equol-producer "cross-overs" (i.e. their equol-producer status was altered from a non-producer either to a producer or vice versa) [191, 192].

As a conclusion, there is a lack of solid knowledge on the collective specific strains of intestinal bacteria, required conditions, dietary factors, possibly other determinants such as genetic factors; that might be cumulatively responsible for metabolising daidzein to equol.

However, it should be noted that research on this matter is challenging because the colon is a highly complex and metabolic organ that is inhabited by a huge variety of bacteria strains that change along the different intestinal regions and other environmental factors such as diet [193].

Effects of diet on equol producer phenotype

Diet may contribute to the association between the intestinal microflora and the ability to produce equol. Diet can influence the dominance of intestinal microflora species [194] and

thus studies have investigated whether diet changes including altered prebiotic intakes induce the equol-producing ability. Studies have found that equol producers had significantly higher energy intake of carbohydrates [195, 196], lower energy intake of fat [196] and fibre intake [195] than those of non-equol producers. Another study of healthy postmenopausal women has shown that urinary equol production was positively associated with high PUFA intake [197]. However, most of these studies are cross-sectional and they do not provide causality.

Feeding studies have also been conducted to investigate whether equol production ability can be influenced by certain dietary interventions such as prebiotics through changing the intestinal microflora profile. However, results of animal and human studies were equivocal; a 1 month intervention of fibre (16 g of wheat bran) with soy protein did not influence equol production [198]. In contrast, it was shown that feeding resistant starch (12% supplemented diet) to ovariectomized mice for six weeks significantly increased urinary equol concentration and, interestingly, it showed markedly higher caecal ratio than those of other animals who were fed only daidzein [199].

1.5 Metabolism and bioavailability of soy isoflavones and equol

1.5.1 Metabolism and absorption

In order to understand bioactivity of isoflavones and their metabolites, there is a need to understand their metabolism and absorption in the body. Isoflavones are found as ß-glycosides, and might also be bound to acetyl and malonyl glucosides (Figure 1.3) in soy food and products [200]. The levels of ß-glycosides, acetyl or malonyl glycosides vary between food and supplements following preparation and processing methods [201]. Isoflavone metabolites exhibit biphasic appearance in the blood; the first phase reflects these glycosides being partially hydrolysed along the proximal small intestine by ß-glucosidases (e.g. lactase phlorizin hydrolase (LPH)) in order to release aglycones that can be absorbed [172, 200]. Afterwards, isoflavones undergo metabolism phase II reactions of glucuronidation and to a lesser extent sulfation by glucuronosyl-transferases (UDP-glucuronyltransferase) and sulfotransferases in the intestine and/or liver [17]. These conjugates get transported to the tissues by circulation and then get excreted by the kidneys, or disposed into the bile and return to the intestine. In the large intestine, they can undergo de-conjugation and their aglycones can be reabsorbed and excreted through the kidneys, or returned to the liver for re-conjugation and more enterohepatic circulation. These recycling steps also contribute to the biophasic pharmacokinetics of isoflavones [202].

A substantial part of the ingested isoflavones that are not hydrolysed in the small intestine reaches the colon, along with the returned part of conjugated isoflavone from the enterohepatic circulation and aglycones that are not reabsorbed [172]. Isoflavones conjugates then undergo intestinal metabolic reactions including dehydroxylation, demethylation, C-ring cleavage, and reduction to produce different metabolites [17, 172, 196, 200]. These previous metabolic steps seem highly variable between subjects [172, 203] and thus may explain the high inter-individual variability of plasma concentrations of isoflavones and their metabolites upon consumption of the same isoflavone dose [176]. Glycitin is the minor isoflavone in soy food. It is quickly hydrolysed to glycitein and is considered metabolically inert [204]. However, there is a recent study that identified

glycitein metabolites such as dihydroglycitein [205]. Genistein is converted to p-ethyl phenol and 4-hydroxy-phenyl-2-propionic acid. Daidzein is metabolised more intensively. It undergoes up to two different metabolic pathways, which might result in the formation of equol via dihydrodaidzein and tetrahydrodaidzein [200](Figure 1.8) or O-desmethylangolensin (ODMA) via dihydrodaidzein, and 2'-Dehydro-O-desmethylangolensin. In a review, it has been suggested that only little percentage of daidzein is metabolised 4% for equol and 7% for O-DMA [202].

Little is known about the metabolism of equol [183]. It was previously thought that equol is an inert metabolic product [17]. However, further research discovered six aromatic hydroxylated metabolites (phase I) with 39-hydroxy-equol and 6-hydroxyequol as main products [172]. Overall, isoflavones are found in plasma mostly as glucuronide conjugates (75%), and to less extent as sulphates (24%), and less than 1% as aglycones [172]. The main metabolites for daidzein and genistein are 7-O-glucuronides, 4'-O- glucuronides, and to a lesser extent 7,4'-O-diglucuronide, genistein 7,4'-O-disulphate [188].
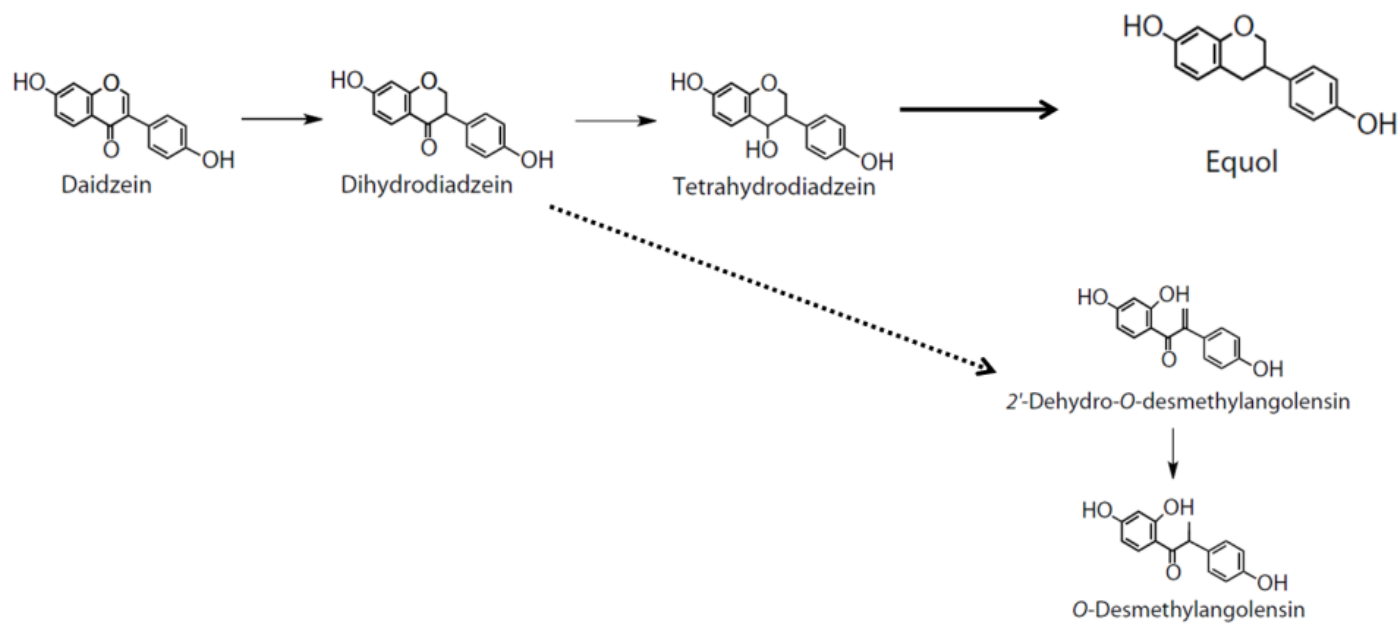
Figure 1.8 Metabolic pathways of daidzein and equol production in the intestine
(Figure adapted from Cassidy *et al.,* 2012 [201]).

1.5.2 Pharmacokinetics of soy isoflavones and equol

Isoflavone consumption plasma concentrations of daidzein and genistein attain a first peak at 1 to 2 h, followed by a second peak at 6-8 h and then start to decrease after 12 h [65, 175, 206, 207] (Figure 1.9)**.** Different isoflavone compounds and metabolites have different bio-availabilities [208]. For instance, plasma genistein levels have been shown to peak more rapidly than those of daidzein (5.2 h; 6.6 h, respectively; [174]). They also had a lower urinary excretion rate than daidzein [206] and this suggests further metabolism of daidzein and conversion to equol [17]. Genistein and daidzein had elimination half-lives of 7 and 9 h after aglycone administration [174] and 3-9 h, 8-11 h after glycoside consumption or food consumption, respectively [174, 202]. After isoflavone intake (soy germ, total isoflavone: 1.20 mg/kg body weight), maximum concentrations (C_{max}) of total isoflavones reached ~2.7 µmol/L in the blood (at 6 h) [208]. In a review of Nielsen *et al.* 2007, it has been show that urinary recovery of isoflavones follows daidzein > glycitein > genistein [209].
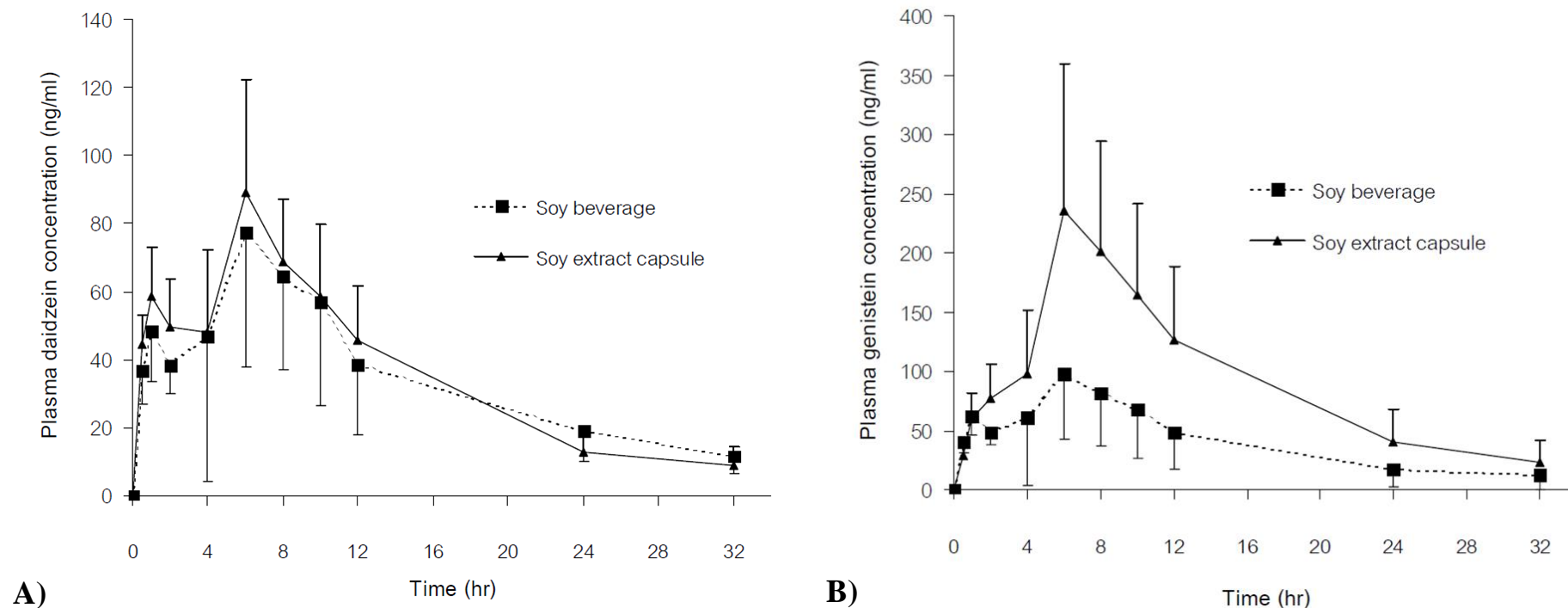
Figure 1.9 Pharmacokinetics of soy isoflavones daidzein and genistein (an example)

Overview of the pharmacokinetics of isoflavones in 12 postmenopausal women following an intake of soy extract capsule (daidzin: genistin, 7.79:22.57 mg) or soy based beverage (daidzin: genistin, 9.27:10.51 mg), A) for daidzein and B) for genistein, plasma concentrations presented as mean±standard deviation, (figure reproduced from Anupongsanugool *et al.,* 2005 [65]).

S-equol is negligible in plasma until 8 h and peaks around 24 h after soy consumption in humans [174, 175, 210] (Figure 1.10). Oral administration of synthetic equol displays different pharmacokinetics with higher bioavailability and rapid absorption than the parent isoflavone, e.g. 50% of equol circulates freely in the plasma compared with only 18.7% as free daidzein [174, 175]. Moreover, T_{max} of equol has been shown at 1-1.5 h after consuming 10 mg/30 mg of SE5-OH (S-equol containing tablets) and attained plasma C_{max} of 2 and 5 μmol/L that are much higher than that of endogenously produced equol (see Figure 1.10 and Table 1.8 for details) [211].

A)
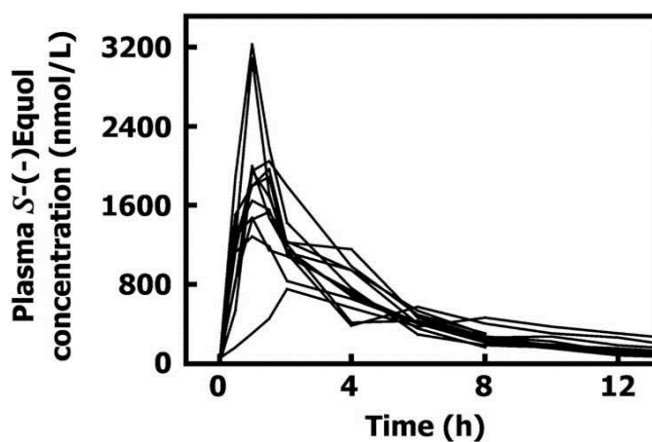
B)
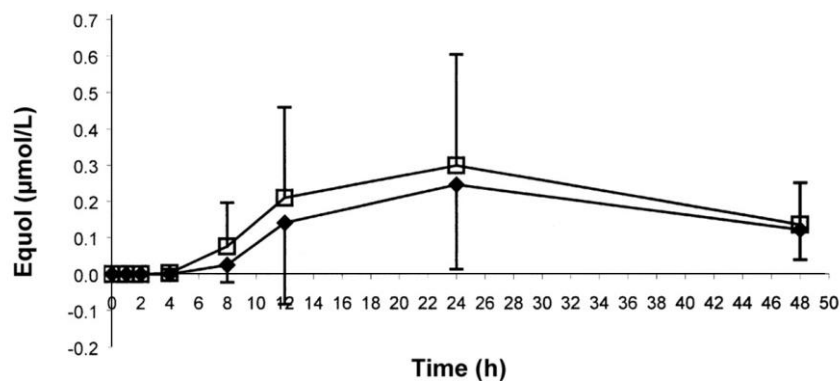
Figure 1.10 Pharmacokinetics of equol in humans
(A) Plasma C_{max} of synthetic S-equol at 2 h after 10mg of S-equol (SE5OH) supplement ingestion 12 women (figure adapted from Setchell *et al.*, 2009 [211]). (B) Plasma C_{max} of endogenously produced S-equol at 24 h after isoflavone capsule ingestion as aglycone (♦) or glucoside (□) in 15 women (figure adapted from Zubik *et al.*, 2003 [175]).

Table 1.8 Plasma levels of equol when consumed as supplements or endogenously produced after daidzein consumption

Ref	Equol dose (mg)	T_{max} (h)/time of measurements	Plasma equol C_{max} (nmol/l)
[211]	10 mg, 30 mg (SE5-OH) supplements	1 h	1907, 4953
[211]	20 mg S- (C^{13}) equol supplements	3 h	991
[141]	14 mg daidzein (500 ml soy milk)	day 4 (3 days challenge)	139
[165]	38 mg daidzein (52 g soy protein powder)	After 2 months	144
[212]	30 mg daidzein (100 mg soya isoflavones concentrate)	day 1	300

Data represented as means. T_{max}, *Peak time;* $C_{max;\ Peak}$ *concentration.*

1.5.3 Factors affecting bioavailability of soy isoflavones

The extent of metabolism and absorption of isoflavones appear to be highly variable between individuals. A daily consumption of 50 mg isoflavones has been shown to achieve plasma concentrations of 50-800 ng/ml [174]. Current human and animal studies propose isoflavone bioavailability of around 20-30% [39] and this can be highly influenced by many factors including delivery medium, different isoflavones sources, food matrix, food processing, diet, dose and frequency of ingestion, age, gender and gut microflora [174, 196, 207, 209, 213]. The chemical composition of isoflavone intake as glycosides or aglycones affects their absorption. It is reported that the absorption of isoflavone glycosides is higher than that of their aglycones and this might be related to the stability and water-solubility of glycosides that might facilitate faster delivery to LPH [209]. In contrast, fermented soy food (rich in aglycones), showed higher bioavailability than non-fermented soybeans (rich in glycosides). However, this was linked in part to the food medium rather than the attaching sugars *per se* [85, 207, 214].

 Previous studies have not found any correlations between age and bioavailability of isoflavones except for equol during the first months compared with adulthood, which is most likely due to the undeveloped intestinal microflora. Further food processing and

storage conditions affect bioavailability, and therefore it is important to measure the isoflavones prior to consumption in trials.

1.6   Summary - identifying research gaps

Multidisciplinary research suggests that consumption of soy isoflavones might reduce CVD risk factors and in particular hemodynamic and vascular-related markers; however, a number of RCTs have reported equivocal data. One potential explanation for the variable response to isoflavones may be metabolic differences, particularly the ability to metabolise the soy isoflavone daidzein to equol. Some *in vivo* studies have shown associations between the equol producer phenotype and reduced endothelial dysfunction and arterial stiffness, and lower blood pressure and this has been supported by *in vitro* work that has identified potential underlying mechanisms, particularly elevated production of the endothelial-derived vasodilator NO.

Despite these notable findings, there is a lack of data to demonstrate: 1) the potential role of equol producer phenotype in the benefits of isoflavone intake on vascular function particularly data derived from RCTs that prospectively recruit equol and non-equol producers, 2) the effects of S-equol supplement intake *per se* on vascular health, specifically in non-equol producers, 3) the biological markers of vascular function that may explain the underlying mechanisms for the vascular protective effects of isoflavones, and 4) the differential efficacy of isoflavones consumption as isolated compounds or dietary sources on vascular function.

1.7 Hypothesis and objectives

The hypothesis of this thesis was that the ability to produce the isoflavone metabolite equol influences the effectiveness of isoflavone intake on vascular function in humans. This research was designed to investigate primarily whether equol producers have greater benefits on vascular function markers (in particular arterial stiffness, endothelial function and blood pressure) in response to an isoflavone intervention than non-equol producers. Our secondary aims were to study: a) the effects of isoflavone containing intervention on circulating biomarkers of vascular and hemodynamic function that might explain the underling mechanisms for vascular bioactivity of soy isoflavones and the metabolite equol, b) if non-equol producers have acute benefits on vascular function following an intake of S-equol supplements, and c) the efficacy of isoflavone intake in different food matrices on vascular function (food versus supplements, providing the same dose).

Figure 1.11 summarises the research objectives and approaches in this thesis. We conducted the followings:

1) a retrospective analysis from a previously completed trial to evaluate whether equol production capability were associated with enhanced vascular function in postmenopausal type 2 diabetic women after a chronic flavonoid intervention. Outcomes included arterial stiffness (PWV), blood pressure (primary outcome measures), and biomarkers of vasodilation (NO) and vasoconstriction (ET-1 and ACE), and insulin resistance assessed by HOMA-IR and resistin (secondary outcome measures) (Chapter 2).

2) an acute study for which we prospectively recruited equol and non-equol producers, to further establish the role of equol producer phenotype on arterial and endothelial function responses to a single dose of soy isoflavone in a male population at elevated risk of CVD. Non-invasive clinical vascular markers such as endothelial function (reactive hyperaemia reaction (RHI)), and arterial stiffness (PWV) (primary outcome measures), augmentation index (AI)), and blood pressure were studied (secondary outcome measures) (Chapter 3).

3) An additional study arm to investigate whether a single intake of S-equol supplements induces acute benefits on vascular markers including RHI, and arterial

stiffness measurements (PWV) (primary outcome measures), AI and blood pressure (secondary outcome measures) in non-equol producers (Chapter 4).
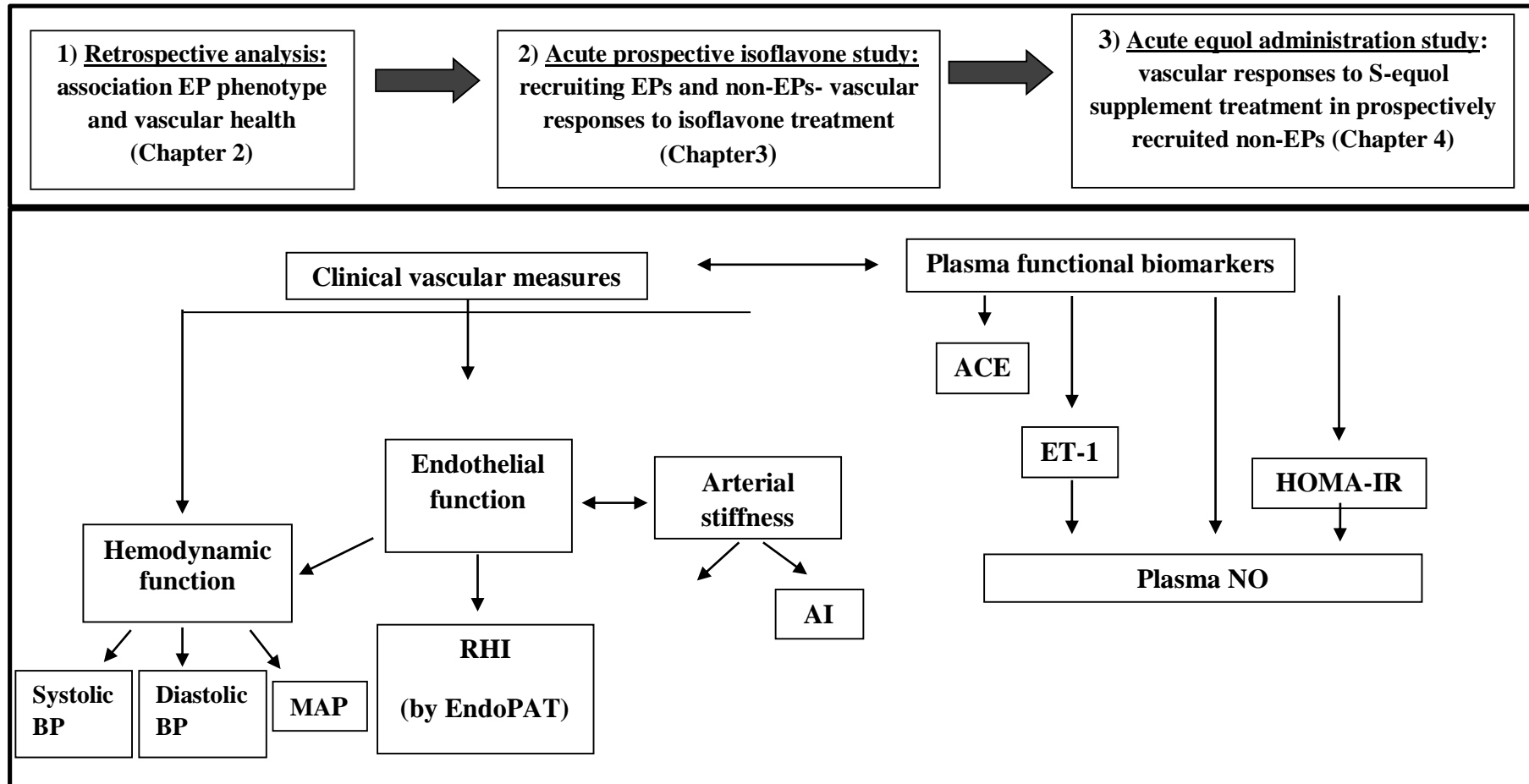
Figure 1.11 Thesis project approaches and vascular-related outcomes

Upper diagram represents chapters' content; lower diagram represents clinical and biological markers of vascular and hemodynamic function. **ACE,** Angiotensin converting enzyme, *AI*, *Augmentation index;* *BP*, *Blood pressure;* *EP*, *Equol producer;* *ET-1*, *Endothelin-1;* *c-fPWV*, *carotid-femoral Pulse wave velocity;* *HOMA-IR*, *Homeostatic model assessment of insulin resistance;* *MAP*, *Mean arterial pressure;* *NO*, *Nitric Oxide assessed as nitrite and nitrate;* *RHI*, *Reactive hyperaemia index.*

Chapter 2   Establishing the association between equol producer phenotype and vascular function: Retrospective analysis of a completed 1-year isoflavone containing intervention.

2.1   Introduction

Soy isoflavones are polyphenol compounds that may have potential health benefits [64]. In particular there is a significant interest in the effects of soy isoflavone intake on cardiovascular disease risk factors but the available data are inconclusive [5, 6, 8-16]. Inter-individual differences in metabolism might offer an explanation [17]. The equol producer phenotype is present in only a third of the Western population, who possess the ability to convert the precursor compound, daidzein, into the unique metabolite equol in the large intestine after consuming soy [18]. Equol showed higher bioavailability in human studies [211], greater antioxidant activity [20, 21], and was suggested to be more anti-atherosclerotic than daidzein *in vitro* [23, 24]. Additional *in vitro* and animal work has shown beneficial vaso-relaxant activity of equol suggesting its potential for the prevention and treatment of hypertension [23, 92-94, 155].

Few recent observational studies of Asian populations have shown positive associations between the equol producer phenotype and cardiovascular risk factors; including blood pressure [25], plasma lipid profiles [25, 26] and inflammatory biomarkers [25, 27].

Using a retrospective analysis approach, several isoflavone interventions have investigated associations between the equol producer phenotype and cardiovascular disease risk factors. Two studies showed benefits of isoflavone intake on vascular function including enhanced endothelial function [6], and a trend towards lower blood pressure [15] in equol producers. Conversely, two other studies have reported no difference between equol and non-equol producers on vascular reactivity [215] or carotid artery intima-media thickness [12]. However, these studies had major limitations in their methodology for identifying equol producers [12, 215] which impacts upon the confidence in the results; in addition, the small

sample size of equol producers was a limitation in the dataset [215]. Overall, further data are required to determine whether the equol producer phenotype might affect the vascular bioactivity of isoflavone interventions.

Furthermore, there is also currently a lack of data regarding the underlying biological mechanisms by which isoflavone intake and the metabolite equol might influence CVD risk factors, in particular endothelial dysfunction, hypertension and arterial stiffness, with an overview provided in Figure 2.1. It has been suggested that the vascular bioactivity of isoflavone intake could be explained by elevated levels of the potent vasodilator Nitric Oxide (NO) [9, 92] which has been shown to play an important role in the regulation of endothelial function, arterial stiffness and development of hypertension. The literature also suggests a potential inhibition of vasoconstrictors such as Endothelin-1 [11, 98] and angiotensin converting enzyme (ACE) [39, 99] (Figure 1.4) by isoflavones. In diabetic populations, isoflavone intake might induce benefits on insulin resistance [112] and this might be correlated with improvements on vascular function [110]. It has been shown that the homeostatic model assessment of insulin resistance (HOMA-IR) [107] is positively associated with premature stiffening of small and large arteries [216] and hypertension [217]. Notably, isoflavones effects on the previously mentioned potential biomarkers have been investigated mostly in cell lines and animal models, while further research is required in human studies.

In order to help address gaps in the current understanding of equol producer phenotype on vascular and hemodynamic functions, and understand the underlying biological mechanisms, we conducted a retrospective analysis using data from a previously completed study in postmenopausal women with type 2 diabetes who had consumed a flavonoid enriched chocolate (containing 100 mg isoflavone as aglycones units and 90 mg epicatechins) for 1 year. The hypothesis of this retrospective analysis was that the equol producers had greater improvements in vascular and hemodynamic markers than non-equol producers, after the isoflavone-containing intervention. The primary outcome measures of our analysis included change from baseline in blood pressure, and arterial stiffness following the flavonoid intervention.

In addition, secondary outcomes included change from baseline in circulating levels of biomarkers of endothelial function (in particular, NO metabolites, ET-1, ACE, and insulin resistance assessed by HOMA-IR and resistin).
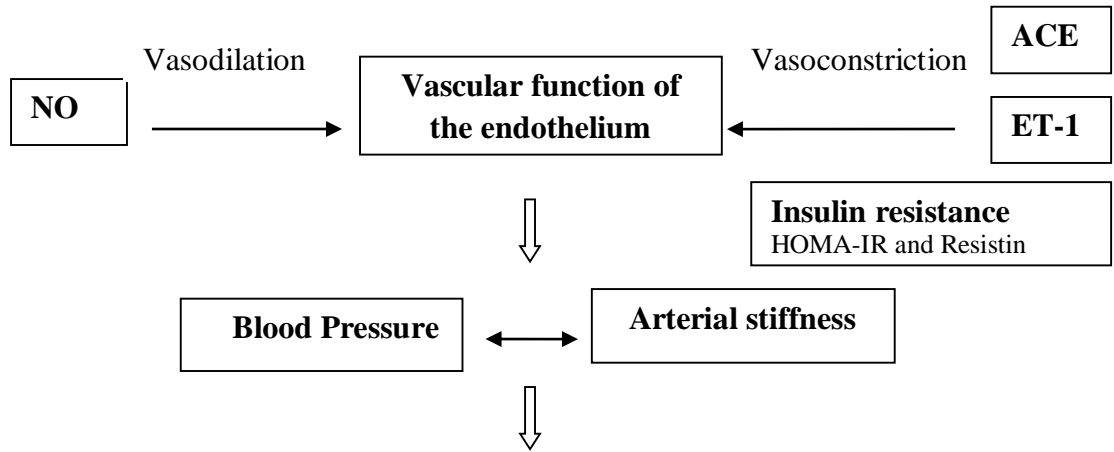
Development and progression of CVD

Figure 2.1 Potential correlated biological and clinical risk factors that explain the underlying mechanisms by which isoflavones might protect against the CVD development

Hypertension and arterial stiffness are established predictors of CVD mortality [29, 218]. Under hypertensive stimuli, functional and structural changes in the blood vessels take place and may cause arterial stiffening [219]. Endothelial dysfunction can explain hypertension and arterial stiffness prior to CVD development [41] and thus the potential benefits of isoflavones/equol on endothelial function by regulating biomarkers of vasoconstriction and vasodilation, may reduce the susceptibility to developing CVD. *ACE, Angiotensin converting enzyme; CVD, Cardiovascular disease; ET-1, Endothelin-1; HOMA-IR, Homeostatic model assessment of insulin resistance; NO, Nitric oxide.*

2.2 Methods

2.2.1 Study design, subjects and intervention dose

This analysis included data and biological samples collected from a previously conducted study (www.clinicaltrials.gov; NCT00677599).

Briefly, the study was a double-blinded RCT with a parallel design. It included 118 postmenopausal women (at least one year since last menstruation) with type 2 diabetes, up to 75 years old, using statins for at least 12 months. On the other hand, exclusion criteria included:

- those who were smokers/ex-smokers within less than 1 year ago
- taking hormone replacement therapy
- clinically diagnosed with vascular disease, or cancer, hypertension with systolic BP ≥ 160 mm Hg, prescribed aspirin for a period of less than 12 months prior to recruitment
- having antibiotic medications or with planned vaccinations, or those consuming flavonoid/vitamin supplements (within 3 months prior to the study).

Participants were randomly allocated into a flavonoid or placebo arm (n=59 for each group) where they were required to consume flavonoid-rich chocolate containing 100 mg isoflavones as aglycone units, and 90 mg epicatechins (850 mg total flavan-3-ols) or matched (appearance and nutrient content) placebo daily for 12 months. Intake was divided in 2 doses a day to sustain sufficient circulating levels of metabolites based on their known half-lives [220]).

2.2.2 Endpoints

Outcomes included hemodynamic and endothelial function in particular blood pressure measurements (ambulatory systolic, diastolic blood pressure (BP), and mean arterial blood pressure (MAP)) and arterial stiffness assessment (central pulse wave velocity (PWV)) that were assessed at baseline, 6 months and 12 months of flavonoid consumption in the previously conducted study [220]. Fasting plasma/serum samples were additionally

analysed for biomarkers of hemodynamic and vascular function such as NO, ACE, ET-1, HOMA-IR, and resistin.

Anthropometric measurements

Body weight was measured by digital electronic weighing scale to the nearest 0.1 Kg and the body height was measured using a stadiometer to the nearest 1 cm following the standard procedure (participants adapted a head position according to the Frankfort plane). Body mass index was calculated (Kg/m²). All anthropometric measurements were carried out following standard operating procedures by research nurses blinded to intervention allocation.

In addition, body composition was estimated by bioelectrical impedance analysis (BIA) using Tanita, Model TBF-305. The machine follows leg-to-leg BIA, where a weak current is passed through the lower body part. BIA determines the electrical resistance of different body tissues to calculate an estimate of whole body fat, and trunk fat [221].

Clinical hemodynamic and vascular function measurements

Ambulatory pressure measurements were obtained over a 2 h daytime observation period using a non-invasive ambulatory monitor (Spacelabs SL90207[2]) approved by the British Society of Hypertension.

The monitor automatically inflated every 10 minutes. An appropriately sized cuff (pneumatic bag 20% wider than the diameter of the upper arm; standard adult (24-32 cm), large adult (32-42 cm) or extra large adult (42-55 cm)) was placed on the least dominant arm at the heart level. For each recording, mean of blood pressure was calculated from average measurements obtained every 10 minutes (in total 12 measurements). Central PWV was measured between 2 recording sites: neck (Carotid) and femoral (thigh) ateries using validated non-invasive device (Vicorder, Skidmore Medical Ltd, Bristol, UK) and appropriate sized cuffs. All measurements were done in triplicate and subjects were in a relaxed supine position and controlled conditions (dim light, quiet atmosphere and suitable temperature between 21° and 24° C). Valid PWV data was only available for a subgroup analysis due to problems with the equipment. It was reviewed by two-blinded independent

researchers for validity and inclusion based on quality assessment criteria and guidance from manufacturer.

The central PWV is derived from the transit time (Δt) between the carotid and femoral pulse waves and the estimated distance between the two sites (ΔL). The pulse wave velocity (m/s) is calculated from the ratio of the estimated distance travelled by the pulse (ΔL in meters) during the pulse transit time over the measured pulse transit time (ΔT in seconds) (Figure 2.2).
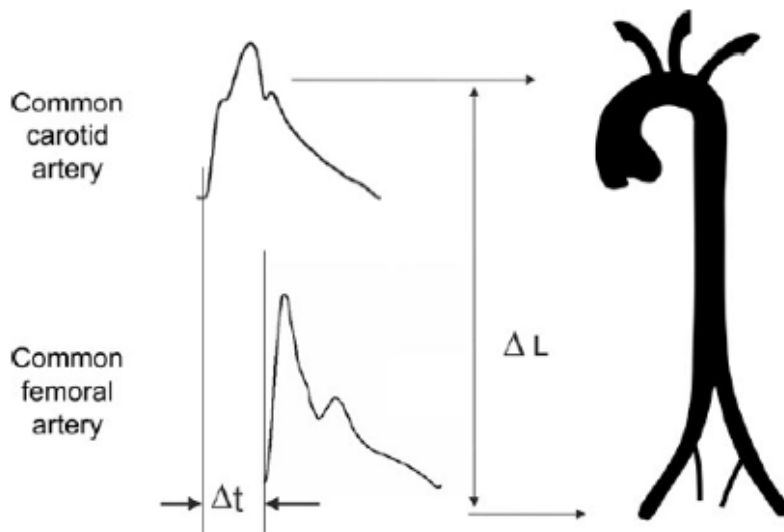
Figure 2.2 Clinical measurement of the central pulse wave velocity (PWV)
The figure shows the arterial tree on the right. Top wave is measured at the carotid artery and the bottom wave is measured at the femoral artery. PWV is calculated from the ratio of the estimated distance travelled by the pulse during the pulse transit time over the measured pulse transit time. *Δt, transit time; ΔL, estimated distance travelled by the pulse* (figure reproduced from [55]).

Analyses of vascular function related biomarkers

Collected blood samples were immediately centrifuged and then the resulting plasma was stored until analysis at -80 ºC. Stored plasma was thawed at room temperature and used for quantification of the vascular biomarker concentrations. To obtain reliable and precise results, researchers were blinded, all samples were analysed in duplicate, any repeated thaw-freeze cycles were avoided, and aliquots of human quality controls were used in every assay. In addition, samples which had showed a coefficient of variation (CV) % of

higher than 10%, were re-run or excluded in order to obtain an adequately precise data [222, 223].

Plasma fasting samples were analysed for insulin, ET-1, ACE, and resistin in duplicate by commercially available ELISA kits (Table 2.1). For every analysis, approvals for COSHH forums and scheme of work for handling human tissues and body fluids were obtained and standard operating procedures (SOPs) were prepared following guidelines from the manufacturers and UEA safety officer.

Fasting plasma glucose concentrations were determined in the pathology labs of Norfolk and Norwich University Hospitals (NNUH) and provided to calculate HOMA-IR, which is a reliable, accurate index for measurement of insulin resistance and has been shown to correlate to the gold standard method euglycemic-hyperinsulinemic clamp. HOMA-IR is calculated from fasting plasma glucose (mmol/L) × fasting serum insulin (mU/L) divided by 22.5 [107]. For NO analysis, defrosted plasma samples were filtered to remove proteins > 10kDa and total nitrate and nitrite (NO) concentration was analysed in triplicate by a colorimetric assay using a Nitric Oxide Quantitation kit (nitrate/ nitrite colorimetric assay, Caymen Chemical). Quantification of the nitrite and nitrate concentrations in plasma, serum and urine, is the most feasible mean to assess NO production *in vivo* [224] because of the short half-life of circulated NO (<0.1 second) that is oxidised to nitrate by oxy-haemoglobin in red blood cells and undergoes autoxidation in haemoglobin-free media to nitrite [224]. This assay includes two steps: first, conversion of nitrate to nitrite by a nitrate reductase and second, a Griess reagent is added that converts nitrite to an azo compound that can be measured spectrophotometrically.

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Table 2.1 Summary of biomarker assays used to determine vascular-related functions.

Biomarker	Assay	Source of assay	Inter-assay, intra-assay	Detection limit
Insulin	ELISA	Human Insulin ELISA, Mercodia, Sweden	4%, 3%	1 mU/l
ET-1	Chemiluminescent ELISA	Endothelin-1 ELISA kit (QuantiGIo), R&D systems, UK	5%, 3%	0.06 pg/ml
ACE	ELISA	Human ACE ELISA, USCNK, China	12%, 7%	0.11 ng/ml
Resistin	ELISA	Human resistin ELISA, Millipore, UK	7%, 2%	0.16 ng/ml
NO	Nitrate/ nitrite colorimetric assay (Griess reagent)	Nitric Oxide Quantitation kit, Caymen Chemical, UK	3%, 3%	2.3 μmol/L

ACE, Angiotensin converting enzyme; ET-1, Endothelin-1; ELISA, Enzyme linked immunsorbent assay; NO, Nitric Oxide assessed as nitrate/ nitrite.

Identification of equol producer phenotype

Urinary concentrations of the isoflavone daidzein, genistein and equol were analysed by HPLC using an in-house validated method in the institute of food research (Norwich, UK). Compliance to intervention was confirmed by flavonoid excretion in 24 h urine and counting of returned chocolate bar wrappers. Subjects were then defined as equol producers by having urinary log10 S-equol/ daidzein > -1.75 [141].

2.3   Statistical analysis

All variables were first evaluated for their normal distribution and equality of variances between groups using Levene's test and sphericity for homogeneity of variance. Data were log transformed when normal distribution assumption was violated.

Independent t-test was used to check any differences in baseline measures between equol producers and non-equol producers. χ^2 test was used for categorical variables and Mann-Whitney test was used for data identified as not normally distributed.

Univariate tests were used with equol production phenotype as a fixed factor and baseline level as a covariant. Data were analysed as changes from baseline levels at 6 month and 12 month observations. A Mann-Whitney test was used for analysis of skewed data that could not be transformed to a normal distribution. For variables that showed a significant effect of equol producer phenotype, Pearson's test was used to test correlation of levels of vascular endpoints and urinary concentrations of equol. For analysis of urinary excretion of isoflavones, data were not normally distributed and could not be transformed to normal distribution, thus a non-parametric test was used (Kruskal-Wallis test) to examine any significant differences of urinary excretion between equol and non-equol producers at baseline, 6 months and 12 months.

Additionally, statistical analyses were conducted to investigate interaction effects of time (baseline, 6 and 12 months) and equol production phenotype on the analysis variables (i.e. vascular markers). These analyses were carried out using a mixed model that included repeated-measures ANOVA to investigate the effect of time (within subject factor), and effect of equol production phenotype (between subject factor).

 All results are expressed as mean±SEM (standard error of mean) and a P value<0.05 was considered statistically significant. SPSS software (PASW, version 18, SPSS) was used. On a blinded review of the data, all outliers of values > mean±3standard deviation (SD) were excluded and for lab analyses, and all data showing coefficient of variation CV% > 10% were excluded. The number of participants in the analysis was for equol producers= 17, non-equol producers= 30 for most variables (check table footnotes for participant numbers).

2.4 Results

The number of participants included in the analysis was limited to the participants who only completed the flavonoid arm, which included 17 equol producers, and 30 non-equol producers. The subjects allocated to the placebo arm could not be included because they did not have any isoflavone intake or soy challenge to assess their equol producer phenotype (Figure 2.3).
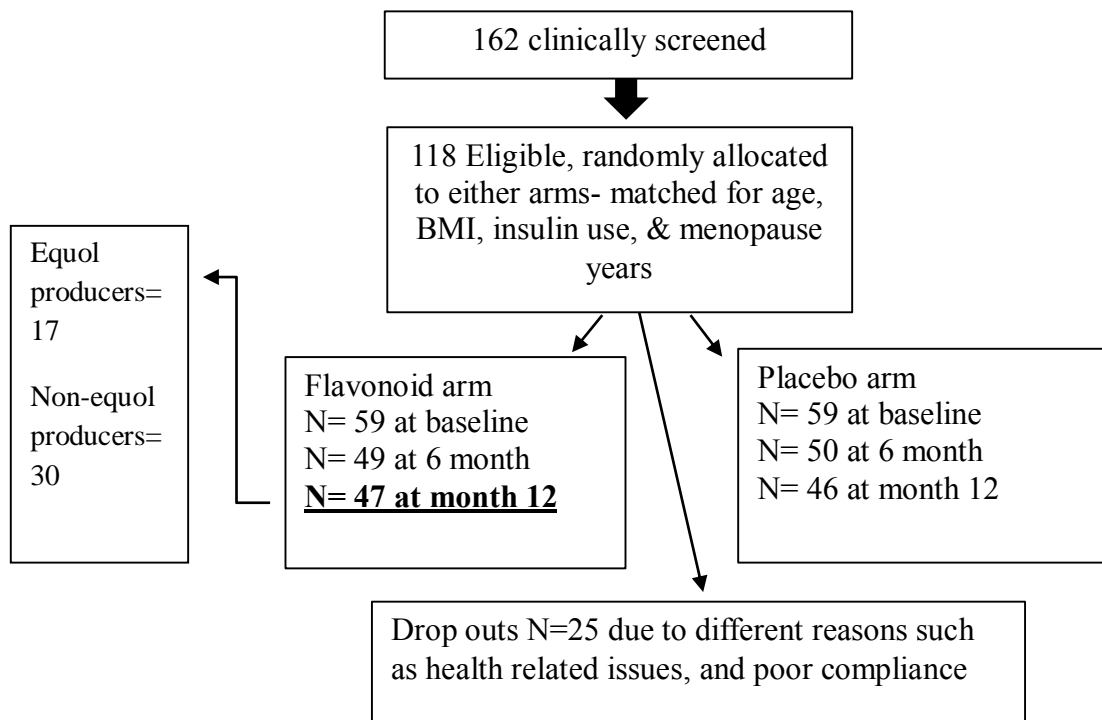
Figure 2.3 Number of enrolled subjects in the 12 month flavonoid intervention

2.4.1 Baseline differences between equol producers and non-equol producers – general health and metabolic characteristics, and vascular function markers

There were no significant differences in baseline levels of general health and metabolic related characteristics between equol and non-equol producers (Table 2.2). Similarly, there were no significant differences in the clinical vascular measures, i.e., ambulatory blood pressure or PWV at baseline (Table 2.3). In contrast, the levels for most analysed vascular-related biomarkers were significantly different between equol and non-equol producers

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with a healthier profile in the equol producers. In particular, plasma concentrations of the vasodilator biomarker NO were significantly higher in equol than non-equol producers (61.1±6.5 vs 43.0±4.2 μmol/L, respectively, P<0.01). Consequently, the baseline ratio of NO to ET-1 was also significantly higher in equol than non-equol producers (37.29±4.66 vs 33.59±6.22, respectively, P<0.05). In addition, baseline plasma levels of the vasoconstrictor ACE were lower in the equol than non-equol producers (102.89±33.01 vs 163.79±104.82 ng/ml, respectively, P<0.05). Lower baseline levels of fasting plasma insulin (mU/L) in equol producers than non-producers (6.1±0.9vs 8.8±1.1 mU/L, respectively, P= 0.05) were indicative of a greater insulin sensitivity.

Table 2.2 Differences in baseline levels of health and metabolic related characteristics of equol and non-equol producers

Baseline characteristics		Non-equol producer (N1)		Equol producer (N2)		P-value
		Mean±SEM	Percent %	Mean±SEM	Percent%	
Age (years)		62±1		62±1		0.67
Diabetes duration (years)		7±1		10±2		0.27
*Years since menopause		14±2		13±2.		0.97
*Weight (Kg)		87.6±3.9		84.5 ± 5.3		0.65
*BMI (kg/ m²)		32.8±1.29		32.5±2.01		0.84
Trunk fat %		38.3 ± 1.4		36.1±1.8		0.35
Insulin use	NO		87		71	0.18
	YES		13		29	
Blood Pressure medicated	NO		43		35	0.59
	YES		57		65	
Ever smoked	NO		70		71	0.97
	YES		30		29	
Diagnosed as hypertensives	NO		27		29	0.84
	YES		73		71	

N1=30, N2=17. Data presented as mean±SEM. P- values of independent T-test represent statistical significance of differences in baseline measures between equol producers and non-producers. χ^2 test was used for categorical variables. *Data found not normally distributed and Mann-Whitney test was used. **BMI**, Body Mass Index.

68

Table 2.3 Differences in baseline levels of hemodynamic and vascular function markers between equol and non-equol producers

Baseline endpoints	Non-equol producer (N1)	Equol producer (N2)	P value
AMBP systolic BP (mmHg)	135 ±2	131±2	0.16
AMBP diastolic BP (mmHg)	77 ±2	74±2	0.25
AMBP MAP (mmHg)	98±2	95±2	0.21
PWV (m/s)	9.1 ± 0.6	8.6±0.5	0.50
***Fasting plasma insulin (mU/L)**	8.8±1.1	6.1±0.9	0.05
***HOMA-IR (mU.mmol/L²)**	2.74±0.34	2.13±0.33	0.16
***plasma resistin (ng/ml)**	16.98±1.73	17.27±2.69	0.48
***Plasma NOx (µmol/L)**	43.0±4.2	61.1±6.5	<0.01
***Plasma ET-1(pg/ml)**	1.64±0.17	1.65±0.16	0.181
***NO/ET-1**	33.59±6.22	37.29±4.66	<0.05
***ACE (ng/ml)**	163.79±104.82	102.89±33.01	<0.05

The number of participants in the analysis was: N1=30, N2=17 for AMBP systolic and diastolic BP, N1=30, N2=16 for PP, N1=29, N2=17 for MAP, N1=29, N2=16 for resistin, insulin and HOMA-IR; N1=8, N2=5 for ACE; N1=19, N2=13 for ET-1 and NO/ET-1. Data presented as mean±SEM (Standard Error of the Mean). P values of T-independent test represent statistical significance of differences in baseline measures between equol producers and non-producers. χ^2 test was used for categorical variables. * Data found not normally distributed and Mann-Whitney test was used. *ACE, Angiotensin converting enzyme; AMBP, Ambulatory blood pressure; BP; Blood pressure; ET-1, Endothelin-1; HOMA-IR, Homeostatic model assessment of insulin resistance, MAP, Mean arterial pressure; NO, Nitric oxide assessed as nitrite/ nitrate ; PP, Pulse pressure; PWV, Pulse wave velocity.*

2.4.2 Urinary concentrations of isoflavones and equol producer frequency

17 volunteers (36%) were identified as equol producers following soy isoflavone intervention. There were no equol cross-overs (i.e. equol producers becoming non-equol or vice versa) identified from the data at either 6 or 12 months.

As expected, after 6 and 12 months consumption of intervention, the urinary excretion of daidzein and genistein ($P<0.01$) significantly increased compared to baseline in equol producers and non-producers. Results indicated good compliance to the flavonoid intervention with significantly increased levels of isoflavones in urine ($P<0.01$ for all analytes) and 88% wrappers returned. It is notable that non-equol producers excreted significantly more daidzein at 6 months than equol producers ($P<0.01$) but this relationship was no longer evident at 12 months ($P= 0.18$) (Figure 2.4).

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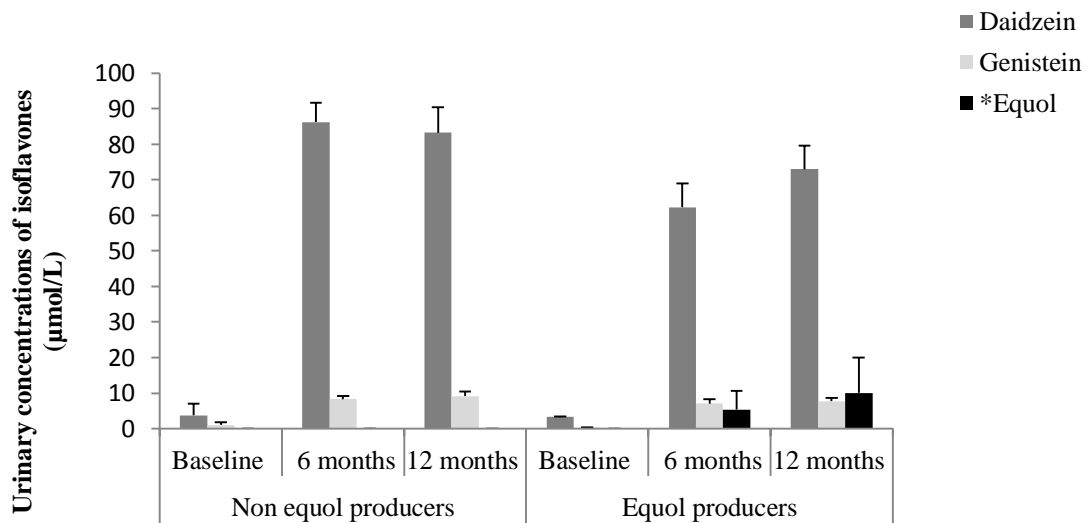
Figure 2.4 Urinary concentrations of isoflavones (μmol/L) after 6 and 12 months of flavonoid intervention

P values represent significant difference of urinary excretion of isoflavones between equol and non-equol producers. P values for daidzein, genistein, and *equol in were, $P> 0.05$ for all at baseline, $P< 0.01$, 0.30, and <0.0001 at 6 months, and $P= 0.18$, 0.86, <0.0001 at 12 months respectively.

2.4.3 Effects of equol production phenotype on blood pressure and arterial stiffness after flavonoid consumption

There were no significant differences in the levels of blood pressure (Systolic BP, Diastolic BP, MAP or pulse pressure), and arterial stiffness (PWV) between equol and non-equol producers following intervention (including baseline, 6 months and 12 months, see Appendix B, page 178, Table I) for P values of repeated measure ANOVA for interaction effect of time and equol production phenotype.

After 6 months of flavonoid consumption, there were no observed effects of equol producer phenotype on blood pressure or arterial stiffness (Table 2.4, Figure 2.5, Figure 2.6, Figure 2.7). However, after 12 months of flavonoid consumption, diastolic blood pressure was significantly decreased in equol producers (change from baseline: -2.2 ± 1.3 mmHg in equol producers *versus* 1.0 ± 0.9 mmHg in non-equol producers, $P<0.01$, Figure 2.5). Mean arterial pressure (MAP) was also significantly lower in equol producers (change from baseline: -1.2 ± 1.3 mmHg in equol producers *versus* 2.2 ± 1.1 mmHg in non-equol producers, $P<0.01$, Figure 2.6). Similarly, arterial stiffness assessed by PWV was significantly decreased in equol producers compared to no change in non-equol producers (change from baseline: -0.7 ± 0.4 m/s in equol producers, *versus* 0.3 ± 0.6 m/s in non-equol producers, $P<0.01$, Figure 2.7) after 12 months. There were no differences in systolic blood pressure and pulse pressure between equol and non-equol producers after 12 months of flavonoid consumption (Table 2.5).

Table 2.4 Effects of equol producer phenotype on blood pressure levels after 6 month flavonoid consumption

Endpoint	Non-equol producers (N1)			Equol producers (N2)			P value
	BL	6 months	6 months– BL	BL	6 months	6 months– BL	
Systolic BP (mmHg)	135±2	137± 2	1.6±2.1	131±2	132±4	1.9±3.1	0.49
PP (mmHg)	58.3±1.8	60.2±2.0	1.9±1.4	56.4±2.2	56.8±2.8	0.3±1.4	0.40

N1=30, N2=17 except for PP N1=30, N2=16. P value for univariate analysis. **BL**, *Baseline;* **BP**, *Blood Pressure;* **PP**: *pulse pressure.* Blood pressure medicated was used as covariant.

Table 2.5 Effects of equol producer phenotype on blood pressure levels after 12 month flavonoid consumption

Endpoint	Non-equol producers (N1)			Equol producers (N2)			P value
	BL	12 months	12 months– BL	BL	12 months	12 months–BL	
Systolic BP (mmHg)	135± 2	138± 2	2.8±1.8	131±2	131±2	0.4±1.6	0.11
PP (mmHg)	58.3±1.8	60.8±1.8	2.5±1.4	56.4±2.2	59.1±2.6	2.6±0.9	0.98

N1=30, N2=17 except for PP N1=30, N2=16. P value for univariate analysis. **BL**, *Baseline;* **BP**, *Blood Pressure;* **PP**, pulse pressure. Blood pressure medicated was used as covariant.

12 months - baseline, P= 0.008
6 months- baseline, P= 0.67

■ **Non-equol**
producers
N1=30

▪ **Equol producers**
N2=17

Change in diastolic BP from baseline

3.
2.
1.
-
-1.
-2.
-3.
-4.

1.0±0.9

-0.4±1.1   -0.4±1.2

6 months - baseline

-2.2±1.3

12 months - baseline
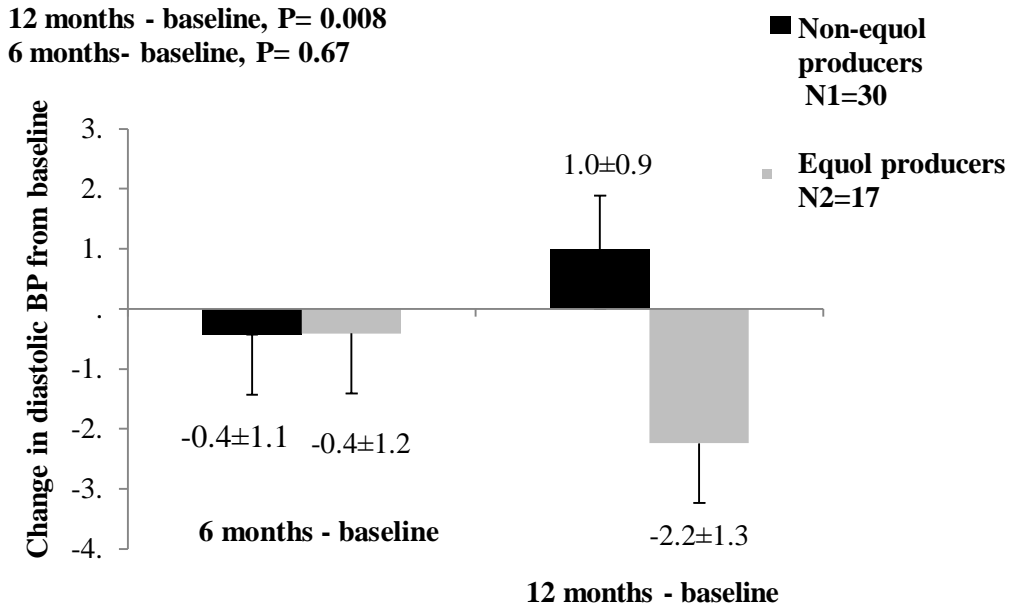
Figure 2.5 Change in diastolic BP in equol and non-equol producers after consuming flavonoids for 6 or 12 months.

Data presented as mean±SEM. P value for univariate analysis. Medicated blood pressure status was used as covariant. *BP, blood pressure.*

12 months - baseline, P= 0.01
6 months- baseline, P= 0.37

■ **Non-equol**
producers
N1= 29

▪ **Equol producers**
N2=17

Change in MAP from baseline

4.
3.
2.
1.
-
-1.
-2.
-3.

2.2±1.1

0.8±1.5   0.4±2.2

6 months - baseline

-1.2±1.3

12 months - baseline
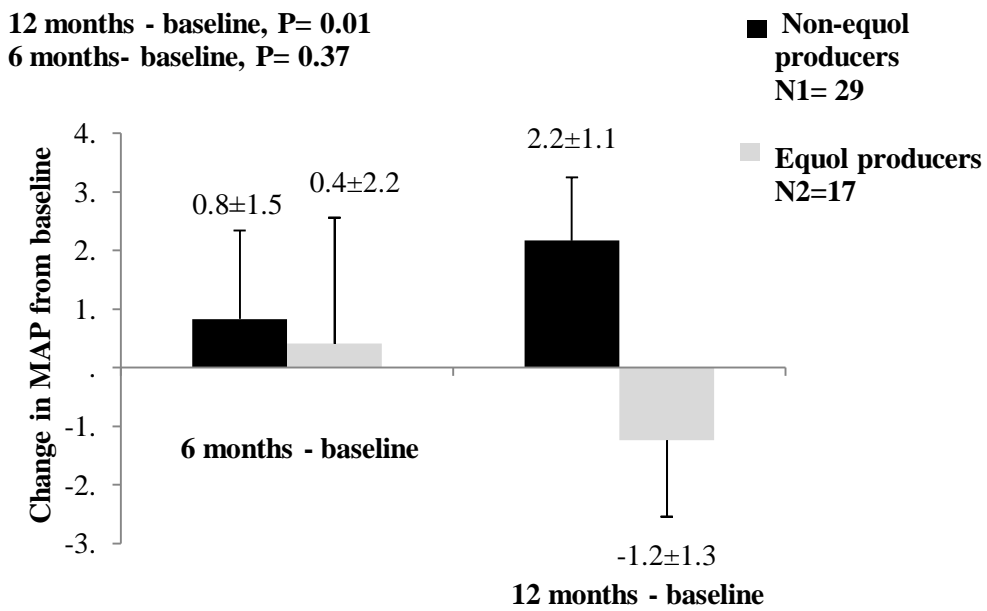
Figure 2.6 Change in MAP in equol and non-equol producers after consuming flavonoids for 6 or 12 months.

Data presented as mean±SEM. P value for univariate analysis. Blood pressure medicated was used as covariant. *MAP, Mean arterial pressure.*
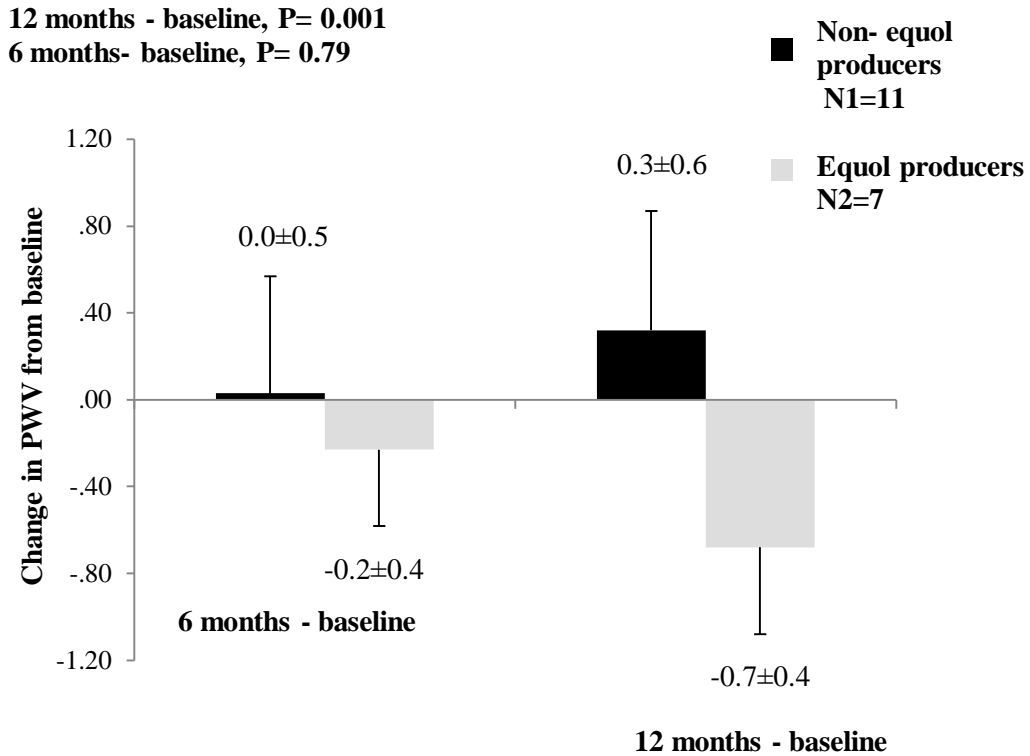
Figure 2.7 Change in PWV in equol and non-equol producers after consuming flavonoid for 6 or 12 months.
Data presented as mean±SEM. P value for univariate analysis. *PWV, Pulse Wave Velocity.*

2.4.4 Effects of equol producer phenotype on circulating levels of vascular function and insulin resistance related biomarkers after flavonoid consumption

Plasma level of NO significantly decreased in equol and non-equol producers ($P<0.01$) during the intervention (Appendix B, Table I, page 178). Specifically, after 6 months, only plasma NO levels decreased significantly in equol producers compared to non-equol producers (change from baseline: -17.8±19.6 vs 0.8±3.9 µmol/L, P= 0.05, respectively, Table 2.6) and there was no difference at 12 months (Table 2.6). There were no significant differences in the other analysed biomarkers of hemodynamic and vascular function between equol and non-equol producers following flavonoid intake neither at 6 month nor 12 month assessment (Table 2.6, Table 2.7).

Table 2.6 Effects of equol producer phenotype on vascular function and insulin resistance related biomarkers after consuming flavonoids for 6 months

Biomarker	Non-equol producers (N1)			Equol producers (N=2)			P value
	BL	6 month	6 month– BL	BL	6 month	6 month– BL	
*NO (μmol/L)	43.0±4.2	43.9±3.4	0.8±3.9	61.1±6.5	43.3±4.0	-17.8± 19.6	<0.01
*Resistin (ng/ml)	16.98±1.73	18.45±1.99	1.47±1.24	17.27±2.69	17.15±2.29	-0.12±1.35	0.91
Insulin (mU/l)	8.80±1.05	7.45±0.72	-1.35±0.6	6.05±0.91	5.93±0.79	-0.11±0.55	0.81
HOMA-IR (mU.mmol/l²)	2.74±0.34	2.40±0.23	0.34±0.2	2.13±0.33	2.07±0.28	-0.06±0.30	0.85

N1=30, N2=17 for NOx; N1=29, N2=16 for resistin, Insulin and HOMA-IR; N1=8, N2=5 for ACE. Data presented as means ±SEM, P values of univariate analysis and baseline values were used as covariant. *Data appeared not to follow normal distribution and presented as mean±SEM and analysed by Mann-Whitney test. **BL,** Baseline; **HOMA-IR**, Homeostatic Model Assessment of Insulin Resistance; **NO**, Nitric Oxide assessed as nitrite/nitrate. Note: ACE and ET-1 were not analysed for 6 months samples because of lack of funds.

Table 2.7 Effects of equol producer phenotype on vascular function and insulin resistance related biomarkers after consuming flavonoid for 12 months

Biomarker	Non-equol producers (N1)			Equol producers (N2)			P value
	BL	12 month	12 month– BL	BL	12 month	12 month– BL	
*NO (μM)	43±4.2	40.7±3.3	-2.2±3.5	61.1±6.5	53.5±5.4	-7.5± 13.1	0.24
*ET-1 (pg/ml)	1.64±0.17	1.98±0.25	0.35±0.13	1.65±0.16	1.57±0.21	-0.08±0.22	0.09
*NO/ ET-1	33.59±6.22	26.38±4.52	-7.20±4.25	37.29±4.66	38.59±5.03	1.30±5.72	0.43
*ACE (ng/ml)	163.79±104.82	151.17±92.68	-12.62±14.17	102.89±33.01	119.85±44.78	16.96±14.86	0.22
*Resistin (ng/ml)	16.98±1.73	19.04±2.67	2.06±1.57	17.27±2.69	15.68±1.72	-1.59±1.42	0.68
Insulin (mU/l)	8.80±1.05	7.90±0.81	-0.90±0.55	6.05±0.91	5.56±0.74	-0.49±0.57	0.55
HOMA-IR(mU.mmol/l²)	2.74±0.34	2.49±1.89	-0.25±-0.10	2.13±0.33	1.89±0.26	-0.23±0.09	0.49

N1=30, N2=17 for NOx; N1=29, N2=16 for resistin, insulin and HOMA-IR; N1=8, N2=5 for ACE; N1=19, N2=13 for ET-1 and NO/ET-1. Data presented as means ±SEM. P values of univariate analysis and baseline values were used as covariant. *Data appeared not to follow normal distribution and presented as mean±SEM and analysed by Mann-Whitney test was used. *ACE, Angiotensin Converting Enzyme; BL, Baseline; ET-1, Endothelin-1; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; NO, Nitric Oxide assessed as nitrite/nitrate.*

In addition, flavonoid intervention for 12 months or equol producer phenotype did not significantly affect body weight, BMI or trunk fat % (P>0.05). When analysing the association between equol excretion and parameters of vascular function in the equol producer group alone, there was a weak trend for a correlation between change in diastolic blood pressure from baseline and urinary concentrations of equol at 12 months (r= -0.44, P= 0.07). Changes in MAP and in arterial stiffness (PWV) were not correlated with urinary equol (µM) (*P*>0.05). In addition, diastolic BP, MAP and PWV were not associated with age, BMI, trunk fat percentage, diabetes duration, insulin use or years at menopause (P>0.05).

2.5   Discussion

This retrospective analysis of a previously completed study [220] showed that the equol producer phenotype was associated with enhanced clinical markers of hemodynamic function and arterial stiffness in postmenopausal women with type 2 diabetes, following long-term isoflavone containing intervention. In particular, equol producers had significantly lower diastolic blood pressure, mean arterial pressure and central pulse wave velocity than non-equol producers after completing a one year intervention.

However, we cannot conclude that these observed vascular and hemodynamic benefits were in response to the 12 month flavonoid intervention, because there was no control arm included in the analysis. Intermediate assessment at 6 months observed no effects of the equol producer phenotype on the analysed clinical and circulating markers of hemodynamic and vascular function except for plasma levels of the endothelial vasodilator NO (measured as plasma nitrite/ nitrate levels) which were surprisingly reduced in equol producers compared to non-equol producers. Collectively, these findings could suggest that long-term flavonoid intakes might be more beneficial in postmenopausal diabetic equol producers.

Using a similar retrospective analysis, another large RCT of postmenopausal women has shown trends towards improvements in systolic and diastolic blood pressure and an enhanced endothelial function (assessed by FMD) in the equol producers (n=25) while these measures deteriorated in the non-equol producers (n=63) after consuming isoflavones (99 mg as aglycones /day) for 12 months [15]. Another parallel-designed RCT of soy germ-rich pasta intervention (33 mg isoflavones/day as aglycones) for 4 weeks has reported significant improvements from baseline levels of endothelial function (assessed by brachial artery FMD) in hypercholesteraemic equol producers (n=9) compared to non-equol producers (n=20) [6].

In contrast, in a recent 2.7 year RCT of 350 postmenopausal women, there were no significant differences on atherosclerosis progression (carotid artery intima-media thickness (CIMT)) in equol and non-equol producers in the soy group (91 mg isoflavone as aglycones/ day) versus placebo group. But this analysis is conflicted by the comparison to the placebo group who were not assessed for their equol producer phenotype [12]. Another 6 week study similarly showed no differential responses to isoflavone plus soy protein (107 mg as aglycone + 25 mg protein) between postmenopausal equol producers

(n=10) and non-equol producers (n=18) on endothelial function (assessed by FMD). However, this intervention showed significantly better vascular reactivity than that after placebo intake (total milk protein), to similar extent in both equol and non-equol producers [215]. Yet, it is worth to note that the observed number of equol producers was small (n=10).

In our study, it is difficult to explain the significant better improvements in the equol producers on PWV, diastolic blood pressure, mean arterial blood pressure, but not on systolic blood pressure and to what extent these observed hemodynamic changes were related to the decreased arterial stiffness assessed from PWV. These findings may be related to the fact that the equol producer group had a mean PWV of 8.6 m/s at baseline (Table 2.3) which falls within the normal expected range for this age group [225]. Subsequently, one could hypothesise that the reflected pulse waves in these subjects might be returning close to the diastole and therefore, the decreased diastolic blood pressure might be related to the decreased PWV, while the systolic blood pressure was not affected. Consistent with our findings, it was previously shown that arterial stiffness indices, such as augmentation index and PWV, were strongly correlated to diastolic blood pressure, but not to systolic blood pressure. However this study sample was limited to young males [226] and further research is needed on other population groups such as our study sample, type 2 diabetic postmenopausal women.

The plausible underlying biological mechanism of the significant better hemodynamic and arterial stiffness in the equol producers might be related to higher vaso-relaxant activity of equol compared to daidzein, as it has been shown by *in vitro* work [23]. Previously, it has also been shown that infusion of dehydroequol has increased blood flow in forearm resistance arteries in humans and this effect diminished by inhibition of eNOS [156]. Most studies suggest that the underlying mechanisms of vascular bioactivity of equol involve phosphorylation of eNOS and thus increasing bio-available NO [94, 155]. However, the current study did not find increased levels of plasma NO metabolites in equol producers, compared to non-equol producers after 12 months of flavonoid consumption. However, these results could have been conflicted by the limitations of the griess reagent method for analysis of NO derivatives (nitrite and nitrate). Specifically, this method is not considered sensitive enough to measure nitrite (NO^{-2}) levels in biological samples and nitrate (NO^{-3}) levels could have been influenced by dietary factors [227], in particular because this trial did not restricted their intake of nitrate.

ET-1 is an another important biomarker and predominant vasoconstrictor peptide that constricts vascular smooth muscle and is responsible along with NO for vascular tone in the endothelium and therefore NO/ET-1 ratio is suggested as a marker of coronary artery disease development [228, 229]. Previous short term RCT of soy enriched cereal bar has showed significant increases in plasma NO/ET-1 ratio in healthy postmenopausal women. On the contrary, our analysis did not show significant changes on NO/ET-1, but the hypotensive effects observed after 12 months intervention might be explained in part by a trend towards a decreased plasma ET-1 (change from baseline, -0.08±0.22 pg/ml in equol producers, P= 0.09, Table 2.7). Other potential plausible underpinning mechanisms could be related to effects of isoflavone and equol on the vasodilator prostacyclin (PGI2) and/or platelet function [24]. *In vitro* work showed that daidzein and genistein induced increased PGI2 production [24, 96, 97] and equol inhibited platelet activation and aggregation that were induced by thromboxane 2 analogs [24].

An *in vitro* study has shown that equol treatment increased relaxation of endothelium in aortic and porcine pulmonary artery rings [155] in a dose-reponse manner [93]. Therefore, the bioactivity of equol *per se* could be related to the observed hemodynamic benefits in our study. This hypothesis can be tested by styding the effects of equol supplements intake on endothelial and hemodynamic functions in human studies and to our knowledge, the literature lacks such studies.

The urinary concentration of isoflavones significantly increased after 6 and 12 months of consumption of study intervention (P values<0.001 for all analytes). Our data did not show any equol producer cross-overs (i.e. their equol-producer status was altered), which agrees with the literature reviewing equol production as a permanent capability except during administration of antibiotic [17, 172, 230]. In contrast, some studies have reported significant cross-overs in equol producer phenotype over longer periods [191, 192, 231, 232]. It has been suggested that equol producer phenotype might significantly change and antibiotic use influences it inconsistently [191, 192].

Our results suggest that the positive associations between equol producer phenotype and the flavonoid intervention on hemodynamic function and arterial stiffness are more profound after long term exposures (12 months rather than 6 months). These findings might explain the epidemiological data on better profiles of cardiovascular risk factors, including

lower blood pressure and plasma lipids [25, 28] in equol producers compared to non-equol producers in Asian populations that have daily dietary intakes of high isoflavones.

Another interesting data was observed at baseline, where equol producers had significantly higher plasma levels of vasodilation markers NO and NO/ET-1 (P<0.01, 0.05, respectively), lower plasma levels of the vasoconstrictor marker ACE (P<0.05), and better profile (but not statistically significant) for almost all studied vascular markers than non-equol producers, Table 2.3. The literature lacks information about plausible explanation for association between equol producer phenotype and better vascular health independent of soy supplementation. It has been suggested that the gut bacterial flora might have an effect on the oestrogen metabolism [200]. Specifically, inter-individual differences in intestinal microflora species may contribute to variation in disease vulnerability through effects on metabolism and subsequent exposure to phytoestrogens. It might be postulated that the capacity to produce equol is a marker for other mechanisms that may have an effect on the vascular function. For instance, the equol producer phenotype has been studied in assessment of breast and prostate cancer risk [125].

Our analysis had strengths in its double-blinded randomised design, long duration of intervention, good compliance to intervention, reproducible and robust clinical methods such as ambulatory blood pressure that produces more representative data than blood pressure measured in clinics [233]. Some baseline characteristics of equol and non-equol producers showed no statistical difference, such as years at menopause, diabetes duration, percentage of women who used blood pressure medications, insulin, and past history of smoking. On the other hand, it is notable that equol producers showed non-significant better profiles for blood pressure, PWV, HOMA-IR, and fasting insulin and significant higher levels of baseline plasma NO, NO/ET-1, and ACE than non-equol producers, which might have masked beneficial effects of flavonoid intake in equol producers compared to non-equol producers.

This trial was not designed to determine the responsiveness to soy interventions in equol producers and a formal sample size calculation was not considered *a priori*. For instance, our data of PWV included only 7 equol producers, and there was not a standard method of soy challenge to properly evaluate equol producer phenotype prior to intervention in the subjects allocated to the control group. The soy challenge is important because soy intakes are not part of the habitual diet of the study general population. Using standard soy

challenge would have enabled us to include the participants in the placebo (control) group in the analysis and investigate the effects of the flavonoid intervention in equol and non-equol producers. We would have also had a larger sample size to study the baseline differences in vascular function between equol and non-equol producers independent of isoflavone intake.

Additional weakness of this analysis lie in the supplementation that included flavan-3-ols in addition to isoflavones which also suggest effects of flavan-3-ols supplementation that might have masked the differential responsiveness of equol producers to isoflavones. The lack of a controlled low-nitrate diet constitutes a limitation of assessment of NO activity measured as plasma nitrite/ nitrate as it is stated that the circulatory concentration of these NO metabolites (nitrate/ nitrite) reflects constitutive eNOS activity *in vivo*, but only under standardized low-nitrate diet prior study assessment [234].

2.6 Conclusion

In summary, our retrospective analysis showed that equol producers had improved hemodynamic markers and arterial stiffness, particularly reduced diastolic BP, MAP and central PWV in comparison to non-equol producers at increased risk of developing CVD after 12 months of isoflavone containing food intake. These effects were not apparent after 6 months intervention, which might reflect the importance of longer-term chronic interventions. However, the observed hemodynamic benefits in equol producers could not be imputed to the 12 month flavonoid consumption because the control arm was not included in the analysis (due to the absence of soy challenge prior to the intervention). Consequently, studies with prospective analysis for equol producer phenotype would provide better research tool that follows hypothesis-driven controlled design, has powered sample size and they should follow a standard method for identification of equol producer phenotype independent of analysis procedure and daidzein intake.

Chapter 3 The effect of soy isoflavone intake on vascular health – potential differential effects in prospectively recruited equol and non-equol producers

3.1 Introduction

There is growing research on the potential health benefits of the equol producer phenotype, which is apparent in 20-30% of Western and 50-60% of Asian populations [141]. In particular, the ability to produce equol has been associated with a reduction in cardiovascular risk factors. In observational studies on Asian populations exposed to isoflavones through their habitual soy intake, equol producers had a more favourable blood pressure profile [28], significantly improved plasma lipids [25, 28], and inflammatory biomarkers [25, 26] compared to non-equol producers. In two intervention trials on Caucasian postmenopausal women, equol producers had lower arterial stiffness, improved endothelial function, lower blood pressure [165, 166] and higher circulating concentrations of the most potent vasodilator nitric oxide (NO) [220]. In support of these findings, equol has also been shown to possess higher bioavailability with a longer half-life and higher circulating concentrations than the major isoflavones daidzein, genistein and glycitein. *In vivo* [19] and *in vitro* studies have also shown that equol has a greater antioxidant [20-22] and vasodilator activity [23, 24] compared to its parent compound, daidzein.

Retrospective analysis of randomised controlled trials (RCT) which have intervened with soy-based foods, or supplements, for between 4 weeks to 12 months [6] [15, 220], have shown significant reductions in risk markers of cardiovascular disease (CVD) in equol producers. In particular, effects on blood pressure [15, 220], arterial stiffness [220] and endothelial function [6] have been reported.

Despite these promising data, there is limited evidence from isoflavone RCTs to explain the potentially beneficial associations between equol producer capacity and vascular function. Almost all previous research on the role of equol producer phenotype on the vascular health is derived from observational studies or retrospective analyses of RCTs. To our knowledge, only one published study to date has prospectively recruited equol

producing versus non-equol producing postmenopausal women [166]. These data suggested that hemodynamic function and arterial stiffness were not affected by 8 week exposure to isoflavone intake (52 g of soy protein drink containing 112 mg of isoflavones). However, this lack of effect may have been due to at least two factors; I) significant differences in vascular function which were observed at baseline between equol and non-equol producers and II) the use of the oestrogen replacement therapy Tibolone by the study population, that is known to affect vascular function [235]. It is also notable that most of the previous RCTs have been limited to postmenopausal equol producers and there is a gap in studying equol producer males. It has also been suggested that the stage of disease development may be related to the bioactive effects of isoflavones; one meta-analysis has shown that chronic isoflavone intake was associated with greater improvements in endothelial function in subjects with initial endothelial dysfunction (i.e. those at elevated CVD risk) [2].

In terms of study design, an important factor, related to bioavailability, is the time of collection of vascular measures and biological samples. It has been suggested that the vasodilation induced by isoflavones could be positively correlated to the bioavailable levels of flavonoids [236-239], isoflavones [9] and potentially their bioactive metabolite equol. Therefore, vascular measurements are likely to be related to the peak levels of isoflavones, highlighting the importance of investigating the effects of isoflavone consumption on vascular function at peak concentrations of equol.

There is also some evidence to suggest that food matrix may impact on the absorption of isoflavones and that isolated forms may be more bioactive [85, 86, 213]; studies are now required to further understand the importance of food matrix on vascular function and to determine the effects of isoflavone intake in prospectively recruited equol producers.

In this chapter, we aimed primarily to examine the acute effects of isoflavone intake on vascular function in prospectively recruited equol and non-equol producer males at moderately elevated risk of heart disease and secondarily to investigate any differential effects of isoflavone matrix on the vascular response.

Particularly, we hypothesised that a single dose of isoflavone intake would induce greater improvements on vascular function related measurements in equol producers than in non-

equol producers, at the anticipated peak times of plasma isoflavones (6 h post intervention) and equol (24 h post intervention).

Our primary outcome measures were endothelial function (Reactive hyperaemia index (RHI)) and arterial stiffness measurements (assessed by Pulse wave velocity (PWV)), while blood pressure assessments and Pulse wave analysis (PWA) were secondary outcome measures.

3.2 Methods

3.2.1 Study design

28 healthy subjects were recruited and completed our double blinded randomised controlled acute isoflavone intervention trial. Written informed consent was obtained from all subjects before participation. The national research ethics committee (REC) approved the study (REC number: 11/EE/0233). The study was conducted at the Clinical Research & Trials Unit (CRTU) at the University of East Anglia (United Kingdom) and followed the principles of the Declaration of Helsinki. It was registered at www.clinicaltrials.gov, NCT01530893 (note: this study was part of a bigger trial - the FASTCHECK study) consisting of different acute studies on isoflavones, flavanones and anthocyanins). Scientist contribution and all trial documents (i.e. poster, participant information sheet, health and lifestyle questionnaire, and consent form) are provided in Appendix C, page 183.

Sample size

Our aim was to prospectively recruit 14 equol producers and 14 non-equol producers. Our sample size calculations included 80% statistical power at 5% significance levels. It assumed expected mean difference±SD of 0.5±0.4 for RHI (endothelial function assessment) [46] and 1.5±1.2 m/sec for PWV [240] (arterial stiffness assessment), respectively. According to these calculations, we required 12 participants per group (Sample size= 16 (σ^2/ Δ^2) +1, σ= standard deviation of variable as estimated, Δ= expected

mean difference, [241]) to complete study. Assuming 15% drop out, we recruited 14 subjects per group. These calculations were based on clinical treatments/studies (not nutrition related interventions) and thus the sample size might be under-powered. Nevertheless, it was deemed appropriate to carry out the study due to the limited study budget and the fact that similar sample sizes (n=10) were used in previous acute cross over flavonoid RCTs showing beneficial effects on vascular function [236-239].

Inclusion and exclusion criteria

The study included men aged 50-75 years, with a 10-year risk of CVD of 10-20% (based on age, lipid profile and systolic blood pressure [36]) and excluded those who were smokers/ ex-smokers for at least 3 months prior to study start, those clinically diagnosed with vascular disease, diabetes, cancer, hepatic, renal, digestive, haematological, neurological and/ or thyroid disorders, those prescribed anti-hypertensive, statin, antibiotic medications or with planned vaccinations, those consuming flavonoid supplements (within 3 months prior to the study), or having a soy allergy.

Recruitment and eligibility

Participants were recruited through local advertisements (i.e. poster and electronic mail out) including Norwich research park (NRP), local media including newspapers, and radio and targeting potential local groups/ societies (e.g. Parish councils), advertising in public events such as science fairs, and through GP practices (posters and hardcopy mail-outs).

The primary eligibility of those showing interest in participating in the study was assessed after completion of a health and lifestyle questionnaire (H&LQ). Eligible subjects then attended a consent visit where they were provided with three soy bars and a urine collection pot to assess their equol producer phenotype (described in more details in 3.2.5). Participants, who had provided consent, attended a clinical screening visit during which blood pressure (BP), pulse, anthropometric measurements and fasted blood samples were collected. Age, systolic BP, and ratio of total cholesterol to HDL-cholesterol were

incorporated into the Cardiac risk assessor (by Joint British Societies' Cardiac Risk Assessor Computer Program) to assess the subject 10 year CVD risk.

Soy challenge for identifying equol producers

Participants undertook a soy challenge to determine their ability to produce equol. A standard method was followed; each volunteer consumed a 63 g soy protein bar providing 161 mg soy isoflavones as aglycone equivalents; 63.5 mg daidzein, 63.9 mg genistein and 33.7 mg glycitein (commercial soy bars, Revival. CO, Kernersville, USA) for 3 consecutive days and collected a mid-stream urine sample (50 - 80 ml) from the first urine voided on the 4th day. Participants were provided with the necessary 3 soy bars, instructions for soy bar intake, urine collection container at the end of the consent visit and returned the urine sample when attending the clinical screen. A questionnaire was administered to determine whether the soy bars were consumed. Urinary isoflavones are stable at room temperature [206]. On receipt, the urine was frozen at -80°C until quantification of isoflavone concentrations.

Blinding and randomisation

Samples from eligible volunteers were quantified for their urinary concentrations of equol and daidzein by HPLC-MS/MS (analysis was done externally by HFL sport science, Fordham, Cambridgeshire, UK), following in-house developed method [150]. The results were sent to an independent researcher at UEA (who was not involved in the study conduction or outcome analysis) to identify equol producers as having urinary log10 S-equol/ daidzein of greater than -1.75 [141]. All researchers involved in conducting the trial and participants taking part were blinded to the intervention order and equol producer phenotype until the end of analysis stage.

A UEA staff member who was not involved in the study conduction or outcome analysis assigned labels A, B, and C to the three study interventions and this information was stored in a protected locked place. The intervention order were randomised using the random

function in MS office Excel to create the three random intervention labels A, B, and C (interventions are described in Table 3.1, page 90) and the intervention order was provided to the research nurse giving the intervention foods who was not involved with the data collection/ analysis.

Intervention allocation

14 equol-producers were identified and then 14 non-equol producers were matched with the identified equol producers for factors that might majorly affect vascular function including body mass index (BMI) and blood pressure. Two volunteers dropped out after the first visit and were replaced by two additional participants (Figure 3.8). Following a randomised crossover design, groups attended 3 assessment visits (isoflavone rich food assessment, isoflavone supplement assessment, and one placebo assessment). A wash out period of 1 week between each acute study day was selected based on other acute phase studies with flavonoids and polyphenols [242-246] and bioavailability studies on isoflavones [175, 213, 242-246] (Figure 3.1).
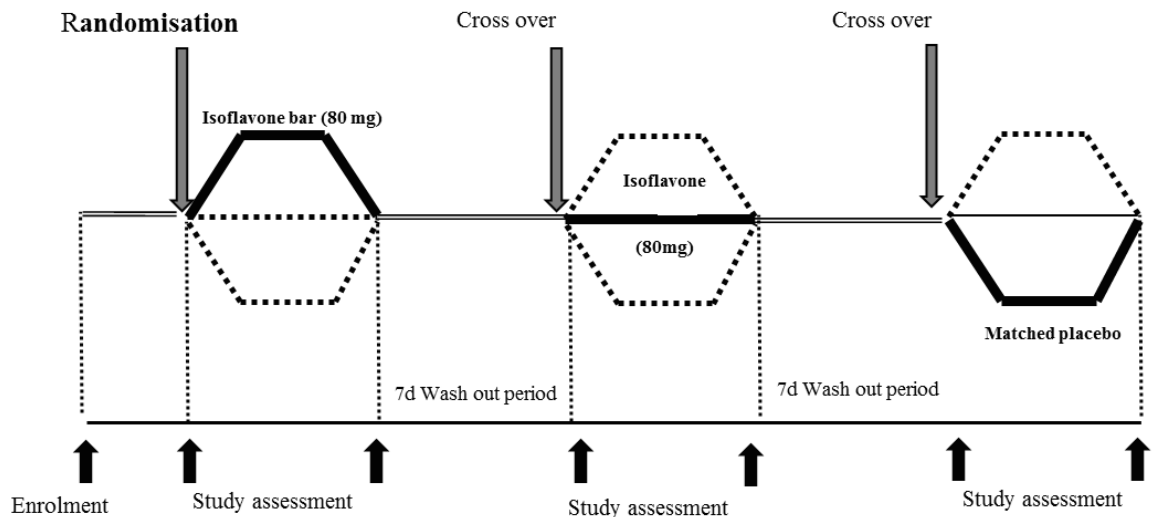
Figure 3.1 Randomisation to study interventions
This figure describes the study design of cross-over randomisation to isoflavone supplement, isoflavone enriched cereal bar or placebo separated by at least 7 d in a randomised order. ***BP, Blood pressure; CVD, Cardiovascular disease; EP, Equol producers.***

Intervention dose

An isoflavone dose of 80 mg was chosen because previous studies have reported enhanced endothelial function following consumption between 50 and 100 mg isoflavone (as aglycone units) per day [9, 63, 81, 220] and there have been no reported side effects for this intake.

The isoflavone interventions included an:

- isoflavone enriched protein bar (a 40 g soy protein bar, providing 80 mg aglycone equivalents of total isoflavone, prepared from SoyLife extract (40%) which has a typical soy germ ratio of genistein : daidzein : glycitein which is 15 : 50 : 35).
- isoflavone supplement (matched for isoflavone content, also containing 80 mg aglycone equivalents of total isoflavone, prepared from SoyLife extract (40%) which has a typical soy germ ratio of genistein : daidzein : glycitein which is 15 : 50 : 35).
- placebo supplement (matched for colour and containing carboxy-methylated cellulose (CMC)) and placebo bar (matched for micro and macronutrient content, flavour and texture) (Table 3.1).

Table 3.1 The content of the study's interventions

Food based intervention	*Supplement intervention*	*Placebo intervention*
Isoflavone enriched bar (80 mg aglycone equivalents of daidzein, genistein and glycitein) + **Placebo supplement**	**Placebo bar** (no isoflavone) + **Isoflavone supplement** (80 mg aglycone equivalents of daidzein and genistein and glycitein)	**Placebo bar** (no isoflavone) + **Placebo supplement**

Lifestyle and dietary control

In order to control for potential confounders (e.g. dietary intake, alcohol consumption and physical activity) that might interfere and affect our analyses, participants were asked to:

1. maintain their habitual lifestyle during the study (e.g. alcohol and dietary intake, physical activity)

2. stop taking flavonoid containing dietary supplements 1 month prior to start of trial

3. maintain typical intake of non-flavonoid supplements (i.e. fish oils, multivitamins) during study duration

4. follow a low flavonoid diet for three days prior to commencing each study day
Phenol explorer 2.0 database was used to identify foods that contain one or more of 4 flavonoid subclasses (flavanols, anthocyanins, flavanones and isoflavones) that might have vascular and hemodynamic effects [13, 237, 239]. For each food, the portion size, which provided 10% of the intervention dose of any of the four flavonoids above, was calculated. Participants were asked to avoid the consumption of foods where one portion provided ≥10% of the intervention dose, and to restrict their intake of foods, where eating ≤12 portions of these foods provided ≥10% of the intervention dose, to one portion for the three days before study assessment. Alternative foods were suggested using dietary restrictions tables, for restricted food including foods if consumed more 12 portions provide 10%. In addition, we also asked participants to restrict the intake of food that contained other compounds [242] which, in previous publications, have been shown to have vascular effects i.e. such as oily fish.

5. refrain from strenuous exercise for 48 hours before each study assessment

6. abstain from alcohol consumption, and caffeinated products (including decaf coffee/ tea due to their flavonoid content) for 24 hours before each study assessment

7. avoid nitrate and nitrite rich foods such as leafy green vegetables, broccoli, radishes, carrots, sausages, other processed and cured meats and bottled waters containing high levels of nitrate. Consumption of these foods elevate circulating NO metabolites concentrations [247].

8. eat a standardised evening meal consisting of a ready meal of low flavonoid and low nitrate/ nitrite content and bottled water (Buxton; containing <0.1mg/L nitrate) in order

to control for any effect of recent background dietary intake on the biological assessments

9. fast for 10 h prior to the clinical visit and advised to consume only the provided bottled water for 10 h prior to the assessment visit.

Analysis of 24 dietary recalls

Compliance to the dietary restrictions was monitored by 24hr food recall conducted by telephone 1-2 days prior to the assessment visit. In addition, 24h dietary recalls were used to calculate daily intakes of macronutrients. All data were analysed using software for nutritional analysis of dietary intakes (WISP version 4.0, Tinuviel software, Anglesey, UK).

The food coding procedure was conducted following a standardised operating procedure conducted by two scientists and 10% of total food entries were re-entered to check accuracy of data analysis. Analysed data were reviewed for data below or above 3 Standard deviation (±3SD) of average daily intakes of macronutrients. Food portion sizes were decided based on food standard agency (FSA), 3rd edition [248].

For unusual food entries, a decision tree was made to look for similar food items with best matching macronutrient percentages of energy>fat>protein>fibre content and all decisions made were justified, kept in a log and reviewed by two additional scientists.

Assessment visits schedule

Subjects underwent a baseline assessment i.e. anthropometric measurements (height and weight), blood pressure measurement, blood sampling, arterial stiffness and endothelial function measurements (Figure 3.2). All measurements were taken in a temperature controlled (22-24°C) and dimly lit clinical room after at least a 15 minute resting period in a supine position.

After the baseline measurements were completed, the intervention was administered by a research nurse (who was not involved in the blinding and randomisation procedure). Blood sampling and study assessment measurements were repeated at 2, 6 and 24 h after intervention administration, which corresponded to the anticipated peak plasma

concentration of the isoflavones of interest (i.e. daidzein, genistein, glycitein and equol) from published literature [175, 201].

A standard lunch comprising of two cheese sandwiches and a vanilla flavoured yogurt (consisting of 31 g of protein, 96 g of carbohydrates and 27 g of fat providing 753 kcal) was administered after the 2 h assessment was finished. Participants were also provided with a standard pasta dinner (Brake Bros Ltd, Kent, UK) and asked to fast for at least 10 h before returning to the facility for the 24 h study assessment (Figure 3.2).
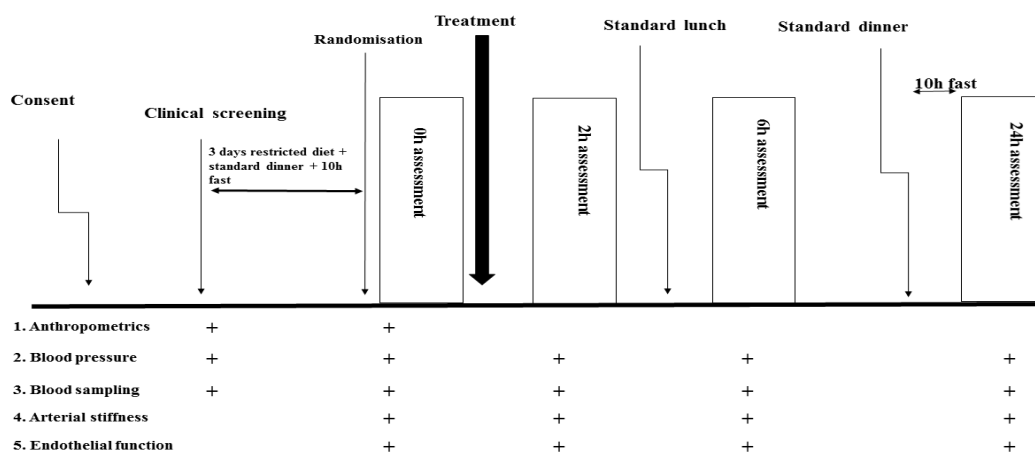
Figure 3.2 Schedule of baseline and study assessments and time scale for visits
Top panel represents timeline and schedule of each study day. Bottom panel represents + collection of blood samples and/ or clinical measurements that were collected at baseline, 2, 6 and 24 hours after intervention administration. *BP, Blood pressure.*

3.2.2 Endpoints

For each time point clinical vascular (CV) markers of endothelial function, arterial stiffness and blood pressure were assessed and blood sampling was conducted in an identical manner at each time point (0, 2, 6 and 24 h) for the analysis of isoflavone concentrations. Prior to the trial start, there were many training and test sessions for all staff involved in the conduction of the study measurements including anthropometrics, blood pressure, arterial stiffness (PWV, and PWA assessed by Vicorder) and endothelial function (RHI assessed by EndoPAT) assessments. In addition, there were training sessions provided by the manufacturer (for Vicorder) and dry runs of the study assessment. However, the intra-observer coefficient of variation in reproducibility tests were not collected.

Blood pressure measurements

Ambulatory blood pressure measurements were obtained over a 24 h observation period using a non-invasive ambulatory monitor (Spacelabs SL902072) approved by the British Society of Hypertension. The monitor automatically inflated every 10 minutes during the daytime and once/ hour between 22:00 pm and 6:00 am to minimally disturb participants at night. Participants were asked to keep their arm relaxed and still down by their side when the cuff inflated until deflation. An appropriately sized cuff (pneumatic bag was 20% wider than the diameter of the upper arm; standard adult (24-32 cm), large adult (32-42 cm) or extra-large adult (42-55 cm)) was placed on the non-dominant arm at the heart level. One off measurement was made at each start of baseline assessment at supine position. The monitor was then attached during the measurement gaps between baseline and 2 h assessments, 2 h and 6 h assessments, and 6 h and 24 h assessments. However, it was detached during CV measurements. Blood pressure measurements included systolic BP, diastolic BP and MAP. Participants were provided with a diary sheet to record any unusual events, their sleeping hours, error messages and times when they removed the ambulatory device. In addition, before each assessment battery, systolic and diastolic BPs were measured in triplicate using a validated Omron device 705IT (Omron Healthcare Co., Kyoto, Japan).

Arterial stiffness

Measurements of arterial stiffness were conducted by carotid- femoral pulse wave velocity (c-f PWV) and augmentation index (AI) using the Vicorder device (Skidmore Medical Ltd, Bristol, UK).

Pulse wave velocity (PWV)

Measurement of PWV is a non-invasive, researcher independent and reproducible method [55]. The aortic (carotid - femoral) PWV is considered the gold standard for assessment of arterial stiffness and greater arterial stiffness is associated with an increased cardiovascular mortality in adults with hypertension and coronary heart disease. PWV has been recommended for the assessment of individual CVD risk and therapy by the European Society of Hypertension [35] [55].

PWV was measured using the Vicorder equipment, which allowed simultaneous oscillometric measurements of the carotid and femoral pulse waves [249, 250]. C-f PWV was assessed by placing BP cuff around the neck at the carotid artery level and placing another BP cuff around the upper thigh at the femoral artery level. Good quality waveforms were recorded simultaneously for 7 seconds by a volume displacement technique. The upstroke to upstroke transit time was calculated using an in-built algorithmic equation. Path length was defined as the distance from the middle of the carotid cuff to the middle of the femoral cuff. PWV (m/s) was calculated from the ratio of the estimated distance travelled by the pulse (ΔL in meters) during the pulse transit time over the measured pulse transit time (ΔT in seconds) (Figure 3.3).
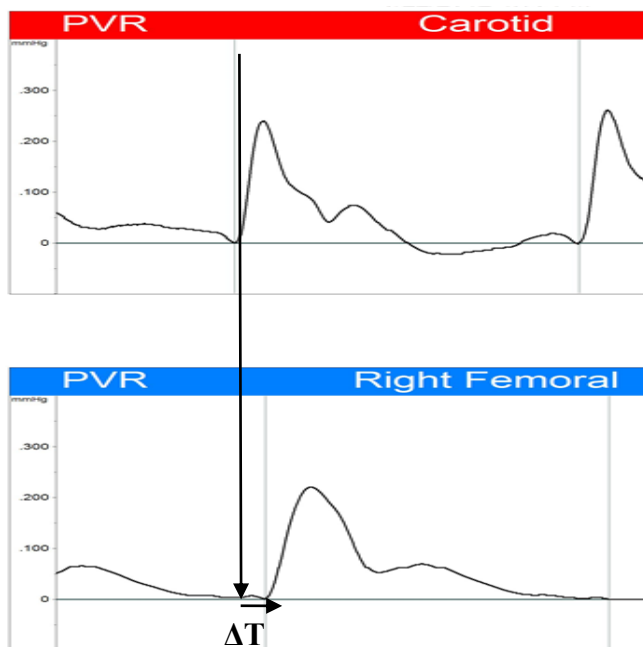
Figure 3.3 Carotid femoral pulse wave (PWV) output using Vicorder
An example of PWV from collected data during the study. The upper wave represents the pulse wave at the carotid artery and the lower one represents the pulse wave at the femoral artery. ΔT is the pulse transit time calculated from the time delay between the two pulse waves.

Pulse wave analysis (PWA)

PWA studies the arterial pressure wave and consists of the wave produced by left-ventricular contraction and the reflected wave from the peripheral arteries [55].

PWA was undertaken using the Vicorder by placing a cuff around the brachial artery to estimate AI and cardiac output (CO). Aortic waveform and central BP were then calculated using the inbuilt brachial-to-aortic transfer function. The AI was calculated from difference in amplitude between the first and second systolic peaks divided by pulse pressure $\times$ 100, while pulse pressure is the difference between aortic systolic and diastolic BP. The cardiac output (CO) was calculated by multiplying the stroke volume by heart rate (Figure 3.4).
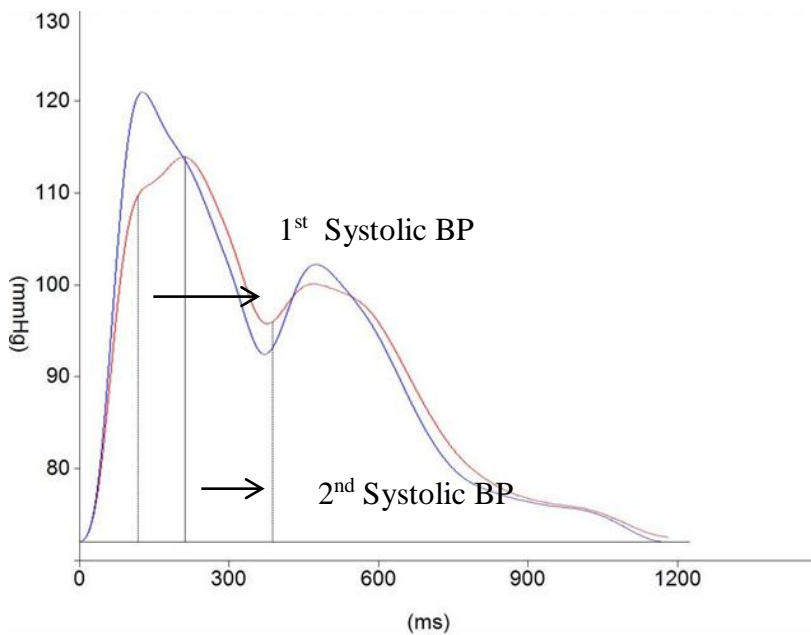
Figure 3.4 Pulse wave analysis (PWA) output using Vicorder
An example of PWA from collected data during the study. PWA represent estimate the aortic pulse wave (red) from the pulse wave at the brachial artery (blue). AI= augmentation pressure (difference the first and second systolic peaks)/ pulse pressure $\times$ 100, and CO= stroke volume x heart rate. **BP,** Blood pressure.

Endothelial function

Endothelial function was assessed using a non-invasive method, whereby a beat-to-beat plethysmographic recording of peripheral arterial tone (PAT) [46, 239] was assessed using finger cuff s by the EndoPAT 2000 equipment (Itamar Medical Ltd, Israel). Each measurement included 5 min baseline, 5 min occlusion (using a minimum 60 mmHg above the systolic blood pressure) and 5 minutes post-occlusion recordings [46, 239] (Figure 3.5). Reactive hyperaemia (RHI) was induced after release of brachial artery occlusion and the other arm was used as control for any confounding effects of the sympathetic system. RHI is calculated using an inbuilt automated algorithm (EndoPAT^{TM} 2000, software 3.5x). Consistently, all PAT measurements were conducted on one arm and the blood collection was taken from the other arm.

$$RHI = \frac{A}{B} / \frac{C}{D} \times \text{Baseline Correction Factor}$$
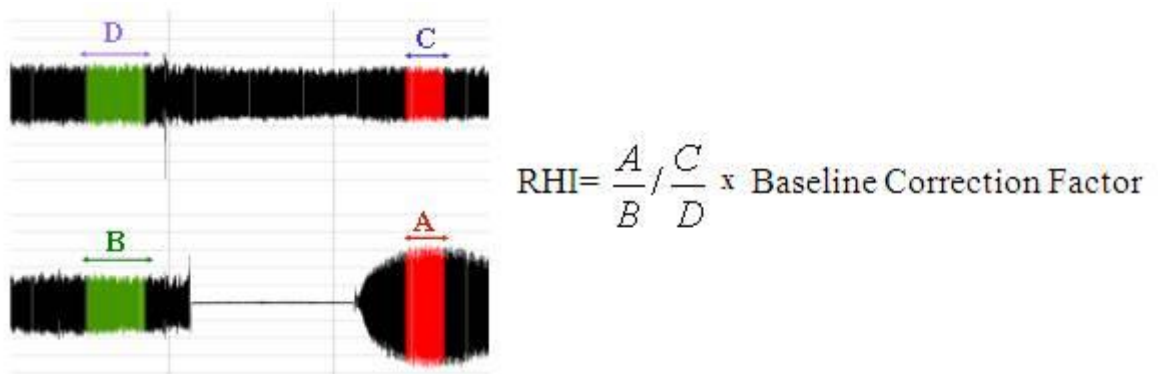
Figure 3.5 Reactive hyperaemia index (RHI) calculations by EndoPAT
A= Mean PAT amplitude between 90-150 seconds post occlusion, and B= Mean PAT amplitude from the baseline period of the assessed arm. C= Mean PAT amplitude between 90-150 seconds post occlusion and D=Mean PAT amplitude from the baseline period of the control arm. Figure reproduced from (http://www.itamar-medical.com/). **PAT**, *peripheral arterial tone;* **RHI**, *reactive hyperaemia index.*

3.2.3 Analysis of isoflavones in blood samples

Plasma concentrations of the isoflavone metabolites of daidzein, genistein, glycitein, and equol were analysed using LC-MS based on a previously developed method [150, 251] and details are described below. With the exception of the plasma samples collected at baseline, all samples were analysed individually. For each participant, the plasma samples

collected at baseline were pooled together before analysis. The baseline sample pooling was done to reduce the required time and consumables for analysis including enzyme hydrolysis, and solid phase extraction of study samples (which were done manually because the 96 well plate extraction was not feasible in our laboratory).

The pooling was done only to the baseline samples because these samples were expected to have negligible concentration of all isoflavones and equol (the participants were on a controlled diet). If any pooled sample had shown substantial isoflavone/equol levels, the individual samples would have been analysed again to identify the outlier. This was not the case and all baseline plasma samples showed negligible amounts of isoflavones (Table 3.8, page 112).

Chemicals

Daidzein, genistein, glycitein, taxifolin, β-glucuronidase type HP-5, and sulfatase type HP-1 from *Helix pomatia* were purchased from Sigma (Sigma-Aldrich Ltd, Dorset, UK). S-equol was purchased from Caymen chemical company (Cambridge Bioscience Ltd, Cambridge, UK). Stock standards were prepared at 50 mmol/L in dimethyl sulfoxide (DMSO), from which sub-aliquots were stored at -80°C to use when preparing mixture of standards.

Sample collection and preparation

Blood samples were collected in Sodium heparin tubes. Within 30 min, samples were centrifuged at 3300 g for 10 min at 4°C and plasma was stored in aliquots at -80°C until further analysis. Sample preparation for the analysis of isoflavones in plasma was performed following the method developed by [251]. Briefly, plasma (200 μL) was thawed before addition of 25 μL of each β-glucuronidase type HP-5 (100,000 U/mL) and sulfatase type HP-1 (1,000 U/mL) from *Helix pomatia*, 200 μL phosphate buffer (pH 5), and 1.2 μL of internal standard taxifolin (1 mg/mL in matched-matrix human serum). Isoflavone conjugates were hydrolysed by incubation at 37°C for 3 h. Thereafter, 200 μL cold methanol was added to each tube, tubes were mixed and left for 20 min at room temperature for equilibration of the two phases. Samples were then spun for at 12,000 rpm for 15 min

at 0°C and then 200 µL supernatant were transferred into solid phase extraction (SPE) cartridges (Strata-X[TM], 1 mL, 30 mg, Phenomenex) for extraction of aglycones. 700 µL of methanol, followed by 700 µL 30% methanol (aqueous) were used to condition the SPE cartridges as stated in the manufacturer's instructions. Samples were then loaded onto the cartridges and washed twice with 600 µL 40% methanol. Afterwards, the aglycones were eluted using 600 µL acetonitrile/ methanol (1:1 v:v). The eluates were dried in Speedvac® centrifugal evaporator (Savant, USA) and reconstituted in 100 µL 40% methanol and stored at −80°C until injection in the LC-MS. A blank was prepared and used to control for the levels of isoflavones found naturally in the β-glucuronidase and sulfatase. Calibration standards were prepared in matched-matrix of fasted human serum, which was extracted as described above, then spiked with known concentrations of isoflavones.

LC-MS conditions

Following hydrolysis and SPE, sample analysis was performed on an Agilent 1200 series HPLC coupled to an AB Sciex 3200 series Q-trap LC/MS/MS system, with samples injected onto a Kinetex pentafluorophenol (PFP) RP-HPLC column (2.6 µm, 100 x 4.6 mm) with PFP Security Guard cartridge (4 x 2.0 mm) from Phenomenex, UK. The column temperature was set at 37°C, and samples were stored in the autosampler at 4°C prior to injection. The system was controlled by Analyst software (v. 1.5, Applied Biosystems/MDS Sciex).

- ▪ ***For analysis of daidzein, genistein and glycitein***

Daidzein, genistein and glycitein were eluted from the column using a flow rate of 1 mL/min over a 22 minute gradient elution as detailed in Table 3.2. A binary solvent system was used consisting of 0.1% formic acid in MilliQ water (solvent A), and 0.1% formic acid in acetonitrile, HPLC grade (solvent B).

Table 3.2 Solvent gradient for analysis of genistein, daidzein, glycitein, and taxifolin

Time (min)	% A	%B
0	99.9	0.1
14	60	40
15	0	100
17	0	100
18	99.9	0.1
22	99.9	0.1

Quantification was conducted in negative mode using multiple reaction monitoring (MRM), with the compound parameters optimised for each standard with m/z of the parent (Q1) and daughter fragments (Q3) as shown in Table 3.3. The MS/MS source parameters included curtain gas 40 psi, ion spray voltage -4000V, temperature 700 °C, nebulizer gas 60 psi and auxiliary gas 60 psi.

Table 3.3 Optimised MRM compound parameters for the target analytes

Analyte	Q1 (m/z)	Q3 (m/z)	Time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
Genistein*	269	133	100	-100	-10	-35	-2
Genistein	269	107	100	-100	-10	-35	-2
Genistein	269	181	100	-100	-10	-35	-2
Genistein	269	224	100	-100	-10	-35	-2
Daidzein*	253	91	100	-97	-10	-51	-1.19
Daidzein	253	133	100	-97	-10	-45	-1.19
Daidzein	253	208	100	-97	-10	-40	-1.19
Daidzein	253	224	100	-97	-10	-35	-1.19
Glycitein	283	184	100	-80	-8	-55	-3.6
Glycitein	283	196	100	-80	-8	-55	-3.6
Glycitein	283	211	100	-80	-8	-55	-3.6
Glycitein	283	240	100	-80	-7	-35	-3.6
Glycitein*	283	268	100	-80	-7	-26	-3.6
Taxifolin	303	285	50	-50	-5	-17	0
Taxifolin	303	151	50	-50	-5	-30	0
Taxifolin*	303	125	50	-32	-5	-26	0

* refers the predominant precursor ions/MS^2 fragments used for single reaction monitoring (SRM) quantification. ***CE,*** *collision energy corresponding to MS2 fragment;* ***CXP,*** *collision cell exit potential;* ***DP,*** *declustering potential;* ***EP,*** *entrance potential;* ***MS***, *Millisecond****; Q1,*** *precursor ion;* ***Q3,*** *MS^2 fragments;* ***V,*** *Volts.*
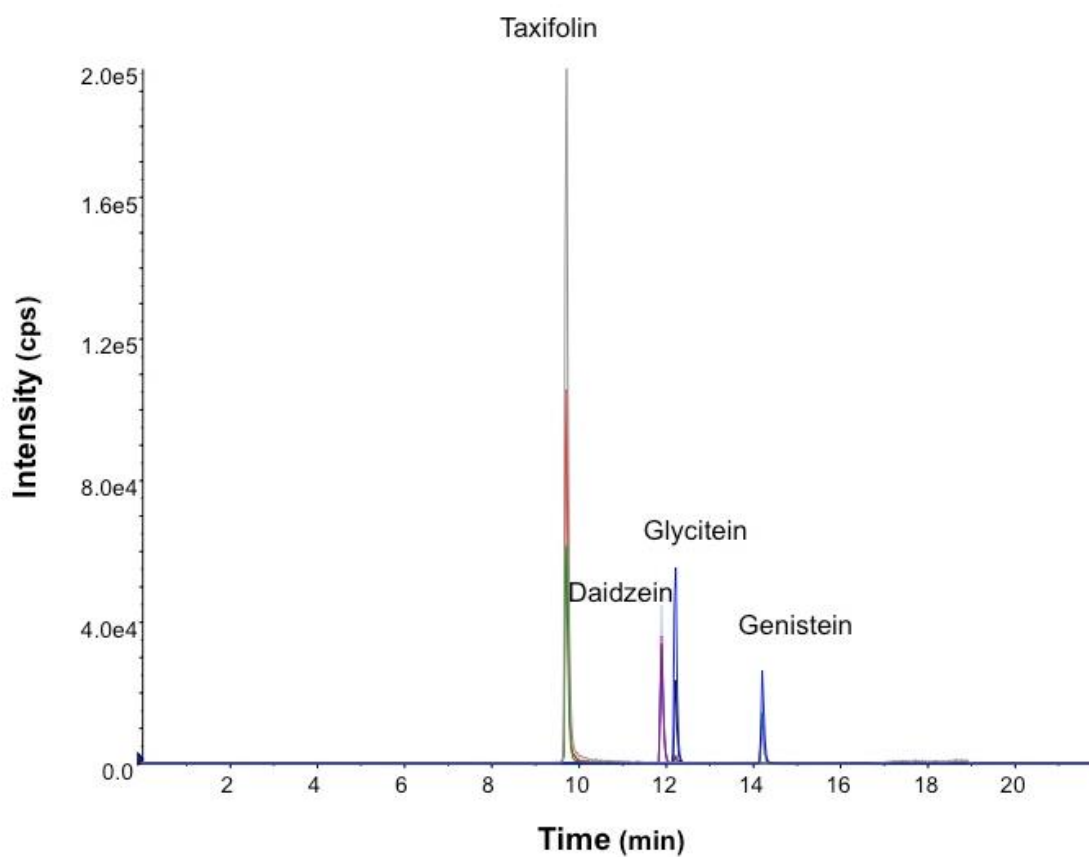
Figure 3.6 Multiple reaction monitoring (MRM) chromatograms of major isoflavones

Standards of daidzein, genistein, and glycitein were spiked in extracted (SPE) fasted serum at 5 μM.

- *For analysis of S-equol*

The method described previously was found to be unsuitable for the analysis of S-equol due to its poor ionisation in acidic conditions (pK$_a$ of S-equol = 9.94), which were required for optimum ionisation of the other analytes. Figure 3.7 illustrates the improvement in the method sensitivity when S-equol was eluted in the absence of any acid modifier within the mobile phase. Therefore, S-equol was eluted using the same conditions as described previously, but with a mobile phase consisting of MilliQ water (solvent A) and acetonitrile (solvent B) and the gradient as detailed in Table 3.4

Table 3.4 Solvent gradient for analysis of S-equol

Time (min)	% A	%B
0	75	25
5	0	100
7	0	100
8	75	25
10	75	25

Quantification of S-equol was conducted in a negative mode, using multiple reaction monitoring (MRM), with the compound parameters optimised for each standard with m/z of the parent (Q1) and daughter fragments (Q3) as shown in Table 3.5. The MS/MS source parameters were as described previously.

Table 3.5 MS compound parameters for analysis of S-equol

Analyte	Q1 (m/z)	Q3 (m/z)	Time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
S-equol	241	93	100	-63	-2	-42	-1
S-equol*	241	119	100	-63	-2	-34	-1
S-equol	241	121	100	-63	-2	-20	-1
S-equol	241	135	100	-63	-2	-26	-1
Taxifolin	303	285	100	-50	-5	-17	0
Taxifolin	303	151	100	-50	-5	-30	0
Taxifolin*	303	125	100	-32	-5	-26	0

Dwell time of 100 ms was constant across all transitions. * refers the predominant precursor ions/MS2 fragments used for single reaction monitoring (SRM) quantification. *CE, Collision energy corresponding to MS2 fragment; CXP, Collision cell exit potential; DP, Declustering potential; EP, Entrance potential*; **MS**, *Millisecond;* **Q1,** *precursor ion;* **Q3,** *MS^2 fragments;* **V,** *Volts.*

A)
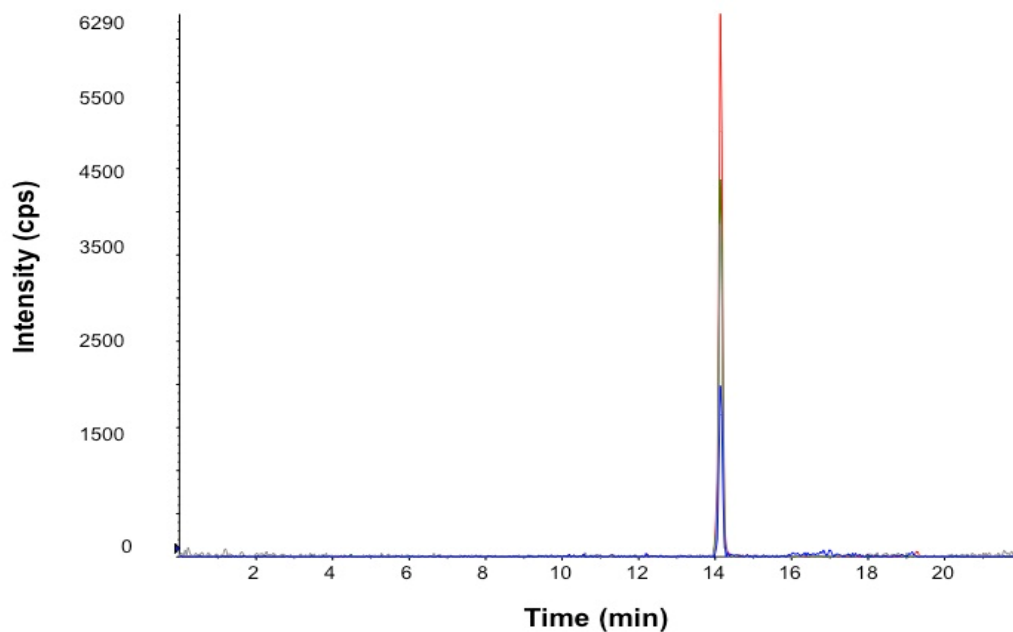
B)
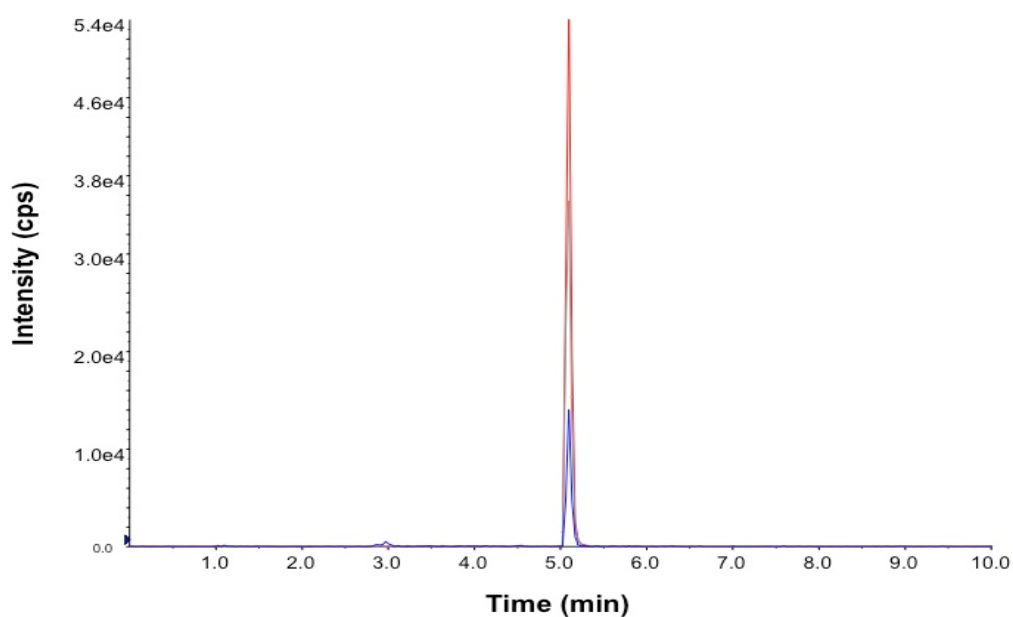
Figure 3.7 Multiple reaction monitoring (MRM) chromatograms showing the ion pairs used for quantification of S-equol

S-equol standard spiked in extracted (SPE) fasted serum. **A)** shows ionisation of S-equol (50 μM) in 0.1% formic acid in MilliQ water (solvent A), and 0.1% formic acid in acetonitrile (solvent B). **B)** shows ionisation of S-equol (5 μM) in MilliQ water (solvent A) and acetonitrile (solvent B).

Method validation

Analytes were quantified using Analyst software (v. 1.5, Applied Biosystems/MDS Sciex). Their identities were confirmed by retention time (using standards) and three ion transitions. The HPLC-ESI-MS/MS method was validated for linearity and precision across all analytes, based on the United States Food and Drug Administration's (U.S. FDA) guidelines for analytical method validation. Six point standard curves ranging from 0.039 to 5 μmol/L were constructed. The inter-assay and intra-assay precisions had overall means of 12% and 11%, respectively (with a range between 6.7% to 18.0%; data were calculated from 4 standard curves and quality controls that were analysed regularly) and linearity of the standard curves was established as $R^2 = 0.9916\text{-}0.9999$ ($CV\% = 12\%$) across all analytes and for both methods. The recovery (extraction efficiency) of the method was evaluated by comparing the analytical results for samples spiked with the analytes prior to extraction, to samples spiked with the analytes post extraction. The extraction efficiencies for the analytes of interest ranged from 81% to 92% (prepared in triplicate). The limit of detection of the method were established by calculating the concentration of the analyte yielding a signal to noise ratio of 3:1, and were established as 0.003 μmol/L for genistein, 0.001μmol/L for daidzein and glycitein, and 0.002 μmol/L for S-equol.

3.2.4 Statistical analysis

All data are presented as mean±standard error of mean (SEM). Results were considered statistically significant at $P < 0.05$. Only volunteers who completed all interventions were included in the analysis. Statistical analyses were performed using SPSS software (version 22). Primary outcomes were the change in endothelial function (RHI) and arterial stiffness (PWV) following isoflavone intervention intake in equol and non-equol producers. Secondary outcomes included effects of interventions on BP, AI and biological markers of CVD risk.

Before start of analysis, a statistical plan was prepared. During the statistical analysis, we were blinded to treatment and equol producer phenotype. For each variable/outcome, outliers (mean±3.5 SD) have been identified and then the case report forms were reviewed for any unusual comments/errors that could explain the outlier result. There were no unusual errors or comments that could explain the outliers and thus all results were

included in the analysis. Shapiro-Wilk test was used to assess normality of data. Levene's test and Mauchly's test were applied to check equality of variances for use of univariate analysis and homogeneity of variance for use of repeated measures analysis of variance (ANOVA), respectively. Baseline characteristic health data were evaluated for statistical differences using Student's independent t test. In addition, baseline data for outcome variables were evaluated for statistical differences between the intervention/ equol producer groups using univariate analysis.

Given the cross-over design of our study in prospectively recruited equol and non-equol producers, the most suitable statistical model is mixed general linear model with repeated measures analysis [252].

First, we conducted our analysis following a repeated measures general linear model that included two within subject factors (treatment at three levels A, B, C and time point at 4 levels baseline, 2 h, 6 h and 24 h) and one between subject factor (equol producing status at 2 levels 1 and 2). The model investigated significant differences of outcomes at baseline, 2 h, 6 h, and 24 h after consuming A, B, C in equol producers and non-equol producers (by including interaction of treatment, time and equol producing status factors). Posthoc tests using Bonferroni correction were done to test pairwise comparisons between groups, time points and treatments and highlight where statistically significant differences occurred. There were no statistical differences for any variables/outcomes and it was difficult to interpret results due to the 4 time points included in the analysis.

Subsequently, we adopted the second option in the statistical plan, which was to separate the analysis based on time points, which enabled us to relate our results to the peak times of major isoflavones daidzein and genistein (outcomes measured at 6 h) or metabolite equol (outcomes measured at 24 h). Outcomes were analysed as changes from baseline to 2 h, 6 h or 24 h post-intervention. Three models were used to investigate significant differences in change from baseline to 2 h, 6 h or 24 h after consuming placebo, isoflavone supplement, or isoflavone rich cereal bar in equol and non-equol producers (by including interaction between intervention and equol producer phenotype factors). Each model included one within-subject factor (repeated ANOVA for intervention at 3 levels: placebo, isoflavone rich cereal bar and isoflavone supplement) and one between-subject factor (equol producer phenotype at two levels: equol vs. non-equol producers). Bonferroni correction was used to test post hoc comparisons and to highlight where statistically significant differences occurred.

Our statistical analyses included different possible confounding factors such as age, BMI, and treatment order. Including BMI and treatment order did not make a difference to the outcomes and it was decided to include only age in the model as it was statistically different between the two groups of equol and non-equol producers.

LCMS data was analysed using univariate analysis in equol and non-equol producers separately to investigate differences in plasma concentrations of daidzein, genistein, glycitein and S-equol following intakes of the three different interventions (placebo, isoflavone rich cereal bar and isoflavone supplement). Additional analyses were run for differences in concentrations between baseline and the different time points. Our plasma analyses revealed that circulating levels of isoflavones after consumption of the isoflavone-enriched cereal bar were not significantly different from circulating levels of isoflavones after consumption of placebo, suggesting that the purchased bars did not contain a sufficient amount of bioavailable isoflavones. Unfortunately, we did not independently test the isoflavone content of the bar prior to commencing the study. Therefore, we amended our statistical analysis on vascular bioactivity to include only data collected after placebo or isoflavone supplement intervention and all presented data are related to a comparison of these two arms of the study. For ambulatory blood pressure data, participant data with less than 70% of the expected number of recordings were excluded [253]. In addition, ambulatory blood pressure measurements were analysed as mean±SE of systolic blood pressure, diastolic blood pressure and mean arterial blood pressure collected at the following time intervals: 2 to 6 h (relating to significant plasma levels of isoflavones) and 8 to 24 h excluding night time data (relating to significant plasma levels of equol) post-intervention.

Spearman nonparametric test was used for bivariate correlations between plasma equol concentrations and the vascular endpoints because the plasma concentrations of equol were not normally distributed.

3.3 Results

3.3.1 Baseline characteristics of equol and non-equol producers

During the recruitment phase of the trial, 190 volunteers were screened, from which 42 participants were identified as eligible to take part in the study based on our inclusion/exclusion criteria. These participants undertook the soy challenge, and 14 (33%) were selected as equol producers and were matched to an equal number of non-equol producers based mainly on criteria related to our primary vascular endpoints, BMI and blood pressure levels (non-equol producers: n = 14 and equol producers: n=14, Figure 3.8).
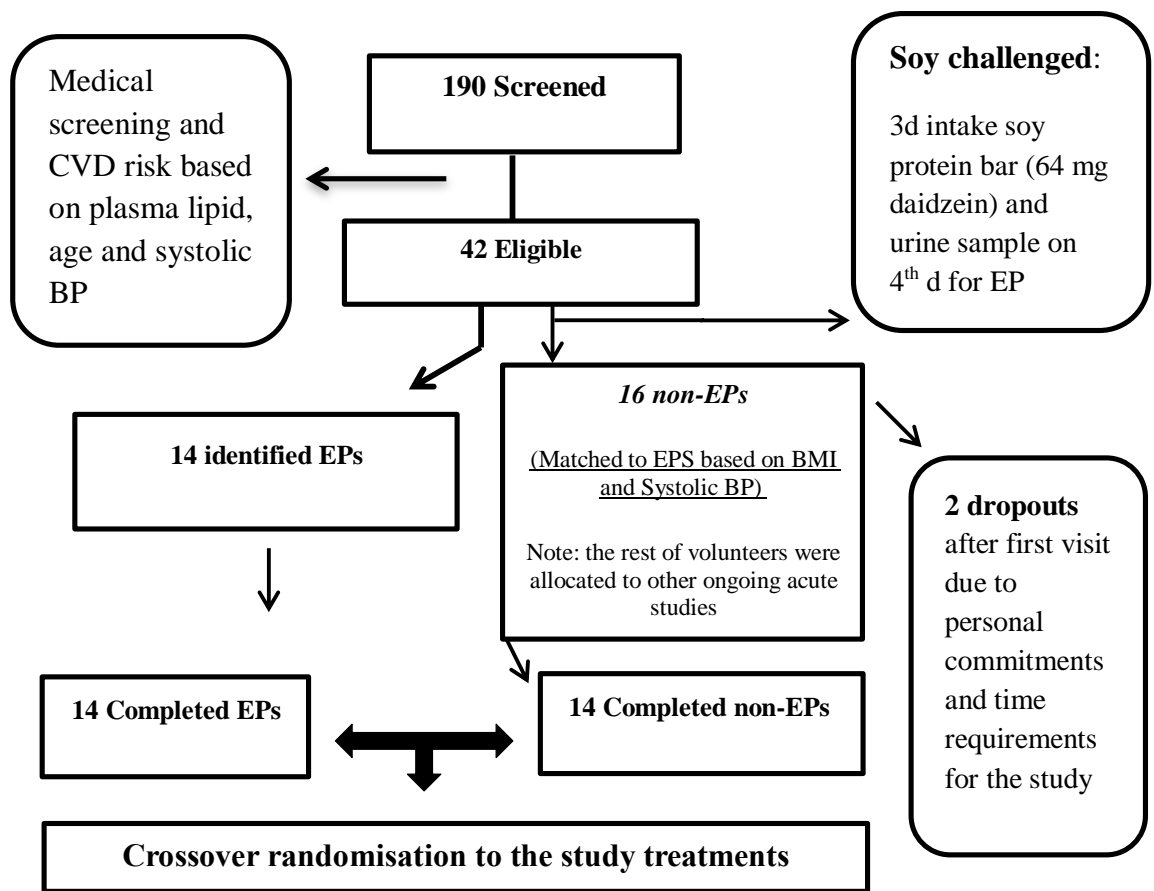
Figure 3.8 Participant recruitment and enrolment
This figure explains the number of participants recruited and enrolled in the study, eligibility according to CVD risk, identification of eligible equol producers and matching them to non-equol producers.

Our study population ranged in age from 50 to 70 years old, BMI of 19 to 35 kg/m^2 and 54 % of them were classified as having phase I pre-hypertension and at 10% to 20 % risk of

developing CVD over the next 10 years (Table 3.6). Baseline characteristics were similar between equol producers and non-producers; except for age - equol producers were approximately 5 years younger than non-equol producers. However due to the limited time frame for completion of the study, it was not feasible to continue recruitment for age-matched groups (Table 3.6).

In addition, the estimated daily dietary macronutrient intakes were similar between the two groups. Equol and non-equol producers showed dietary intakes consisting on average of 18.6± 1.1 %, 18.6±0.9 % protein; 47.6±1.7 %, 46.1±2.6 % carbohydrates; and 34.2±1.6 %, 35.7±2.1 % fat, respectively (Table 3.7).

Table 3.6 Baseline characteristics for the participants who completed the trial

Baseline characteristics	Non-equol producers (n=14)	Equol producers (n=14)
Age (range in years)	54-70	50-69
Height (m)	1.77±0.01	1.80±0.02
BMI (kg/m2)	25.0±1.0	26.4±0.9
LDL-C (mmol/l)	3.82±0.20	3.86±0.21
TG- (mmol/l)	1.24±0.15	1.49±0.20
Systolic BP (mmHg)	133±2	131±3
Diastolic BP (mmHg)	80±1	81±2
CVD risk (%)	16±1	14±1

All values presented as mean±SEM, with the exception of age (presented as a range). All $P> 0.05$ (Student's independent t test), except age $P=0.01$. **BMI,** *Body mass index***; BP,** *Blood pressure***; CVD,** *Cardiovascular disease***; LDL-C,** *Low density lipoprotein cholesterol***; TG,** *Triglycerides.*

Table 3.7 Daily macronutrient intakes calculated from 24 h dietary recalls

Macronutrient	*Non-equol producers (n=14)*	*Equol producers (n=14)*	P value
Energy (kcal/d)	1622±100	1777±119	0.36
Protein (g/d)	76.0±6.1	80.3±4.0	0.54
Carbohydrate (g/d)	194±15	200±13	0.74
Sugars (g/d)	77.8±6.3	73.6±7.5	0.67
Starch (g/d)	114±11	124±10	0.47
Total Fat (g/d)	61.9±5.1	73.2±8.66	0.27
Saturated fatty acids (g/d)	23.7±2.3	29.7±3.2	0.14
Monounsaturated fatty acids (g/d)	20.2±1.7	23.5±3.0	0.34
Polyunsaturated fatty acids (g/d)	8.99±0.73	10.4±1.5	0.40
Percentage energy intake from			
Protein	18.6±1.1	18.6±0.9	0.96
Carbohydrates	47.6±1.7	46.1±2.6	0.62
Fat	34.2±1.6	35.7±2.1	0.56

Data presented as mean±SEM, P value for Student's independent t test.

3.3.2 Isoflavone concentrations in plasma

Plasma concentrations of genistein, daidzein, and glycitein (post-hydrolysis quantified as aglycones) significantly increased 6 h after intake of isoflavone supplement compared with placebo (for all analytes, $P< 0.01$) with concentrations (mean±SEM) of 301±40, 1692±269, 291±48 nmol/L in non-equol producers and 200±33, 1688±215, and 237±36 nmol/L in equol producers, respectively (Table 3.8). Total plasma concentrations of isoflavone concentration decreased 24 h after consumption of the isoflavone supplement group but remained significantly higher than placebo ($P< 0.01$) (Table 3.8).

In equol producers, plasma concentrations of equol showed a similar pattern, reaching a significantly higher mean concentration (236±81 nmol/L) at 24 h following intake of isoflavone supplements than that after placebo (range:12 nmol/L-1000 nmol/L) (Table 3.8).

Plasma concentrations of isoflavones after intake of the isoflavone-enriched cereal bars were low and ranged for daidzein from 0 to 238 nmol/L compared to a range from 253 to 3436 nmol/L after isoflavone supplement intake. They were not significantly different from the placebo data (Table 3.8, and for P values see Appendix B, Table IV) and therefore we excluded this arm of the study from all subsequent analysis. A validated LCMS method was used for the plasma analysis (for further details section 3.2.3, page 97) and all study samples were extracted, and analysed in random order. In addition, prior to the sample analysis at the method development stage for the isoflavone analysis in plasma, these bars were used in experiments to develop LCMS methods that would quantify isoflavone metabolites and these tests failed three times repeatedly to identify any isoflavones or metabolites in the samples. The company had assured us that their cereal bars were isoflavone rich, and provided us the content (40 g soy protein bar, providing 80 mg aglycone equivalents of total isoflavone, prepared from SoyLife extract (40%) consisting of a ratio, 15 : 50 : 35 of genistein : daidzein : glycitein). Unfortunately, these data were not independently confirmed prior to commencing the trial. It is therefore likely that there was an error in the preparation procedure of the cereal bar and/or used soy germ extract used to produce the bars. The method development experiments and plasma data support no isoflavones in the soy bars.

Table 3.8 Plasma concentrations of total isoflavones at baseline, 6 h and 24 h in non-equol and equol producers (n=28) after intake of either placebo or isoflavone intervention (80 mg isoflavone as aglycone equivalents)

		Intervention										
	Plasma	*Placebo*			*Isoflavone supplement*			*Isoflavone bar*			*P value*	*P value*
EP phenotype	*isoflavone (nmol/L)*	*BL*	*6 h*	*24 h*	*BL*	*6 h*	*24 h*	*BL*	*6 h*	*24 h*	*(6 h)*	*(24 h)*
Non-equol producers (n=14)	*Genistein*	4±3	15±9	7±5	4±3	301±40	72±37	4±3	45±17	1±1	<0.001	0.093
	Daidzein	4±2	16±7	8±4	4±2	1692±269	170±46	4±2	54±16	6±4	<0.001	0.002
	S-equol	ND	ND	ND	ND	ND	ND	ND	ND	ND	NA	NA
	Glycitein	21±7	20±13	3±2	21±7	291±48	54±12	21±7	28±10	ND	<0.001	<0.001
Equol producers (n=14)	*Genistein*	ND	ND	3±3	ND	200±33	28±15	ND	7±3	ND	<0.001	0.11
	Daidzein	1±1	3±2	4±2	1±1	1688±215	190±31	ND	39±11	4±2	<0.001	<0.001
	S-equol	ND	ND	ND	ND	ND	236±81	ND	ND	11±5	<0.001	0.007
	Glycitein	19±6	7±4	6±4	19±6	237±36	35±9	18±6	23±7	2±2	<0.001	0.009

All values presented as mean±SEM. Univaraite analysis used. Presented P value*s* for difference between placebo and isoflavone supplement intervention at 6 h timepoint (6 h), and at 24 h timepoint (24 h). Isoflavone or S-equol concentrations were significantly different from baseline at 6 h and/or 24 h after consumption of isoflavone supplement, while these concentrations were not significantly different from baseline at 6 h or 24 h after consumption of placebo (P values are shown in Appendix B, Tables II&III). *BL, Baseline; NA: Not applicable; ND, non-detectable or below limit of detection.* Note: isoflavone concentrations were not significantly different between isoflavone enriched bar and placebo in both equol and non-equol producers at 6 h and 24 h (P values are shown in Appendix B, Table IV).

3.3.3 The role of the equol producer phenotype in explaining the potential acute effects of isoflavone intake on vascular function

There were no significant differences in the assessed vascular measures between equol and non-equol producers at baseline (Table 3.6) or 2 h after isoflavone consumption (Appendix B, Table V). At 6 h after isoflavone intake, there were no effects of isoflavone supplement intake on the assessed vascular measures; including endothelial function (assessed by peripheral arterial tone (RHI)), arterial stiffness (PWV, and AI), or systolic and diastolic BP in either equol or non-equol producers (Table 3.9).

At 24 h after isoflavone supplement intake, arterial stiffness assessd by PWV significantly decreased in equol producers 24 h after intake of isoflavone supplement (change from baseline in PWV: -0.2 ± 0.2 m/s after isoflavone intake, versus 0.6 ± 0.2 m/s after placebo intake, P= 0.002, Figure 3.9). Notably, these changes in PWV were significantly correlated with plasma equol concentrations 24 h post-isoflavone supplement consumption (r= -0.36, P = 0.01, Figure 3.9). However, there were no additional observed effects on RHI, AI, CO, systolic and diastolic BP (Table 3.10). No significant changes were observed in non-equol producers 24 h after intake of isoflavone supplement (change from baseline in PWV: 0.3 ± 0.2 m/s after isoflavone, versus -0.1 ± 0.2 m/s after placebo intake, P= 0.21, Figure 3.9).

After plotting the hourly means for all collected ambulatory BP dataset (Figure 3.10), it was noticeable that the systolic BP in non-equol producers had decreased levels between 14:00 and 18:00 pm after isoflavone consumption while this pattern seems reversed in equol producers. However, when the average of systolic BP measurements collected between 2 and 6 h after isoflavone consumption (≈12:00 to 18:00 pm) were compared, there were not significantly different between equol and non-equol producers (Table 3.11). Overall, there were no significant effects on ambulatory systolic, diastolic BP and MAP assessed from the average of measurements collected between 2 and 6 h (Table 3.11), and 8 and 24 h (Table 3.12) after intake of isoflavone supplements in either equol and non-equol producer groups.

Table 3.9 Acute effect on hemodynamic and vascular measures at 6 h after isoflavone consumption in equol and non-equol producers

Equol producer phenotype	Intervention	Time (h)	RHI**	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	CO (L/min)	AI (%)	PWV (m/s)
Non-equol producers (n=14)	Placebo	0	2.57±0.15	128±2	76±2	4.30±0.18	26±2	9.9±0.2
		6	2.92±0.20	126±3	72±2	4.54±0.24	24±1	9.8±0.3
		6-0*	0.39±0.22	-2±3	-4±2	0.18±0.15	-2±1	-0.1±0.2
	80 mg isoflavone supplement	0	2.58±0.15	128±3	76±2	4.32±0.17	26±1	9.7±0.3
		6	2.51±0.16	122±3	70±2	4.39±0.23	25±2	9.6±0.3
		6-0*	-0.05±0.26	-7±3	-5±2	0.03±0.24	-2±1	-0.3±0.2
Equol producers (n=14)	Placebo	0	2.72±0.16	122±6	82±3	3.92±0.21	24±1	9.9±0.3
		6	2.39±0.17	120±4	78±4	4.40±0.20	21±1	10.1±0.3
		6-0*	-0.38±0.23	-3±3	-5±2	0.54±0.15	-3±1	0.2±0.2
	80 mg isoflavone supplement	0	2.62±0.17	123±6	83±3	4.09±0.21	25±1	10.0±0.3
		6	2.19±0.21	121±5	77±4	4.61±0.32	22±1	10.4±0.4
		6-0*	-0.46±0.27	-3±3	-6±2	0.56±0.24	-3±1	0.5±0.2
P value			0.816	0.227	0.544	0.654	0.970	0.311

Data presented as Mean±SEM. *Adjusted means, age used as covariate. For RHI**, equol producers (n=13), and Non-equol producers (n=14). P value for the interaction of intervention and equol producer phenotype factors (mixed general linear model, repeated ANOVA for intervention). *AI, Augmentation index; BP, Blood pressure; CO, Cardiac output; PWV, Pulse wave velocity; RHI, Reaction hyperaemia index.*

Table 3.10 Acute effects on hemodynamic and vascular measures at 24 h after isoflavone consumption in equol and non-equol producers

Equol producer phenotype	Intervention	Time (h)	RHI**	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	CO (L/min)	AI (%)	PWV (m/s)
Non-equol producers (n=14)	Placebo	0	2.57±0.15	128±2	76±2	4.30±0.18	26±2	9.9±0.2
		24	2.75±0.16	130±3	78±2	4.27±0.21	25±1	9.9±0.3
		24-0*	0.05±0.19	2±3	2±1	-0.06±0.18	-1±1	-0.1±0.2
	80 mg isoflavone supplement	0	2.58±0.15	128±3	76±2	4.32±0.17	26±1	9.7±0.3
		24	2.59±0.13	130±3	77±2	4.48±0.21	24±1	10.1±0.3
		24-0*	-0.07±0.18	2±3	0±2	0.23±0.17	-2±1	0.3±0.2
Equol producers (n=14)	Placebo	0	2.72±0.16	122±6	82±3	3.92±0.21	24±1	9.9±0.3
		24	2.80±0.24	124±5	85±4	4.20±0.23	23±1	10.4±0.3
		24-0*	0.22±0.20	2±3	3±1	0.31±0.18	-1±1	0.6±0.2
	80 mg isoflavone supplement	0	2.62±0.17	123±6	83±3	4.09±0.21	25±1	10.0±0.3
		24	2.87±0.12	124±5	83±4	4.27±0.23	22±1	9.7±0.4
		24-0*	0.33±0.18	2±3	0±2	0.11±0.17	-3±1	-0.2±0.2
P value			0.552	0.993	0.558	0.076	0.952	0.007

Data presented as Mean±SEM. *Adjusted means, age used as covariate. For RHI**, equol producers (n=13), and Non-equol producers (n=14). P value for the interaction of intervention and equol producer phenotype factors (mixed general linear model, repeated ANOVA for intervention). *AI, Augmentation index; BP, Blood pressure; CO, Cardiac output; PWV, Pulse wave velocity; RHI, Reaction hyperaemia index.*
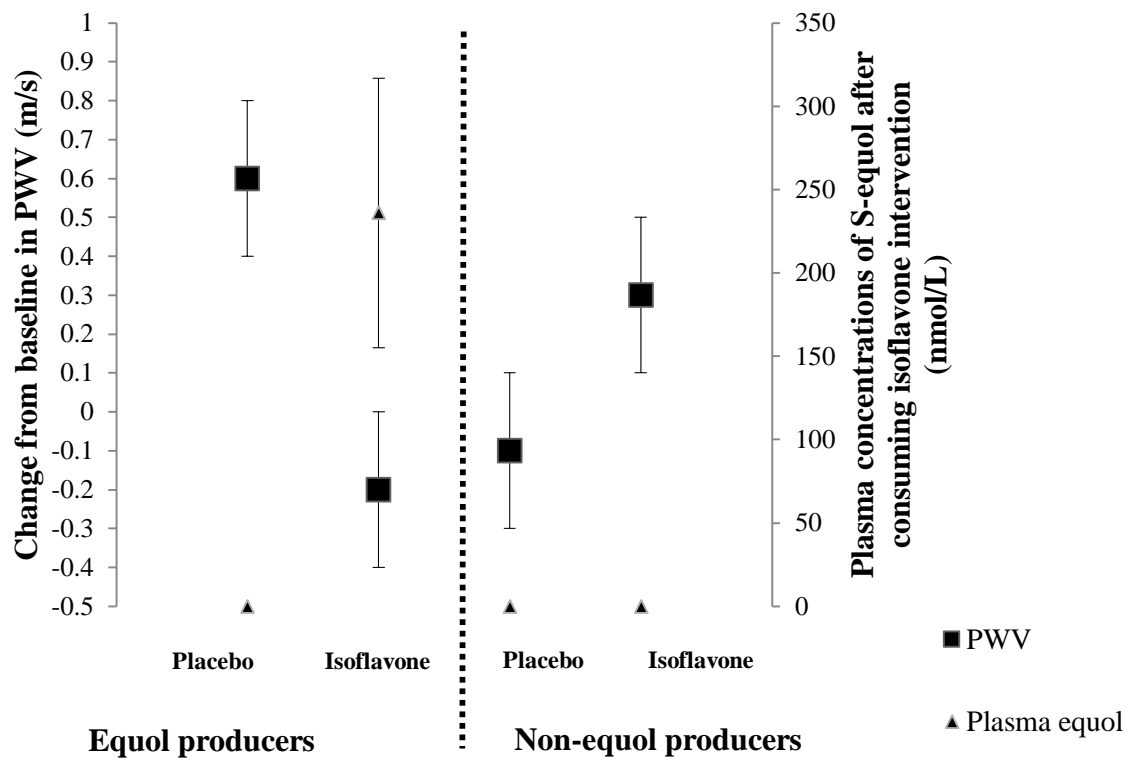
Figure 3.9 Acute effect on 24 h PWV after isoflavone consumption on PWV and plasma equol concentrations in equol and non-equol producers

Data presented as mean±SEM. For PWV data, an overall P value for mixed general linear model for interaction effect between intervention and equol producer phenotype was 0.007, as shown in Table 3.10. Further analysis using repeated ANOVA for intervention effect only showed P= 0.002 in equol producers and P= 0.209 in non-equol producers. *PWV, Pulse wave velocity*.

Table 3.11 Acute effect of isoflavone consumption on average ambulatory blood pressure measurements (time interval between 2 and 6 h post treatment) in equol and non-equol producers

EP phenotype	Intervention	Systolic BP (mmHg)	Diastolic BP (mmHg)	MAP (mmHg)
Non-equol producers (n=11)	Placebo	130±4	81±3	98±3
	Isoflavone supplement	127±5	82±3	96±4
Equol producers (n=10)	Placebo	128±5	85±3	98±3
	Isoflavone supplement	127±5	84±3	96±4
P value		0.882	0.729	0.957

Data presented as Mean±SEM. P value for the interaction of intervention and equol producer phenotype factors (mixed general linear model, repeated ANOVA for intervention). Participant data with less than 70% of the expected number of recordings were excluded as explained in the statistical analysis section 3.2.4. **BP**, *Blood pressure;* **MAP**, *mean arterial blood pressure.*

Table 3.12 Acute effect of isoflavone consumption on average ambulatory blood pressure measurements (time interval between 8 and 24 h post treatment) in equol and non-equol producers

EP phenotype	*Intervention*	*Systolic BP (mmHg)*	*Diastolic BP (mmHg)*	*MAP (mmHg)*
Non-equol producers (n=11)	*Placebo*	122±5	76±3	91±4
	Isoflavone supplement	121±5	74±3	90±4
Equol producers (n=10)	*Placebo*	125±6	79±3	93±4
	Isoflavone supplement	128±6	82±3	95±4
P value		*0.225*	*0.117*	*0.180*

Data presented as Mean±SEM. P value for the interaction of intervention and equol producer phenotype factors (mixed general linear model, repeated ANOVA for intervention). Participant data with less than 70% of the expected number of recordings were excluded as explained in the statistical analysis section 3.2.4. Night data were excluded too because most volunteers turned the ambulatory blood pressure monitor off during sleep time (data collected through night time were available only for 5 participants). *BP, Blood pressure; MAP, mean arterial blood pressure.*
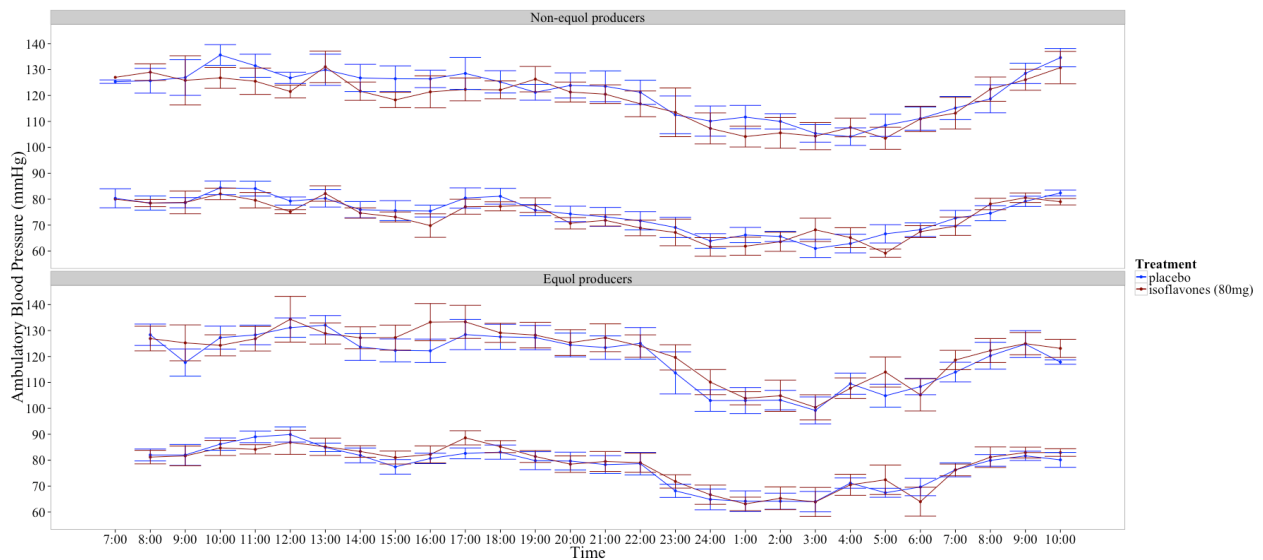
Figure 3.10 Acute effect of isoflavone consumption on 24 ambulatory blood pressure measurements in equol and non-equol producers.
Non-equol producers (n=11), Equol producers (n=10). Participant data with less than 70% of the expected number of recordings were excluded as explained in the statistical analysis section 3.2.4

3.3.4 The acute effects of isoflavone intake on vascular and hemodynamic function

Overall, there were no significant acute effects of isoflavone intake on vascular function after either at 6 h assessments (Table 3.13 shows data for 6 h timepoint).

Table 3.13 Acute effect of isoflavone consumption 6 hours on hemodynamic and vascular measures

Intervention	*Timepoint (h)*	*RHI**	*Diastolic BP (mm Hg)*	*Systolic BP (mm Hg)*	*CO (L/min)*	*AI (%)*	*PWV (m/s)*
	0	2.64±0.11	79±2	125±3	4.11±0.14	25±1	9.9±0.2
Placebo	6	2.73±0.15	82±2	129±3	4.31±0.20	23±1	10.0±0.3
	6-0*	*0.02±0.17*	*-4±1*	*-2±2*	*0.36±0.10*	*-3±1*	*0.0±0.1*
	0	2.60±0.11	79±2	126±3	4.21±0.14	25±1	9.9±0.2
80 mg isoflavone supplement	6	2.55±0.12	79±2	125±3	4.13±0.15	24±1	10.0±0.2
	6-0*	*-0.25±0.18*	*-6±1*	*-5±2*	*0.30±0.17*	*-2±1*	*0.1±0.2*
P value		0.271	0.334	0.188	0.695	0.429	0.807

Data presented as Mean±SEM.*Adjusted means, age used as covariate. Total n=28 except for RHI* n=27. P value for repeated ANOVA for intervention effect. *AI*, Augmentation index; *BP*, Blood pressure; *CO*, Cardiac output; *PWV*, Pulse wave velocity; *RHI*, Reaction hyperaemia index.

3.4 Discussion

This study investigated the acute effects of isoflavone supplement intake on vascular function in prospectively recruited equol and non-equol producer males at moderately elevated risk of cardiovascular disease. Although the acute intake of 80 mg isoflavone supplement did not significantly affect endothelial function (assessed by EndoPAT) or blood pressure in either equol nor non-equol producers equol producers, it did show an improved response in PWV at 24 hours after isoflavone supplement intake. This significant decrease could be related to the peak plasma concentrations of equol at 24 hours.

Human studies have been conducted to investigate the vascular effects of isoflavones in equol producers, but the most commonly used approach has been retrospective data analysis of studies, which were not appropriately designed to address the subject. Interestingly, our previous analysis (presented in Chapter 2) observed similar improvements on PWV in postmenopausal equol producers after 1 year of a combined isoflavone and flavan-3-ols intervention. Additionally, a previous parallel-designed RCT of soy germ-rich pasta has shown positive association between equol producer phenotype and endothelial function after 4 week isoflavone intake [6], in particular FMD significantly improved in hypercholesteromic equol producers compared to non-equol producers. On the other hand, contrasting data was shown in one study, where supplementation with isoflavone soy protein had no effect on atherosclerosis progression [12]. However, this study used retrospective analysis and included subjects that were not analysed properly for their equol producer phenotype. The current study was novel in its prospective recruitment design and also the investigation of the acute effects of isoflavone consumption on vascular function at 24 hours, which related to the peak plasma concentration of equol [175, 210].

Our acute study showed no benefits on blood pressure in equol producers, while in our retrospective analysis (presented in chapter 2), equol producers showed significantly lower blood pressure following long-term isoflavone intervention (12 months). Similarly, another acute study providing a single dose of isoflavones (80 mg) did not report changes on blood pressure 6 h post-intervention [9]. In addition, a study showed significant reductions in diastolic blood pressure after intake of orange juice or hesperidin (another subgroup of flavonoids) for 4 weeks and this hemodynamic benefit was not apparent following acute intervention intake despite there were microvascular endothelium reactivity [254]. A recent review suggested that isoflavone supplement interventions that

are longer than 3 months showed greater benefits on reducing blood pressure [1] however further data are required to investigate the plausible underlying mechanisms. In addition, gender-dependent responses could have played a role and equol may induce greater benefits for vascular function in females than males because of its suggested selective oestrogen receptor modulator (SERM) activity [255].

Our analysis of isoflavone concentrations in plasma confirmed the peak plasma levels of equol at 24 h post supplement intake. Vedrine et al. [212] had reported comparable maximum plasma concentrations of 1.68 µmol/L for daidzein after consumption of 100 mg isoflavone (containing up to 40 mg daidzein that is similar to our provided dose of daidzein). In addition, their maximum plasma concentration of equol reached 0.31 ± 0.27 µmol/L which is comparable to the equol concentrations of 0.24 ± 0.81 µmol/L observed in our study 24 h post intake of isoflavone supplement (Table 3.8). We observed high inter-individual variability in equol concentrations (between-subject range of plasma concentrations of equol at 24 h was 0.012- 1.029 µmol/L; CV= 27%) which also agrees with previous pharmacokinetic studies on isoflavones and equol [141, 174, 176, 212] (e.g. CV for plasma concentrations of equol was 110% in equol producers [176]). Additionally, concentrations of total isoflavones significantly increased at 6 h after supplement intake and these levels were decreased at +24 hours but remained higher than placebo. This observed pattern agrees with isoflavone pharmacokinetics reported in the literature [174, 212, 213].

Interestingly, Vedrine *et al*. [212] also showed that the peak concentrations of equol increased threefold after 30 days of isoflavone consumption and this might be a result of an adaptation of the gut microbiota that is responsible for equol production [28]. This could be one of the reasons for our null findings after single exposure on the assessed vascular endpoints such as endothelial function, augmentation index and blood pressure; therefore a longer duration intervention could potentially result in higher and sustained plasma concentrations of equol and presumably increased benefits on vascular function.

Only at the 24 h time point post-intervention, there was a significant decrease in PWV, while we found no significant changes 6 h post-intervention, at a time when equol levels were negligible. It has previously been shown that equol appears in the blood only ~8 h after ingestion as daidzein requires time to reach the intestine and be metabolised to equol by the microbiota (212). A plausible explanation for our observed improvement in pulse wave velocity following soy supplementation in the equol producers might be related to

higher vascular bioactivity of equol compared to daidzein, which has been suggested by previous *in vitro* work [23, 24].

Studies have suggested that the underlying mechanisms of vascular bioactivity of equol involve phosphorylation of eNOS and consequent increased levels of bio-available NO [93, 94]. Moreover, it has also been shown that infusion of dehydroequol (equol precursor) increased blood flow in forearm arteries in humans and this effect was diminished by inhibition of eNOS [156].

Arterial stiffness is linked to structural changes induced by increased collagen to elastin ratio or dynamic changes in the tone of smooth muscle cells induced by decreased release of NO [53]. Thus, the possible underpinning mechanisms for our positive findings on reduced PWV in equol producers with the lack of effect on blood pressure, could be related to increased levels of NO that caused peripheral changes in vascular resistance and consequently reduced PWV. In support of this hypothesis, it has been shown previously that inhibition of eNOS resulted in a significant acute increase in PWV independent of changes in blood pressure [256].

Central PWV is a direct measurement of arterial stiffness of the thoracic and abdominal aorta. It is the gold standard method for assessment of arterial stiffness and it is non-invasive, simple, quick, and reproducible [35]. Interestingly, it has been reported that reductions in PWV of 1 m/s might result in a reduction in heart disease risk of 13-15%) [50]. Thus our results could be clinically important if sustained, as the equol producers showed central PWV reductions of -0.8 m/s after isoflavone consumption compared to placebo consumption, and thus our results might lead to 11-12% reduction in CVD risk.

Our study, with prospective recruitment of equol producers, provides a better insight on the potential effect of equol production compared to other previous retrospective studies because its hypothesis-driven design, use of reliable standard method for analysing equol producer phenotype correctly, matched equol and non-equol producers, and assessment for vascular function at a time correlating to equol bioavailability. Our study participants had similar characteristics and profiles of CVD risk factors at baseline and the prevalence of equol producer phenotype in our recruited sample was found to be 33% which is a similar proportion to previous trials [18].

Our study subjects were men because many of the previously conducted RCTs have been limited to postmenopausal women [10, 15, 171, 220]. However, further studies are required to include both males and females and investigate any gender related variations in the effects of isoflavone intake and equol producer phenotype on CVD risk.

Our study had a number of limitations, one of which could be the use of the EndoPAT system to analyse microvascular reactivity/ endothelial function, instead of using a more robust common standard method such as ultrasound flow mediated dilatation (FMD). EndoPAT have recently been reviewed [49, 257, 258] and there is some suggestion that EndoPAT assessed endothelial function does not significantly correlate with CVD risk score (Framingham score) or with flow mediated dilatation (FMD) [49, 257].

Another limitation is the lack of independent verification of the isoflavone content in the soy bars prior to commencing the trial, which resulted in unusable data and an inability to investigate the impact of food matrix on the effectiveness of acute isoflavone intake on vascular function. The LCMS method used for analyzing our samples followed a validated method [150] and our extraction and preparation steps were the most commonly used for isoflavone analysis in biological samples. Moreover, our LCMS method identified significant levels of isoflavones in the plasma samples collected after ingestion of isoflavone supplement at 6 h and 24 h and the resulting plasma concentrations were comparable with previous bioavailability studies that used a similar dose [212]. Therefore, it is most likely reason that the cereal bars did not contain isoflavones and it might be due to a processing or preparation error in the production phase.

NO metabolites in plasma samples were not analysed due to lack of PhD time and it is a research gap that could be done in the future to investigate more on the underlying mechanism for our findings on PWV.

3.5 Conclusion

In summary, we showed that the acute intake of isoflavones improved central PWV in equol producers and this improvement coincides with the peak 24 h equol plasma concentration. These results suggest that equol might be more effective in modifying vascular function than parent compounds and/or the endogenous capacity of producing equol may play an additional role in the effectiveness of soy isoflavones on arterial stiffness. This finding supports further investigations on the direct effects of equol supplement intake on vascular function in non-equol producers and also provides preliminary data for longer term interventions to further understand the efficacy of isoflavone intake on endothelial function, arterial stiffness and hemodynamic function in equol producers and the plausible underlying mechanisms to explain their bioactivity.

Chapter 4 The effect of S-equol (SE5OH) supplement intake on vascular health in non-equol producers

4.1 Introduction

There has been recently a research interest in the benefits of the isoflavone metabolite equol on various health aspects, such as postmenopausal symptoms [64, 167], metabolic syndrome [27, 168], cancer [125] and cardiovascular disease (CVD) [63]. Equol is a gut metabolite of the isoflavone precursor daidzein and it has a similar chemical structure to oestrogen. This structural similarity might explain the results from *in vitro* studies that have observed a higher binding affinity of equol to oestrogen receptor α, and β than daidzein [146]. Additionally, equol has similar binding affinity to the major bioactive isoflavone, genistein, to both oestrogen receptors, more preferably β than α. Equol has also been shown to activate oestrogen-receptor dependent transcription activity more strongly than other isoflavones [132]. Equol has additionally showed higher vascular [20, 23, 24] and antioxidant bioactivity [20-22] compared with its parent compound daidzein.

Equol, out of all major isoflavones tested including; genistein, daidzein and glycitein, was found to have the highest antagonistic effects to the thromboxane A2 (TXA_2) receptor that is implicated in endothelial dysfunction [24]. In addition, equol has been shown to induce vasodilation in carotid arteries isolated from hypertensive rats whilst there was no response to daidzein [23]. In a liver cancer cell model (HepG2 human hepatocellular carcinoma cells), equol stimulated activity and protein expression of potent antioxidant proteins, catalase and total superoxide dismutase more than daidzein [21]. Equol also induced greater oxidation inhibition of serum lipoproteins than genistein and approximately 10 times greater than daidzein [22].

Notably, equol has shown vasodilation effects in cell culture models. Treatments of equol caused rapid relaxation in human endothelial cells in a dose-dependent manner and this effect was inhibited by pre-treatment with eNOS inhibitor (L-arginine methyl ester (L-NAME)) [93, 94].

In support of these *in vitro* findings, several recent observational studies have shown that the ability to metabolise isoflavones to equol is associated with reduced levels of cardiovascular risk factors such as lower blood pressure [28], improved plasma lipids and lower inflammatory biomarkers [112], lower arterial stiffness and improved endothelial function [166]. Likewise, retrospective analyses of several isoflavone randomized controlled trials (RCTs), which quantified equol levels reported improvements in blood pressure [15, 220], endothelial function [6], and arterial stiffness [220] in equol producers after 4 weeks to 1 year of daily isoflavone consumption compared with non-equol producers. In addition, as detailed in chapter 3, when we prospectively recruited equol and non-equol producers, we observed an improvement in arterial stiffness after isoflavone intake only in the equol producers at the time of the expected peak plasma concentration of equol. Collectively, these data further suggest that equol could be the key to the efficacy of isoflavone interventions on vascular function and one explanation for this may be that equol affects vascular function causing higher vasodilation in the endothelium [23, 156].

To date, there have been few RCTs that have investigated the direct effects of equol supplements and those that have been published, have predominantly focused on the potential benefits in relation to postmenopausal symptoms [167, 169, 170]. Overall, the current data suggest that daily consumption of between 10 mg and 40 mg S-equol supplement for durations between 4 weeks and 12 months significantly reduces the frequency of hot flushes, neck and shoulder muscle stiffness [167, 169] and bone density loss in postmenopausal women [170]. Notably, the dose of 40 mg/day was more efficient in reducing the frequency of hot flushes than soy isoflavones in postmenopausal women experiencing more than 8 episodes per day [167]. To our knowledge, only one study considered effects of administered equol on metabolic syndrome-related and secondary cardiovascular biomarkers. This study showed that daily intake of 10 mg/d of synthesised S-equol supplements by overweight/obese subjects for 12 weeks, significantly improved glycaemic control (decreased glycated haemoglobin HbA1c levels) and secondary CVD-related outcomes such as circulating LDL concentrations and arterial stiffness (measured by cardio-ankle vascular index (CAVI) scores) [168]. However, this study had limitations in both study design (i.e. lack of a wash-out period) and the method used for assessing equol producer phenotype (i.e. not following the standard method suggested by Setchell et al. [141]). Therefore, 'fit-for-purpose' research is required to establish the effects of equol supplements on vascular function in non-equol producers and to identify plausible

explanations for the previously reported beneficial effects of isoflavone intake on vascular health in equol producers.

Thus, we conducted a study where we fed an equol supplement to non-equol producers and hypothesised that equol intake would have beneficial effects on vascular function.

Based on the assumption that vasodilation induced by isoflavones is correlated to their bioavailiability, we related our study assessment timing to the previously published data on the pharmacokinetic profile of S-equol in blood following equol supplement intake (T_{max}: 1-1.5 h, $T_{1/2:}$ 7.4- 8 h, C_{max}/dose: 50-191 nmol/L per mg, [19]) and we followed an acute phase design. Our study aimed to assess the effects of an equol supplement on a range of biomarkers for CVD risk (including endothelial function (reactive hyperaemia index (RHI), arterial stiffness measurements (PWV) (primary outcomes), augmentation index (AI), and blood pressure (secondary outcomes)), measured at around the anticipated T_{max} of equol in middle-aged males who were non-equol producers and at elevated cardiovascular risk.

4.2   Methods

4.2.1 Subjects and study design

This study was an additional study arm to the clinical trial (described in detail in section 3.2, page 86). It followed the same study design as a randomized, double-blind, placebo-controlled, crossover study and was conducted at the Clinical Research & Trials Unit (CRTU) at the University of East Anglia (United Kingdom), with approval from the local Ethics Committee (www.clinicaltrials.gov; NCT01530893). The same subjects that were recruited for the isoflavone study (Chapter 3) were asked to complete this additional study arm and all investigations were conducted according to the Declaration of Helsinki (Appendix C, page 183). 12 non-equol producers were required to complete study and 14 subjects were recruited assuming 15% dropouts (Details for the sample size calculations are presented in Chapter 3, see page 86).

The participants included 42 men aged 50-75 years who had a 10-20% risk of CVD over the following 10-years (same as described in detail in section 3.2.1, page 87) [36].

16 prospectively recruited non-equol producers were identified using a standard soy challenge, out of which 14 subjects completed the study (as described in page 88), Figure 4.1.

Briefly, a 63 g soy protein bar providing 161 mg soy isoflavones as aglycone equivalents (63.5 mg daidzein, 63.9 mg genistein and 33.7 mg glycitein (commercial soy bars, Revival. CO, Kernersville, USA)), was consumed daily for three consecutive days. Urinary concentrations of equol and daidzein were then quantified in a fasted urine sample collected on the fourth morning using a validated LC-MS/MS methodology [150]. Non-equol producers were defined as the ones having a urinary log10 S-equol/daidzein ratio < -1.75 [141] (as described in chapter 3 in more details).

The non-equol producer participants were afterwards cross-over randomised to a 6.6 g SE5-OH supplement (containing 40 mg of S-equol as aglycones equivalents), or a matched placebo dose with a wash out period of minimum 1 week.

Participants followed the same dietary and exercise restrictions as described in chapter 3, section 3.2.1, page 91. Briefly, before the study visit participants were asked to follow a number of dietary and lifestyle restrictions; maintaining intake of non-flavonoid supplements, excluding intake of flavonoids (for 72 h) and strenuous exercise (for 48 h), and avoidance of caffeine and alcohol (for 24 h). We also controlled for other potential factors including nitrate/ nitrite intake that might affect circulatory levels of NO metabolites [247], therefore subjects were asked to avoid nitrite/ nitrate rich foods (for 24 h) and drink only low nitrite / nitrate containing bottled water (Buxton, Nestle Ltd UK). In addition, the same standardised pasta meal (Brake Bro Ltd, Kent, UK) was consumed for dinner before the assessment visit. Habitual dietary intakes were also assessed by 24 h dietary recalls (using food intake software, WISP version 3.0, Tinuviel, UK).

A)
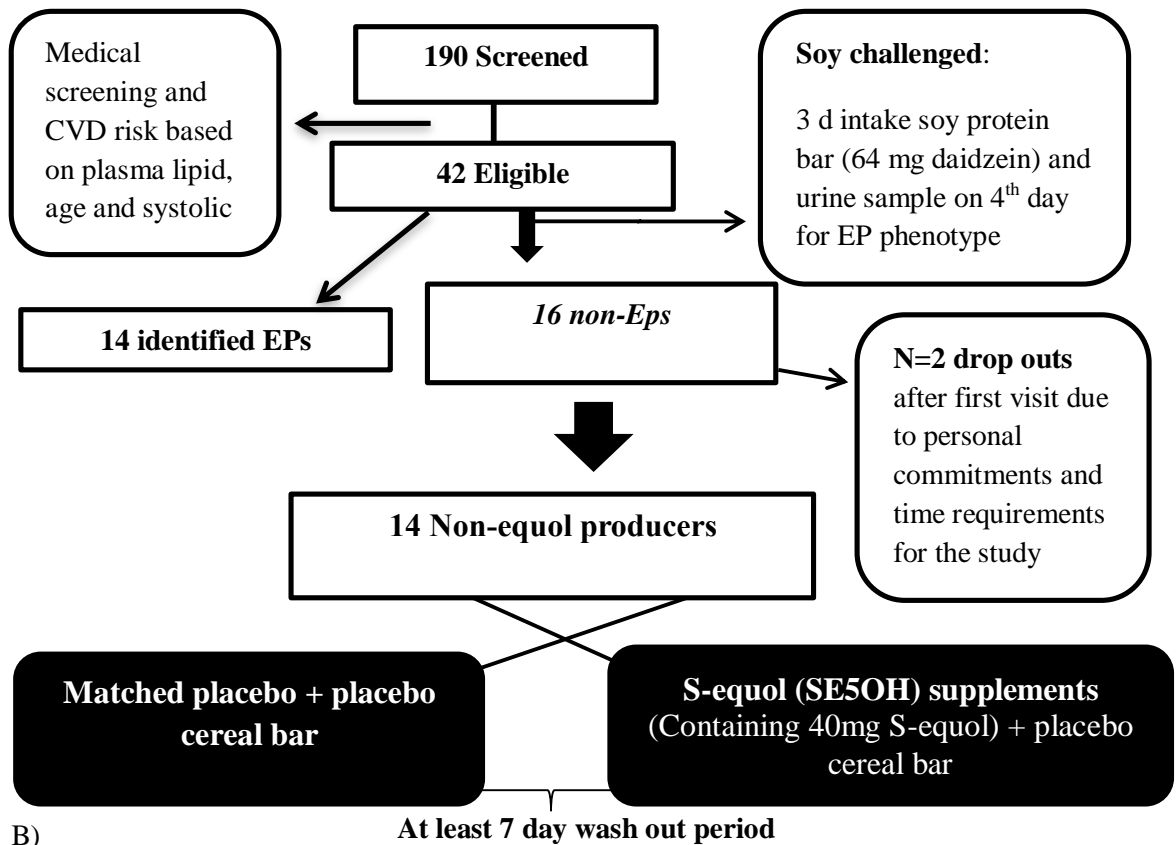
B)
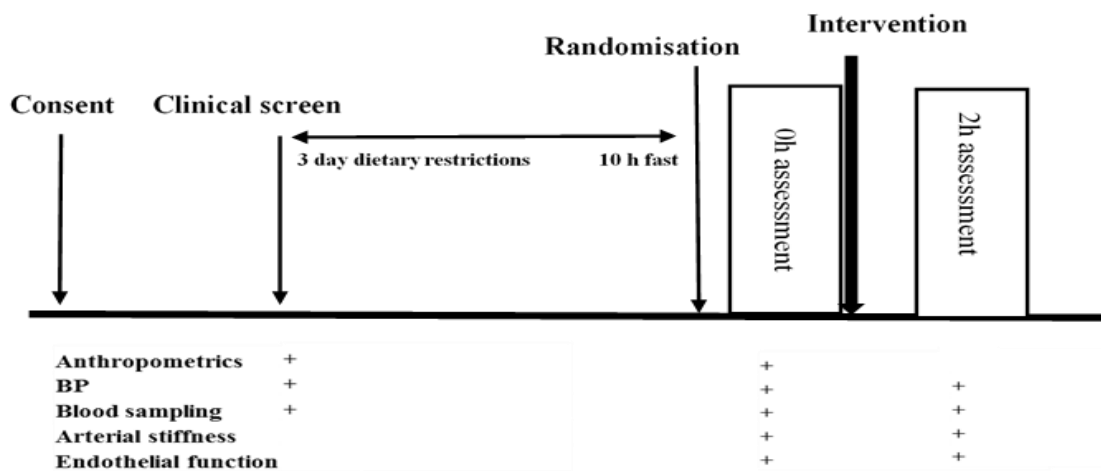
Figure 4.1 Study design and assessment schedule

A) This study included only non-equol producers (the same subjects recruited to study in Chapter 3), who were crossover randomised to S-equol supplement (intervention group) or matched placebo (placebo group). Only one volunteer attended the first visit for this study arm from the 2 participants who dropped out and it was not included in the analysis because of the used statistical analysis (the repeated measures ANOVA requires completed datasets for each volunteer).

B) + represents collection of blood samples and/ or clinical measurements that were collected at baseline, and 2 h after intervention administration. *BP*, blood pressure.

4.2.2 Study interventions

The study interventions were 6.6 g SE5-OH given in gelatine capsule (Otsuka Pharmaceutical Co. Ltd., Japan) which delivered a total of 40 mg of S-equol as aglycones equivalents, or a matched placebo dose (6.6 g) of carboxyl-methyl cellulose (CMC) in a gelatine capsule.

The dose of 40 mg of S-equol was chosen as this dose has been previously reported to be bioactive [167]. Although it is important to note that this dose was found effective on postmenopausal symptoms in an 8 week intervention, unlike our study that investigated the effects of a single dose of equol on vascular function in men.

Due to the lack of available literature for an effective dose of S-equol on vascular function derived from human trials when we set up our study design, we considered *a priori* that 40 mg would achieve physiological plasma concentrations comparable with previous *in vitro* work. These studies have reported NO-dependent relaxation in endothelium intact aortic rings following a dose-response relationship at concentrations between 0.1-10 µmol/L [93]. In addition, data from a human study has shown NO-dependent vasodilation in brachial arteries following infusion of 3 µmol/L dehydroequol (a potential equol precursor found in human urine) [156].

Table 4.1 Content of isoflavones and S-equol in SE5OH supplements

Isoflavones/S-equol	*Content (mg/g)*
S-equol	6.03
Daidzin	0.12
Acetyl-daidzin	0.09
Dihydrodaidzein	0.03
Genistin	0.12
Genistein	0.61
Dihydrogenistein	1.35
Glycitin	2.18
Acetyl-glycitin	0.08
Glycitein	1.04

4.2.3 Endpoints- clinical markers of vascular function and plasma isoflavones

Assessment schedule and vascular function measurements

Subjects attended the CRTU in the morning after fasting for ≥ 10 h whilst consuming nitrate-low mineral water only (Buxton mineral water co, Buxton, UK). After a 15 min supine rest in a standardized quiet, light and temperature monitored clinical room (21–24°C), baseline blood pressure (BP) measurements were taken in triplicate (3 min between assessments) using an automated sphygmomanometer (Omron Healthcare Ltd, UK). Subsequently, carotid-femoral pulse wave velocity (c-f PWV) and augmentation index (AI) were assessed using a Vicorder according to manufacturer's instructions (Smart Medical, UK) as indices of arterial stiffness. Endothelial function was also measured using an EndoPAT 2000 device (Itamar Medical, Israel), whereby the response to a 5 min restriction of arterial blood flow (brachial artery), inducing reactive hyperaemia, was monitored using peripheral arterial tonometry (PAT) of the index finger. In the contra lateral arm to that used for all vascular measures, blood samples were taken and the isolated plasma stored in cryovials at -80 °C until subsequent analysis of biomarkers of vascular function and circulatory isoflavone levels. Interventions were then administered by a research nurse that was not involved in the study analysis, and subsequently all vascular measurements and biological sampling procedures were repeated 2 h after intervention administration (around the time of peak equol concentration in plasma 1-1.5 h after intake). The 2 h assessment was chosen to allow time for study assessments to be conducted on other volunteers on the same day (each battery of vascular measurements took around 60-90 minutes and after the baseline assessment, there was around 90 minutes break that was enough for another volunteer study assessment, for details refer to Table 4.2). The vascular measurement equipments, research nurses and clinical room facilities were shared among our study, and other clinical trials taking place in the CTRU.

Plasma isoflavones and equol were analysed by HPLC-ESI-MS/MS (Agilent1200 series HPLC (Agilent Technologies UK Ltd., Stockport, UK) coupled with a 3200 QTRAP® LC/MS/MS System (AB Sciex UK Ltd, Warrington, UK)) using a previously validated method as described in Section 3.2.3. Note: the plasma concentrations of isoflavone and equol in non-equol producers 2 h after consuming placebo were not analysed due to the time restriction and study funds.

Table 4.2 Study assessment times and order

Example time	Time taken	Assessment
8:30-8:45 am	15 minutes	Baseline anthropometric measurements
8:45-9:00 am	15 minutes	Baseline relaxing period
9:00-9:10 am	10 minutes	Baseline blood pressure measurement (by Omron)
9:10- 9:25 am	5-10 minute	Baseline blood collection
9:25- 9:55 am	15-25 minutes	Baseline arterial stiffness (by Vicorder)
9:55- 10:20 am	20-25 minutes	Baseline endothelial function (by EndoPAT)
10:25 am	5 minutes	Treatment administration
12:00 am	90 minutes	Break
12:00-12:15 am	15 minutes	Relaxing period
12:15- 12:25 am	10 minutes	Blood pressure measurements (by Omron)
12:25- 12:40 am	5-10 minute	2 h blood collection
12:40-13:10 am	15-25 minutes	2 h arterial stiffness (by Vicorder)
13:10- 13:40 am	20-25 minutes	2 h endothelial function (by EndoPAT)

Note: equipment used for vascular measurements (Vicorder and EndoPAT) were shared for study assessments of at least two participants a day.

4.2.4 Statistical analysis

Data are presented as means ± standard error of means (SEM). Results were considered to be statistically significant at P< 0.05. Only volunteers who completed the trial were included in the analysis. Statistical analyses were performed using SPSS software (version 22, IBM Corporation, Armonk, NY, USA). The primary outcomes were the changes in endothelial function (reactive hyperaemia index (RHI)) and arterial stiffness (c-f PWV)) post-intervention. Secondary outcomes included the effects of the intervention on blood pressure, AI and isoflavone concentrations in plasma.

Shapiro-Wilk test was used to assess normality of data. Levene's test and Mauchly's test were applied to check equality of variances for use of univariate analysis and homogeneity of variance for use of repeated measures analysis of variance (ANOVA), respectively. Baseline data for all outcome variables were evaluated for statistical differences between the intervention groups using Student's paired t-test.

For the main data analysis, similar analysis plan was followed as in the study arm in Chapter 3 (for details refer to Statistical analysis section, page 105); a repeated measures ANOVA model was used with one within-subject factor (intervention at 2 levels: placebo, and SE5OH supplement). Data was analysed as change from baseline at 2 h post-intervention and age was included as a covariate.

LCMS data was analysed using univariate analysis to investigate differences in plasma concentrations of daidzein, genistein, glycitein and S-equol following intakes of the SE-5OH isoflavone supplement and additional repeated measures ANOVA analysis was run for differences in concentrations between baseline and 2 h time point.

4.3 Results

At baseline, there were no significant differences in all assessed clinical vascular measurements (Table 4.4).

4.3.1 Isoflavone concentrations in the plasma samples

As expected, plasma concentrations of equol (post-hydrolysis quantified as aglycones) significantly ($P<0.001$) increased 2 h after SE5OH intake in the non-equol producers: at baseline plasma concentrations of S-equol were below the detection limit versus a mean± SEM of 3.22±0.47 µmol/L (Range: 1.77-7.9 µmol/L) at 2 h (Table 4.3).

Table 4.3 Plasma concentrations of total isoflavone at baseline and 2 h post-intervention in non-equol producers (n=14) after intake of SE5OH intervention (40mg S-equol)

Equol producer phenotype	Isoflavone (nmol/L)	BL	2 h	P value
Non-equol producers	*Genistein*	4±3	137±15	<0.001
40mg S-equol	*Daidzein*	4±2	29±7	0.005
	S-equol	ND	3217±469	<0.001
	Glycitein	21±7	192±28	<0.001

All values presented as mean±SEM. Univariate analysis was used. Presented P value is for differences from baseline in plasma concentrations of isoflavone and equol in non-equol producers 2 h after consuming SE5OH. **BL**, *Baseline;* **ND**, *non-detectable or below limit of detection.*

4.3.2 The acute effects of equol supplements on vascular and hemodynamic function in non-equol producers

There was no significant acute effects on any of the assessed hemodynamic and vascular measures in the non-equol producers 2 h after SE5OH supplement consumption (Table 4.4).

Table 4.4 Acute effect of SE5OH supplement consumption after 2 h on hemodynamic and vascular measures in non-equol producers.

Intervention	Timepoint (h)	RHI*	Diastolic BP (mm Hg)	Systolic BP (mm Hg)	CO (L/min)	AI (%)	PWV (m/s)
	0	2.57±0.15	76±2	129±2	4.30±0.18	26±2	9.9±0.2
Placebo	2	2.87±0.27	79±2	132±4	4.31±0.20	24±2	9.9±0.3
	2-0*	0.20±0.25	3±2	3±4	0.01±0.15	-2±1	-0.1±0.2
	0	2.78±0.14	80±2	134±3	4.60±0.25	24±1	9.8±0.3
40 mg SE5OH	2	3.17±0.18	81±2	135±3	4.46±0.21	24±1	9.9±0.3
	2-0*	0.43±0.20	1±2	1±2	-0.14±0.19	0±1	0.1±0.2
P value		0.205	0.627	0.665	0.593	0.936	0.804

Data presented as Mean±SEM.*Adjusted means, age used as covariate. Total n=28 except for RHI* n=27. P values for repeated ANOVA for intervention effect. There were not significant differences in the vascular measurements at baseline between equol and non-equol producers (P> 0.05). *AI, Augmentation index; BP, Blood pressure; CO, Cardiac output; PWV, Pulse wave velocity; RHI, Reaction hyperaemia index.*

4.4   Discussion

This study suggests that acute intake of 40 mg S-equol (SE5OH) supplement did not have significant acute effects on vascular function, in particular endothelial function, arterial stiffness and blood pressure in non-equol producer males at elevated CVD risk.

A previous crossover RCT provided a lower dose of S-equol supplements (SE5OH) (10 mg) to 54 overweight or obese subjects (16 men and 38 women having a mean age of 59.4 years) for 12 weeks [168] and in contrast to our results, observed benefits of S-equol supplements on glycaemic control (assessed from HbA1c levels, the change from baseline was -0.2±0.1 mmol/L), blood lipids (low density lipoprotein (LDL), the change from baseline was -0.2±0.1 mmol/L) and arterial stiffness measured by CAVI score (the change from baseline was -0.2±0.1 m/s). However, after stratification based on gender and equol producer phenotype, their analysis showed that these effects were only statistically significant in non-equol producer women. The observed lack of effect in males suggests that equol supplements may exert greater benefits for vascular function in women more than in men. This phenomenon might be due to the suggested selective oestrogen receptor modulator (SERM) activity of equol [64] and potential gender-dependent differences in protein expression of oestrogen receptor (ER) subtypes [255]. An *in vitro* study has shown up-regulated protein expression of both ER subtypes ERα and ERβ in isolated neutrophils from women in response to incubation with 17β-oestradiol, while only ERα expression was up-regulated in isolated neutrophils from men [255] and this is of particular interest as S-equol possesses higher binding affinity to ERβ than ERα [129]. Similarly, Diarylpropionitrile is a synthetic SERM that causes rapid NO-dependent vasodilation and possesses 30 to 70-fold agonist activity to ERβ than ERα [259].

Another factor could be gender differences in isoflavone metabolism. Watanabe *et al* [260] reported maximum plasma concentrations of 1.4 µmol/L in males and 2.5 µmol/L in females 1 h after ingestion of 30 mg of S-equol (as SE5OH supplements) and Setchell et al [19] showed average levels of around 3 µmol/L at 2 h after ingestion of the same dose and supplement (30 mg) in postmenopausal women. Our plasma analysis of total equol concentrations showed mean concentrations of 3.2 µmol/L (1.8-7.9 µmol/L) in non-equol producer males 2 h after intake of 40 mg SE5OH supplement (Table 4.3). These figures are comparable with the previously reported pharmacokinetic data [19, 149, 260] but there

is an assumption that higher levels could have been achieved in female participants and this might be worth to investigate in future studies.

It has been shown previously that S-equol at 0.1-10 μmol/L can induce vasodilation in an *in vitro* model [93], and 3 μmol/L of an isoflavone metabolite (potential precursor of equol found in human urine [261], dehydro-equol) significantly increased forearm blood flow in humans [156]. In this study, infusion of dehydro-equol into the brachial artery at an increasing dose of 0.1-3 μmol L^{-1} min^{-1} resulted in a dose-dependent increase in vasodilation of the forearm arteries (i.e. forearm blood flow after occlusion) in six healthy men. The vasodilation response was inhibited by the use of LNAME (an eNOS inhibitor) indicating that the underlying mechanism for dehydro-equol-induced vasodilation was mediated by an increase in endogenous NO [156]. This latter study confirms the importance of investigating potential dose-response relationships for the vascular effects of equol to investigate an efficient dose of S-equol and it also highlights the need for further research work on bioactivity of equol related metabolites *in vitro* and *in vivo*. These findings also relate to previous *in vitro* work on isolated rat aortic rings where it was shown that dehydro-equol was the most potent vasodilator compared to other daidzein metabolites and its bioactivity was comparable to that of 17β-oestradiol [262].

In our previous study, we have shown that equol producers had reduced arterial stiffness (assessed by c-f PWV) after isoflavone intake and this effect correlated positively with the plasma concentrations of equol (Chapter 3). This subsequent study now suggests no benefits of feeding equol supplements to non-equol producers for arterial stiffness.

These contradictory findings might be explained by higher bioactivity of the endogenously produced S-equol. It is found that the endogenously produced S-equol shows much slower clearance rate from the body compared to that of the supplemented S-equol (see Figure 1.10). Alternatively, it is likely that other features of the equol producer phenotype may also contribute to the observed effects on vascular function.

It has been proposed that the inter-individual differences in intestinal microflora species may contribute to variation in disease vulnerability through effects on metabolism and subsequent exposure to isoflavones or equol [172]. In addition, single nucleotide polymorphisms (SNPs) were suggested to be linked to the equol producer phenotype: a genome-wide association study in a Korean population has identified an association between 5 SNPs in HACE1 (a gene related to immune responses) and equol production[28]. One of the SNPs in HACE1 (rs17065302 C > G) has been proposed to modulate the expression of HACE1 in the gut, which might lead to changes in the host

immune responses in the colon. The resulting intestinal environment might more suitable to sustain equol-producing microbiota [28]. This study also reported statistically significant lower diastolic blood pressure and non-statistically significant lower systolic blood pressure in equol producers compared to non-equol producers. Further analysis showed an interaction effect between the equol producer phenotype and one of the aforementioned HACE1 SNPs (rs6927608 A>C, located in an intron) on blood pressure (both diastolic and systolic). This interaction effect was stronger than the effect of equol producer phenotype alone. For instance, equol producers who had major allele homozygotes (AA) of rs6927608, showed significantly lower systolic blood pressure than non-equol producers. It is unknown why the association between SNP rs6927608, blood pressure and equol producer phenotype occurred and further investigations are needed.

The association between isoflavone intakes, equol producer phenotype and potential vascular benefits could also involve direct or indirect regulation of the expression levels of genes related to CVD development. A recent study has shown that isoflavone supplement intake (94 mg/d for 8 weeks, compared to a placebo) significantly altered gene expression of 357 genes in white blood cells isolated from equol producers. In particular, gene sets related to inflammation and oxidative phosphorylation, that might be involved in atherosclerosis development, were down-regulated [263].

The limitations of our study included the measurement of endothelial function by EndoPAT that might have weakened our observation of improved endothelial function (assessed by EndoPAT, described in detail in section 3.4) and the recent publication regarding its lack of power to assess acute changes in endothelial function in relatively healthy populations [258]. The use of ultrasound flow mediated dilatation (FMD) (the most used non-invasive method for measurement of endothelial function) would have been more senstive but this method was not available to us at the time of the study. Our study could be also underpowered because of our sample size calculations as discussed in Chapter 3 (page 86). Additionally, this study arm would have benefited from more frequent measurements to coincide with published pharmacokinetics of equol. [19, 149, 260]. Especially, an earlier endpoint measurement 1 h after S-equol intake would have been useful as a range between 1 to 1.5 h has been reported to be the peak time of S-equol in plasma [141]. However, this was not possible due to the time restrictions on use of clinical equipment and research unit facility (the time required for conduction of vascular measurements was around 60-90 min to complete each test battery, for more details refer

to page 131). And maybe another endpoint measurement at 4 h when S-equol levels decreases in the blood, would be done to investigate any potential time course for bioactivity.

In addition, our selection of the dose of S-equol (40 mg) was based on bioactivity in reducing postmenopausal symptoms in an 8 week intervention unlike our acute phase trial in men [167]. However, this was the available literature at the time of the study design.

4.5 Conclusion

In this study arm we showed that feeding a single dose of equol supplements (40 mg) did not have a significant impact on vascular responses in non-equol producers. Although this is an acute feeding study, assessing vascular and hemodynamic function only at two time points (baseline and 2 h post-intake), the results suggest that the endogenous capacity to produce equol may have an important role on vascular benefits beyond the production of equol.

These study findings could have been affected by limitations such as the use of EndoPAT for endothelial function assessment, the small sample size, the selected dose and measured time points (as discussed previously). Yet, the investigation of the benefits of natural equol (SE5OH) supplements on vascular markers in non-equol producers is novel and our study followed a hypothesis driven design, prospectively recruiting non-equol producers using a standard methodology.

Further studies on dose-response and/or time-course relationship, and larger long term trials are required to establish the influence of equol supplement intake on arterial stiffness in equol producers versus non-equol producers including males and females. In summary, future human trials may investigate the potential effect of equol producer phenotype on bioavailability of equol supplements and subsequent vascular function, in addition to the possible gender-dependent differential effects.

Chapter 5   Main discussion and future work

5.1   Main discussion

Cardiovascular disease is a major public health burden and nutrition plays an important role in the prevention and treatment of cardiovascular disease (CVD) [29]. Clinical nutrition and dietary strategies have been shown to modify the development of CVD. Dietary constituents such as soy isoflavones were suggested to lower CVD risk factors, although the evidence is inconsistent [5-16]. This thesis focused on the potential importance of the ability to produce the isoflavone metabolite equol in explaining the benefits of soy isoflavones on reducing major CVD risk factors such as endothelial dysfunction, arterial stiffness, and hypertension. Additionally, we examined the vascular effects of a single dose of S-equol containing supplements in subjects who lack the endogenous capacity to produce the gut metabolite equol.

Equol producers had significant improvements in arterial stiffness and blood pressure, compared with non-equol producers, following 1-year of an isoflavone containing intervention (assessed through a retrospective analysis approach (Chapter 2)). Our acute study, which prospectively recruited equol producers, has further substantiated improvements in arterial stiffness following a single dose of an isoflavone supplement at the expected peak time of plasma equol (Chapter 3). These data suggest a higher vascular bioactivity of equol, than the parent compound daidzein. However, no significant vascular benefits were shown by non-equol producers following single-dose intake of an S-equol supplement (Chapter 4), which suggests that the endogenous equol producer phenotype may contribute more than equol *per se* in explaining the efficacy of isoflavone intake on vascular function.

Our first analysis (Chapter 2) included type-2 diabetic population that may have a 2 to 4 fold higher risk of CVD mortality relative to non-diabetics [217]. In particular, the benefits of lowering blood pressure in type 2 diabetic patients on cardiovascular mortality have been established in human studies [264] and it has been estimated that a 2-3 mmHg reduction of diastolic blood pressure (BP) in 50–69 years old subjects, would reduce the risk of coronary artery disease and stroke by 10%-20% [58]. Therefore, the observed

reductions of - 2.4±1.3 mmHg in diastolic BP in our analysis could result in an estimated decrease of 14% in CVD risk.

We also found positive effects on an important predictor of CVD, arterial stiffness assessed by the gold standard method PWV [35]. It has been estimated that an elevation of PWV of 1 m/s would result in a substantial increase of 14-15% in CVD risk [50]. Our findings also suggest that isoflavone interventions in equol producers might be associated with decreased carotid-femoral PWV and the observed reductions (-0.7 m/s in postmenopausal women (Chapter 3) and -0.8 m/s in males at elevated CVD risk (Chapter 4), respectively) could be of clinical importance leading to 10-12% decrease in CVD risk if sustained.

Our findings could be therefore clinically relevant; however, they are influenced by the limitations of our studies as discussed earlier (in Chapters 2, 3, and 4). Further studies are required to confirm benefits of the isoflavone intake and equol producer phenotype on reducing CVD disease risk factors. Subsequently, if evidence is established, further public health research could investigate dietary recommendations of soy isoflavone intake.

As our knowledge about the interaction of nutrition, genetics, metabolism, gut microflora profile and other environmental factors increases, the tendency is for clinical nutrition to move from an old paradigm of rigid guidelines to a new paradigm where nutritional indications are custom-designed according to individual characteristics to promote health. In fact, dietary recommendations are already established for specific group of populations with different age, gender and health status [265]. In addition, nutrition research has suggested a concept called 'nutritional profile', which is a combined set of genetic, metabolic, proteomic, behavioural and functional factors that are studied to assess the interaction between diet and other factors, to affect health/disease status. This concept is considered as a good start to the intervention of personalised nutrition [266].

Our current knowledge about equol producer phenotype suggests that it is a metabolic profile defined by isoflavone intake and could be influenced by the gut bacterial community. In addition, it is known that human capability to produce equol varies between different populations and the frequency of equol producers in Asian populations is much higher than in Western populations [139]. However, the biological and environmental explanations for this metabolic phenotype are still lacking, all related microbiota profiles are yet to be identified and any associated genetic variation or interaction. Interestingly, two previous studies have shown that equol producer phenotype was associated with some genetic alterations [267] and variations [28]. Given all the available information on the

equol producer phenotype, we envisage that it might become an integral part of the nutritional profiles and further developments on personalised/stratified dietary recommendations for optimal health.

Interestingly, equol *per se* has already attracted the attention of supplements industry and research has been conducted for almost 18 years to produce S-equol supplement. In 2002, Otsuka Pharmaceutical Co., Ltd has managed to develop a natural S-equol supplement (SE5-OH) by fermentation of a soy germ solution with the lactic acid bacterium Lactococcus garvieae (20-92 strain). Afterwards, few human studies and clinical trials have been conducted to confirm its safety and usefulness for women health. In 2014, SE5-OH supplements have been launched by Otsuka Pharmaceutical Co., Ltd and it is currently being advertised as a natural supplement for menopausal and postmenopausal women (http://www.otsuka.co.jp/en/company/globalnews/2014/0402_01.html). However, this supplement is directed towards women during or post-menopause because of its oestrogenic bioactivity. Potentially, the synthesised equol supplements could also target different population subgroups such as non-equol producer men at high CVD risk. However, further clinical trials investigating effects of equol supplements on cardiovascular health are still required. If more evidence becomes available, there could be also an interest from food industries to develop different types of supplements associated with greater equol production such as probiotics of equol producing bacteria.

Overall, this thesis supports the potential importance of equol producer phenotype on the effectiveness of soy isoflavone interventions to improve arterial stiffness, and hemodynamic function. However, further evidence is still needed to elucidate the relative benefits of equol producer phenotype and/ or equol on vascular function.

5.2 Research gaps and future work

Previous RCTs have shown that longer term interventions with isoflavones e.g. 12 months, may be required to elicit cardiovascular improvements, [15, 171, 220]); studies which assess vascular function in equol producers compared to nonproducers would therefore benefit from such a longer-term intervention. There is also a need for assessment of the vascular function using multiple robust techniques, in particular, for assessments of endothelial function and arterial stiffness. Regarding endothelial function, ultrasound FMD at brachial artery is considered the most robust non-invasive method, which has been shown to be associated with the Framingham risk score in predicting CVD risk, and is thought to provide more sensitive assessment of endothelial function than that of EndoPAT [43, 257]. In addition, it would be interesting to further confirm our positive PWV findings in equol producers, by methods other than c-f PWV. Whilst aortic PWV is the non-invasive gold standard method, and estimating it between carotid to femoral is still the most accepted approach [38], there are other techniques such as MRI measurements of aorta PWV which have been shown to be associated with c-f PWV [268]. As MRI technologies might provide more insights regarding the structural and/or functional changes associated with improved arterial stiffness, then this technique could be an informative target for future research.

It is also suggested that different isoflavone metabolites have different bioactivities; for instance dehydro-equol, the potential precursor of S-equol found in human urine [261] has been shown to significantly increase forearm blood flow in humans [156]. Therefore, establishing the differential bioactivity of isoflavone metabolites, and determining the compounds, which have the greatest impact on vascular health, is worth further investigation.

Regarding the benefits of S-equol *per se* on vascular function, there is also a need for further human interventions of S-equol supplements that investigate dose-response relationship, gender-dependent responses and long-term exposures. Although, *in vitro* work has shown that S-equol at 0.1-10 µmol/L can induce vasodilation [93], the *in vivo* dose of S-equol that induces vascular-related benefits is unknown. In a previous study, women showed significantly decreased arterial stiffness (measured via a CAVI device) after 12 week, daily intake of 10 mg S-equol containing (SE5OH) supplements [168]; there

was, however, no effect in males. There is, therefore, a possibility that females might benefit more from S-equol intake than males and further studies are required to elucidate gender related variations. In addition, previous cross-sectional studies in Asian populations with habitual intakes of 25- 50 mg soy isoflavone/ day, have shown equol producers to have a better cardio-metabolic profile than non-equol producers (as reviewed in Chapter 1, Table 1.5, page 39). The findings of this thesis support the latter data in part and there might be beneficial features of the endogenous equol producer phenotype which could be related to health benefits of specific intestinal microflora, and/or genetic factors that are associated with the endogenous equol producer phenotype (Figure 5.1). Previous studies have shown that the ability to produce equol could be determined by specific intestinal microflora (Table 1.5) and it has been proposed that inter-individual differences in intestinal microflora species may contribute to variations in disease vulnerability [125, 269]. Faecal microbiota extracted from equol producers were able to produce equol from incubated daidzein *in vitro* [176, 186], however it is still unknown if the ability to produce equol could be acquired by faecal infusions *in vivo* and if the changed capability is related to any benefits on vascular health. Alternatively, or in combination with a specific microbiota profile, genetic variations might contribute to equol producer phenotype and interact with equol to improve vascular function. In particular genetic differences (5 SNPs) in the human genome were found to be associated with the equol producer phenotype by a GWAS study in a Korean population [28]. However, research in this area is at a very early stage and further genetic studies are required in other Asian and Western populations to identify potential SNPs related to the ability to produce equol and search for any interactions with specific microbiota profiles and vascular health. A strategic plan of future research directions based on these identified research gaps is provided in Figure 5.2.
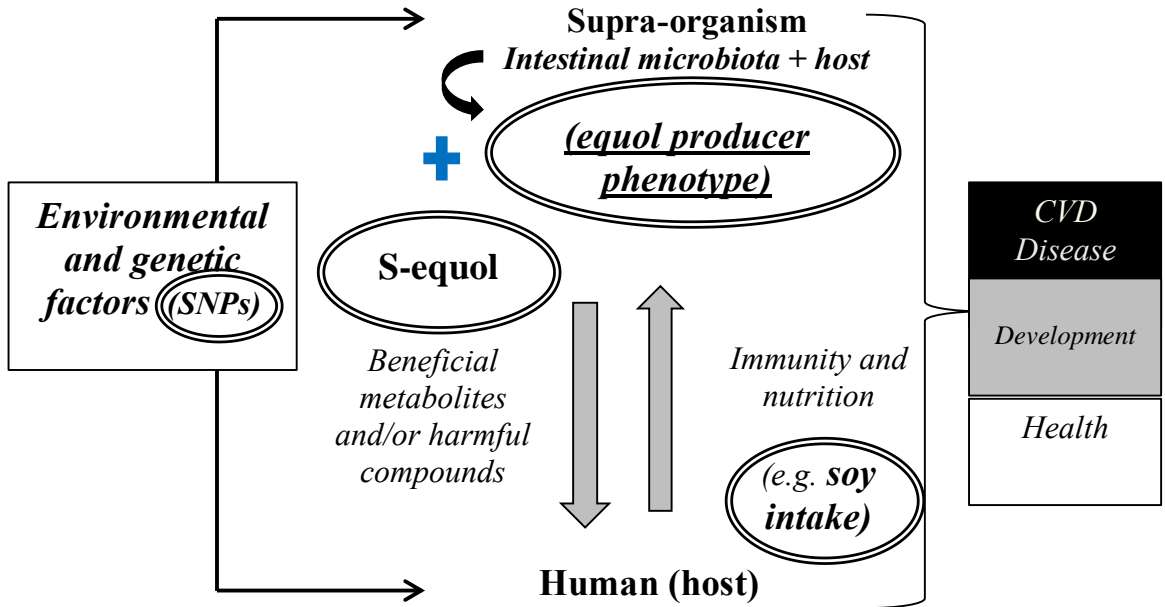
Figure 5.1 Illustration of the interactions between gut microflora, and human health and the potential role for intestinal metabolite equol.

Human health is influenced by many environmental and genetic factors. One of the major environmental contributors is food. Another factor within the human body is the gut microbiota that affects the physiology and metabolism. Recently, it has been suggested that the intestinal microbiota plays a role in the development of diseases such as CVD. The intestinal microflora is responsible for the production of several metabolites from food sources such as equol from the ingested soy isoflavone that is shown to possess vascular bioactivity. In addition, the host genotype might interact with diet to contribute to human health and susceptibility to CVD disease (figure adapted from Zhao et al., 2013 [269]).

1) Long term isoflavone intervention that prospectively recruiting equol and non-equol producers to elicit if extended exposure to isoflavones exert greater benefits of isoflavone intake on vascular function in equol producers compared to nonproducers and confirm

the potential role of equol producer phenotype in the effectiveness of isoflavone intake on vascular health

2) Dose-response study prospectively recruiting EPs and non-EPs to further study the vascular effects of S-equol supplements and EP phenotype, identify the most efficient dose of S-equol supplement on vascular function, effects of long-term intake, and any gender-dependent differential responses.

3) Pilot human trial infusing microbiota species (faecal transplant) from equol producers into non-equol producers to investigate if equol producer phenotype could be acquired and subsequent RCT to study the vascular benefits of isoflavone intake after acquiring microbiotic equol producer phenotype.

4) GWAS and intestinal microbiome observational studies in Asian and Western populations to study the biological explanations and factors that may contribute to ability to produce equol.

Figure 5.2 Plan for future research directions and approaches

Future research could include 1) large long term trials to establish the role of equol producer phenotype in the benefits of isoflavone intake on vascular function 2) human studies feeding S-equol supplements to investigate dose-response relationships, if more evidence has become available, 3) faecal infusion studies to transfer equol producing bacteria into the gut of non-equol producers, 4) GWAS and observational studies to investigate association between equol producer capacity and genetic variations. *EP, Equol producer.*

1. **<u>Long term isoflavone intervention with prospectively recruited equol and non-equol producers</u>**, in order to elicit if extended exposure to isoflavones exert greater benefits of isoflavone intake on vascular function in equol producers compared to nonproducers.

1.1   This trial would be a long term parallel-designed RCT in individuals prospectively recruited as equol producers and non-producers. Populations at elevated risk of CVD, such as postmenopausal women and prehypertensive men could be selected, as previous data suggests 'at-risk' groups may gain clinical benefits from isoflavone intervention [2]. Based on previous evidence of cardiovascular benefits [15, 161, 171, 220], participants would be supplemented with a dose of 80-100 mg/day (as isoflavone aglycones equivalents). The vascular measurements would include blood pressure; endothelial function measured by brachial ultrasound FMD and arterial stiffness by c-f PWV and MRI-derived PWV taken at baseline, 3 months and 12 months. To elucidate the plausible biological mechanisms by which isoflavones or equol induce improvements on arterial stiffness and hemodynamic function, biological analyses could include plasma lipids (Total cholesterol, HDL-cholesterol, LDL- cholesterol [6, 161, 171] and biomarkers of vascular function. In particular, analyses of plasma levels of NO and other modulators of vaso-reactivity produced by endothelial cells such as ET-1 [98], prostacyclin/TXA_2 [24] and NADPH oxidase activity [20] (see rationale, section 1.3.4, page 21) would help in developing further insight into the mechanisms of action. Effects on plasma levels of hydrogen sulphide that is also known to induce relaxation of vascular smooth muscles in the endothelium [270]) could also be determined. The collected blood samples could be analysed for different isoflavone and equol metabolites and these compounds could be investigated for significant correlations with vascular benefits.

1.2   Complementary *in vitro* bioactivity assays in aortic rings or endothelial cell lines could be done to identify isoflavones or equol metabolites with the highest bioactivity on vascular-related biomarkers such eNOS and /or NADPH oxidase expression and activity.

Collectively, these studies could confirm the role of the equol producer phenotype in the effectiveness of isoflavone intake on cardiovascular health, particularly in long term inteventions reflecting effects of habitual intakes. In adidition, this work might identify biological mechanisms of action and help establish the relative bioactivity of various isoflavone metabolites.

2.   Assessing the cardiovascular effects and dose-response relationship of providing exogenously produced S-equol to non-equol producers

2.1   A dose-response pilot study would assign participants (including postmenopausal women and prehypertensive men) to one of five treatments: 2.5, 5, 20, 40, 60 mg S-equol/day (i.e. the anticipated range for plasma levels of S-equol following these doses would range from 0.125 μM to 11 μM [93]). The low S-equol doses are expected to attain similar plasma levels to the ones reported in previous studies that have shown cardiovascular benefits in equol producers. Particularly, these studies have shown mean plasma concentrations of S-equol of 0.24 µmol (our acute study, Chapter 3), 0.11 µmol/L [6] and 0.19 µmol/L [171] after consumption of 80 mg (50% daidzein), 33 mg (~50% daidzein), and 105 mg (daidzein content was not defined) of total isoflavones (as aglycone units), respectively. Vascular function measurements including endothelial function (ultra-sound brachial FMD) and arterial stiffness (c-f PWV) would be employed. Blood samples would be collected for analysing levels of S-equol and plasma biomarkers of vascular function such as NO, ET-1 [98], NADPH oxidase activity [20] that might explain mechanisms of bioactivity. In term of endpoint time collections, they should be at 1, 2, 4 h after dose ingestions, to cover the anticipated pharmacokinetics of S-equol [19].

2.2   If the suggested study identifies an optimal efficient dose, a subsequent larger parallel design RCTs would investigate vascular responses to S-equol intake (longer term, i.e. 12 months, rationale in page 146), isoflavone intake (daidzein-rich supplements, 80-100 mg, same rationale as in approach 1, page 146), or matched placebo in prospectively recruited equol producers and non-producers.

These planned studies would provide data about effects of S-equol intakes on cardiovascular function, and help establish differential effects of S-equol as endogenously produced or supplements.

3.   Faecal infusion approach to transfer equol producing bacteria into the gut of non-equol producers, in order to determine the ability to aquire the equol producer phenotype and its associated vascular benefits.

3.1   A human study could investigate if infusing microbiota species (faecal transplant) from equol producers into non-equol producers would induce the ability to produce equol in non-producers. Briefly, this future RCT would prospectively recruit non-equol and equol producers; the non-producers would be blinded to treatment, and would be randomly allocated to a single faecal infusion of microbiota either collected from equol producers (allogenic microbiota infusion refers to transplant of microbiota to a non-identical recipient from the same species) or collected from own faeces (autologous microbiota infusion means transplant of microbiota to a genetically identical recipient). These infusions could be done through a gastro-duodenal tube or colonoscopy into the intestine. Faecal samples will be collected at baseline and 6 weeks after infusion to compare changes in microbiota compositions after both infusions (previously research has shown that the colonic microflora is capable of significant changes at 6 weeks after a single faecal infusion [271]. The soy challenge would be repeated at 6 weeks to assess if the equol producer capacity is acquired in the subjects undergoing the allogenic microbiota infusion.

3.2   A follow-up RCT could investigate vascular responses to isoflavone intake in non-equol producers who have had a microbiota transfer from an equol producer donor. This study would include a cross-over randomisation to an intervention with isoflavones (80-100 mg isoflavone aglycones equivalents, same rationale as in approach 1, page 146) or matched placebo for 6 weeks. Vascular functions (endothelial function (ultrasound FMD) and arterial stiffness (c-f PWV)) would be assessed at baseline and 6 weeks after intervention. Previously, 6 weeks have shown induced changes in the intestinal microflora profile after a single allogeneic microbiota infusion *in vivo*. This change was also

associated with cardio-metabolic benefits and according to previous isoflavone intervention, short term studies would be still sufficient to improve vascular reactivity in equol producers [6, 161] and it would be a good start for such primary research in this area, Figure 5.3.

These study approaches would elucidate if equol ability could be acquired in the intestine of non-equol producers and subsequently if there is any interactive role between equol producing microbiota and isoflavone intake to induce benefits on vascular health.
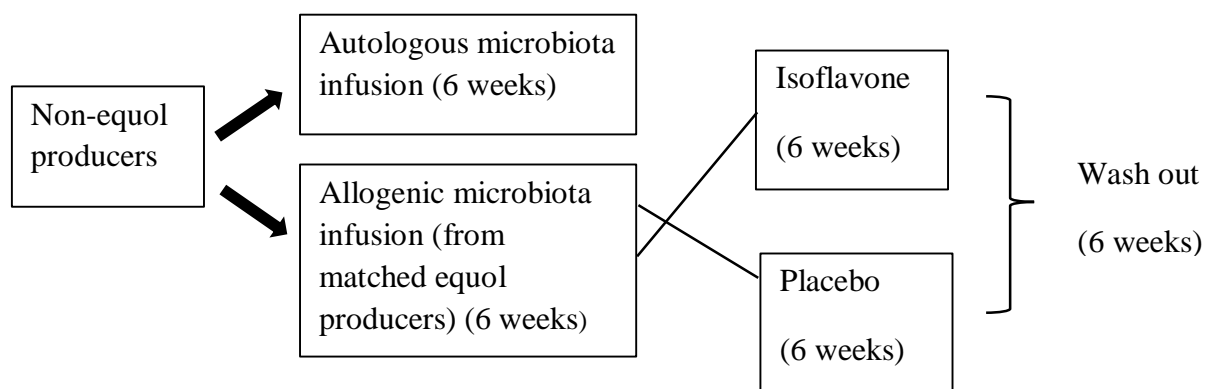
Figure 5.3 Study design for a suggested future isoflavone intervention to investigate the importance of the endogenous microbiota profile of equol producer phenotype on vascular function.
If strong evidence for benefits of the equol producer phenotype on cardiovascular health become available, an interesting future research would be a human study that investigates if infusing microbiota species (faecal transplant) from equol producers into non-equol producers would induce the ability to produce equol in non-producers, and if vascular benefits will be associated with this transplant.

Faecal microbiota transplant procedures are used in clinical practice as a useful treatment for treating infections such as Clostridium difficile [272] and also faecal transplant from lean donors has shown improvements on insulin sensitivity in subjects with metabolic syndrome [271]. Even though serious side events in faecal microbiota transplant are unusual, it is an invasive operation that has risks associated with the administration methods (such as colonoscopy) and the screening tests for pathogens before transplant [272, 273]. In

conclusion, the proposed study above would be worthwhile only following strong evidence for benefits of the equol producer phenotype on cardiovascular health.

4. **Understanding the association between equol producer capacity and genetic variation,** in order to investigate any genetic variations, and / or specific intestinal microbiota which are associated with the equol producer phenotype.

One suggestion would be to conduct good quality observational GWAS studies with large sample sizes in Asian and/or Western populations (e.g. at least 1000 paticipants per study to end up with the minimum required numbers for valid GWAS analysis, [28]). These studies could be done as parts of on-going cohorts. Participants would undergo a soy challenge to identify their equol producer phenotype as part of the screening procedure and then they would attend one assessment visit where blood and faceal samples would be collected for genotyping for GWAS analysis and pyrosequencing for intestinal bacteria analysis. It would be interesting if a comparative analysis could be conducted in Asian and Western subjects to investigate any genetic and /or microbiotic differences between the two populations where equol producer phenotype are different (50-60% and 20-30%, respectively).

These observational studies might be able to identify genetic factors, and potentially specific intestinal microflora that are associated with equol producer phenotype and any interactions with better vascular health.

5.3 Conclusion

The data in this thesis contributes to the literature research gap regarding the potential effect of the equol producer phenotype in modulating the impact of dietary isoflavones intake on vascular health. First our retrospecive analysis was conducted on a large double-blinded randomised design with a long term (12 months) intervention and the assessd outcomes included robust clinical markers of hemodynamic and vascular function (ambulatory blood pressure and carotid-femoral PWV). Second, our acute study is one of the first human studies that prospectively recruit based on the equol producer phenotype. In addition, equol and non-equol producers were matched based on high priority CVD predictors (blood pressure and BMI) and the method used to identify their equol producer phenotype was a validated standard method. This study was also well-designed as a double blinded randomised placebo-controlled cross over intervention trial and included dietary restrictions on flavonoid and dietary nitrate intake, as well as restrictions for strenuous physical activity that might affect vascular function, on the days prior to the clinical visit. Finally, we were the first to investigate the vascular responses on feeding synthetic S-equol (SE5OH) supplements to non-equol producers and to suggest a potentially important role for the equol phenotype rather than equol *per se* in the vascular reponses to isoflavone intake.

The limitation in the research of this thesis include assessment of endothelial function using EndoPAT, which recently has been reviewed to have limitations [257, 258]. In addition, the isoflavone content in the provided treatment bars was not independently confirmed prior to commencing the trial, resulting in that arm feeding negligible levels of isoflavones and therefore hampering our ability to compare the effects of different matrices of isoflavones on vascular function which we had set out to do. This research question is yet to be investigated in future studies.

In conclusion, equol producers had significant benefits on blood pressure and arterial stiffness after long-term intake of an isoflavone intervention; and improvements in arterial stiffness (assessed by the gold standard method, carotid-femoral PWV) after acute intake of isoflavones. The latter coincided with increased equol concentrations in plasma and was not observed when synthetic S-equol (SE5OH) supplements were provided to non-equol

producers. Our findings suggest that equol might be more effective on vascular function than the isoflavone parent compounds and the endogenous capacity to produce equol might be more important than equol *per se* in explaining the effectiveness of soy isoflavones on arterial stiffness. In contrast, we did not observe significant benefits on endothelial function as assessed by EndoPAT after single intake of isoflavones in equol producers and this finding needs further verification using the most common used method of brachial FMD.

Overall, this thesis work supports the hypothesis that equol producer phenotype may partly explain the effectiveness of isoflavone interventions on arterial stiffness. Further longer-term interventions are still required to elucidate the relative benefits of the equol producer phenotype on efficacy of isoflavone intake on vascular function and how to achieve similar benefits in non-equol producers.

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Appendices

Appendix A   Scientific contribution and acknowledgments

A.1   Scientist contribution for retrospective analysis (covered in Chapter 2)

Trial was designed, conducted, completed by Prof. Aedin Cassidy and Dr. Peter Curtis. Urinary isoflavone and equol was quantified externally in institute of food research, Norwich, UK. During the 1st Phd year, Phd student Sara Hazim prepared a scientific rationale to the retrospective analysis including clinical vascular endpoints, biomarkers, and statistical methods to be used with guidance of supervisors. SH and PC conducted the laboratory analysis for the analysed biomarkers for insulin, ET-1, ACE and resistin.

A.2   Scientist contribution for acute studies (parts of the FASTCHECK study that are covered in Chapter 3 and 4)

During 1st year, supervisory team with PhD student SH did study concept and design. PC, Manuel Schaer (MS) and SH prepared FASTCHECK study protocol, mainly SH provided the scientific rationale for study design of the acute studies providing isoflavone and S-equol. Prof John Potter provided guidance on the selection of clinical measurements for the study, sample size calculations, and clinical judgment for screening results and eligibility of participants. During the 2nd PhD year, PC, MS and SH prepared ethics documents including poster, participant information sheet, and filling IRAS except submission of IRAS documents were done by PC &MS. During the 2nd and 3rd PhD years, the Fastcheck team PC, SH, MS, Luisa Ostertag (LO) and Kathleen McGrath (KM) recruited the volunteers and conducted the trial (SH conducted most of the vascular function assessment for the acute studies feeding isoflavone and S-equol). Colin Kay (CK)

did the treatment randomisation. During first half of the 4th PhD year, SH did the laboratory analyses with guidance on LCMS method by CK, Rachel De Ferras (RF), LO and PC. SH and placement students (KM, and Esme Ward) did food data entry and analysis for the FASTCHECK study. SH performed statistical analysis with guidance of PC.

Appendix B   Statistical analyses: supplementary data

Table I. Effect of flavonoid supplementation on the vascular function in equol producers and non-equol producers

Outcome	Non equol producers (N1)			Equol producers (N2)			P value
	Baseline	6 mo	12 mo	Baseline	6 mo	12 mo	
PWV (m/s)	9.1±0.6	9.1± 0.4	9.4 ± 0.2	8.6±0.5	8.4±0.3	7.9± 0.2	0.30
Systolic BP (mmHg)	135± 2	137±2	138± 2	131±2	132±4	131±2	0.65
Diastolic BP (mmHg)	77±2	77±2	78±2	74±2	74±2	72±2	0.98
MAP (mmHg)	98±2	99±2	100±2	95±2	95±2	94±2	0.28
PP (mmHg)	58.3±1.8	60.2±2.0	60.8±1.8	56.4±2.2	56.8±2.8	59.1±2.6	0.66
*NO (M)	43.0±4.2	43.9±3.4	40.7±3.3	61.1±6.5	43.3±4.0	53.5±5.4	<0.01

Table continues in the next page.

*Outcome	Non equol producers (N1)			Equol producers (N2)			P value
	Baseline	6 mo	12 mo	Baseline	6 mo	12 mo	
ET-1 (pg/ml)	1.64±0.17	-	1.98±0.25	1.65±0.16	-	1.57±0.21	0.08
NO/ET-1 (µmol/ng)	33.59±6.22	-	26.38±4.52	37.29±4.66	-	38.59±5.03	0.22
Resistin (ng/ml)	16.98±1.73	18.45±1.99	19.04±2.67	17.27±2.69	17.15±2.29	15.68±1.72	0.89
Insulin (mU/l)	8.8±1.1	7.5±0.7	7.9±0.8	6.1±0.9	5.9±0.8	5.6±0.7	0.81
HOMA-IR(mU.mmol/l²)	2.74±0.34	2.40±0.23	2.49±1.89	2.13±0.33	2.07±0.28	1.89±0.26	0.91
ACE (ng/ml)	163.79±104.82	-	151.17±92.68	102.89±33.01	-	119.85±44.78	0.54

The number of participants in the analysis was: N1=30, N2=17 for AMBP systolic and diastolic BP, N1=30, N2=16 for PP, N1=29, N2=17 for MAP, N1=29, N2=16 for resistin, insulin and HOMA-IR; N1=8, N2=5 for ACE; N1=19, N2=13 for ET-1 and NO/ET-1. Data presented as mean±SEM (Standard Error of the Mean). P values represent the significance of the repeated measures ANOVA for effect of flavonoid treatment over time accounting for an interaction of time and equol production status. *data for outcomes was log-transformed to achieve normality. *ACE, angiotensin converting enzyme; AMBP, Ambulatory blood pressure; BP; Blood pressure; ET-1, Endothelin-1; HOMA-IR, Homeostatic model assessment of insulin resistance, MAP, Mean arterial pressure; NOx, Nitric oxide metabolites; PP, Pulse pressure; PWV, Pulse wave velocity.*

Table II. Plasma concentrations of isoflavones at baseline, 6 h and 24 h in non-equol and equol producers (n=28) after intake of placebo

EP phenotype	Isoflavone (nmol/L)	Placebo BL	6 h	24 h	P value (6 h vs BL)	P value (24 h vs BL)
Non-equol producers (n=14)	Genistein	4±3	15±9	7±5	0.21	0.51
	Daidzein	4±2	16±7	8±4	0.08	0.38
	S-equol	ND	ND	ND	NA	NA
	Glycitein	21±7	20±13	3±2	0.95	0.02
Equol producers (n=14)	Genistein	ND	ND	3±3	NA	0.33
	Daidzein	1±1	3±2	4±2	0.19	0.21
	S-equol	ND	ND	ND	NA	NA
	Glycitein	19±6	7±4	6±4	0.14	0.12

All values presented as mean±SEM. Univaraite analysis used. Presented P value for difference from baseline in plasma isoflavone concentrations for both equol and non-equol producers at 6 h and 24 h after consumption of placebo. **BL**, *Baseline;* **NA**: *Not applicable;* **ND**, *non-detectable or below limit of detection.*

Table III. Plasma concentrations of isoflavones at baseline, 6 h and 24 h in non-equol and equol producers (n=28) after intake of isoflavone intervention *(80 mg isoflavone as aglycone equivalents)*

EP phenotype	Isoflavone (nmol/L)	Isoflavone supplement BL	6 h	24 h	P value (6 h vs BL)	P value (24 h vs BL)
Non-equol producers (n=14)	Genistein	4±3	301±40	72±37	<0.001	0.08
	Daidzein	4±2	1692±269	170±46	<0.001	<0.001
	S-equol	ND	ND	ND	NA	NA
	Glycitein	21±7	291±48	54±12	<0.001	0.02
Equol producers (n=14)	Genistein	ND	200±33	28±15	<0.001	0.08
	Daidzein	1±1	1688±215	190±31	<0.001	<0.001
	S-equol	ND	ND	236±81	NA	0.007
	Glycitein	19±6	237±36	35±9	<0.001	0.15

All values presented as mean±SEM. Univariate analysis used. Presented P value for difference from baseline in plasma isoflavone concentrations in both equol and non-equol producers at 6 h or 24 h after consumption of isoflavone supplement. **BL**, *Baseline;* **NA**: *Not applicable;* **ND**, *non-detectable or below limit of detection.*

Table IV. Plasma concentrations of isoflavones at baseline, 6 h and 24 h in non-equol and equol producers (n=28) after intake of placebo or isoflavone enriched cereal bar

EP phenotype	Isoflavone (nmol/L)	Placebo			Isoflavone cereal bar			P value (6 h)	P value (24 h)
		BL	6 h	24 h	BL	6 h	24 h		
Non-equol producers (n=14)	**Genistein**	4±3	15±9	7±5	4±3	45±17	1±1	0.71	0.98
	Daidzein	4±2	16±7	8±4	4±2	54±16	6±4	0.99	1.00
	S-equol	ND	ND	ND	ND	ND	ND	NA	NA
	Glycitein	21±7	20±13	3±2	21±7	28±10	ND	0.98	0.96
Equol producers (n=14)	**Genistein**	ND	ND	3±3	ND	7±3	ND	0.97	0.98
	Daidzein	1±1	3±2	4±2	ND	39±11	4±2	1.00	0.98
	S-equol	ND	ND	ND	ND	ND	11±5	ND	0.99
	Glycitein	19±6	7±4	6±4	18±6	23±7	2±2	0.85	0.87

All values presented as mean±SEM. Univariate analysis used. Presented P value for difference in plasma concentrations of isoflavones and equol between placebo and isoflavone enriched cereal bar intervention at 6 h timepoint (6 h), and at 24 h timepoint (24 h). **BL**, *Baseline;* **NA**: *Not applicable;* **ND**, *non-detectable or below limit of detection.*

Table V. Acute effect on hemodynamic and vascular measures at 2 h after isoflavone consumption in equol and non-equol producers

Equol producer phenotype	Intervention	Time (h)	RHI*	Diastolic BP (mm Hg)	Systolic BP (mm Hg)	CO (L/min)	AI (%)	PWV (m/s)
Equol producers (n=14)	Placebo	0	2.72±0.16	82±3	122±6	3.92±0.21	24±1	9.9±0.3
		2	2.59±0.13	84±4	126±5	4.31±0.35	23±1	10.2±0.5
		2-0*	-0.02±0.25	2±2	4±3	0.45±0.19	-1±1	0.4±0.2
	80 mg isoflavone supplement	0	2.62±0.17	83±3	123±6	4.09±0.21	25±1	10.0±0.3
		2	2.53±0.15	82.9±3.9	124±5	3.93±0.23	23±1	10.0±0.3
		2-0*	-0.11±0.24	1±2	2±3	-0.14±0.13	-2±1	0.2±0.2
Non-equol producers (n=14)	Placebo	0	2.57±0.15	76±2	129±2	4.30±0.18	26±2	9.9±0.2
		2	2.87±0.26	79±2	132±4	4.31±0.20	24±2	9.9±0.3
		2-0*	-0.02±0.24	3±2	3±3	-0.05±0.19	-3±1	-0.2±0.2
	80 mg isoflavone supplement	0	2.58±0.15	76±2	128±3	4.32±0.17	26±1	9.7±0.3
		2	2.84±0.01	75±2	127±4	4.32±0.20	24±2	9.9±0.3
		2-0*	0.01±0.23	-1±2	-2±3	-0.03±0.13	-2±1	-0.0±0.2
P value			0.761	0.355	0.472	0.58	0.68	0.33

Data presented as Mean±SEM. *Adjusted means, age used as covariate. For RHI*, equol producers (n=13), and Non-equol producers (n=14). P value for mixed general linear model, repeated ANOVA for intervention. *AI, Augmentation index; BP, Blood pressure; CO, Cardiac output; PWV, Pulse wave velocity; RHI, Reaction hyperaemia index.*

Appendix C   FASTCHECK study documents

C.1   Poster

CAN YOU HELP?

Scientists at the University of East Anglia (UEA) are looking for healthy men to take part in a study looking at the health effects of oranges, blackberries, chocolate or soy.

The study aims to test if these foods, <u>or</u> a capsule containing the compounds in these foods, improve blood pressure control.

Volunteers will be asked to attend 3 to 4 study days, lasting between 5 and 10 hours, and have blood samples and blood pressure control measurements taken. Participants will only be required periodically during the 10 hour visits and may leave the facility for up to 5 hours at a time. For some volunteers, a 2 hour visit will also be held the morning after eating the soy snack bar.

There will be at least 1 week between each assessment visit.

You can be in the study (lasting 20-31 days), if you are:

△ male 50 to 75 years △ non smoker (or ex-smoker, having stopped for at least 3 months) △ without a history of heart disease, diabetes or cancer △ not taking blood pressure, cholesterol-lowering drugs or flavonoid food supplements

and for 3 days before each assessment you agree to:

△ avoid dark chocolate, citrus fruit, berries, and soya foods △ <u>limit</u> some other fruits, vegetables and drinks (e.g. red wine, tea)

For further information, contact the research team (led by Dr Peter Curtis) on 01603-591063 or fastcheck@uea.ac.uk
This study will take place at the Clinical Trials Research Unit and travel expenses will be reimbursed

UEA University of East Anglia

NHS
Norfolk and Norwich University Hospital NHS Trust

C.2   Participant information sheet

University of East Anglia

INVITATION TO PARTICIPATE IN A RESEARCH PROJECT

Short title: Flavonoids, blood pressure control and blood vessel function

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

This study has been approved by the NRES Committee East of England – Norfolk and the Research Governance Committee at the Norfolk & Norwich University Hospitals NHS foundation trust.

The Chief investigator of the study is:
Professor Aedín Cassidy

The scientists in charge of the study can be contacted at:

Dr. Peter Curtis: Research **Fellow (P.Curtis@uea.ac.uk)**
Dr. Luisa Ostertag: **Senior** Research Associate (**L.Ostertag@uea.ac.uk**)
Ms. Sara Hazim: PhD Student (**S.Hazim@uea.ac.uk**)
Mr. Manuel Schär: PhD Student (**M.Schar@uea.ac.uk**)
Study e-mail: fastcheck@uea.ac.uk
Telephone number: 01603 591063

What is the study about?

As we age our risk of heart disease increases, with 1 in 3 men over the age of 75 in the UK suffering from heart disease. The result of this is often ongoing illness or premature death.

Diet and cardiovascular disease

It is well known that what we eat and drink can have a major role in protecting us from disease. In particular, eating more plant based foods e.g. fruits, vegetables and cereals appear to reduce the risk of heart disease. This may be, in part, due to natural elements in these types of food, such as a group of natural compounds called 'flavonoids'.

Research to date, has shown that after eating 'flavonoids', or the foods that they are found in (for example chocolate, berries, orange and soy food), the blood vessels in our system can become more 'relaxed' which helps the blood flow more freely. This could help lower blood pressure which in turn might lower the risk of damage to our major organs (e.g. heart).

Lots of research now shows that blood pressure benefits can be achieved by eating more of these compounds (and the foods they are found in), and they may be even more effective if your blood pressure is a little higher than ideal.

What's still not clear, however, is:

- HOW these benefits occur?

- HOW the body makes changes to our blood pressure control when we eat these food components?

- WHETHER different types of flavonoids are more effective than others?

We'd like your help to find this out.

Which type of flavonoids is this study interested in?

We are interested in four types of flavonoids that are part of the UK diet:

- **A**: 'anthocyanins' (found in berries and other fruits and vegetables)

- **B**: 'flavan-3-ols' (found in grapes, wine, cocoa and apples)

- **C**: 'flavanones' (found in citrus fruits)

- **D**: 'isoflavones' (found in soybeans).

We've chosen these types of flavonoids because they have been shown to have an effect on blood pressure after consuming them regularly.

What do we aim to do?

We plan to study the effects of eating a single portion of flavonoids on blood vessel activity (e.g. how relaxed the vessel is) and blood pressure control. We would also like to find out if these effects differ when the flavonoids are taken as supplements (e.g. in a capsule) or as part of a food (e.g. berries, orange juice or soy snack bar (like a cereal bar)) **or in a supplement containing all the flavonoids found in a food (e.g. chocolate)**.

To do this, we will ask all volunteers to visit us on 3 test-days, as shown below

(1) to eat a flavonoid food **(or for the 'chocolate' group; consume a chocolate based supplement containing all the flavonoids found in chocolate)**

(2) to consume a capsule with the same amount of flavonoid as the food (**(or for the 'chocolate' group; consume a chocolate based supplement without a flavonoid called 'epicatechin')**

(3) to consume items without flavonoids in them (placebo test)

In addition, volunteers in the soy snack bar group will be asked to attend a 4th test day;

(4) at which (a) volunteers who do not naturally produce a compound called '**equol**'*, when they eat soy, will consume a capsule containing natural **equol*** from soy-germ based ingredients and (b) volunteers who do produce '**equol**'*, will consume a capsule containing no equol.

The test foods and capsules will be prepared so that the researchers and the subjects will find it difficult to tell whether they contain flavonoids. **Initially, v**olunteers will be placed in one of the four groups and the foods **(*or food based supplements)** we

will test are**:**

- **A:** A glass of blackberry drink
- **B:** ***A chocolate based supplement**

At least 1 week gap — Test day 1* — At least 1 week gap — Test day 2* — Test day 3*

Consume 1 of these flavonoid foods

Consume flavonoids in a supplement

Consume **no** flavonoids

Study lasts 20 to 31 days
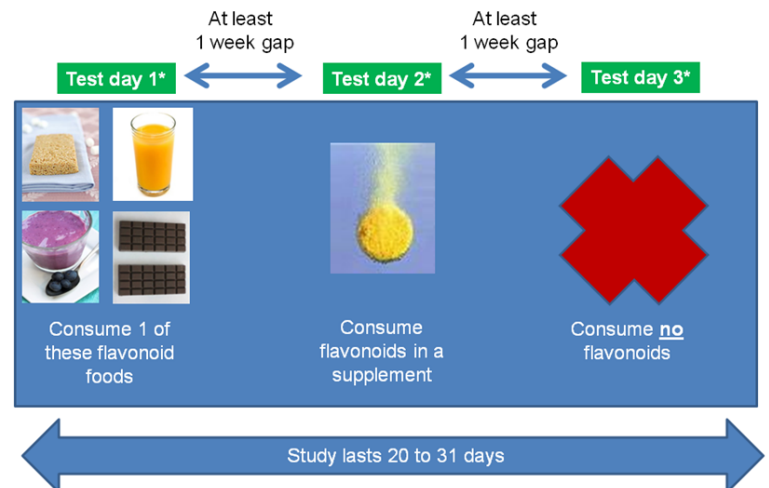
*The order of the test days will be at random

- **C:** A glass of Orange juice
- **D**: A soy snack bar (approx.. 40g)

This will be done at random and unfortunately, you will be unable to request which group to belong to. **If interested, volunteers can participate in a second group after a minimum of 1-month break.**

* Equol is a natural compound that is produced by the body after eating soy, in around 1/3rd of the UK population. So far, research has suggested that people who produce this compound may have better blood vessel function. We'd like to work out if giving this compound to people who do not naturally produce it, can help heart health. The equol supplement is produced by Otsuka Japan and the natural S-equol is produced by fermenting an isoflavone compound found in soy called 'daidzein' (using fermenting bacteria from the same family used in the dairy industry to manufacture cheese). After S-Equol is produced, the fermenting bacteria are deactivated by heat, which also sterilises the product.

What do I have to do?
During the study, which lasts at least **20 to 31 days**, there are times when you will be asked to stop eating dark

chocolate, berries, citrus fruits and soy products and to eat less of some other foods and drinks (depending on your normal eating habits) as these might affect our results. You will be asked to follow these instructions for 3 days before each test-day (see Table 1 for details (on page 195).

We strongly recommend that you consider these dietary restrictions before volunteering for this study. Some examples of food and drink that can be consumed during the study are included in Table 1 and more advice will be given upon request.

What will be measured?
Three or four times during the study (at visit 1, 2, 3 and 4), we will do measurements that are linked to blood pressure control and we will also collect blood samples at times when the tested compound is thought to appear in the blood.

The blood pressure measures will tell us the amount of blood your heart pumps each minute, and how much your blood vessels are able to relax. We'll also be able to measure how flexible your arteries are (the blood vessels carrying oxygen rich blood) and how signals from the brain can control your blood pressure. All of these measures are non-invasive (which means they do not enter the body).

The blood samples will be taken by qualified nurses and from these important samples we will be able to measure substances which show how blood pressure is controlled by the body. We will also use the blood to look for non-disease related genetic differences which might explain why some people naturally produce equol or seem to have better blood vessel responses after eating flavonoids.

Each test day will start in the morning and, as shown below, will last between 6 and 8 hrs. After taking our fasted set of measures, we'll ask you to eat the test food (or supplement) and then we'll repeat our measures to see how things change. The number of times we do this is shown in the figure below.
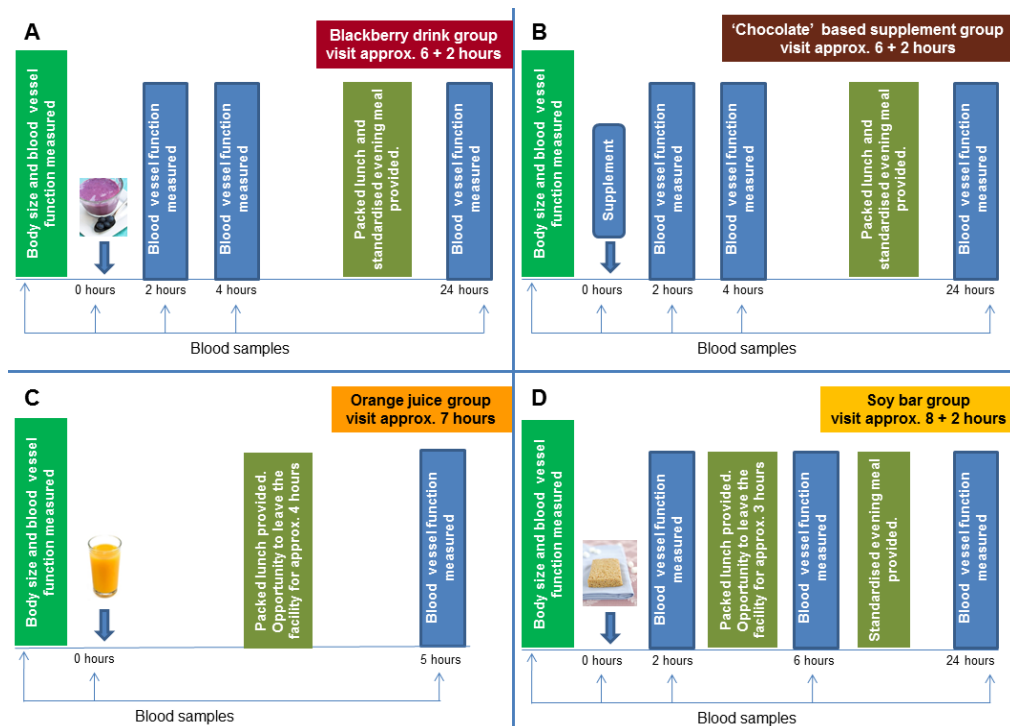
In the orange juice and soy bar groups, participants will only be required periodically during the 10 hour visits and may leave the facility for up to 4 hours at a time (advice will be provided on what activities are permitted during this time). A packed lunch will be provided for volunteers in these groups.

For ALL subjects other than the orange juice group, you will **ALSO** need to return (fasted, e.g. consuming only water that we will provide for 10 hours) on the next morning for another 2 hours assessment at the Clinical Research and Trials Unit (CRTU) in the UEA.

For all groups, we'll provide you with a meal to eat after the visit.

How much blood and other samples will I provide?
At each visit, you will provide up to 104ml of blood (this is around 6 tablespoons). Over the three to four visits (during the 20 to 31 days you are taking part) you will give up to 377ml. This is less than a pint and is around the amount generally provided at a single blood donor session (470ml; source: The National Blood Service). We will endeavour to make your stay as comfortable as possible.

Who can enter this study?
We are aiming to recruit men who are 50 to 75, who are generally healthy.

At our health check visit, we'll use your blood pressure and blood fat results (and your age) to work out if you fall into our target group of men who are at mild to moderate risk of heart disease over the next 10 years.

- If your GP has told you that you have a disease related to your heart, liver, kidney, gut, blood, brain, cancer or diabetes

- You are allergic to chocolate, milk, blackberries, orange juice or soy food

- You are having vaccinations (including the flu vaccination) or antibiotics 3 months prior to and during the trial

- Your GP has prescribed you medication for high cholesterol or high blood pressure

- You wish to take flavonoid-containing dietary supplements or are taking part in another study

If you are in doubt about whether or not you are suitable to volunteer please do not hesitate in contacting the research scientists listed on the front page.

Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason.

If you chose not to take part in the study, or withdraw from the study at any time, this will not affect your future healthcare.
An expression of interest does not commit you to participation.
What happens next?

You will **NOT** be able to volunteer if:

- You smoke (or have stopped smoking within 3 months)
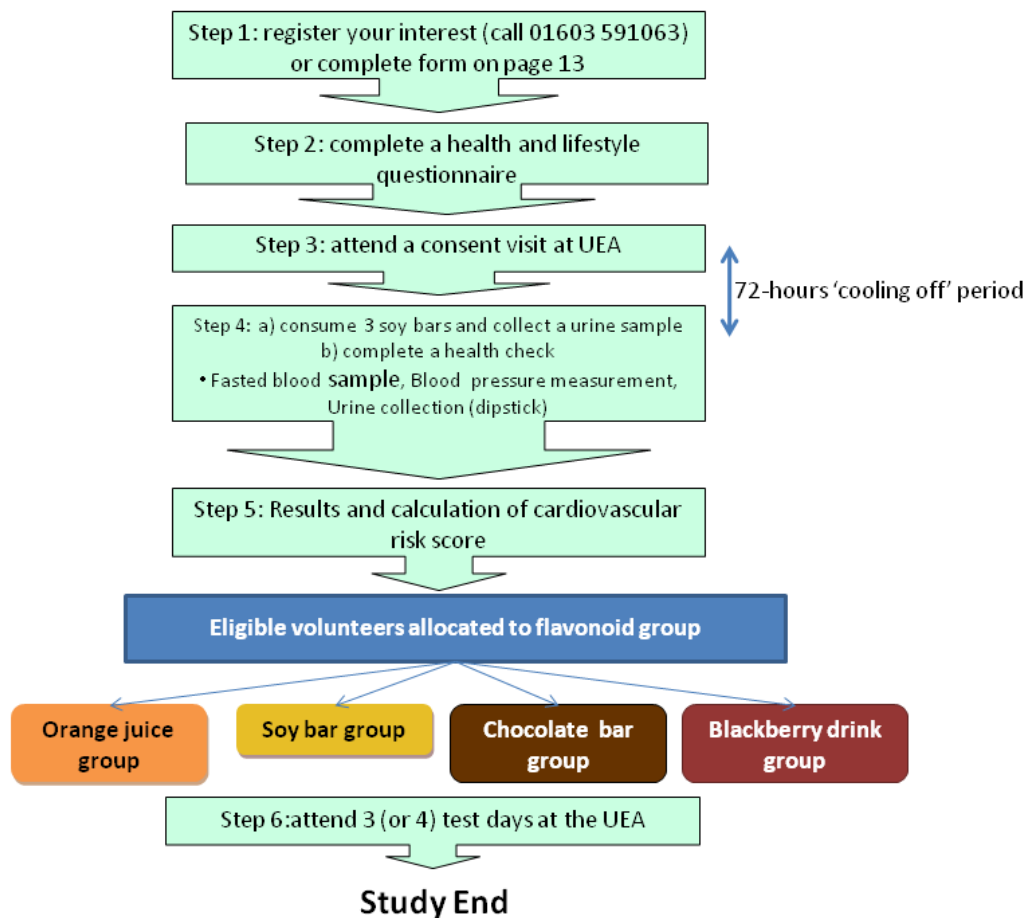
Step 1: Register an interest
Please complete the form at the back of this leaflet and return it in the pre-paid envelope (enclosed).

Step 2: We'll call you!
One of the study scientists will contact you (by telephone, or e-mail) to ask some general questions about your health and lifestyle (i.e. smoking, drinking, food habits) and will answer any questions you may have.

Step 3: Ready to proceed?
If you're eligible for our study, ***and happy to proceed***, we'll arrange a visit to our research facility at the University of East Anglia.

Don't worry if you're unsure how to find us, we'll send you a map and reimburse your travelling expenses.

At the consent visit, we'll explain the study in greater detail and answer any questions you may have. If you then wish to volunteer for the study, we'll ask you to fill in a consent form. A signed consent form allows us to proceed with the study.

We won't take any samples (e.g. blood or urine) at this visit and we'll give you time (at least 3 days) to think about whether you still want to take part after hearing more about the study.

What am I consenting to?
As part of the consent process, you will confirm that you wish to take part in the present research study, having read and understood the study information and

having had any questions satisfactorily answered by the research team. You will also confirm that we can inform your GP that you are taking part.

Please be aware that you consent to participate in **ANY** of the 4 intervention groups (i.e. chocolate **based supplement**, milk-based blackberry drink, orange juice or soy snack bar). Additionally, the consent form will ask whether you consent to your samples and / or data being available for inclusion in research studies in the future, which relate to nutrition and heart health. You are free to decide whether to give this consent and you can still take part in the present study if you decide NOT to give consent for your samples to be used in future research.

Step 4: Complete a health check
Before coming to have a health check, we'll ask you to;

a) eat 1 soy protein bar (each day) for the 3 days before the health check

b) collect some urine on the morning of your health check.

We'll give you instructions and a specimen pot, along with the soy bars. From your urine sample we can tell if your gut produces equol, the compound derived from eating soy.

Before coming to the health check, we will need you to not eat or drink anything other than water for 10 hours, as this can affect the blood results.

Health check: During this visit at the research facility at the UEA, the research team will test your urine sample, measure your blood pressure and your heart rate and also measure your body size. A qualified nurse will then take a small *FASTED* blood sample (between 2 and 3 teaspoons) to make sure that you are healthy.

From your blood sample we'll be able to check some basic blood chemistry e.g. whether you are anaemic, and we'll also measure your blood fats (e.g. your cholesterol level), and see if your kidneys and liver are functioning properly.

Finally, once we have collected your blood sample we will provide you with breakfast.

Step 5: Waiting for the results
It may take 1-2 weeks for the results of the blood test to become available. Sometimes blood results can fall outside the anticipated range. This does not mean you are unwell, it may be perfectly normal for you. With our clinical adviser, we will discuss the study results and whether we need to repeat any tests. If our clinical adviser has any concerns about your blood results, and thinks that your health might be affected by taking part, then we will not be able to recruit you to the study.

The same will apply if the clinical advisor feels that the results of the urine test are unusual or if your blood pressure needs looking at by your GP.

Notification that you have volunteered for this study and all blood and urine results will be forwarded to your GP (with your consent).

From your blood pressure and cholesterol we will calculate your future heart risk according to the British Hypertension Society risk assessment. Men who are at mild to moderate risk of heart disease over the next 10 years are eligible to take part.

What happens if I am eligible?
You will be invited to take part in this study if the screening results are within an acceptable range and you meet all the listed criteria to take part.

If you accept our invitation to take part, we will arrange for you to attend the same research facility at the UEA for 3 or 4 test days (each separated

Preparing for the trial
For **3 days** before each test-day, we will ask you to follow our diet guidelines in **Table 1** which involves stopping eating certain foods (like dark chocolate, berries, citrus fruits and soy) and limiting the intake of some other food and drinks. Our diet guidelines (**Table 1**) include examples of foods and drinks you can continue to eat. We can give you further advice if you are unsure.

Assessments; randomised order of placebo, supplement and food source intervention

≥ 1 week wash-out   ≥ 1 week wash-out   ≥ 1 week wash-out

24hr food recall, AE* and medication check — 1-2 days before assessment — Assessment #1 — Assessment #2 — Assessment #3 — Isoflavone Assessment #4

low flavonoid diet (3-day run-in)

Standard meal followed by fasting period (10hr)

Measure conducted at all assessment visit;
- BRS (baroreflex sensitivity)
- BP (blood pressure) – Oscillometric BP
- PWV (pulse wave velocity)
- Endothelial function (EndoPAT2000)
- Plasma biomarkers (blood and urine samples)
- Anthropometric measurements (e.g. weight, height)

*AE, adverse event

Study lasts 20 to 31 days; low flavonoid diet for 3 days before each test-day

Again, we will reimburse your travel expenses.

An overview of the measurements that we will make is shown at the bottom of this page.

some questions about the food that you have eaten the day before and about your health.

For 48-hrs before your assessment visit, we'll ask you to avoid strenuous exercise (e.g. competitive sports).

For 24-hrs before your assessment visit, we'll ask you not to drink alcohol and foods that contain caffeine (such as tea, coffee, coke, energy drink, hot chocolate) and some other foods that could interfere with our measures (e.g. such as leafy green vegetables, broccoli, radishes, carrots, sausages, other processed and cured meats and some varieties of bottled water); see **Table 2** for details. We'll provide you with bottled water (Buxton) to drink during the 24hrs before you attend an assessment.

The evening before, we'll provide you with a low flavonoid evening meal which you will need to eat at least 10 hrs before your appointment. You will then be unable to have food or drink thereafter, except water (which we will provide for you).

Are there any side effects or risks of taking part in the study?
The 'flavonoid' compounds in our study are found in many foods and products (like cocoa, berries, oranges and soybeans), that are commonly consumed in the UK, and we are providing them in doses that you could get from your normal diet. The flavonoid supplements that we will use are commercially sourced food ingredients / plant-based food compounds that are fit for human consumption. In this respect, we do not anticipate any side effects.

It is normal that you may feel some discomfort whilst a blood sample is taken and there is a slight risk of bruising. Using experienced nursing staff and trained research scientists minimises these risks.

In addition, one of our measures of your blood vessel function will involve temporarily stopping the flow of blood in one arm (for a 5 minute period); whilst this test is harmless, it may cause some discomfort and tingling in the

fingers and there is a chance of some bruising.

What are the possible benefits of taking part?
There will be no direct benefit to you in taking part in this research study. However, our results will help increase our understanding of flavonoids affect blood pressure control. This could help design better studies in the future to test if these compounds can improve heart health.

Will my details be confidential?
Any personal information (e.g. name, address, contact details) supplied by you during the study, will be handled by trained research staff and will be treated as strictly confidential (e.g. stored in locked filing cabinets, within a room with restricted access). All volunteers will be assigned a random 3 digit code number when they enrol into the study, and all paperwork, samples and results will be coded with this number to protect their identity. Any documents that link your name to this code will be stored securely in locked filing cabinets which are only accessible to the Chief investigator; delegated members of the research team and regulatory bodies for audit/monitoring purposes. Personally identifiable information and study results (coded with a 3 digit number) will be held in separate locations (so that you cannot be linked to the results). Any transfer of raw data from our tests, which may be provided to our product suppliers to establish product effectiveness, will be fully anonymised (e.g. not be traceable to volunteers). Any data we hold, or results we generate from your involvement during the study will be used for the purpose of this study and only the averaged results across participants will be reported (protecting your identity). The biological samples we collect will be analysed within 5 years from collection and disposed safely within this time. Data from the

study will be used for up to 10 years after collection and disposed of according to UEA confidential information disposal routes.

For those also consenting to the future use of their samples and / or data, appropriate ethics committee approval will be sought prior to use of these resources.

Is there any insurance arranged?
Yes, if you are harmed by the negligence of anyone in the research team.

What will happen to the results of the research study?
The results of this research study will be published in scientific journals and presented at national and international scientific meetings.

It is our policy to provide the volunteers who take part in the study a summary of our findings for the whole study population. We do not report on the findings of individuals alone.

Who has reviewed the study?
The research study has been approved by the Research Governance Committee at the Norfolk & Norwich University Hospitals NHS foundation trust and the NRES Committee East of England - Norfolk ethics committee.

Expense Payments
You will be reimbursed for travel expenses incurred as part of the study. Expenses include travel to and from UEA on production of a receipt or at 45p/mile for private cars (up to a 25 mile radius) and car parking.

Please be aware that travel payments are liable to tax, and the University of East Anglia is not responsible for informing the Inland Revenue.

Contact for further information
If you would like further information about the study then you can contact the study team at:

fastcheck@uea.ac.uk
Tel: 01603 591063

Please complete the reply slip overleaf (**p13.**) and return it in the PRE-PAID envelope provided if you wish to take part in this study.

Thank you for showing interest in this study.

An expression of interest does not commit you to take part!

Table 1: Food restriction for 3 days prior to each assessment visit

Foods to avoid	Restrict		Alternatives (*see table 2, for foods to be avoided 24hr before assessment)
Fruit: including fruit juices and products (e.g. desserts, jams, yoghurts) containing:			
Citrus fruit – **for example: oranges and tangerine / satsuma, grapefruit, lemon, lime and mixed tropical fruit items** *Berry fruit* – **for example blackberry, blueberry, strawberry, raspberry, cranberry, blackcurrant, redcurrant** *Other fruit* - **Black / red grapes, raisins, plum, prune, cherry, pomegranate**	**Apple** **Nectarine** **Nuts (e.g. almond, hazelnut, peanut, macadamia nut, walnut, cashew nut, chestnut, pecan nut)**	**1 per 3 days** **2 per 3 days** **2 handful per 3 days**	**Mango, banana, melon, pineapple, kiwi, pear, peach apricot, green grapes, fig, date gooseberry, avocado**
Vegetables, salad and products (e.g. quiche, pizza, casserole) containing:			
Broad bean, red onion, aubergine, red cabbage, red skinned potatoes, purple carrot	**Black bean and black olives** **Kidney beans** **Garlic cloves**	**2 handful per 3 days** **1 handful per 3 days** **4 per 3 days**	**Onion (excluding red), shallot, green bean, tomato, broccoli*, carrot*, cauliflower*, celery*, cucumber*, peas, leek, green/white cabbage*, potato (excluding red skinned)*, pumpkin, radish*, parsnip, pepper, beetroot*, lentil, sweet corn, lettuce*, Brussels sprouts*, green olive**
Other foods: including products (e.g. drinks, desserts, vegetarian meat alternatives) containing the following:			
Dark chocolate, baking chocolate and cocoa products **Soy, soy milk and soy containing products (e.g. quorn, tofu)** **Cider, red wine**	**White or milk chocolate** **Oily fish (e.g. tuna, mackerel, kippers, salmon, sardines, herring)**	**2 chunks per 3 days** **1 portion per 3 days**	**Sugar based and/or dairy desserts and snacks, egg based desserts (e.g. plain biscuits, cereal bar, ice cream, custard)** **Milk and milk containing products (e.g. non berry fruit yoghurt, cheese)** **Water, carbonated drinks (e.g. coca-cola, sprite), beer, white wine, spirits**

Table 2: Further food restriction 24 hrs prior to each assessment visit

Foods to avoid for 24hr (*In addition to Table 1)	Alternatives
Vegetables, salad and products (e.g. quiche, pizza, casserole) containing:	
Beetroot, broccoli, cabbage, carrot, cauliflower, celery, cucumber, lettuce, Brussels sprouts, parsley, potato, radish, spinach	Onion (excluding red), shallot, green bean, tomato, peas, leek, pumpkin, parsnip, pepper, lentil, sweet corn, green olive
Other foods: including products (e.g. roll, sandwich) containing the following:	
Drinks containing caffeine e.g. tea, coffee, hot chocolate, coke, energy drinks (such as RedBull) Drinks containing berry fruit and citrus fruit (including cordials) Bottled water (excluding Buxton mineral water) Drinks / foods containing alcohol Cured and canned meat (e.g. bacon, ham, sausages, corned beef) Smoked fish	Milk (excluding soya milk), Buxton water (provided by the researchers for the 24hr before you attend an assessment visit) Fresh meat (e.g. chicken, turkey, beef, pork, lamb) Fresh fish (excluding oily fish)

Are the cardiovascular responses of participants at mild to moderate cardiovascular risk influenced by dietary flavonoid sub-class and dietary form?

I am interested in taking part and/ or finding out more information about this study (please complete the personal details below).

Name:

Address:

Date of Birth:

		Can we contact you this way? (please tick)	
		YES	NO
Daytime telephone no:			
Evening telephone no:			
Mobile phone no: (if applicable)			
E-mail address: (if applicable)			

Please return this form in the pre-paid envelope provided to:
Peter Curtis – Flavonoids, blood pressure control and blood vessel function
Clinical Research and Trials Unit
MED2 building
Chancellors Drive
University of East Anglia
NR4 7TJ
Expressing an interest does not commit you to taking part in the study!

C.3 List of prohibited medications during the trial

Blood pressure medication

Ca2+-channel blockers

Adalat Capsules (Nifedipine)	Adalat LA (Nifedipine)	Adipine XL (Nifedipine)	Amlodipine (Amlodipine)	Angitil SR (Diltiazem)	Beta-Adalat (Atenolol, Nifedipine)
Calchan MR (Nifedipine)	Cardene (Nicardipine)	Cardene SR (Nicardipine)	Cardioplen XL (Felodipine)	Coracten XL (Nifedipine)	Diltiazem (Diltiazem)
Dilzem XL (Diltiazem)	Exforge (Amlodipine, Valsartan)	Fortipine LA (Nifedipine)	Istin (Amlodipine)	Lercanidipine	Motens (Lacidipine)
Nicardipine (Nicardipine)	Nifedipine (Nifedipine)	Nimotop (Nimodipine)	Plendil (Felodipine)	Prescal (Isradipine)	Securon I.V. (Verapamil)
Securon SR (Verapamil)	Sevikar (Amlodipine, Olmesartan)	Slozem (Diltiazem)	Syscor MR (Nisoldipine)	Tarka (Trandolapril, Verapamil)	Tenif (Atenolol, Nifedipine)
Tensipine MR (Nifedipine)	Tildiem LA (Diltiazem)	Triapin (Felodipine, Ramipril)	Univer (Verapamil)	Valni XL (Nifedipine)	Verapamil (Verapamil)
Vertab SR (Verapamil)	Viazem XL (Diltiazem)	Zanidip (Lercanidipine)	Zemtard XL (Diltiazem)	Zolvera (Verapamil)	

α-blockers

Baratol (Indoramin)	Cardura XL (Doxazosin)	Carvedilol (Carvedilol)	Combigan (Brimonidine, Timolol)	Combodart (Dutasteride, Tamsulosin hydrochloride)	Contiflo XL (Tamsulosin)
Doralese (Indoramin)	Doxazosin (Doxazosin)	Eucardic (Carvedilol)	Flomaxtra XL (Tamsulosin)	Hypovase (Prazosin)	Hytrin (Terazosin)
Indoramin (Indoramin)	Labetalol (Labetalol)	Opilon (Moxisylyte)	Pinexel PR (Tamsulosin)	Raporsin XL (Doxazosin)	Rogitine (Phentolamine)
Tabphyn MR (Tamsulosin)	Tamsulosin (Tamsulosin)	Terazosin (Terazosin)	Trandate (Labetalol)	Xatral XL (Alfuzosin)	

β-blockers

Acebutolol (Acebutolol)	Atenolol (Atenolol)	Azarga (Brinzolamide, Timolol)	Beta-Adalat (Atenolol, Nifedipine)	Beta-Cardone (Sotalol)	Betagan (Levobunolol)
Celectol (Celiprolol)	Celiprolol (Celiprolol)	Combigan (Brimonidine, Timolol)	Corgard (Nadolol)	Cosopt (Dorzolamide, Timolol)	Co-tenidone (Co-tenidone)
DuoTrav (Timolol, Travoprost)	Emcor (Bisoprolol)	Eucardic (Carvedilol)	Ganfort (Bimatoprost, Timolol)	Inderal Injection (Propranolol)	Inderal LA (Propranolol)
Kalten (Amiloride, Atenolol, Hydrochlorothiazide)	Labetalol (Labetalol)	Levobunolol	Lopresor (Metoprolol)	Metoprolol (Metoprolol)	Myocardial infarction, LVD
Nebilet (Nebivolol)	Nyogel (Timolol)	Prestim (Bendroflumethiazide, Timolol maleate)	Propranolol (Propranolol)	Sectral (Acebutolol)	Slow-Trasicor (Oxprenolol)
Sotacor (Sotalol)	Syprol (Propranolol)	Tenif (Atenolol, Nifedipine)	Tenoret 50 (Atenolol, Chlortalidone, Co-tenidone)	Tenoretic (Atenolol, Chlortalidone, Co-tenidone)	Tenormin (Atenolol)
Teoptic (Carteolol)	Timolol (Timolol)	Timoptol-LA (Timolol)	Trandate (Labetalol)	Trasicor (Oxprenolol)	Trasidrex (Co-prenozide, Cyclopenthiazide, Oxprenolol)
Viskaldix (Clopamide, Pindolol)	Visken (Pindolol)	Xalacom (Latanoprost, Timolol maleate)			

ACE Inhibitors

Accupro (Quinapril)	Accuretic (Hydrochlorothiazide, Quinapril)	Capoten (Captopril)	Capozide (Captopril, Co-zidocapt, Hydrochlorothiazide)	Captopril (Captopril)	Carace 10 Plus (Hydrochlorothiazide, Lisinopril)
Coversyl Arginine (Perindopril)	Coversyl Arginine Plus (Indapamide, Perindopril)	Enalapril (Enalapril)	Enalapril + hydrochlorothiazide (Enalapril, Hydrochlorothiazide)	Fosinopril (Fosinopril)	Gopten (Trandolapril)
Innovace (Enalapril)	Innozide (Enalapril, Hydrochlorothiazide)	Lisinopril (Lisinopril)	Lisinopril + hydrochlorothiazide (Hydrochlorothiazide, Lisinopril)	Perdix (Moexipril)	Perindopril (Perindopril)
Quinapril (Quinapril)	Ramipril (Ramipril)	Tanatril (Imidapril)	Tarka (Trandolapril, Verapamil)	Triapin (Felodipine, Ramipril)	Tritace (Ramipril)
Vascace (Cilazapril)	Zestoretic 20 (Hydrochlorothiazide, Lisinopril)	Zestril (Lisinopril)			

Lipid lowering medication

Statins

Crestor (Rosuvastatin)	Fluvastatin	Lescol XL (Fluvastatin)	Lipitor (Atorvastatin)	Lipostat (Pravastatin)	Pravastatin (Pravastatin)
Simvastatin (Simvastatin)	Zocor (Simvastatin)				

Antibiotics

Beta Lactam Medicines

Amoxicillin	Amoxicillin + clavulanic acid	Ampicillin	Benyathine benzylpenicillin	Benzylpenicillin	Cefalexin
Cefazolin	Ceftriaxone	Cloxacillin	Phenoxymethylpenicillin	Procaine benzylpenicillin	Cefotaxime
Ceftazidime	Imipenem + cilastatin				

Other antibacterials

Azithromycin	Chloramphenicol	Ciprofloxacin	Doxycycline	Erythromycin	Metronidazole
Nitrofurantoin	Spectinomycin	Sulfamethoxazole + trimethoprim	Clindamycin	Vancomycin	

C.4   General health and lifestyle questionnaire (HLQ)

Please fill in the questionnaire below:

	DAY		MONTH			YEAR			
Date questionnaire completed									
Example	1	0	J	U	N	2	0	1	1

Title: **Forename(s):** **Surname:**	**Address:** **Postcode:**
Daytime telephone no.	**Evening telephone no.**
Mobile telephone no.	**E-mail address:**
Best method (e.g. landline, mobile, e-mail) and time to contact you:	

Date of Birth:	Age today	50 - 75yrs?	Proceed? Yes / No
		Office use only	

Q: Question, A: Answer
Q. Do you smoke?   If you are an ex-smoker, when did you stop? A.
BRIEF MEDICAL HISTORY
Q. Has your doctor told you that you suffer from heart disease (e.g. Angina), stroke or any other disease of the circulation (i.e. Reynaud's disease)? A.
Q. Has your doctor diagnosed you as having high blood pressure (hypertension)? A.
Q. Has your doctor diagnosed you as having high cholesterol? A.
Q. Has your doctor diagnosed you as having diabetes (either type 1 (insulin dependent) or type 2 (age-onset diabetes))? A.
Q Has your doctor diagnosed you with cancer? A.
Q. Has your doctor told you that you suffer from any other illness (e.g. illness related to kidney, stomach, colon, brain, thyroid, or liver)? A.
Q. Do you have any known food allergies? Any other allergies? A.

MEDICATIONS
Q. Have you been prescribed cholesterol lowering drugs (e.g. statins, simvastatin, atorvastatin)? A.
Q. Have you been prescribed blood pressure medications (e.g. alpha blockers, beta blockers, ACE inhibitors, Ca^{2+} channel blocker)? A.
Q. Have you taken antibiotics (either oral or topical) within the last 3 months? A.
Q. Have you been vaccinated (including the flu vaccination) within the last 3 months? A.
Q. Do you have any vaccinations planned (including the flu vaccination)? If yes, indicate the date (day/ month/ year) A.
Q. Are you currently on any long-term medication; including aspirin / steroids, antihistamines, anti-inflammatory medication, pain relief? A.
Q. Do you regularly take non prescribed pain relief, anti-inflammatory, anti-histamines? A.

Dietary intake and supplement use
Q. Do you take minerals/vitamins or 'dietary' supplements (containing flavonoids)? If so, please state the product name. A.
Q. Do you regularly consume any of the following; Dark chocolate, baking chocolate and other rich sources of cocoa (e.g. hot chocolate, chocolate desserts), soy food and products? If so, please estimate how much you eat, and how often (per week). A.
Q. Do you regularly consume berries (such as blueberry, raspberry, blackberry, black/redcurrant, strawberry, cranberry), and citrus fruits/ juices (such as orange, grapefruit, tangerine, satsuma, lemon)? If so, please estimate how much you eat, and how often (per week). A.
Q. Do you regularly consume red wine/cider? If so, please estimate how many cups per day. A.

Q. Do you regularly consume any of the following;

Apples, milk and white chocolate, nuts (e.g. almond, hazelnut, pecan nut, peanut, macadamia nut, walnut, cashew nut, and chestnut), garlic, black and kidney beans, olives, oily fish (e.g. tuna, mackerel, kippers, salmon, sardines, and herring)?

 If so, please estimate how much you eat, and how often (per week).

A.

Q. Do you regularly consume coffee?

 If so, please estimate how many cups per day.

A.

Q. Do you regularly consume tea?

If so, please estimate how many cups per day.

A.

Q. Do you regularly consume any of the following;

Broccoli, beet, cabbage, carrot, cauliflower, celery, cucumber, lettuce, brussel sprouts, parsley, potato, radish, spinach?

 If so, please estimate how much you eat, and how often (per week).

A.

Q. Do you regularly consume canned and cured meats such as sausages, bacon, ham, hot dogs, corned beef and smoked fish?

If so, please estimate how much you eat, and how often (per week).

A.

Q. Do you drink alcohol?

(how many units per week; see below *for answer)

A.

*A unit of alcohol is defined as: about half a pint (300ml) of ordinary-strength (around 3-4% alcohol) lager, beer or cider,

- a 25ml pub measure of spirit or a small glass of fortified wine, such as sherry or port (17.5% ABV),
- a small glass (125 ml) of 8% ABV wine.

NB: Alcohol content has increased in popular drinks; e.g. A typical (175ml) glass of Australian Chardonnay (at 12% ABV) is nearer two units than one.

Q. Have you been involved in a dietary intervention trial in the last 6 months? Or a trial which has included giving blood?

A.

This section for office use only

Is the volunteer eligible to proceed in the trial?   YES ☐   NO ☐

Name of scientist / research nurse (PRINT): …………………………………….

Signature of the scientist / research nurse: …………………………………

DATE:

	D	D	M	M	M	Y	Y	Y	Y
E.g.	0	1	J	A	N	2	0	1	1

COMMENTS:

C.5 Soy bar consumption record and urine collection during soy-challenge

REC Study ID:
11/EE/0233
_____ Volunteer
Study ID: ☐☐ ☐

Please remember to keep the wrappers from the soy bars, and return them to the study team.

Please collect you're urine sample 'mid-stream' – instructions are provided below.

1. Please complete the following by circling the correct answers:

	Task	Did you consume the bar?		Did you keep the wrapper? (Tick if YES)	Date (DD MM MY
Day 1	Eat Soy bar 1	YES	NO		
Day 2	Eat Soy bar 2	YES	NO		
Day 3	Eat Soy bar 3	YES	NO		

	Task	Did you collect a urine sample?		Was this a mid-stream collection? (Tick if YES)	Date (DD MM MY
Day 4	Collect Urine sample	YES	NO		

A mid-stream urine sample is collected as follows:

Pass 1 to 2 seconds of urine into the toilet as normal, then without stopping the flow of urine, catch the rest of the urine into the bottle provided. If you fill the container, finish off passing the rest of your urine into the toilet as normal.

C.6　24hr dietary recall sheet

VOL_ID:	Date:					Day of week (circle below)			Typical? (circle)		Other important detail:
	Mon	Tue	Wed	Thu	Fri	Sat	Sun	YES	NO	
Meal / eating event	Time (24hr format)	location	Food item – identify full description, brand, type (i.e. low fat, sugar free etc.)			Preparation method (e.g. boiled, grilled etc.)	Estimate amount	Leftovers? how much		Additional info identified during multiple pass

Instructions to the researcher

The recall should follow these steps

1. **Snapshot-** Volunteer is asked to recall main meals, food and beverages consumed the day before the interview (morning to morning).
2. **Playback** – recall the foods consumed back to the participant – remembering to include time and location;
   a. Look for long gaps between eating events and ask specifically about this
   b. Look for food items that are usually eaten together i.e. toast + fat spread + preserve
   c. Drinks, snacks, accompaniments (i.e. sauce, salt, pepper), alcoholic beverages are often missed – **ask about these if they are not on the list**
3. **Detail Cycle** - For each food, a detailed description, amount eaten, and additions to the food are collected.
4. **Usual dietary practices / forgotten Foods –** volunteer is asked about their normal eating habits, with the intention to cross-reference normal habits against reported 24hr intake. Consumption of foods commonly forgotten during the snapshot and probes will be used as time and eating occasion are collected for each food (See table 1 below)
5. **Final Probe -** Additional foods not remembered earlier are collected (further probe questions)

List of questions for further food intake detail:

Select (√)	List of probes questions (for all questions, cross-check responses against reported 24hr intake)
	Do you normally eat breakfast? Did you do that yesterday?
	Do you drink hot drinks? If so, what type and how many do you usually have? Did you drink that many yesterday?
	Do you drink milk in hot drinks and / or on cereal? What type?
	Do you add sugar / artificial sweetener to hot drinks? If so, what type and how much?
	Do you normally have biscuits or snacks with hot drinks? If yes, did you do that yesterday? (**cross check with dietary restrictions**)
	What fat spread do you use? Do you add this to sandwiches etc.
	What oils do you cook with? Do you deep fry items?
	Do you tend to buy full fat, reduced fat or low fat foods? Were any of the foods you ate yesterday any of the reduced or low fat type?

	Did you drink any alcohol yesterday? Do you usually drink? What kind (e.g. beer, **red wine**)? How much in glasses/ pints? Did you go to bar/café/ pub yesterday?
	Was the amount of food consumed on the recall day more than usual, usual, or much less than usual?

Restricted foods cross-check:

Food	Consumed in last 3 days (circle)		Amount consumed during last 3d
Berries, berry products (e.g. Blueberry, strawberry, raspberry, cranberry, blackcurrant, redcurrant**),** **If yes, which;** **Or any of the following fruit and vegetables:** Black / red grapes, raisins, plum, prune, cherry, pomegranate, Broad bean, red onion, aubergine, red cabbage, red skinned potatoes, purple carrot **If yes, which;**	**Yes** **Yes**	**No** **No**	
Citrus fruits (e.g. orange, tangerine, satsuma, Clementine, grapefruit, lime, lemon) **If yes, which;**	**Yes**	**No**	
Soy and soy based products (e.g. soya milk, soya spreads, tofu, vegetarian meat) **If yes, which;**	**Yes**	**No**	
Dark chocolate and cocoa products **If yes, which;**	**Yes**	**No**	

Food	Consumed in last 3 days (circle)		Amount consumed during last 3d
Red wine or tea? **If yes, which;**	Yes	No	
Oily fish (e.g. tuna, kippers, mackerel, salmon, sardines, herring) **If yes; which:**	Yes	No	

Name of scientist / research nurse (PRINT): …………………………………

Signature of the scientist / research nurse: …………………………………

	D	D	M	M	M	Y	Y	Y	Y
DATE:									
E.g.	*0*	*1*	*J*	*A*	*N*	*2*	*0*	*1*	*2*