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Does genotype and equol-production status affect response to isoflavones? Data from a pan-European study on the effects of isoflavones on cardiovascular risk markers in post-menopausal women

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The increase in CVD incidence following the menopause is associated with oestrogen loss. Dietary isoflavones are thought to be cardioprotective via their oestrogenic and oestrogen receptor-independent effects, but evidence to support this role is scarce. Individual variation in response to diet may be considerable and can obscure potential beneficial effects in a sample population; in particular, the response to isoflavone treatment may vary according to genotype and equol-production status. The effects of isoflavone supplementation (50 mg/d) on a range of established and novel biomarkers of CVD, including markers of lipid and glucose metabolism and inflammatory biomarkers, have been investigated in a placebo-controlled 2×8-week randomised cross-over study in 117 healthy post-menopausal women. Responsiveness to isoflavone supplementation according to (1) single nucleotide polymorphisms in a range of key CVD genes, including oestrogen receptor (ER) α and β and (2) equol-production status has been examined. Isoflavones supplementation was found to have no effect on markers of lipids and glucose metabolism. Isoflavones improve C-reactive protein concentrations but do not affect other plasma inflammatory markers. There are no differences in response to isoflavones according to equol-production status. However, differences in HDL-cholesterol and vascular cell adhesion molecule 1 response to isoflavones *v.* placebo are evident with specific ERβ genotypes. In conclusion, isoflavones have beneficial effects on C-reactive protein, but not other cardiovascular risk markers. However, specific ERβ gene polymorphic subgroups may benefit from isoflavone supplementation.

Isoflavones: CVD: Post-menopausal women: Oestrogen receptor β: Single nucleotide polymorphisms: Nutrient–gene interaction

Oestrogen loss at the menopause has been associated with increased risk of CVD that is partly attributed to an adverse lipoprotein profile and arterial dysfunction. Hormone-replacement therapy (HRT) has been widely used to counteract the adverse effects of oestrogen deficiency. However, the recently reported lack of HRT efficacy in relation to CVD progression (Nelson *et al*. 2002) and evidence of adverse effects (Cushman *et al*. 1999; Manning *et al*. 2002) has led to increased interest in alternative therapies such as isoflavones. Isoflavones are phyto-oestrogens bearing a similar structure to mammalian oestrogen and, therefore, could act as oestrogen mimics or selective oestrogen-receptor (ER) modulators. It is not clear from the present literature whether isoflavones have lipid-lowering properties or if they have any effect on novel cardiovascular risk factors such as inflammatory biomarkers or markers of endothelial dysfunction. Furthermore, it is not known whether variability in response to

Abbreviations: CRP, C-reactive protein; ER, oestrogen receptor; HDL-C, HDL-cholesterol; HRT, hormone-replacement therapy; VCAM-1, vascular cell adhesion molecule 1.

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isoflavones exists among individuals according to genotype or according to their capacity for equol production, since equol is thought to be one of the active isoflavone metabolites.

The present paper will give a brief review of the current literature concerning the effects of isoflavones on markers of lipid and glucose metabolism and inflammatory biomarkers in man and will present the results of a pan-European study (ISOHEART) that has investigated the effects of isoflavone supplementation on established and novel risk markers for CVD in a large group of healthy post-menopausal women, including the analysis of differences in response to isoflavones according to single nucleotide polymorphisms in key genes relevant to CVD and according to equol-production status.

CVD and menopause

Susceptibility to CVD can be partially characterised by a range of risk factors, including impaired lipid and glucose metabolism and endothelial dysfunction. For example, elevated triacylglycerol and LDL-cholesterol and decreased HDL-cholesterol (HDL-C) levels are associated with increased risk of CVD (Hopkins *et al*. 2005). High lipoprotein (a) concentrations also seem to be an independent risk factor for CVD (Burman *et al*. 2004). Disturbed glucose and insulin metabolism, which is a feature of the metabolic syndrome, is associated with subsequent development of diabetes and CVD (Lakka *et al*. 2002). Endothelial dysfunction, characterised by a chronic vascular inflammation and increased expression of cell adhesion molecules such as intracellular cell adhesion molecule 1, vascular cell adhesion molecule 1 (VCAM-1), E-selectin and chemokines such as monocyte chemotactic protein 1, is also considered to be predictive of cardiovascular risk (Hwang *et al*. 1997). Other markers of inflammation, including C-reactive protein (CRP) and von Willebrand factor (which plays an important role in platelet aggregation and adhesion), are also considered to be predictive biomarkers of coronary risk (Blann *et al*. 1993; Ridker *et al*. 2000). Furthermore, increased concentrations of the vasoconstrictor molecule, endothelin 1, indicate endothelial dysfunction associated with chronic inflammation (Tousoulis *et al*. 2005).

The decreased ovarian function and subsequent oestrogen deficiency at the menopause predisposes post-menopausal women to a high CVD risk (Mobasseri *et al*. 2004) as a result of a less favourable blood lipid profile, decreased insulin sensitivity and impaired endothelial function. Previously, HRT was advocated as an effective means of delaying the progression of atherosclerosis in post-menopausal women. HRT has been shown to improve the lipoprotein profile by decreasing plasma concentrations of LDL-cholesterol and increasing concentrations of the beneficial HDL-C (Erberich *et al*. 2002) and improves insulin sensitivity (Sites *et al*. 2005). Evidence also exists that is suggestive of a protective role for HRT on endothelial function (Krasinski *et al*. 1997). HRT has been suggested to decrease cell adhesion molecules (Koh *et al*. 1997) and endothelin 1(Wilcox *et al*. 1997) concentrations. However, recent reports have shown adverse effects of HRT on the vasculature, including increased concentrations of CRP (Cushman *et al*. 1999; Manning *et al*. 2002) and increased risk of thrombosis (Nelson *et al*. 2002). In addition to the latter, the reported association between hormone-dependent cancers and HRT (Beral *et al*. 2005), as well as unexpected reports of increased CVD rates with HRT (Grady *et al*. 2002), has led to investigation of alternative therapies to counteract oestrogen deficiency at menopause.

Isoflavones

Interest has been focused on isoflavones as a natural alternative to traditional oestrogen therapies, and in particular as a means of delaying CVD incidence in post-menopausal women. Isoflavones are plant-derived compounds with structural similarity to oestrogen (phyto-oestrogens), able to bind to ER and therefore induce transcription of oestrogen-responsive genes (Kuiper *et al*. 1998). The most important dietary source of isoflavones is soyabean. Epidemiological evidence in human subjects suggests that increased soyabean consumption is cardioprotective. This effect may be a result of the ability of the isoflavones found in soyabean (genistein, daidzein and glycitein) to act as oestrogen mimics or selective ER modulators. Although evidence from *in vitro* studies suggests that isoflavones can be cardioprotective through a range of mechanisms, the current data from human intervention studies are insufficient to support this role.

Isoflavones and cardiovascular health: the existing evidence

Isoflavones and markers of lipid and glucose metabolism

It is currently uncertain whether isoflavones can exert beneficial effects on lipoprotein profile similar to that reported for oestrogen. The small number of studies that have investigated the effects of isolated isoflavones on lipoprotein profiles have to date produced negative results (for example, see Nestel *et al*. 1997; Hodgson *et al*. 1998; Simons *et al*. 2000; Dewell *et al*. 2002; Nikander *et al*. 2004), although there is evidence from a single study for beneficial effects of isoflavones on lipoprotein profiles (Han *et al*. 2002). Only a few studies have investigated the effects of pure isoflavones on insulin concentrations, giving equivocal findings (Blakesmith *et al*. 2003; Cheng *et al*. 2004; Nikander *et al*. 2004). Some of these studies have low statistical power, have administered isoflavones as capsules and only a handful have included a randomised double-blind placebo-controlled cross-over design. Thus, the question of the effect of isoflavones on lipoproteins and on glucose metabolism remains open. A summary of human intervention studies on the effect of isolated isoflavones on circulating lipoproteins is shown in Table 1.

Isoflavones and inflammatory biomarkers

The effect of isoflavones on novel cardiovascular risk markers, such as inflammatory markers, is unclear. Although evidence from *in vitro* studies suggests that

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Table 1. Human intervention studies on isoflavones and lipoprotein profiles

Reference	Subjects and study design	Duration	Isoflavone supplementation (mg/d)	Outcome
Nestel *et al.* (1997)	Seven peri-menopausal W, fourteen PMW RSBP, CO	15 weeks per arm	80 isoflavones (45 genistein, 33 daidzein, 2 glycitein), in tablets	NC in TC, LDL-C, HDL-C and TAG
Hodgson *et al.* (1998)	Forty-six middle-aged M, thirteen PMW RDBP, Pa	8 weeks	55 clover (*Trifolium subterraneum*) isoflavones (16 biochanin A, 30 genistein, 8 formononetin, 1 daidzein), in tablets	NC in TC, LDL-C, HDL-C or TAG
Nestel *et al.* (1999)	Twenty-one menopausal and peri-menopausal W RDBP, Pa	15 weeks	RC isoflavones, in tablets; placebo, 40 isoflavone and 80 isoflavone sequentially for 5 weeks each (*n* 14) or placebo for 15 weeks (*n* 3)	NC in TC, LDL-C, HDL-C and TAG
Howes *et al.* (2000)	Sixty-six HC PMW, sixty-six on increasing dose treatment, nine on placebo RDBP	5 weeks on each dose	Placebo followed by 43·5 and then 87 (mg/g; 600 biochanin A, 370 formononetin, 20 genistein, 10 daidzein), in tablets	NC in TC, TAG, HDL-C or LDL-C
Simons *et al.* (2000)	Twenty PMW RDBP, CO	8 weeks	80, in tablets	NC in TC, LDL-C, HDL-C or TAG
Han *et al.* (2002)	Eighty menopausal W RDBP, Pa	16 weeks	100 (70 genistein, 19 daidzein, 11 glycitein), in capsules	↓ in TC and LDL-C NC in TAG or HDL-C
Dewell *et al.* (2002)	Thirty-six HC PMW RP, Pa	24 weeks	150 (90 aglycones (40 genistein, 50 daidzein and glycitein), 60 glycosides), in tablets	NC in TC, HDL-C or TAG
Squadrito *et al.* (2002)	Sixty PMW RDBP, Pa	6 months	54 genistein, in tablets	NC in TC, HDL-C, LDL-C or TAG
Squadrito *et al.* (2003)	Seventy-nine PMW RDBP, Pa	12 months	54 genistein	NC in TC, HDL-C, LDL-C or TAG
Nikander *et al.* (2004)	Fifty-six PMW RDBP, CO	3 months	114 (66 glycitein, daidzein, 6 genistein), in tablets	NC in TC, HDL-C, LDL-C or TAG
Atkinson *et al.* (2004)	Twenty-eight premenopausal W, twenty-six peri-menopausal, 117 PMW RSBP, Pa	12 months	43·5 RC isoflavones (26 biochanin, 16 formononetin, 1 genistein, 0·5 daidzein), in tablets	NC in TC, LDL-C, HDL-C ↓ in TAG (peri-menopausal W only)
Campbell *et al.* (2004)	Sixteen premenopausal W, 7 PMW RDBP, CO	28 d	86 RC isoflavones (50 biochanin, 16 formononetin, 8 genistein, 10 daidzein), in pills	↓ in HDL-C NC in TC or TAG

NC, no change; HC, hypercholesterolaemic; M, men; W, women; PMW, post-menopausal women; RDBP, randomised double-blind placebo-controlled; RSBP, randomised single-blind placebo-controlled; RP, randomised placebo-controlled; CO, cross-over design; Pa, parallel design; RC, red clover (*Trifolium pratense* L.); TC, total cholesterol; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; TAG, triacylglycerol; ↓, decrease.

Table 2. Human intervention studies on isoflavones and inflammatory biomarkers

Reference	Subjects and study design	Duration	Isoflavone supplementation (mg/d)	Outcome
Squadrito *et al.* (2002)	Sixty PMW RDBP, Pa	6 months	54 genistein, in tablets	↑ in nitrites or nitrates and ↓ in ET-1
Squadrito *et al.* (2003)	Seventy-nine PMW RDBP, Pa	12 months	54 genistein	↑ in nitrites or nitrates and ↓ in ET-1
Nikander *et al.* (2003)	Fifty-six PMW RDBP, CO	3 months	114 (66 glycitein, 42 daidzein, 6 genistein), in tablets	NC in CRP, E-selectin and nitrites or nitrates
Teede *et al.* (2003)	Thirty-four PMW RDBP, CO.	6 weeks	80 RC isoflavones (80 biochanin or formotenin), in tablets	↓ in VCAM-1 (following formotenin)
Atkinson *et al.* (2004)	Twenty-six peri-menopausal women, 117 PMW RSBP, Pa	12 months	43·5 RC isoflavones (26 biochanin, 16 formononetin, 1 genistein, 0·5 daidzein), in tablets	NC in fibrinogen and ↓ in PAI-1 (in peri-menopausal women only)

NC, no change; PMW, post-menopausal women; RDBP, randomised double blind placebo controlled; RSBP, randomised single blind placebo controlled; CO, cross-over design; Pa, parallel design; RC, red clover (*Trifolium pratense* L.); CRP, C-reactive protein; ET-1, endothelin 1; VCAM-1, vascullar cell adhesion molecule 1; PAI-1, plasminogen activator inhibitor 1; ↑, increase; ↓, decrease.

isoflavones possess anti-inflammatory effects, including inhibition of cell adhesion molecule expression (Gottstein *et al*. 2003; Rimbach *et al*. 2004), there have been few human intervention studies reporting on the effects of isoflavones on inflammatory biomarkers for cardiovascular risk. Some studies have reported no effect for fibrinogen, CRP, E-selectin and NO (Nikander *et al*. 2003; Teede *et al*. 2003; Atkinson *et al*. 2004), but others have shown beneficial effects of isoflavones on plasminogen activator inhibitor 1, endothelin 1, VCAM-1 and NO (Squadrito *et al*. 2002, 2003; Teede *et al*. 2003, Atkinson *et al*. 2004). A summary of human isoflavone supplementation studies on inflammatory biomarkers is shown in Table 2.

Inter-individual variability in response to isoflavones

The efficacy of isoflavones could vary between individuals, and studies are needed to identify and verify the factors that define responsiveness to isoflavones. Factors that may predict responsiveness to isoflavone supplementation include equol-production status and genotype.

Equol, the gut bacterial metabolite of daidzein, is a molecule with great biological importance since it exhibits higher binding affinity to ER and greater antioxidant capacity than the parent compound (Rimbach *et al*. 2003; Muthyala *et al*. 2004). Epidemiological and observational studies suggest a relationship between equol production and decreased risk of certain diseases (Atkinson *et al*. 2005). Evidence shows that there is an inter-individual variability in equol-synthesising capacity and studies have suggested that the response to isoflavone supplementation may vary according to an individual's equol-synthesising capacity (Rowland *et al*. 2000; Setchell *et al*. 2002).

In addition, there is emerging evidence from genetic studies to suggest that the response to diet can be affected by inter-individual differences in the genetic background of a population (Masson & McNeil, 2005). Thus, variability in response to isoflavones may be observed in relation to single nucleotide polymorphisms in cardiovascular risk genes or genes relevant to oestrogen action, as has been shown for the HDL-C and E-selectin response to HRT therapy for ER polymorphisms (Herrington *et al*. 2002*a*,*b*).

Aims

Based on the existing literature on the cardiovascular effects of isoflavones at the time (i.e. 2001), a pan-European research project (ISOHEART) was launched to investigate the effect of isoflavones on cardiovascular health of post-menopausal women. The specific aims of the ISOHEART study were: (1) to examine the effects of isolated soyabean isoflavone intake, provided within a food vehicle, on a range of cardiovascular risk markers including plasma biomarkers of lipoprotein and glucose metabolism and circulating inflammatory markers, by means of a well-powered randomised double-blind placebo-controlled cross-over dietary intervention design; (2) to evaluate differences in response to isoflavone supplementation according to genotype and to equol-production status.

Materials and methods

Study protocol

Each study centre obtained ethical approval from their local ethics and research committees and written consent was obtained from all volunteers before the beginning of the study. Healthy post-menopausal women (117; 45–70 years old) were recruited from the surrounding areas of the University of Reading (Reading, UK), the German Institute of Human Nutrition (Nuthetal, Germany), the Royal Veterinary and Agricultural University (Copenhagen, Denmark) and the Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (Rome, Italy). Participants were randomly assigned to isoflavones (50 mg/d) or placebo by stratified randomisation according to baseline age, BMI and triacylgycerol in a placebo-controlled 2×8-week double-blind cross-over design with an 8-week washout period. The

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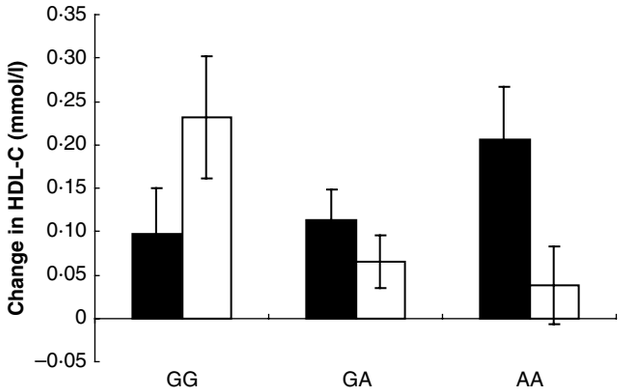

Fig. 1. Changes in plasma HDL-cholesterol (HDL-C) according to oestrogen receptor βcx *Tsp*509I genotype (GG, GA and AA) for healthy post-menopausal women following treatment with isoflavones (■) and placebo (□). For details of subjects and procedures, see p. 109. Values are means with their standard errors represented by vertical bars. The genotype × treatment interaction was significant (linear mixed-modelling approach; $P = 0{\cdot}009$). Differences from baseline (only subjects with no missing data points included in the analysis) were used as the response variable. There was no significant difference between treatments for the GG genotype group (*n* 26) or the AG genotype group (*n* 56), but there was a significant treatment effect in the AA genotype group (*n* 32; slice test; $P = 0{\cdot}003$).

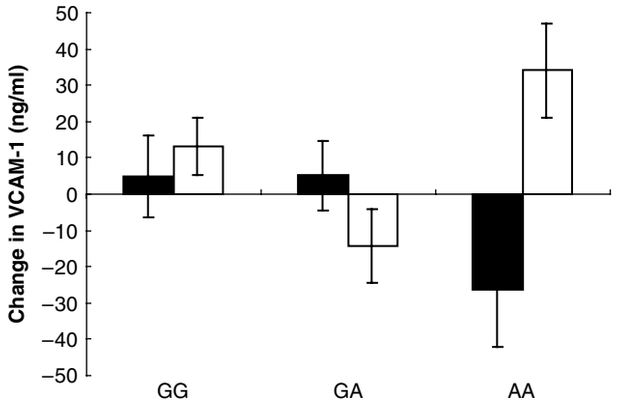

Fig. 2. Changes in plasma vascular cell adhesion molecule 1 (VCAM-1) according to oestrogen receptor β *Alu*I genotype (GG, GA, AA) for healthy post-menopausal women following treatment with isoflavones (■) and placebo (□). For details of subjects and procedures, see p. 109. Values are means with their standard errors represented by vertical bars. The genotype × treatment interaction was significant (linear mixed-modelling approach; $P = 0{\cdot}023$). Differences from baseline (only subjects with no missing data points included in the analysis) were used as the response variable. There was no significant difference between treatments for the GG genotype group (*n* 46) or the AG genotype group (*n* 47), but there was a significant treatment effect in the AA genotype group (*n* 18; slice test; $P = 0{\cdot}016$).

source of isoflavones was an extract (Solgen 40; Solbar Plant Extracts Ltd, Ashdod, Israel; genistein : daidzein 2 : 1) that was incorporated into cereal bars. The method used to determine the isoflavone content of the enriched and unenriched cereal bars has been described by Hall *et al.* (2005). The average nutrient content of cereal bars was (g): energy 652 kJ, protein 2·6, carbohydrate 17·3, fat 8·5, fibre 1·8, Na 0·012. Food diet diaries (3 d) were used to evaluate the dietary intake at baseline and midway during each intervention period at 4 weeks.

Sample collection and biochemical analysis

Fasting blood samples were taken at weeks 0, 4 and 8 of the isoflavone and placebo interventions. The protocols and methods used for blood collection and subsequent biochemical analysis have been described elsewhere (Hall *et al.* 2005; WL Hall, K Vafeiadou, J Hallund, S Bugel, C Koebnick, F Zunft, M Ferrari, F Branca, D Talbot, J Powell, AM Minihane, A Cassidy, M Nilsson, K Dahlman-Wright, J-Å Gustafsson and CM Williams, unpublished results). Briefly, fasting blood samples were collected for the determination of total cholesterol, HDL-C, triacylgycerol, lipoprotein(a), LDL subclasses, glucose, NEFA, endothelin 1, CRP, von Willebrand factor, insulin, E-selectin, VCAM-1, intracellular cell adhesion molecule 1 and monocyte chemotactic protein 1. A formula was used to describe insulin resistance, i.e. the homeostasis model assessment: insulin resistance = (fasting glucose × fasting insulin)/22·5 (Matthews *et al.* 1985).

Genotyping

Genotypes for single nucleotide polymorphisms in ERα (*Xba*I and *Pvu*II), ERβ (*Alu*I and ERβcx *Tsp*509I), endothelial NO synthase (Glu298Asp), apoE (apoE_2, E_3 and E_4), cholesteryl ester transfer protein (*Taq*IB) and leptin receptor (Gln223Arg) were characterised using methods described in detail elsewhere (Hall *et al.* 2005; WL Hall, K Vafeiadou, J Hallund, S Bugel, C Koebnick, F Zunft, M Ferrari, F Branca, D Talbot, J Powell, AM Minihane, A Cassidy, M Nilsson, K Dahlman-Wright, J-Å Gustafsson and CM Williams, unpublished results).

Statistical analysis

SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses (for details, see Hall *et al.* 2005). Data are expressed as the means and standard deviations, except in Figs. 1 and 2, in which the means with their standard errors are given. SPSS for windows (version 12.0.1; SPSS Inc., Chicago, IL, USA) was used to calculate the difference between dietary intakes at baseline, mid-isoflavone (week 4) and mid-placebo (week 4) intervention arms using repeated-measures ANOVA. $P<0{\cdot}05$ was considered significant.

Results

No changes in body weight were evident following the dietary intervention. Subject mean age, BMI, blood pressure and fasting plasma lipids and glucose at baseline were as follows: age 57·7 (SD 5·4) years, BMI 25·0 (SD 2·9) kg/m^2, systolic blood pressure 120·6 (SD 15·4) mmHg,

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diastolic blood pressure 76·1 (SD 8·3) mmHg, total cholesterol 5·88 (SD 0·93) mmol/l, LDL-cholesterol 3·59 (SD 0·80) mmol/l, HDL-C 1·79 (SD 0·38) mmol/l, triacylgycerol 1·10 (SD 0·47) mmol/l, fasting glucose 5·17 (SD 0·47) mmol/l. Compliance was assessed using study diaries, number of empty cereal-bar packets and serum and urinary isoflavone analysis. Following the isoflavone treatment plasma genistein and daidzein concentrations increased 20-fold and 36-fold respectively, with no significant increase in concentrations evident following placebo treatment.

The classification of volunteers as 'equol producers' has been described elsewhere (Hall *et al*. 2005). Thirty-three of the 117 subjects (28·2%) were classified as equol producers.

Dietary intake was assessed at baseline and week 4 of the intervention and placebo arms. There were no significant differences in energy intake or macronutrient intake across the treatments nor compared with baseline. Urinary isoflavones concentrations, equol production and differences in nutrient intake after both placebo and isoflavone periods are reported elsewhere (Hall *et al*. 2005).

Effects of isoflavones on markers of lipid and glucose metabolism and inflammatory biomarkers

There were no significant differences in plasma biomarkers of lipid and glucose metabolism or in the majority of plasma inflammatory biomarkers after the two intervention periods. However, there was a beneficial effect of isoflavones intake on CRP concentrations ($P<0·05$; Hall *et al*. 2005; WL Hall, K Vafeiadou, J Hallund, S Bugel, C Koebnick, F Zunft, M Ferrari, F Branca, D Talbot, J Powell, AM Minihane, A Cassidy, M Nilsson, K Dahlman-Wright, J-Å Gustafsson and CM Williams, unpublished results).

Effect of genotype on response to isoflavone supplementation

Isoflavones–genotype interactions were evident for polymorphisms in ERβ gene. The change in plasma HDL-C (week 8 – baseline) was significantly different according to the ERβcx *Tsp*509I genotype ($P<0·01$; Fig. 1; WL Hall, K Vafeiadou, J Hallund, S Bugel, C Koebnick, F Zunft, M Ferrari, F Branca, D Talbot, J Powell, AM Minihane, A Cassidy, M Nilsson, K Dahlman-Wright, J-Å Gustafsson and CM Williams, unpublished results). The change in plasma VCAM-1 was significantly different according to the ERβ *Alu*I genotype ($P<0·05$; Fig. 2; Hall *et al*. 2005).

Effect of equol-production status on response to isoflavone supplementation

There were no differences between the responses of plasma markers of glucose and lipid metabolism and inflammatory biomarkers to isoflavones and those to the placebo between equol producers (*n* 33) and non-equol (*n* 84) producers ($P<0·05$; Hall *et al*. 2005; WL Hall, K Vafeiadou, J Hallund, S Bugel, C Koebnick, F Zunft, M Ferrari, F Branca, D Talbot, J Powell, AM Minihane, A Cassidy, M Nilsson, K Dahlman-Wright, J-Å Gustafsson and CM Williams, unpublished results).

Discussion

The ISOHEART study was a pan-European study on the cardiovascular effects of dietary soyabean isoflavones. The aims of ISOHEART were to determine the impact of isoflavones on a range of established and novel biomarkers for cardiovascular risk in healthy post-menopausal women and to investigate whether genotype or equol production status can affect the response to isoflavone supplementation. The results obtained from the ISOHEART study demonstrate few beneficial effects of isoflavone supplementation on biomarkers of lipoprotein and glucose metabolism or on the majority of inflammatory markers in a group of healthy post-menopausal women. However, data from the ISOHEART study suggest that isoflavones improve CRP concentrations. The response to isoflavones was not affected by equol-production status. However, when the response to isoflavones was evaluated according to genotype, significant isoflavones-genotype associations were evident for polymorphisms in ERβ gene.

The ISOHEART study has shown that isoflavones have no beneficial effects on plasma lipids, including total cholesterol, HDL-C, LDL-cholesterol or triacylgycerol, in healthy post-menopausal women. The outcome confirms previous findings that consumption of isolated isoflavones has no effect on plasma lipids (for example, see Nestel *et al*. 1997; Hodgson *et al*. 1998; Dewell *et al*. 2002; Nikander *et al*. 2004). Previous studies have shown that isolated isoflavones have no effect on lipoprotein(a) (Hodgson *et al*. 1998; Simons *et al*. 2000; Blakesmith *et al*. 2003; Nikander *et al*. 2004), a finding that has been confirmed by the results of the ISOHEART study. The ISOHEART study is the first to examine the effect of isoflavone consumption on LDL subclass distribution and the results suggest that isoflavones have no effect on percentage small dense LDL. In addition, the ISOHEART study suggests that isoflavones have no effect on plasma insulin and glucose, which is in agreement with the limited number of previous studies that have addressed this issue (Blakesmith *et al*. 2003; Nikander *et al*. 2004).

Before the unequivocal findings of the present study it was difficult to draw conclusions about the effect of isoflavones on lipoprotein profile, since a number of the previous studies lacked adequate statistical power and appropriate design. In addition, in most cases encapsulated isoflavones were administered, which raised the possibility of poor intestinal absorption in the absence of a food vehicle. The ISOHEART study was a well-powered randomised double-blind placebo-controlled cross-over design in which isoflavones were administered within a food and analysis of isoflavones in serum and urine has confirmed that isoflavones were well-absorbed.

Increased production of inflammatory factors associated with endothelial dysfunction is integral to the progression of atherosclerosis. Although isoflavones have been suggested to attenuate inflammation of the endothelium *in vitro* (Rimbach *et al*. 2004), the ISOHEART study has

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found no beneficial effects of isoflavone supplementation on circulating biomarkers of endothelial function including intracellular cell adhesion molecule 1, VCAM-1, E-selectin, monocyte chemotactic protein 1 and endothelin 1. Previously, data on the effects of isoflavones on markers of endothelial function have been equivocal. Nikander *et al*. (2003) have shown that 114 mg isoflavones/d in capsules has no effect on plasma E-selectin concentrations. However, Teede *et al*. (2003) have observed a decrease in VCAM-1 following 80 mg formononetin/d, a precursor of daidzein found in red clover (*Trifolium pratense* L.), in a large group of subjects. An important factor that could explain the discrepancy between the two studies is that the dose of daidzein used in the ISOHEART study was much lower than that used by Teede *et al*. (2003; mg/d; 17 daidzein, 33 genistein). Genistein intake (54 mg/d) has been shown previously to reduce endothelin 1 concentrations in post-menopausal women following 6 and 12 months of supplementation (Squadrito *et al*. 2002, 2003). The results from the ISOHEART study do not support these findings, perhaps because of a lower dose of genistein or a shorter duration of intervention. To the authors' knowledge only one study in the past has investigated the effects of isoflavones on von Willebrand factor, an important indicator of endothelial dysfunction. The results from the ISOHEART study are in agreement with findings from the study by Hermansen *et al*. (2001), which show that isoflavones have no beneficial effect on von Willebrand factor.

Although isoflavones did not improve the majority of the inflammatory biomarkers, the results of the ISOHEART study suggest that isoflavone intake may improve CRP concentrations. CRP has been shown to increase after HRT (Manning *et al*. 2002), but CRP concentrations were not increased following supplementation in the ISOHEART study, and therefore isoflavones do not appear to mimic oestrogen action, at least as far as the effects on CRP are concerned. On the contrary, although the data for CRP was highly skewed and the statistical processing was problematic, thorough statistical analysis of the data by logistic regression has demonstrated that isoflavone consumption has a beneficial effect on CRP concentrations compared with the placebo treatment (see Hall *et al*. 2005). This result does not agree with the findings from a recent study in which CRP was not found to change following isoflavone supplementation (Nikander *et al*. 2004). The discrepancy between the two studies may be because Nikander *et al*. (2004) used an isoflavone supplement with a different aglycone profile from the one used in the ISOHEART study, providing (mg/d) 66 glycitein, 42 daidzein and only 6 genistein, in the form of capsules. In the ISOHEART study 50 mg isoflavones (genistein:daidzein 2:1)/d were consumed by subjects as part of a food vehicle.

There are several factors to consider in interpreting the observed lack of efficacy of isoflavones on the majority of the cardiovascular risk factors in the ISOHEART study. First, the period of exposure to isoflavones used may have been too short to observe any beneficial outcomes. Epidemiological data has suggested that countries that habitually consume higher amounts of isoflavones show lower rates of heart disease. The volunteers in the ISOHEART study consumed isoflavones twice daily for 8 weeks. Whilst this intervention period is regarded as a biologically-acceptable length of time for a clinical intervention study, it may not be enough to observe benefits from weak oestrogenic plant compounds compared with the lifetime exposure observed in epidemiological studies. Second, it is possible that the dose of isoflavones used in the ISOHEART study (50 mg/d, with genisteinbe: daidzein 2:1), although representative of a typical dietary intake in countries where soyabean is a staple, is not sufficient to exert any major beneficial effects during the 8-week exposure. Third, the study group consisted of healthy post-menopausal women; it is possible that isoflavones may have beneficial cardiovascular effects on subgroups of population that are in greater risk of developing CVD, such as individuals who are dyslipidaemic or insulin-resistant.

Evidence from *in vivo* studies is supportive of an association between equol production and health benefits (Atkinson *et al*. 2005). In addition, data from *in vitro* studies suggests that equol is biologically more active than the parent isoflavones (Morito *et al*. 2001; Muthyala *et al*. 2004). Since exposure to equol might have biological effects, the ISOHEART study examined whether response to isoflavone supplementation differed according to equol-production status. Of the post-menopausal women 28 % were defined as 'equol producers', a rate that is in agreement with the prevalence rate that has previously been reported (30 %) for other populations (Lampe *et al*. 1998). However, equol-production status was not found to be a determinant of response to isoflavone supplementation in the ISOHEART study. This finding is in contrast to recent evidence that suggests that responsiveness to isoflavones may vary according to an individual's equol-synthesising capacity (Setchell *et al*. 2002). The lack of any effect of equol-production status on the response to isoflavones in the ISOHEART study may again be because the exposure to isoflavones lasted for only 8 weeks, compared with lifelong exposure in certain Asian populations in whom the strongest associations between equol production capacity and beneficial health effects have been reported (Nagata *et al*. 2001).

Differences in response to isoflavone supplementation may be related to variation in the genetic background of a population; for example, single nucleotide polymorphisms in key cardiovascular genes. Apart from one study that has examined the effect of apoE genotype on lipid response to isoflavones (Atkinson *et al*. 2004), the ISOHEART is the first to investigate the effect of various polymorphisms in genes relevant to oestrogen action and lipoprotein metabolism on the response to isoflavone supplementation. In the ISOHEART study preliminary evidence for diet–gene interactions has been observed for isoflavones and *Alu*I and *Tsp*509I polymorphisms in ERβ gene. More specifically, isoflavones reduce plasma VCAM-1 in the variant AA genotype but not the other genotypes (GG or GA) of ERβ *Alu*I polymorphism. Given the fact that VCAM-1 expression has been shown to be regulated by oestrogen via ER-mediated mechanisms (Mori *et al*. 2004), it may be speculated that variation in the function of the

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ER as a result of differences in ligand–receptor activity may influence the expression of VCAM in response to oestrogen or phyto-oestrogens, including isoflavones. It should, however, be noted that the ERβ *Alu*I polymorphism is positioned in the non-coding 3′ untranslated region in exon 8 of the ERβ gene, and therefore the functional implications are unclear (Rosenkranz *et al*. 1998). It is possible that the ERβ *Alu*I polymorphism may be in linkage disequilibrium with polymorphisms of other, as yet unidentified, genes flanking ERβ that modulate VCAM-1 expression directly or indirectly.

The second evident ERβ genotype–isoflavone association has been observed in women with the ERβcx *Tsp*509I AA genotype, who show a greater than 3-fold increase in HDL-C following isoflavone supplementation compared with the placebo, which is not observed in the other genotypes (see Hall *et al*. 2005; WL Hall, K Vafeiadou, J Hallund, S Bugel, C Koebnick, F Zunft, M Ferrari, F Branca, D Talbot, J Powell, AM Minihane, A Cassidy, M Nilsson, K Dahlman-Wright, J-Å Gustafsson and CM Williams, unpublished results). It is a rather complex task to attempt an interpretation of this putative ERβcx *Tsp*509I genotype–isoflavone association, since the effect of ERβcx isoform in the cardiovascular system is currently unknown. ERβcx *Tsp*509I polymorphism is located in exon 9 of ERβcx, a splice variant of ERβ that utilises an alternative exon of the gene (Nilsson *et al*. 2004). The importance of ERβcx has been emphasised previously in a study in which it has been shown to influence the response to anti-oestrogen therapy, therefore acting as a potential predictive molecule in the response to oestrogen-like compounds (Palmieri *et al*. 2004). ERβcx lacks the amino acid residues of the ERβ that are required for optimal oestrogen binding and subsequent oestrogen-induced transcriptional activation (Ogawa *et al*. 1998). The ISOHEART data suggest that isoflavones act on the *Tsp*509I AA variant to a greater extent than the other genotypes of ERβcx (GG or GA). As the ERβcx *Tsp*509I polymorphism is positioned in the 3′ untranslated region, which is a non-coding region, it is possible that this area influences the translational efficiency, e.g. by affecting mRNA stability (Nilsson *et al*. 2004). Another explanation could be that the ERβcx *Tsp*509I polymorphism may be in linkage disequilibrium with another gene variant that may be involved in the metabolic pathways of HDL synthesis, such as apoA-I synthesis and hepatic lipase activity (Brinton,1996). The isoflavones–ERβ genotype associations observed in the ISOHEART study are a potential novel area of research and clearly warrant further investigation.

Conclusions

In summary, in the ISOHEART study it has been shown that isoflavone-enriched foods do not have any beneficial effect on markers of lipoprotein and glucose metabolism and cannot improve the concentrations of the majority of circulating inflammatory biomarkers in healthy post-menopausal women. However, isoflavones may have a beneficial effect on CRP concentrations. Although isoflavones fail to improve the concentrations of the majority of cardiovascular risk factors, the findings for beneficial effects of isoflavones on CRP suggest that there may be some basis for the recommendation of isoflavone supplements to healthy post-menopausal women for the reduction of inflammatory cardiovascular risk factors. Most importantly, certain subgroups may respond more beneficially to isoflavone supplementation, as already demonstrated by the decrease in plasma VCAM-1 concentrations in one of the genotypes of the ERβ *Alu*I polymorphism and an increase in HDL-C in one of the genotypes of the ERβcx *Tsp*509I polymorphism. Isoflavones can act as oestrogen mimics and show high binding affinity for ERβ. It is biologically plausible that variance in ERβ genotype, and consequent variation in isoflavone interactions with the receptor, contributes to inter-individual differences in response to isoflavones. The isoflavone–genotype associations observed in the ISOHEART study are of particular interest and deserve further investigation.

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