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Effect of soy in men with type 2 diabetes mellitus and subclinical hypogonadism – a randomised controlled study.

Soy and testosterone levels in type 2 diabetes

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Context: Isoflavones found in soy products have a chemical structure similar to estrogen, leading to concerns of an adverse estrogenic effect in men, particularly in those with type 2 diabetes mellitus (T2DM) who have low testosterone levels due to hypogonadism.
Objective: The primary outcome was change in total testosterone levels. The secondary outcomes were the changes in glycaemia and cardiovascular risk markers.
Design: Randomised double blind parallel study.
Setting: Secondary care setting in UK.
Participants: 200 men with T2DM with a total testosterone level≤12nmol/L
Intervention: 15g soy protein with 66mg of isoflavones (SPI) or 15g soy protein alone without isoflavones (SP) daily as snack bars for three months.
Results: There was no change in either total testosterone or in absolute free testosterone levels with either SPI or SP. There was an increase in TSH and reduction in fT4 ($p<0.01$) after SPI supplementation.
Glycaemic control improved with a significant reduction in HbA1c (-4.19(7.29)mmol/mol,$p<0.01$) and HOMA-IR after SPI. Cardiovascular risk improved with a reduction in triglycerides, CRP and diastolic BP ($p<0.05$) with SPI versus SP supplementation. There was 6% improvement in 10-year coronary heart disease risk after three months of SPI supplementation. Endothelial function improved with both SPI and SP supplementation ($p<0.01$) with an increased reactive hyperemia index that was greater for the SPI group ($p<0.05$).
Conclusions
Testosterone levels were unchanged and there was a significant improvement in glycaemia and cardiovascular risk markers with SPI compared to SP alone over three months. There was significant increase in TSH and a reduction in fT4.

PRECIS: Soy has no effect on testosterone levels in men with type 2 diabetes and subclinical hypogonadism; it improves glycaemia and cardiovascular risk markers but may impair thyroid function.

Production and consumption of soy foods within Western countries have increased dramatically in the last decade with the postulated health benefits including improvement in bone health, relief of menopausal symptoms and reduced risk of certain types of cancers (1). In addition, habitual intake of soy isoflavones has also been associated with a reduced risk of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (2), which is of particular relevance given the increasing global prevalence of diabetes.

However, there are concerns as isoflavones have a chemical structure similar to estrogens and can transactivate estrogen receptors (ER) exerting estrogen-like effects *in vitro* and *in vivo* (3). Isoflavones have been shown to affect reproductive health in animals. Male rats exposed to maternal dietary isoflavones through gestation and lactation exhibited a decrease in the anogenital distance, testis size and serum testosterone levels (4). Dietary feeding of isoflavones in male mice resulted in reduced plasma testosterone concentrations, atrophy of seminiferous epithelium, atrophy of accessory sex glands, and squamous metaplasia of seminal vesicles (5). Infant marmoset monkeys when fed with soy resulted in increase in testicular size and lower testosterone levels suggesting a potential "compensated Leydig cell failure"(6). This has raised concerns regarding possible adverse effects of isoflavones in men including feminization and infertility (7). Testosterone levels in men with T2DM are lower due to a combination of factors including insulin resistance and obesity that may make these individuals more susceptible to isoflavone-mediated adverse effects (8). Therefore, given the estrogenic effect of phytoestrogens, we hypothesised that a detrimental estrogenic effect of soy with and without isoflavones on testosterone levels would be exaggerated in men who had low testosterone levels; therefore, this randomised double-blind, parallel study was undertaken in men with T2DM and compensated hypogonadism.

Research Design and Methods

Two hundred men aged between 45 to 75 years with an early morning total testosterone level <12 nmol/L (normal range 12-25 nmol/L) (at least on two different occasions) with normal gonadotrophins and HbA1c of <9 % (normal - hbA1c <6.5%) were recruited after screening 412 Caucasian male patients with T2DM. They were randomised either to 15 g soy protein with 66 mg of isoflavones (SPI) per day or 15 g soy protein alone without any isoflavones (SP) per day for three months.

Studies of isoflavones intakes in Western countries indicate an average daily intake of approximately 2 mg isoflavones, vegetarians have a higher daily isoflavone intake of 16 mg and Asian population consuming high soy diet or people consuming soy supplements have a daily isoflavone intake of around 50-90 mg (9). The isoflavone concentrations used in this study reflected the daily intake of an Asian population consuming high soy diet or people consuming soy supplements (10).

Soy protein alone without any isoflavones (SP) contained less than 300 parts per billion of isoflavones, achieved by serial alcohol washing (Dishman Ltd, India) and confirmed analytically by FERA, Sand Hutton, York, UK. Subjects were on stable medications for T2DM, hyperlipidaemia and hypertension for at least three months prior to the study. Subjects with significant hepatic or renal impairment, who were allergic to soy products, receiving testosterone replacement or who had received antibiotics three months prior to the study were excluded.

The primary outcome of this study was a change in testosterone levels. The secondary outcomes for this study were changes in glycemic control and cardiovascular risk markers including insulin resistance, lipid profile, hsCRP and endothelial function.

At randomisation and during study visits, subjects were instructed to maintain their level of physical activity throughout the study. In addition, subjects were required to avoid food products containing soy, alcohol, vitamin or mineral supplementation, and over-the-counter medications. Dietary reinforcement was undertaken at each visit, together with measurement of plasma isoflavone levels to ensure adherence. Adherence with study preparation was calculated by counting the returned bars. All subjects gave their written informed consent. The study was given ethical approval by the Research Ethics Committee (East Yorkshire & North Lincolnshire Research Ethics Committee, ref: 09/H1304/45).

Study product

A bar containing 7.5 g isolated soy protein powder (Solcon F, Solbar Industries, Ashdod, Israel) with 33 mg of isoflavones (Solgen 40, Solbar Industries Israel) (SPI) or 7.5 g of the isoflavone-free soy protein (SP) was consumed twice daily for three months. The soy protein and the phytoeostrogens were supplied by Solbar Industries Israel from a single batch that was designated for the study. The trial product was packaged by Halo Food Ltd, Swindon, UK. Randomisation based on a computer generated randomisation list was performed by Essential Nutrition Ltd, Brough, UK, who held the randomisation codes.

Study measurements

At the beginning and end of the study, following an overnight fast, weight and blood pressure were measured and blood samples were collected. Blood pressure was measured after the subjects had been seated quietly for at least five minutes with the right arm supported at heart level. Blood pressure measurements were performed using an automated device (NPB-3900; Nellcor Puritan Bennett, Pleasanton, CA) during each study visit. Two readings were obtained at the beginning of each visit at least one minute apart and the average of the readings was taken. Waist circumference was measured using a specific abdominal circumference tape measure. The tape measure was wrapped around the participant's waist at the midway point between the bottom of the ribs and the top of the iliac crest. The participants were encouraged to breath naturally during the procedure, relax their abdominal muscles and not hold their breath. Fasting venous blood samples were collected, separated by centrifugation at 2000 g for 15 minutes at 4^{o}C, and the aliquots stored at -80^{o}C within one hour of collection. Plasma glucose was measured using a Synchron LX20 analyser (Beckman-Coulter, High Wycombe, U.K.), and serum insulin was assayed using a competitive chemiluminescent immunoassay performed using the DPC Immulite 2000 analyser (Euro/DPC, Llanberis, UK). The coefficient of variation of this method was 8%, calculated using duplicate study samples. The analytical sensitivity was 2µU/mL Insulin resistance was calculated using HOMA-IR (Insulin x glucose)/22.5) (11).

Blood samples for testosterone levels were taken before 9 am. Serum total testosterone was measured by isotope dilution liquid chromatography tandem mass spectrometry. Serum free testosterone was measured using equilibrium dialysis by adding a tracer amount of tritium-labelled testosterone to serum and then dialyzed against a buffer whose ionic composition was similar to that of serum. The percent of tracer that crossed the dialysis membrane at equilibrium was taken as percent free fraction. Absolute free testosterone was calculated by multiplying total testosterone with percent free testosterone.

Total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-C) levels were measured enzymatically using a Synchron LX20 analyser (Beckman-Coulter, UK). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation. Serum hsCRP was measured by the high-sensitivity method on a Beckman DXC analyser. All thyroid assays were performed on an Abbott Architect i4000 immunoassay analyser (Abbott Diagnostics Division, Maidenhead, UK).

The phytoestrogens in serum were extracted and analysed by LGC, Fordham, Cambridgeshire, UK using isotope-dilution LC-MS/MS (12). LC-MS/MS was conducted using a Sciex 4000 Qtrap with separation achieved using a C18 column and mobile phases of water and acetonitrile, both containing acetic acid. No column switching was used. The calibration range for all analytes was 0.5 ng/mL to 200 ng/mL, with quality control samples prepared at low (1.5 ng/mL), medium (80 ng/mL) and high (150 ng/mL) concentrations and analysed to confirm the assay performance. Incurred QC samples of serum were also run in the sample batches. The assay sensitivity for equol, daidzein and genistein were all 0.5 ng/mL. The inter assay CVs were less than 6.8% for daidzein, less than 6.1% for genistein and less than 7.4% for equol. The intra assay CVs were less than 7.2% for daidzein (3.9% at the lower limit of quantification (LLOQ)), less than 3.6% for genistein (7.5% at the LLOQ) and less than 8.0% for equol (8.9% at the LLOQ).

Reactive hyperaemia peripheral arterial tonometry (RH-PAT) to assess peripheral microvascular endothelial function was measured using an Endopat 2000 (Itamar Ltd, Caesarea, Israel) according to the manufacturer's instructions. The reactive hyperaemic index (RHI), which is a measure for endothelial function, and the augmentation index (AI), which is a measure for arterial stiffness, was also assessed using the EndoPAT 2000 device. Both measures were calculated using a computerised automated algorithm (software version 3.1.2) provided with the device. The subjects were in supine position for a minimum of 20 minutes before measurements, in a quiet, temperature-controlled (22°C) room with dimmed lights. They were asked to remain as still as possible and silent during the entire measurement period. Each recording consisted of five minutes of baseline measurement, five minutes of occlusion measurement, and five minutes post occlusion measurement (hyperaemic period). Occlusion of the brachial artery was performed on the non-dominant upper arm. The occlusion pressure was at least 60 mmHg above the systolic blood pressure. This technique determines functional endothelial change by measuring changes in digital pulse volume during reactive hyperaemia in an operator-independent manner. RHI is the ratio of the average pulse wave amplitude measured over 60 seconds, starting one minute after cuff deflation, to the average pulse wave amplitude measured at the baseline. The other arm served as a control and the ratio was corrected for changes in the systemic vascular tone. The AI is an indirect measure of arterial stiffness and is calculated as augmentation pressure divided by pulse pressure × 100 to give a percentage.

Cardiovascular risk was estimated by using UKPDS Risk Engine, which is a type 2 diabetes specific risk calculator based on 53,000 patients years of data from the UK Prospective Diabetes Study (13).

Breast ultrasound was undertaken and assessed for each subject before and after the study by a consultant radiologist who was blinded to treatment, as a safety measure to assess enlargement of breast tissue since excess estrogen can stimulate breast enlargement in males.

Statistical analysis

Baseline continuously distributed data is presented as median (25^{th}/75^{th} centiles); categorical data by n (%). Based on the variability of the testosterone (14), for a one nmol change in total testosterone, it was calculated that 150 subjects would be required to give 90% power and an alpha value of <0.0l. Assuming a drop out rate of 33%, two hundred patients were recruited for this study. Given randomisation to treatment, baselines were not compared statistically (15-17). Within-group differences (difference between 12 week values and baseline values) are shown for each treatment group separately by a mean and a standard deviation (SD). Between-group comparisons were performed using the independent sample t-test. The t-test assumes equal variance between groups, which is reported for the two within-treatment groups separately. For all statistical analyses, a two-tailed P <0.05 was considered to indicate statistical significance. Bonferroni corrections were not applied (18). Statistical analysis was

performed using the STATA statistical computer package (StataCorp. 2013. *Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP, USA).

Results

Two hundred patients with T2DM and low testosterone levels were recruited after screening 412 patients (Figure 1). Adherence of completed patients by counting returned bars was 98%. Twenty nine patients dropped out (15 patients in the SPI group and 14 patients in the SP group; due to gastrointestinal intolerance to the bars felt by the participants (19 patients (SPI=10; SP=9)), non-adherence (6 patients (SPI=3; SP=3)), the need to take antibiotics for concurrent illness (3 patients (SPI=2; SP=1)); and one patient (SP=1) was initiated on testosterone replacement for his erectile dysfunction. The baseline anthropometric, hormonal, biochemical parameters and isoflavone levels of the two groups are given in Table 1.

There were no changes in weight, body mass index and waist circumference with either SPI or SP supplementation. There was a significant reduction of diastolic blood pressure after both SPI and SP supplementation but there were no significant differences between groups. There was no change in systolic blood pressure in either group. There was a significant increase (improvement) in RHI with SPI supplementation compared to SP supplementation alone (Table 2).

The UK PDS cardiovascular risk engine showed a significant 6% reduction in non-fatal and fatal coronary heart disease risk, a significant 9% reduction in fatal coronary heart disease risk and suggested a significant reduction in fatal stroke risk with three months of SPI supplementation (Table 3).

There were significant reductions in triglycerides, hsCRP with SPI compared to SP supplementation, however there were no changes in total cholesterol, LDL cholesterol and HDL cholesterol with SPI supplementation compared to SP supplementation. (Table 2)

There were no changes in serum total testosterone, absolute free testosterone, FSH, LH or estradiol with either SPI or SP supplementation (Table 2). There was a significant reduction in HbA1c, fasting glucose, fasting insulin and HOMA-IR with SPI versus SP supplementation (Table 2).

There was a significant increase in TSH with 3 months of SPI supplementation (Mean (SD)) (1.81 (0.92) vs. 3.23 (1.03) mU/L) compared to SP supplementation (1.82 (0.93) vs. 1.96 (1.11) mU/L). There was also a significant decrease in free T4 with 3 months of SPI supplementation (12.68 (1.90) vs. 11.09 (2.00) pmol/L) compared to 3 months of SP supplementation (13.06 (1.74) vs. 12.74 (1.62) pmol/L. However, there were no significant changes in fT3 with 3 months of either SPI or SP supplementation.

There was a significant increase in TSH and a reduction in fT4 after SPI versus SP supplementation, but this did not reflect in any changes in fT3 after either SPI or SP supplementation (Table 2).

There were significant increases in daidzein, genistein and equol with SPI supplementation confirming adherence, whilst no changes from baseline were seen with SP supplementation (Table 4).

Discussion

There were no significant changes in testosterone measured by the gold standard techniques of tandem mass spectrometry and absolute free testosterone measured by equilibrium dialysis, by SP with and without isoflavones after three months treatment. Previous studies on soy supplementation have showed conflicting results (19). However, the previous studies

were not designed to investigate testosterone as the primary endpoint (19), and most used immunoassay (20) rather than gold standard testosterone measurements.

There was also no significant change in SHBG or albumin with either SPI or SP supplementation. Testosterone is bound to sex hormone binding globulin (SHBG) and albumin in circulation. There were also no changes in either FSH or LH after both preparations suggesting that there was no significant alteration of hypothalamic-pituitary-gonadal axis with either preparation. There were also no significant changes in estrogen levels in either group. Testosterone is converted into estrogen in various tissues including adipose tissue by aromatase enzyme (21) and could potentially cause gynaecomastia. There were no changes in volume of breast tissue in either group.

There was a significant improvement in glycemic control as evidenced by a reduction in HbA1c after three months of SP and 66 mg isoflavone supplementation. There was also a significant reduction of fasting glucose and insulin after SPI, which was reflected by a 65% reduction in HOMA-IR in this group of men with T2DM and low testosterone levels. *In vitro* studies have suggested several mechanisms for a direct pharmacological action of soy on glycemic control, including inhibiting intestinal brush border uptake of glucose, having α-glucosidase inhibitor actions, tyrosine kinase inhibitory action, changes in insulin receptor numbers and affinity, intracellular phosphorylation, and alterations in glucose transport (22). Estrogen has been suggested to participate in glucose homeostasis by modulating the expression of genes that are involved in insulin sensitivity and glucose uptake (23). Further, estrogen is a major regulator of adipocyte development and adipocyte number and inhibits lipogenesis by reducing the activity of lipoprotein lipase, an enzyme that regulates lipid uptake by adipocytes (24). Isoflavones may also affect glucose metabolism by non-estrogen receptor-mediated mechanisms. For example, isoflavones have been reported to have an anti-diabetic effect through activation of PPAR, nuclear receptors that participate in cellular lipid homeostasis and insulin action (25).

There were no changes in weight after three months of either SPI or SP alone indicating that insulin resistance was decreased independent of a change in weight that has been suggested by others (26). This is supported by epidemiological data showing that compared to Japanese in Tokyo on a traditional soy diet, Japanese-Americans have a higher prevalence of T2DM and insulin resistance despite similar BMI levels (27).

There was a significant reduction in diastolic blood pressure of around 2mmHg with both SPI and SP for three months. Hypertension, estimated to affect approximately one billion individuals worldwide, is a major risk factor for cardiovascular disease (CVD). A 4–5 mmHg reduction in systolic blood pressure (SBP) and a 2–3 mmHg reduction in diastolic blood pressure (DBP) can reduce CVD risk by 8–20% (28). Dietary soy isoflavones have been suggested to result in arterial vasodilatation, improvement in endothelial function, and decreased blood pressure, perhaps by nitric oxide (NO) dependent mechanism in animal experiments (29). A meta-analysis of eleven trials demonstrated that soy isoflavone intake resulted in a mean decrease of 2.5 mmHg for SBP and 1.5 mmHg for DBP compared to placebo (30) though there was a significant heterogeneity between the studies; however, the reduction of diastolic blood pressure here is in accord with those reports on a vasodilation mechanism. Such an antihypertensive effect has been suggested to be intrinsic to the amino acid composition of protein, especially arginine in soy, through multiple mechanisms perhaps accounting for the decrease in blood pressure that was independent of isoflavones treatment (31)

The UKPDS risk engine (13) that calculates coronary heart disease and stroke risk in patients with type 2 diabetes showed a significant reduction in non-fatal and fatal coronary heart disease risk reduction of 6% and reduction of fatal coronary heart disease risk of 9% with three months of SPI supplementation. There was a statistically significant reduction of

fatal stroke risk with both SPI and SP supplementation but there were no changes between the two groups. This is particularly important in patients with type 2 diabetes who are at in increase risk of heart disease and stroke.

There was a significant increase in the RHI with SPI supplementation compared to SP supplementation. Endothelial dysfunction is an early predictor of cardiovascular disease (32) and can be measured using Endo PAT which is non-operator dependent. In the Framingham study, a significant inverse relation was observed between endothelial function as determined by the EndoPAT (RHI) and multiple cardiovascular risk factors (33). The RHI was reported to be significantly decreased in patients with coronary artery disease, hypertension, hyperlipidemia and diabetes (34), and several studies have demonstrated an improvement in endothelial function as a result of lifestyle modification including diet (35). In a small study, soy nuts has been shown to have a modest effects on attenuating endothelial dysfunction over four weeks in adults with features of metabolic syndrome (36). In the current study on patients with type 2 diabetes, there was a significant increase in RHI suggesting potential favourable effects of SPI combination on endothelial function and cardiovascular risk.

There was a significant reduction in triglycerides with SPI compared to the SP group. There were no significant changes in total cholesterol, LDL-C and HDL-C with SPI supplementation compared to SP supplementation. Isoflavones, were shown to be hypo-lipidemic in both cynomolgus monkey (37) and humans (38) in some studies but not all studies (39). Ingestion of ethanol extract rich in isoflavones increases the abundance of hepatic mRNA for cholesterol 7α-hydroxylase (CYP7a) (40) and LDL receptors in rats, which play important roles in cholesterol catabolism. A recent meta-analysis indicated that soy isoflavones significantly reduced total cholesterol and LDL-C but does not change HDL-C (41). However, a positive association between isolated soy isoflavones and HDL-C has been documented in postmenopausal women (42). On the other hand, SP enhances the expression of the LDL receptor in hypercholesterolemic patients with T2DM (43) and animal studies (44). Moreover, some previous studies have demonstrated that SP rather than isoflavones contributes to the lipid-lowering and hypocholesterolemic properties of soy (45). These discrepancies could be attributed to basal lipid profile or different study designs and treatment composition, such as the use of mixed isoflavones of poor purity, glucoside instead of aglycone forms, and the concomitant presence of proteins.

There was a significant reduction of hsCRP that is an important cardiovascular risk marker with three months of combined SPI preparation compared to SP supplementation. The effect of soy on CRP is variable. SPI supplementation significantly improved CRP in post-menopausal women with T2DM (2) whereas isoflavone supplementation alone was ineffective (39) suggesting a potential matrix effect, i.e. the combination of both soy protein and isoflavones rather than either alone, between SP and isoflavones on CRP.

There was a significant increase in TSH and reduction in fT4 with three months of combined SPI supplementation. Soy consumption is associated with thyroid disorders such as hypothyroidism, goitre, and autoimmune thyroid disease (46). *In vitro* studies have demonstrated that isoflavones inhibit thyroid peroxidase (TPO), an enzyme involved in the synthesis of T_3 and T_4 (47). In a study examining the effects of soy isoflavones, Fischer rat thyroid cells (FRTL) were treated with a combination of SPI; it was found that there was a dose dependent suppression of iodide uptake in FRTL cells whereas isoflavone alone was ineffective. SPI combination and isoflavone (genisten) alone increased the 40 kDa thyroglobulin fragment (P40, a known autoimmunogen) and non-glycosylated sodium/iodide symporter (NIS) in the FRTL cells that might contribute to the higher incidence of thyroid dysfunction (48). In patients with subclinical hypothyroidism, SPI combination has shown to increase the risk of developing overt hypothyroidism (49). In women during their early menopause SPI supplementation has shown to significantly increase TSH and reduce free

thyroxine suggesting a detrimental effect on thyroid function (50). In the current study, none of the patients developed subclinical or overt hypothyroidism, however, all patients had normal thyroid function tests at baseline.

The dropout rate during the intervention was less than expected (14.5 %), with the most frequent reason for participant withdrawal in both groups attributable to palatability; however, these numbers are comparable to other nutrition trials in patients with T2DM (51). There was a significant rise in plasma isoflavone levels with SPI, confirming adherence, whilst plasma isoflavone levels did not differ from baseline in the SP group confirming that they had not taken any exogenous isoflavones during the study.

In conclusion, SP with and without 66 mg isoflavone per day for three months did not have an effect on testosterone levels in men with T2DM confirming its safety. In addition, there were significant improvements in both glycemic control and cardiovascular risk markers including diastolic blood pressure, triglycerides and hsCRP. These reflected in a significant improvement in the calculated coronary heart disease risk with SPI supplementation where as there were no changes seen with SP supplementation. However, there was a significant increase in TSH with a reduction in free T4 after high dose isoflavone in combination with SP supplementation suggesting a potential adverse effect of soy on the thyroid.

Acknowledgement and conflict of interests

All authors have no conflict of interests to disclose relating to this article.

SLA, ESK, NJT and TS conceived the study, all authors involved in reviewing the proposal and conducting the study, ASR involved in statistical analysis, SB did the testosterone assays, TS drafted the first draft, all authors reviewed the manuscript and had access to all trials data. TS is the guarantor for this article.

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Figure 1. Flow diagram of participants through the study. *SPI – Soy protein + isoflavone group; SP – Soy protein without isoflavone group*

Table 1. Baseline parameters between the SPI and SP groups. Values are provided as medians (25^{th}/75^{th} centiles).

Parameters	SP1 group (n=100)	SP group (n=100)
Age (years)	52.0 (50.0, 55.0)	52.0 (50.0, 55.0)
Weight	100.1(88.5,112.3)	98(85.7,111.9)
Body Mass Index (kg/m^2)	31.8(28.8,34.7)	31.6(29.2,35.0)
Duration of diabetes (years)	7.3 (4.2,8.8)	7.9 (4.4, 9.1)
HbA1c (mmol/mol)	56 (52,60)	58(53,64)
Total testosterone (nmol/L)	10.0 (8.6, 11.0)	9.4 (7.9, 11.0)
% Free Testosterone	2.7(2.2,3.0)	2.6(2.3,3.2)
Absolute free testosterone (nmol/L)	0.209(0.183,0.236)	0.202(0.167,0.238)
SHBG (nmol/L)	29.0 (23.0, 40.0)	31.0 (21.3, 39.0)
TSH (mU/L)	1.6 (1.2, 2.4)	1.6 (1.2, 2.5)
fT4 (pmol/L)	12.0 (12.0, 14.0)	13.0 (12.0, 14.0)
fT3 (pmol/L)	4.6 (4.2, 5.0)	4.6 (4.2, 4.9)
Fasting glucose (mmol/L)	139.5 (118.8, 160.7)	135.9 (115.2, 154.4)
Fasting insulin (µIU/mL)	16.5 (9.9, 25.3)	18.0 (10.4, 28.6)
HOMA-IR	5.6 (3.6, 9.0)	6.2 (3.8, 9.7)
hsCRP (mg/L)	2.1 (0.8, 3.9)	1.9 (0.9, 4.1)
TC (mmol/L)	3.9 (3.4, 4.6)	3.8 (3.4, 4.5)
LDL-C (mmol/L)	2.0 (1.7, 2.9)	2.0 (1.6, 2.7)
HDL-C (mmol/L)	1.1 (0.9, 1.3)	1.0 (0.9, 1.2)
Triglycerides (mmol/L)	1.4 (1.0, 2.1)	1.3 (0.9, 2.0)
FSH (IU/L)	5.7 (4.0, 9.4)	6.0 (4.8, 8.5)
LH (IU/L)	3.7 (2.4, 5.6)	3.8 (3.0, 5.0)
Oestradiol (pmol/L)	89.0 (72.0, 110.0)	81.5 (69.0, 99.3)
Daidzein (ng/mL)	1.4 (0.6, 2.7)	1.9 (0.7, 4.3)
Genistein (ng/mL)	2.6 (0.7, 5.7)	2.9 (1.3, 7.2)
Equol (ng/mL)	0.1 (0.1, 0.1)	0.1 (0.1, 0.1)

SPI (15 g soy protein with 66 mg of isoflavones); SP (15 g soy protein alone without any isoflavones)
To convert values for glucose to milligrams per decilitre, divide by 0.056.
To convert values for insulin to picomoles per litre, multiply by 6.
To convert values for cholesterol to milligrams per decilitre divide by 0.0259.
To convert values for triglycerides to milligrams per decilitre divide by 0.0113.
TC - Total cholesterol; LDL-C - LDL-cholesterol; HDL-C - HDL cholesterol; TG-Triglycerides; HbA1c – glycated haemoglobin; HOMA – Homeostasis model of assessment – insulin resistance; HsCRP – highly sensitive C-reSPI protein; FSH – follicle stimulating hormone; LH – Luteinising hormone; SHBG – sex hormone binding globulin; TSH – thyroid stimulating hormone; fT4 – free thyroxine; fT3 – free tri-iodo thyronine;

Table 2. Anthropometric, metabolic and hormonal parameters: comparison between SPI and SP supplementation at end of study.

Parameter	SPI group		SP group		Difference of the difference between groups (95% CI)	p-value
	Paired difference 3 months vs baseline Mean (SD)	p-value	Paired difference 3 months vs baseline Mean (SD)	p-value		
Weight (kg)	0.60 (6.83)	0.39	0.39 (9.23)	0.30	0.21 (-2.1, 2.6)	0.85
Body mass index (kg/m^2)	0.17 (2.03)	0.39	0.15 (3.32)	0.29	0.02 (-0.79, 0.83)	0.86
Waist (cm)	0.68 (7.12)	0.36	-0.70 (5.42)	0.50	1.38 (-0.54, 3.32)	0.15
Systolic blood pressure (mmHg)	-2.32 (15.84)	0.09	-4.1 (15.05)	0.07	1.77 (-2.79, 6.34)	0.44
Diastolic blood pressure (mmHg)	-2.47 (11.28)	0.03	-1.04 (11.46)	0.05	-1.42 (-4.79, 1.93)	0.40
RHI	0.30 (0.60)	<0.01	-0.11 (0.56)	<0.01	0.19 (0.02, 0.36)	0.02
AI	0.01 (0.14)	0.72	-0.05 (0.12)	0.07	0.05 (-0,01, 0.089)	0.09
HbA1c (mmol/mol)	-4.19 (7.29)	0.01	1.63 (7.62)	0.06	-5.82 (8.09, -3.56)	<0.001
Fasting glucose (mmol/L)	-1.44 (1.61)	<0.01	0.59 (2.02)	0.09	-2.03 (-2.58, -1.49)	<0.001
Fasting insulin (µIU/mL)	-10.96 (13.51)	<0.01	-0.70 (25.78)	0.83	-10.25 (-16.36, -4.14)	0.001
HOMA-IR	-4.42 (5.73)	<0.01	0.78 (17.67)	0.69	-5.21 (-9.1, -1.32)	0.009
TC (mmol/L)	-0.05 (0.60)	0.41	0.14 (0.55)	0.02	-0.19 (-0.36, -0.02)	0.08
LDL-C (mmol/L)	-0.05 (0.49)	0.34	0.11 (0.47)	0.05	-0.16 (-0.31, -0.77)	0.09
HDL-C (mmol/L)	-0.01 (0.13)	0.38	0.02 (0.14)	0.25	-0.03 (-0.07, 0.01)	0.14
Triglycerides (mmol/L)	-0.78 (0.80)	<0.01	0.08 (0.82)	0.37	-0.86 (-1.1, -0.62)	<0.001
hsCRP (mg/L)	-2.55 (4.35)	<0.01	-0.13 (3.92)	0.74	-2.41 (-3.65, -1.18)	<0.001
Total testosterone (nmol/L)	0.05 (0.5)	0.30	0.11 (0.55)	0.06	-0.06 (-0.22, 0.09)	0.42
% free testosterone	-0.01 (0.44)	0.92	0.05 (0.37)	0.12	-0.07 (-0.2, 0.05)	0.22
Absolute free testosterone (nmol/L)	0.02 (0.04)	0.81	0.02 (0.06)	0.08	0 (-0.01, 0.02)	0.80
SHBG (nmol/L)	-0.26 (5.91)	0.67	-1.10 (4.46)	0.07	0.84 (-0.72, 2.40)	0.29
FSH (Iu/L)	0.26 (1.59)	0.12	0.09 (1.06)	0.56	0.19 (-0.21, 0.61)	0.34
LH (Iu/L)	0.34 (1.17)	0.07	0.17 (1.34)	0.26	0.17 (-0.20, 0.54)	0.37
Oestradiol (pmol/L)	2.15 (23.23)	0.39	0.36 (20.43)	0.80	1.79 (-4.80, 8.38)	0.59
TSH (mU/L)	1.42 (0.83)	<0.01	0.16 (0.64)	0.06	1.26 (1.04, 1.49)	<0.001
fT4 (pmol/L)	-1.53 (2.69)	<0.01	-0.36 (1.98)	0.09	-1.16 (-1.89, -0.44)	0.002
fT3 (pmol/L)	0.05 (0.71)	0.54	-0.09 (1.05)	0.43	0.14 (-0.13, 0.42)	0.30

SPI (15 g soy protein with 66 mg of isoflavones); SP (15 g soy protein alone without any isoflavones); RHI – reSPI hyperaemia index; AI – Augmentation index; FSH – follicle stimulating hormone; LH – Luteinising hormone; DHEAS – dehydroepiandrostenedione sulphate; SHBG – sex hormone binding globulin;); HbA1c – glycated haemoglobin; HOMA – Homeostasis model of assessment – insulin resistance; HbA1c - from mmol/mol to % *-(x/10.929)+2.15;* HbA1c - From % to mmol/mol - *(y-2.15)x10.929; Glucose – from mmol/L – mg/dL (x*18); Glucose – from mg/dL – mmol/L (y/18); TC - Total cholesterol; LDL-C - LDL-cholesterol; HDL-C - HDL cholesterol; TG-Triglycerides; hsCRP – highly sensitive C-reactive protein; TSH – thyroid stimulating hormone; fT4 – free thyroxine; fT3 – free tri-iodo thyronine*
p-values from independent t-test. Within-pair SD in parentheses.

Table 3. 10 year cardiovascular risk reduction using UKPDS risk engine after three months of soy protein + isoflavone supplementation and soy protein alone supplementation

Parameters	SPI			SP			SPI-SP difference
	Pre vs. post SPI	% Change	p value	Pre vs. post SP	% Change	p value	p value
Non-fatal and fatal CHD risk %	21.14 ± 9.34 vs. 19.86 ± 8.84	-6 ± 12	0.01	22.13 ± 7.65 vs. 22.24 ± 7.86	0.02 ± 0.19	0.82	0.04
Fatal CHD risk %	14.07 ± 7.18 vs. 12.86 ± 6.95	-9 ± 0.11	<0.01	16.82 ± 17.27 vs. 16.89 ± 18.22	0.02 ± 0.22	0.86	0.03
Non-fatal and fatal stroke risk %	10.02 ± 5.67 vs. 9.84 ± 5.65	-2 ± 0.11	0.13	10.53 vs. 4.35 vs. 10.27 vs. 4.14	-0.01 ± 0.10	0.07	0.98
Fatal Stroke risk %	1.60 ± 1.12 vs. 1.51 vs. 1.04 ±	-0.06 ± 0.10	0.04	1.69 ± 0.88 vs. 1.55 ± 0.77	-0.04 ± 0.23	0.05	0.76

SPI (15g soy protein with 66mg of isoflavones); SP (15g soy protein alone without any isoflavones)
CHD – Coronary heart disease

Table 4. Comparison of plasma phytoestrogen levels with SPI and SP supplementation.

Parameter	SPI group		SP group		Difference of the difference	p-value
	Paired difference 3 months vs. baseline Mean (SD)	p-value	Paired difference 3 months vs. baseline Mean (SD)	p-value	Between groups (95% CI)	
Daidzein (ng/mL)	6.61 (7.24)	<0.001	0.91 (3.28)	0.34	7.24 (4.48, 17.70)	<0.001
Genistein (ng/mL)	13.19 (9.77)	<0.001	0.90 (3.59)	0.26	14.58 (8.11, 27.66)	<0.001
Equol (ng/mL)	1.69 (3.52)	<0.001	1.03 (1.25)	0.20	1.65 (1.26, 2.15)	<0.001

SPI (15g soy protein with 66mg of isoflavones); SP (15g soy protein alone without any isoflavones)
Diadzein, Genistein and Equol are natural log transformed.