

## ORIGINAL ARTICLE

## Large inter-individual variation in isoflavone plasma concentration limits use of isoflavone intake data for risk assessment

V van der Velpen<sup>1</sup>, PC Hollman<sup>1,2</sup>, M van Nielen<sup>1</sup>, EG Schouten<sup>1</sup>, M Mensink<sup>1</sup>, P van't Veer<sup>1</sup> and A Geelen<sup>1</sup>

**BACKGROUND/OBJECTIVES:** Isoflavones are present in soy foods and soy-based supplements. Despite low plasma isoflavone concentrations in the general Western population, concentrations in supplement users exceed those suggested to be beneficial for health in Asian populations, raising concerns for adverse effects. To aid risk assessment, quantification of the relation between isoflavone intake and plasma concentrations is essential.

**SUBJECTS/METHODS:** Plasma samples were collected from postmenopausal women in three placebo-controlled crossover studies with 8-week periods for supplements (two studies, ~100 mg isoflavones/day,  $n=88$ ) or 4-week periods for soy foods (one study, ~48 mg isoflavones/day,  $n=15$ ). Plasma isoflavone concentrations (daidzein, equol, genistein and glycitein) were quantified using high-performance liquid chromatography and electrochemical detection. The association between plasma concentrations and isoflavone intake, equol producer status, intake–producer interaction and background dietary intake was assessed based on the assumption of a log-linear relation.

**RESULTS:** Median plasma total isoflavone concentrations after the soy food and supplement interventions were respectively 2.16 and 3.47  $\mu\text{mol/l}$  for equol producers and 1.30 and 2.39  $\mu\text{mol/l}$  for non-producers. Regression analysis showed that doubling isoflavone intake increased plasma concentrations by 55–62% ( $\pm$  s.e. 1–2%,  $R^2 > 0.87$ ) for daidzein, genistein, equol (only for producers) and total isoflavones; for glycitein the association was weaker ( $15 \pm 1\%$ ,  $R^2 = 0.48$ ). Adjustments for energy, carbohydrate and fat intake did not affect these estimates. Inter-individual variation, estimated based on repeated measures in one of the studies, was 30–96%.

**CONCLUSIONS:** Although the relation between isoflavone intake and plasma concentrations was adequately quantified, the use of isoflavone intake data for risk assessment needs caution due to large inter-individual variation in plasma concentrations.

*European Journal of Clinical Nutrition* (2014) 68, 1141–1147; doi:10.1038/ejcn.2014.108; published online 18 June 2014

## INTRODUCTION

Isoflavones, present in soy products, are suggested to relieve menopausal complaints<sup>1,2</sup> and to have a number of other beneficial health effects, such as prevention of osteoporosis and cardiovascular disease.<sup>3–5</sup> At higher doses, uncertainty exists regarding potentially detrimental effects on thyroid function and risk of breast and endometrium cancer, because long-term human trials are currently lacking.<sup>6–8</sup>

The glucosides daidzin, genistin and glycitin are the main isoflavones in soy, whereas their malonyl and acetyl equivalents as well as the aglycones daidzein, genistein and glycitein occur in much lower quantities.<sup>9</sup> The various isoflavones have specific pharmacokinetic characteristics, for example, isoflavone glucosides are more bioavailable than their aglycones;<sup>10</sup> daidzein is rapidly excreted in urine, whereas genistein enters enterohepatic recycling.<sup>10,11</sup> In addition, 20–30% of the Western population are so-called equol producers; they can convert daidzein into the more active metabolite equol by their gut bacteria.<sup>12</sup> Furthermore, isoflavone plasma concentrations can be influenced by isoflavone source, food matrix, diet, frequency of ingestion, gender and age.<sup>10</sup>

Mean isoflavone intake is low (0.5–0.8 mg/day) across the European population and remains well below the intake in Asian countries even for vegetarians and vegans (22.4 mg/day).<sup>13</sup> Mean

plasma concentrations were estimated to be  $< 0.01 \mu\text{mol/l}$  in the European population and 0.23  $\mu\text{mol/l}$  for vegetarians and vegans.<sup>14</sup> In Japan, mean intakes of daidzein and genistein are 18.3 and 31.4 mg/day, resulting in plasma concentrations of 0.12 and 0.48  $\mu\text{mol/l}$ , respectively.<sup>15</sup> On average, supplement users in Western countries consume 50 mg of isoflavones/day, but soy-based supplements can contain up to 107 mg aglycone equivalents of isoflavones.<sup>16–18</sup> Two intervention studies demonstrated that an intake of 100 mg isoflavones/day resulted in total circulating isoflavones of 1.12 and 4.50  $\mu\text{mol/l}$ .<sup>19,20</sup> This illustrates that postmenopausal women taking isoflavone supplements, for example, to relieve their menopausal complaints can be exposed to higher isoflavone concentrations than Asians who regularly consume soy products.

For risk assessment, insight into isoflavone plasma concentrations resulting from these higher isoflavone intake ranges is important. Isoflavone pharmacokinetics as well as positive correlations and dose-response curves between intake and circulating isoflavones have previously been thoroughly characterized.<sup>21–26</sup> However, because of the small sample sizes ( $n=10–18$ ), these data are not suitable for risk assessment and a quantitative description of the relation between isoflavone intake and plasma concentrations in a much larger group of

<sup>1</sup>Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands and <sup>2</sup>RIKILT, Wageningen University & Research Centre, Wageningen, The Netherlands. Correspondence: A Geelen, Division of Human Nutrition, Wageningen University, 6700 EV Wageningen, The Netherlands.

E-mail: anouk.geelen@wur.nl

Received 17 December 2013; revised 4 April 2014; accepted 23 April 2014; published online 18 June 2014

postmenopausal women is needed. We aimed to quantify this relation based on intakes of two different isoflavone supplements and a soy food, taking into account relevant factors like equol-producing phenotype and background dietary intake.

## MATERIALS AND METHODS

### Trials and subjects

For this study, data from three randomized crossover trials were used: the ISO study, the ISO II study and the SOY study. All studies were conducted at the Department of Human Nutrition of Wageningen University and were performed according to the guidelines laid down in the Declaration of Helsinki. All procedures were approved by the medical ethical review board of this University and written informed consent was obtained from all subjects.

Thirty postmenopausal women participated in the double-blind ISO study of which all participants were equol producers, characterized at screening. The study was registered at [clinicaltrials.gov](http://clinicaltrials.gov) under NCT01232751.

The ISO II study, hereafter named the GD (genistein/daidzein) study, was a double-blind trial with two arms in which 72 postmenopausal women participated, 36 in each arm. The two arms were either a daidzein-rich supplement (DAI) or a genistein-rich supplement (GEN), both against placebo. Equol producers, characterized at screening, were randomized over the two arms. The study was registered at [clinicaltrials.gov](http://clinicaltrials.gov) under NCT01556737.

Both double-blind trials included two 8-week intervention periods with one 8-week washout period in between and had the same in- and exclusion criteria, previously reported in van der Velpen *et al.*<sup>27</sup> In the ISO study, postmenopausal status was defined as 3 months absence of menses and if shorter than 1 year this was complemented with an follicle stimulating hormone test. For the GD study, 1-year absence of menses defined postmenopausal status (see Table 1 for details on subjects).

The SOY study was a strictly controlled dietary intervention with a soy protein diet. Sixteen postmenopausal women with abdominal obesity, as defined by a waist circumference  $\geq 80$  cm, were included. During each 4-week intervention period, the participants consumed a soy protein-rich diet or a mixed protein diet (control diet), with a 4-week washout period in between. The study was registered at [clinicaltrials.gov](http://clinicaltrials.gov) under NCT01694056.

Equol-producing capacity of the participants of the ISO and the GD studies was verified before entering the study by a 3-day challenge with the daidzein-rich supplement, after which equol was detected in a spot urine sample. For exact criteria used, see van der Velpen *et al.*<sup>27</sup> In the ISO study, 42 equol producers (27%) out of 150 postmenopausal women were characterized of which 30 producers entered the study. In the GD study, 73 postmenopausal women were screened and 17 (23%) were equol producers; 16 producers and 54 non-producers were included in the study. At the end of the isoflavone intervention of the GD study, two non-producers switched producer status. In the SOY study, 4 of 15 women (27%) were characterized as equol producers after the soy diet intervention period.

### Isoflavone intervention

Both isoflavone supplements used in the studies consisted of soy extracts and predominantly contained daidzein, genistein and glycitein as glycosides and as aglycones. The isoflavone supplements of the ISO study (October 2010), which were also used in the DAI arm of the GD study (November 2011), were previously described.<sup>27</sup> The capsules from the two different batches contained 14 mg daidzein, 3–4 mg genistein and 6–7 mg glycitein (aglycone equivalents) per supplement, as quantified in our laboratory (Table 1). The participants were asked to consume four capsules/day; two in the morning and two in the evening.

The supplements in the GEN arm of the GD study were provided by Springfield Nutraceuticals (Oud-Beijerland, the Netherlands) based on the commercially available supplement Mega Soja with an isoflavone content of 25 mg daidzein, 21 mg genistein and 5 mg glycitein per supplement (aglycone equivalents, Table 1). The identical-looking placebo capsules were provided by the same supplier; these consisted of hydroxypropylmethyl cellulose, were not coloured and were filled with microcrystalline cellulose. The participants in this study consumed two capsules/day; one in the morning and one in the evening. A run-in period of 4 weeks preceded both studies. The participants were asked not to eat soy foods during the run-in and the study period.

Before the strictly controlled dietary intervention (SOY study), participants were assigned to an energy group based on the results of a validated 177-item food frequency questionnaire<sup>28</sup> and their height and weight. The soy and mixed protein diet were isoenergetic with the same

**Table 1.** Subject characteristics, isoflavone content of supplements and soy foods and background dietary intake

	ISO (n = 21)		GD DAI (n = 32)		GD GEN (n = 35)		SOY (n = 15)	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Age (years) <sup>a</sup>	60.3	6.2	62.2	5.3	62.7	5.4	61.2	5.4
BMI (kg/m <sup>2</sup> )	25.7	4.0	25.2	3.1	25.0	3.6	25.4	4.1
Body weight (kg)	73.0	13.3	71.4	9.8	69.5	11.0	69.4	12.6
No of equol producers (%) <sup>b</sup>	21	100	8	25	9	26	4	27
Total isoflavone intake (mg/day) <sup>c</sup>	93.9		100.1		104.2		48.3	
Daidzin	53.2		52.0		50.8		15.9	
Daidzein	3.2		3.7		0.5		2.3	
Genistin	11.4		16.5		42.0		21.4	
Genistein	0.4		0		0.7		6.0	
Glycitin	23.8		26.3		9.8		2.3	
Glycitein	2.0		1.5		0.4		0.5	
<i>Energy intake<sup>d</sup> (kJ/day)</i>								
Intervention	8726	2304	7626	2244	7869	1946	9126	1801
Placebo	8377	2264	7819	2675	7359	1938	9021	1674
<i>Carbohydrate intake (g/day)</i>								
Intervention	237	62	197	67	217	65	263	57
Placebo	228	60	208	80	203	62	274	55
<i>Fat intake (g/day)</i>								
Intervention	87	32	73	29	73	27	67	13
Placebo	81	32	76	32	68	24	63	12

Abbreviations: BMI, body mass index; DAI, daidzein-rich supplement; GD, genistein/daidzein; GEN, genistein-rich supplement. <sup>a</sup>Age, BMI and body weight are determined at the start of the studies. <sup>b</sup>Number of equol producers as determined at the end of the intervention. <sup>c</sup>Glucosides daidzin, genistin and glycitin calculated as aglycone equivalents. <sup>d</sup>Diet data for GD DAI arm for  $n = 31$ .

macronutrient composition (21 energy% protein, 26 energy% fat and 51 energy% carbohydrates). The soy protein diet contained ~30 gram of soy protein/day, in the form of soy-based meat analogues and soy nuts provided by Alpro (Ghent, Belgium) containing ~48 mg isoflavones (18 mg daidzein, 26 mg genistein and 3 mg glycitein, Table 1). During the 1-week run-in period and the 4-week washout period, participants were not allowed to eat soy foods.

### Background dietary intake

To monitor background dietary intake during the ISO and GD studies, the participants were asked to fill out a semi-quantitative validated food frequency questionnaire consisting of 125 items at the end of each 8-week intervention period.<sup>28</sup>

Background dietary intake in the SOY study was derived from duplicate portions of both experimental diets in which energy, fat, protein, ash and dry matter contents were analysed. Carbohydrate content was calculated by subtracting protein, fat, ash and moisture content from the total sample weight. Mean energy and nutrient intake per participant was calculated from food tables<sup>29</sup> and adjusted for the duplicate diet analysis.

### Sample collection

In the ISO study, plasma samples were collected halfway and at the end of each 8-week intervention period. During the GD study and SOY study, plasma was collected after each intervention period. Fasting venous blood samples were collected into 6 ml EDTA vacutainers (Becton Dickinson (BD), Breda, the Netherlands) and centrifuged for 10 min at 1190 *g* at a temperature of 4 °C to obtain plasma. The samples were stored at -80 °C in aliquots until analysis.

### High-performance liquid chromatography analysis of isoflavones

Isoflavones in plasma, supplements and soy foods were determined after enzymatic deconjugation using reversed-phase high-performance liquid chromatography with electrochemical detection as previously described for the ISO study.<sup>27</sup> For all isoflavones, except glycitein, the limit of detection and limit of quantification were 0.04 and 0.08 µmol/l, respectively. For glycitein, these values were 0.11 and 0.22 µmol/l, respectively. The within-run and between-run variability coefficients of variation (CV in %) were 7.6 and 16.8%, respectively, for daidzein, 7.6 and 23.8% for equol, 14.7 and 30.6% for genistein and 24.2 and 28.0% for glycitein.

### Data analysis

In the ISO study, one outlier was detected for all plasma concentrations (8 s.d. from mean) and excluded from the analysis. The GD study had three dropouts and the SOY study had one dropout. Ten subjects participated in more than one of the studies; these subjects were excluded from the study with most equol producers, resulting in 103 subjects, that is, ISO study *n*=21, GD study DAI arm *n*=32, GEN arm *n*=35 and SOY study *n*=15. Each subject contributed two data points (206 observations), one after the placebo or mixed protein period (unexposed) and one after the intervention period of each trial (exposed). Although these data are paired, they were considered statistically independent because plasma concentrations for unexposed were close to zero and not correlated to the concentration for exposed and there was no overlap in study subjects.

Linear regression analysis was used to explain the total and component-specific concentrations of isoflavones in plasma by intake (in µmol/kg body weight (BW)). Both dose and plasma concentrations (µmol/l) were log<sub>e</sub> transformed and all models were adjusted for study (ISO study, GD study and SOY study). The data were log<sub>e</sub> transformed as this improved the R<sup>2</sup> of the model compared with normal linear regression. For daidzein, equol and total isoflavones, the model also accounted for equol producer status (prod; 1 for producer, 0 for non-producer) and its interaction with intake:

$$\log_e(\text{conc}) = \beta_0 + \beta_1 \times \log_e(\text{dose}) + \beta_2 \times \text{prod} + \beta_3 \times \text{prod} \times \log_e(\text{dose}) + \varepsilon$$

when original values for concentration were 0, 0.5 times the limit of detection was used to enable log<sub>e</sub> transformation of the data, that is, 0.02 for daidzein, equol, genistein and total isoflavones and 0.055 for glycitein. When intake was zero, a dose of 0.01 µmol/kg BW was used. To account for the role of the background diet, a second model was further adjusted for energy intake (continuous in kJ/day), carbohydrate intake (g/day) and fat intake (g/day). Dietary intake data from one participant in the GD DAI arm was missing.

To estimate inter- and intra-individual variation at a high supplement dose, log<sub>e</sub>-transformed plasma isoflavone concentrations after 4 and 8 weeks of isoflavone supplementation were used (ISO study, *n*=29). Variance between subjects and total variance were obtained by the varcomp procedure and the mean square error of the regression model (MSE<sub>model</sub>). Coefficients of variation were obtained as CV<sub>between</sub> (%) = sqrt(exp(Variance<sub>between</sub>)-1) × 100 and analogously for CV<sub>total</sub> and MSE<sub>model</sub>.<sup>30</sup> Differences between plasma concentrations after 4 and 8 weeks of exposure to 94 mg isoflavones/day in the ISO study were tested with a paired *t*-test (*P*-value < 0.05, SAS v9.2, SAS Institute Inc., Cary, NC, USA).

**Table 2.** Mean (±s.d.) plasma isoflavone concentrations in equol producers and non-producers after the interventions

	ISO study <sup>a</sup> (EP <i>n</i> = 21)		GD study DAI (EP <i>n</i> = 8, NP <i>n</i> = 24)		GD study GEN (EP <i>n</i> = 9, NP <i>n</i> = 26)		SOY study (EP <i>n</i> = 4, NP <i>n</i> = 11)	
	Mean (µmol/l)	s.d.	Mean (µmol/l)	s.d.	Mean (µmol/l)	s.d.	Mean (µmol/l)	s.d.
Daidzein								
EP	1.75	0.79	1.22	1.06	1.07	0.56	0.60	0.40
NP	NA	—	1.53	0.64	1.27	0.69	0.43	0.19
Equol								
EP	1.20	0.52	0.72	0.58	0.61	0.28	0.39	0.15
NP	NA	—	NA	—	NA	—	NA	—
Genistein								
EP	0.69	0.52	0.40	0.30	1.19	0.95	1.16	1.08
NP	NA	—	0.43	0.33	1.33	0.90	0.87	0.64
Glycitein								
EP	0.44	0.40	0.19	0.17	0.02	0.05	0.00	0.00
NP	NA	—	0.15	0.12	0.06	0.08	0.00	0.00
Total <sup>b</sup>								
EP	4.08	1.55	2.53	1.25	2.90	1.48	2.16	1.48
NP	NA	—	2.11	0.84	2.65	1.49	1.30	0.80

Abbreviations: DAI, daidzein-rich supplement; EP, equol producer; GD, genistein/daidzein; GEN, genistein-rich supplement; NA, not applicable; NP, non-producer. <sup>a</sup>The ISO and GD studies provided ~100 mg isoflavones/day (aglycone equivalents) as supplements for 8 weeks, the SOY study provided ~48 mg/day as soy protein diet for 4 weeks. <sup>b</sup>Total isoflavones are the sum of daidzein, equol, genistein and glycitein.

**Table 3.** Outcomes of the linear regression model on log<sub>e</sub>-transformed intake (μmol/kg BW) and log<sub>e</sub>-transformed plasma concentrations (μmol/l)<sup>a</sup>

	Non-producers <sup>b</sup>					Producers <sup>c</sup>					R <sup>b</sup>
	β <sub>0</sub>	95% CI	β <sub>1</sub> × dose	95% CI	% Change at doubling	β <sub>0</sub> +β <sub>2</sub>	95% CI	(β <sub>1</sub> +β <sub>3</sub> ) × dose	95% CI	% Change at doubling	
Daidzein	-0.54	-0.70, -0.38	0.66	0.61, 0.71	58	-0.49	-0.74, -0.24	0.64	0.59, 0.70	56	0.87
Equol	-3.92	-3.98, -3.85	0.00	-0.02, 0.02	0	-1.12	-1.23, -1.02	0.63	0.61, 0.66	55	0.96
Genistein	-0.66	-0.78, -0.54	0.70	0.67, 0.74	62	—	—	—	—	—	0.90
Glycitein	-2.14	-2.26, -2.01	0.20	0.17, 0.24	15	—	—	—	—	—	0.48
Total	-0.39	-0.55, -0.23	0.67	0.63, 0.72	59	-0.06	-0.31, 0.18	0.74	0.68, 0.79	67	0.90

Abbreviations: BW, body weight; CI, confidence interval. <sup>a</sup>The log-linear regression model: log<sub>e</sub>(conc) = β<sub>0</sub>+β<sub>1</sub> × log<sub>e</sub>(dose)+β<sub>2</sub> × prod+β<sub>3</sub> × prod × log<sub>e</sub>(dose)+ε. <sup>b</sup>The model was adjusted for study (ISO, GD or SOY). <sup>c</sup>The model was adjusted for study (ISO, GD or SOY), equol producer status (β<sub>2</sub>) and log<sub>e</sub>-transformed intake–producer status interaction (β<sub>3</sub>).

## RESULTS

In the studies with isoflavone supplements (~100 mg/day), the mean total isoflavone concentration after 8 weeks exposure to isoflavones (daidzein, equol, genistein and glycitein) was 3.47 μmol/l for equol producers (*n*=38) and 2.39 μmol/l for non-producers (*n*=50). After the 4-week intervention with soy protein (~48 mg isoflavones/day), the mean total isoflavone concentration was 2.16 μmol/l for equol producers (*n*=4) and 1.30 μmol/l for non-producers (*n*=11, Table 2). For all studies at the end of the placebo or mixed protein period, 86% of the measured daidzein concentrations were below the quantification limit of the method; this was respectively 99, 96 and 100% for equol, genistein and glycitein.

Significant linear associations between natural logarithm (log<sub>e</sub>) of plasma concentration and log<sub>e</sub> of intake per kg BW (dose) were observed in non-producers for daidzein (β<sub>1</sub>=0.66), genistein (β<sub>1</sub>=0.70), glycitein (β<sub>1</sub>=0.20) and total isoflavones (β<sub>1</sub>=0.67, Table 3). In these non-producers, the regression coefficient of equol concentration on daidzein intake was β<sub>1</sub>+β<sub>3</sub>=0.0, whereas in equol producers this was β<sub>1</sub>+β<sub>3</sub>=0.63 (data from literature). Furthermore, for equol producers, the association between plasma daidzein and intake was β<sub>1</sub>+β<sub>3</sub>=0.64 and for total isoflavones it was β<sub>1</sub>+β<sub>3</sub>=0.74. This linear model on the log<sub>e</sub>–log<sub>e</sub> scale can be interpreted on a normal scale as concentration = e<sup>β<sub>0</sub>+β<sub>2</sub></sup> × dose<sup>β<sub>1</sub>+β<sub>3</sub></sup>, with β<sub>0</sub> (intercept) and β<sub>1</sub> (dose, Figure 1). β<sub>2</sub> (producer status) and β<sub>3</sub> (intake–producer status interaction) were only relevant for equol producers for daidzein, equol and total isoflavone plasma concentrations. When isoflavone intake doubles from 1.5 to 3 μmol/kg BW, the equation is as follows (1.5/3.0)<sup>β<sub>1</sub></sup> = (2)<sup>0.67</sup> = 1.59-fold or 59%. So, total isoflavone plasma concentrations in non-producers increase by 59% when their intake doubles.

Except for glycitein (R<sup>2</sup>=0.48), the explained variances of the models were higher than 0.87.

Inclusion of energy (kJ/day), carbohydrate and fat intake (g/day) in the model as background dietary intake did not change the associations (data not shown).

Figure 2 shows that the relation between intake and plasma concentration, calculated as the concentration divided by the dose, for soy foods is not different than that for isoflavone supplements.

Among the equol-producing women in the ISO study, plasma concentrations of the individual isoflavones after 4 and 8 weeks of exposure were similar (results not shown). In this study, within-person CV is smaller than between-person CV for all individual and total isoflavones, resulting in a large intraclass correlation coefficient (Table 4). The CVs between persons were 30–45% for all isoflavones, except for genistein (96%). The CV for MSE<sub>model</sub>, comprising the inter- and intra-individual variation, was 31% for equol and ranged from 59 to 87% for the other isoflavones.

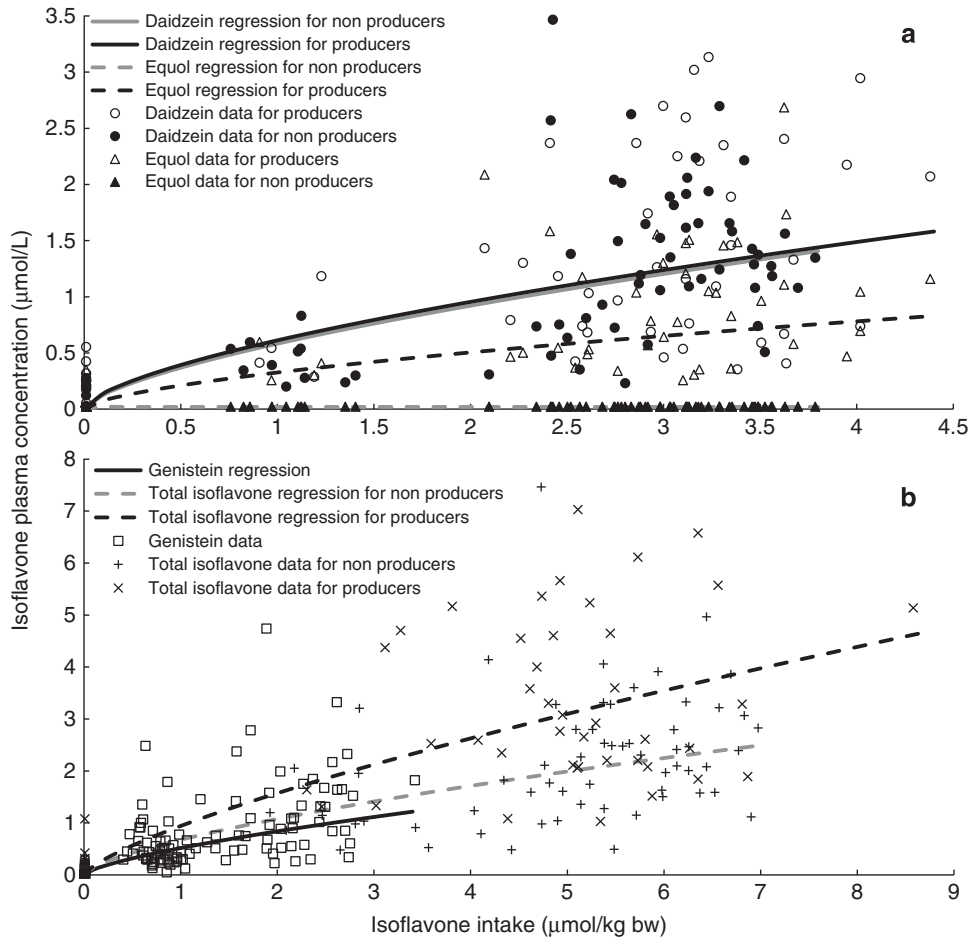
## DISCUSSION

In this study, plasma isoflavone concentrations from 103 postmenopausal women participating in three intervention studies were evaluated after exposure to either isoflavone supplements or soy foods. A log-linear regression model showed that over a range of total isoflavone intakes from 0 to 100 mg/day, doubling of the dose (per kg BW) increased plasma concentrations from 55 to 62% (± s.e. 1–2%) for daidzein, genistein, equol (only in producers) and total isoflavones (R<sup>2</sup>>0.87). For glycitein, with an intake range of 0–28 mg/day, the observed increase was much smaller (15 ± 1%, R<sup>2</sup>=0.47). Including background dietary intake in the model did not affect the associations. Both visual inspection of the regression model and quantification with ISO study data indicated large inter-individual variation of isoflavone plasma concentrations at this intake range.

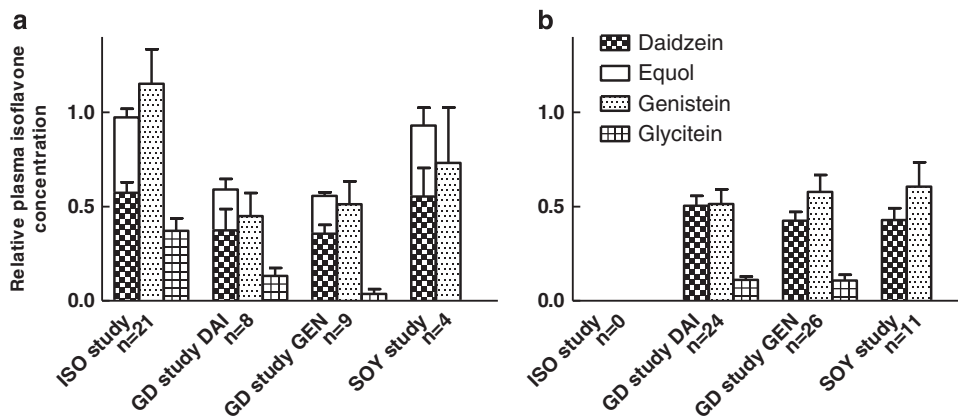
We studied the isoflavone dose–concentration relation among 103 postmenopausal women, which is a relatively large sample size compared with other studies (*n*=39–76).<sup>19,20</sup> The percentage of equol producers in the studies (23–27%) was similar to that in the data from literature.<sup>12</sup> Results from three intervention studies are presented, covering isoflavone intake from 0 to 100 mg/day. The three studies, having small differences in duration and dose, are comparable because they were conducted in postmenopausal women using similar determination of isoflavones with regard to lab, methods and reference materials. We demonstrated that data from 4 and 8 weeks of exposure could be combined in the repeated measurements of the ISO study, also supported by calculations from literature,<sup>31</sup> and that food matrix did not influence the comparability of the studies.

The outcomes of our model suggested that production of equol did not affect daidzein plasma concentrations, as these concentrations increased equally for producers and non-producers (56 and 58%, respectively) on doubling the daidzein intake (Table 3). Equol plasma concentrations, only relevant for producers, did depend on daidzein intake as doubling intake increased plasma equol concentration by 55%. Together, this resulted in higher increases in total plasma isoflavone concentrations in equol producers (67%) than in non-producers (59%) on doubling daidzein intake. Previous literature is not consistent whether equol is produced at the expense of daidzein plasma concentrations,<sup>32–34</sup> which might partly be explained by differences in the pharmacokinetics of daidzein and equol.<sup>35</sup>

Our model was limited to daidzein, equol, genistein and glycitein, because other isoflavones or metabolites, like the gut metabolite O-desmethylangolensin, were not measured in these studies. In the model, we assumed that the ability to produce equol would influence daidzein, equol and total isoflavone concentrations and that a linear relation between the natural logarithms of intake and plasma concentrations existed. By using log<sub>e</sub> transformations of both intake and concentration,



**Figure 1.** Exponential regression lines for the association between isoflavone intake (μmol/kg BW) and plasma concentration (μmol/l) combined with individual data. Isoflavone intake and plasma concentration on the x and y axes refer to specific isoflavones; (a) shows daidzein intake with daidzein plasma concentration and daidzein intake with plasma equol concentration; (b) shows genistein intake with genistein concentration and total isoflavone intake with total isoflavone concentration.



**Figure 2.** Relative plasma isoflavone concentrations of equol producers (a) and non-producers (b) in the three studies. Relative concentrations were calculated by dividing plasma concentrations in μmol/l by intake in μmol/kg BW. The bar for equol concentration is placed on top of the bar for daidzein concentration.

homogeneity of the variance of error terms was allowed for while the back-transformed curve flattened down at higher doses. Without these  $\log_e$  transformations and inclusion of the unexposed data, the model fit would be less (data not shown). The high explained variances of the models ( $R^2 > 0.87$ ) indicated a good model fit, except for glycitein (Table 3), although plasma

isoflavone concentrations varied substantially between individuals (Figure 1). This variation proved to be large when quantified by CV and intraclass correlation coefficient with ISO study data and the  $MSE_{\text{model}}$  for the regression model (Table 4). Differences between these measures of variance can be explained by the number of studies and therefore supplements included. In previous studies

**Table 4.** Between-person and total variance, coefficient of variation (%) and ICC of isoflavone concentrations 4 and 8 weeks after supplementation with 94 mg isoflavones (aglycone equivalents) in the ISO study in 29 equol-producing postmenopausal women

	Var <sub>between</sub>		Var <sub>total</sub>		ICC	MSE model <sup>a</sup>	
	CV <sub>between</sub> <sup>b</sup>	%	CV <sub>total</sub>	%		CV	%
Daidzein	0.18	44	0.27	56	0.67	0.52	83
Equol	0.18	45	0.27	56	0.67	0.09	31
Genistein	0.65	96	0.81	112	0.80	0.32	61
Glycitein	0.19	46	0.56	87	0.34	0.30	59
Total	0.09	30	0.14	39	0.64	0.56	87

Abbreviations: CV, coefficient of variation; ICC, intraclass correlation coefficient; MSE, mean square error; Var, variance. <sup>a</sup>For comparison of the coefficients of the ISO study, the MSE of the linear regression model is shown. <sup>b</sup>Coefficient of variation between persons from log<sub>e</sub>-transformed concentration data calculated as the square root of the exponent of the variance minus 1.

with isoflavone supplements, inter-individual variation in plasma isoflavone concentrations was quantified as 162–1596-fold for the individual isoflavones.<sup>19,20</sup> Inter-individual variation might be caused by differences in uptake efficiency and metabolism, which in turn might be influenced by microbiota<sup>10</sup> or genetic variation in transporter genes.<sup>36</sup> Other studies hypothesized that fat, carbohydrate and fibre intake could explain inter-individual variation.<sup>37,38</sup> Therefore, we complemented our model with energy, fat and carbohydrate intake data. Fibre intake data could not be extracted from the food frequency questionnaire used. This adjustment did not change the observed associations and suggests that background dietary intake did not explain the observed inter-individual variation. However, the relatively high CV% of the method might have influenced the observed inter-individual variation to a limited extent.

Postmenopausal women produce little endogenous estradiol and are therefore considered susceptible to the potentially beneficial or even adverse health effects of isoflavones when using these supplements. Our study confirmed that supplement intake by postmenopausal women led to high concentrations of circulating isoflavones compared with soy food intake in Japanese populations.<sup>15</sup> For risk assessment purposes, plasma isoflavone concentrations from 103 postmenopausal women in this study could be explained by isoflavone intake and equol producer status at a relevant intake range of 0–100 mg isoflavones/day.

Despite the adequate explanation of plasma isoflavone concentrations over this intake range, the large inter-individual variation will restrict the use of this model for future risk assessment to the population level and cannot be applied to predict plasma concentrations in individuals.

#### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

#### ACKNOWLEDGEMENTS

We would like to thank Dini Venema for her excellent technical assistance with the HPLC analysis and Els Siebelink, Corine Perenboom, Saskia Meyboom and Karin Borgonjen for providing the FFQ data. Furthermore, thanks to Springfield Nutraceuticals and Alpro for providing the supplements and soy foods. The ISO and ISO II (GD) studies were funded by the Dutch Food Safety Authority (nVWA), and the SOY study was funded by the Alpro Foundation.

#### REFERENCES

- Bolano R, Del Castillo A, Francia J. Soy isoflavones versus placebo in the treatment of climacteric vasomotor symptoms: systematic review and meta-analysis. *Menopause* 2010; **17**: 660–666.
- Taku K, Melby MK, Kronenberg F, Kurzer MS, Messina M. Extracted or synthesized soybean isoflavones reduce menopausal hot flash frequency and severity: systematic review and meta-analysis of randomized controlled trials. *Menopause* 2012; **19**: 776–790.
- Messina M. Insights gained from 20 years of soy research. *J Nutr* 2010; **140**: 2289S–2295S.
- Taku K, Melby MK, Nishi N, Omori T, Kurzer MS. Soy isoflavones for osteoporosis: an evidence-based approach. *Maturitas* 2011; **70**: 333–338.
- Beavers DP, Beavers KM, Miller M, Stamey J, Messina MJ. Exposure to isoflavone-containing soy products and endothelial function: a Bayesian meta-analysis of randomized controlled trials. *Nutr Metab Cardiovasc Dis* 2012; **22**: 182–191.
- Wuttke W, Jarry H, Seidlova-Wuttke D. Isoflavones—safe food additives or dangerous drugs? *Ageing Res Rev* 2007; **6**: 150–188.
- Andres S, Abraham K, Appel KE, Lampen A. Risks and benefits of dietary isoflavones for cancer. *Crit Rev Toxicol* 2011; **41**: 463–506.
- Messina M, Redmond G. Effects of soy protein and soybean isoflavones on thyroid function in healthy adults and hypothyroid patients: a review of the relevant literature. *Thyroid* 2006; **16**: 249–258.
- Yuan JP, Liu YB, Peng J, Wang JH, Liu X. Changes of isoflavone profile in the hypocotyls and cotyledons of soybeans during dry heating and germination. *J Agric Food Chem* 2009; **57**: 9002–9010.
- Nielsen IL, Williamson G. Review of the factors affecting bioavailability of soy isoflavones in humans. *Nutr Cancer* 2007; **57**: 1–10.
- Manach C, Williamson G, Morand C, Scalbert A, Remesy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* 2005; **81**: 230S–242S.
- Setchell KD, Clerici C. Equol: history, chemistry, and formation. *J Nutr* 2010; **140**: 1355S–1362S.
- Zamora-Ros R, Knaze V, Lujan-Barroso L, Kuhnle GG, Mulligan AA, Touillaud M *et al*. Dietary intakes and food sources of phytoestrogens in the European Prospective Investigation into Cancer and Nutrition (EPIC) 24-hour dietary recall cohort. *Eur J Clin Nutr* 2012; **66**: 932–941.
- Peeters PH, Slimani N, van der Schouw YT, Grace PB, Navarro C, Tjonneland A *et al*. Variations in plasma phytoestrogen concentrations in European adults. *J Nutr* 2007; **137**: 1294–1300.
- Yamamoto S, Sobue T, Sasaki S, Kobayashi M, Arai Y, Uehara M *et al*. Validity and reproducibility of a self-administered food-frequency questionnaire to assess isoflavone intake in a Japanese population in comparison with dietary records and blood and urine isoflavones. *J Nutr* 2001; **131**: 2741–2747.
- Boniglia C, Carratu B, Gargiulo R, Giammaroli S, Mosca M, Sanzini E. Content of phytoestrogens in soy-based dietary supplements. *Food Chem* 2009; **115**: 1389–1392.
- Sturtz M, Lander V, Schmid W, Winterhalter P. Quantitative determination of isoflavones in soy based nutritional supplements by high-performance liquid chromatography. *J Consum Protect Food Safety* 2008; **3**: 127–136.
- Boucher BA, Cotterchio M, Curca IA, Kreiger N, Harris SA, Kirsh VA *et al*. Intake of phytoestrogen foods and supplements among women recently diagnosed with breast cancer in Ontario, Canada. *Nutr Cancer* 2012; **64**: 695–703.
- Wiseman H, Casey K, Bowey EA, Duffy R, Davies M, Rowland IR *et al*. Influence of 10 wk of soy consumption on plasma concentrations and excretion of isoflavonoids and on gut microflora metabolism in healthy adults. *Am J Clin Nutr* 2004; **80**: 692–699.
- Mathey J, Lamothe V, Coxam V, Potier M, Sauvart P, Bennetau-Pelissero C. Concentrations of isoflavones in plasma and urine of post-menopausal women chronically ingesting high quantities of soy isoflavones. *J Pharm Biomed Anal* 2006; **41**: 957–965.
- Ritchie MR, Morton MS, Deighton N, Blake A, Cummings JH. Plasma and urinary phyto-oestrogens as biomarkers of intake: validation by duplicate diet analysis. *Br J Nutr* 2004; **91**: 447–457.
- Bhakta D, Higgins CD, Sevak L, Mangtani P, Adlercreutz H, McMichael AJ *et al*. Phyto-oestrogen intake and plasma concentrations in South Asian and native British women resident in England. *Br J Nutr* 2006; **95**: 1150–1158.
- Franke AA, Hebshi SM, Pagano I, Kono N, Mack WJ, Hodis HN. Urine accurately reflects circulating isoflavonoids and ascertains compliance during soy intervention. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 1775–1783.
- Setchell KD, Brown NM, Desai PB, Zimmer-Nechimias L, Wolfe B, Jakate AS *et al*. Bioavailability, disposition, and dose-response effects of soy isoflavones when consumed by healthy women at physiologically typical dietary intakes. *J Nutr* 2003; **133**: 1027–1035.

- 25 Setchell KD, Faughnan MS, Avades T, Zimmer-Nechemias L, Brown NM, Wolfe BE *et al*. Comparing the pharmacokinetics of daidzein and genistein with the use of <sup>13</sup>C-labeled tracers in premenopausal women. *Am J Clin Nutr* 2003; **77**: 411–419.
- 26 Shinkaruk S, Durand M, Lamothe V, Carpaye A, Martinet A, Chantre P *et al*. Bioavailability of glycitein relatively to other soy isoflavones in healthy young Caucasian men. *Food Chem* 2012; **135**: 1104–1111.
- 27 van der Velpen V, Geelen A, Schouten EG, Hollman PC, Afman LA, van 't Veer P. Estrogen receptor-mediated effects of isoflavone supplementation were not observed in whole-genome gene expression profiles of peripheral blood mononuclear cells in postmenopausal, equol-producing women. *J Nutr* 2013; **143**: 774–780.
- 28 Siebelink E, Geelen A, de Vries JH. Self-reported energy intake by FFQ compared with actual energy intake to maintain body weight in 516 adults. *Br J Nutr* 2011; **106**: 274–281.
- 29 NEVO-tabel. Dutch Food Composition Table. RIVM: Bilthoven, The Netherlands 2011.
- 30 Souverein OW, Dullemeijer C, van't Veer P, van der Voet H. Transformations of summary statistics as input in meta-analysis for linear dose-response models on a logarithmic scale: a methodology developed within EURRECA. *BMC Med Res Methodol* 2012; **12**: 57.
- 31 Setchell KD, Brown NM, Desai P, Zimmer-Nechemias L, Wolfe BE, Brashear WT *et al*. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J Nutr* 2001; **131**: 1362S–1375S.
- 32 Vergne S, Titier K, Bernard V, Asselineau J, Durand M, Lamothe V *et al*. Bioavailability and urinary excretion of isoflavones in humans: effects of soy-based supplements formulation and equol production. *J Pharm Biomed Anal* 2007; **43**: 1488–1494.
- 33 Karr SC, Lampe JW, Hutchins AM, Slavin JL. Urinary isoflavonoid excretion in humans is dose dependent at low to moderate levels of soy-protein consumption. *Am J Clin Nutr* 1997; **66**: 46–51.
- 34 Lampe JW, Karr SC, Hutchins AM, Slavin JL. Urinary equol excretion with a soy challenge: influence of habitual diet. *Proc Soc Exp Biol Med* 1998; **217**: 335–339.
- 35 de Pascual-Teresa S, Hallund J, Talbot D, Schroot J, Williams CM, Bugel S *et al*. Absorption of isoflavones in humans: effects of food matrix and processing. *J Nutr Biochem* 2006; **17**: 257–264.
- 36 Wakeling LA, Ford D. Polymorphisms in genes involved in the metabolism and transport of soy isoflavones affect the urinary metabolite profile in premenopausal women following consumption of a commercial soy supplement as a single bolus dose. *Mol Nutr Food Res* 2012; **56**: 1794–1802.
- 37 Lampe JW, Gustafson DR, Hutchins AM, Martini MC, Li S, Wahala K *et al*. Urinary isoflavonoid and lignan excretion on a Western diet: relation to soy, vegetable, and fruit intake. *Cancer Epidemiol Biomarkers Prev* 1999; **8**: 699–707.
- 38 Rowland IR, Wiseman H, Sanders TA, Adlercreutz H, Bowey EA. Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. *Nutr Cancer* 2000; **36**: 27–32.



This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/3.0/>