No Effect of Red Clover–Derived Isoflavone Intervention on the Insulin-Like Growth Factor System in Women at Increased Risk of Colorectal Cancer

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Abstract

Background: **Increased insulin-like growth factor (IGF)-I and IGF-II concentrations are related to increased colorectal cancer risk. Isoflavones have been associated with reduced colorectal cancer risk, and may affect the IGF system because of their weak estrogenic activity. The aim of the study was to investigate the effect of isolated isoflavones on serum concentrations of IGF system components.**

Materials and Methods: **We conducted a randomized, placebo-controlled, double-blinded, crossover trial in four hospitals in the Netherlands to investigate the effect of an 8-week supplementation with red clover–derived isoflavones (84 mg/d) on serum IGF-I concentrations. In addition, serum concentrations of IGF-II and IGF binding proteins (IGFBP)-1, IGFBP-2, and IGFBP-3 were assessed. Normal colorectal tissue biopsies were obtained after the first intervention period and mRNA expression of *IGF-I*, *IGF-II*, *IGFBP-3*, and *IGF-IR* was evaluated. Our study population consisted of 34 postmenopausal women with a family history of colorectal cancer or a personal history of colorectal adenomas.**

Results: **Isoflavone supplementation did not significantly affect serum concentrations of total IGF-I (mean relative within-person difference; IGF-I, −2.0%; 95% confidence interval, −8.0% to 3.9%). IGF-II and IGFBPs were also not significantly altered after isoflavone supplementation. Colorectal tissue mRNA expression of *IGF* system components did not significantly differ between individuals on isoflavone supplementation and those who received placebo.**

Conclusions: **The results of our trial, supported by a qualitative review of soy trials published to date, suggest that isoflavones do not significantly affect circulating levels of IGF system components. Increased levels of IGF-I, as observed in most of these trials, are likely due to simultaneous protein supplementation.** (Cancer Epidemiol Biomarkers Prev 2008;17(10):2585–93)

Introduction

Evidence is accumulating for a protective effect of estrogenic substances against colorectal cancer (1). In line with these observations, the incidence of colorectal cancer is consistently lower in women than in men. Sex hormone replacement therapy in postmenopausal women has been shown to decrease colorectal cancer risk as well as the development of colorectal adenomas (2-4). Both sex hormone replacement therapy (5, 6) and selective estrogen receptor modulators, e.g., tamoxifen (7, 8), have been shown to reduce serum insulin-like growth factor-I (IGF-I) concentrations.

IGF-I and IGF-II are involved in cell proliferation and apoptosis, and are important in both normal and tumor growth (9). Prospective epidemiologic studies have shown that relatively high circulating concentrations of IGF-I and IGF-II are associated with increased colorectal cancer risk (10). IGF binding protein-3 (IGFBP-3), which binds 90% of circulating IGF-I in humans, is not related to risk of colorectal cancer (10, 11). However, relatively low concentrations of IGFBP-1 and IGFBP-2, which may enhance IGF bioavailability, might be associated with increased colorectal cancer risk (12, 13). Experimental evidence from mouse models has supported the rationale for cancer prevention through lowering the circulating levels of (bioavailable) IGF-I (14).

The lower incidence of several cancers including colorectal cancer in Asian countries has been attributed to the substantially higher consumption of soy foods in these countries (15, 16). Isoflavones, the main bioactive substances in soy, are a class of phytoestrogens that structurally resemble estrogens and also possess weak estrogenic activity (17). Isoflavones have been hypothesized to influence colorectal cancer risk, although data reported on this subject have thus far been inconclusive (18). *In vitro* and *in vivo* animal studies have shown that (soy) isoflavones may decrease (circulating) IGF-I concentrations (19). For humans, however, the results from

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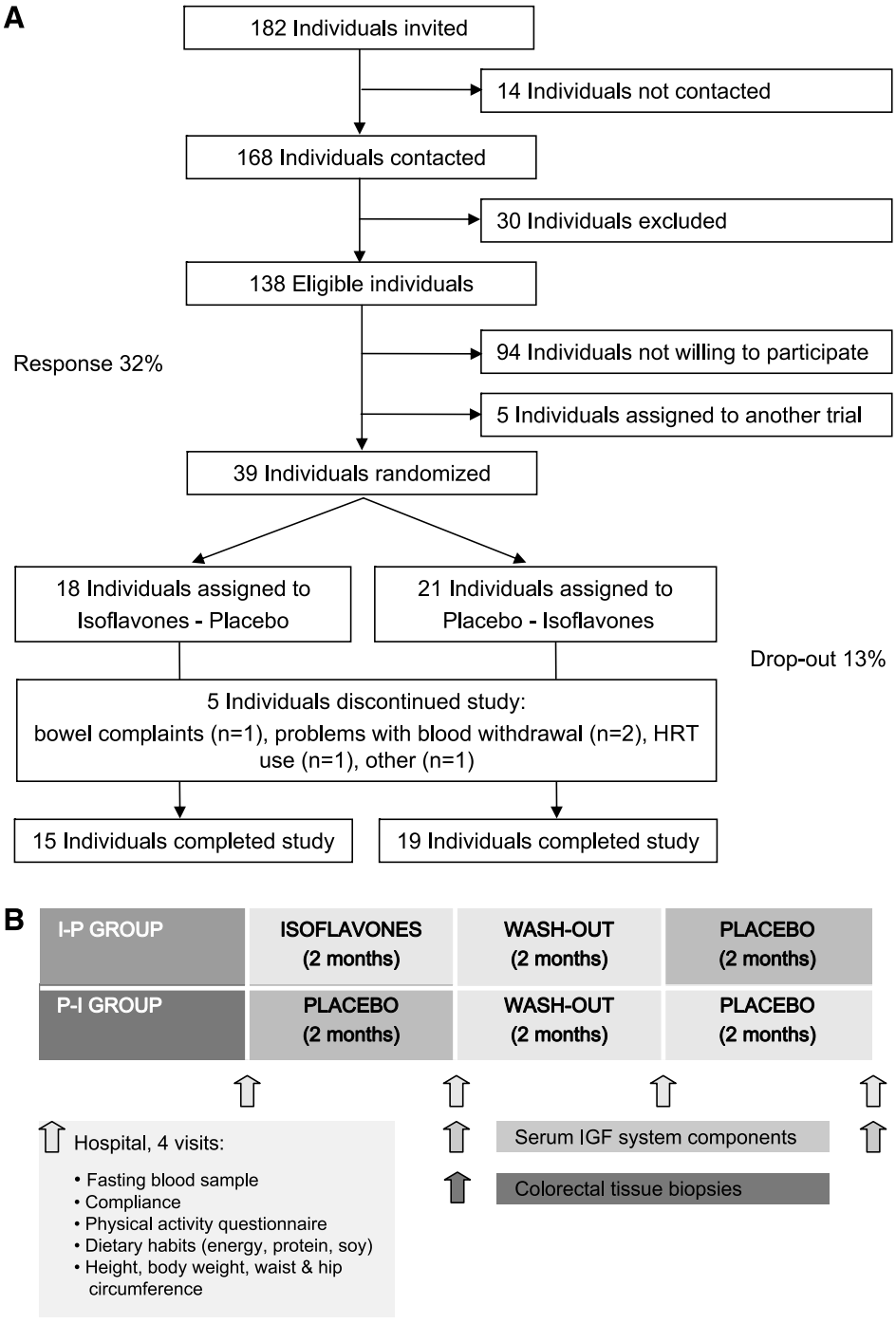

Figure 1. Flow chart of study recruitment and follow-up (**A**), and study scheme of crossover design (**B**).

various intervention studies are conflicting, possibly due to opposite effects of soy protein and soy isoflavones on serum total IGF-I concentrations (14).

We hypothesized that isolated isoflavones, through their estrogenic properties, may induce a reduction of (bioavailable) IGF-I in the circulation in postmenopausal women. Such endocrine changes may be accompanied by changes in the expression of *IGF* system components in normal colorectal tissue. In this randomized, controlled, crossover trial, we investigated the effect of isolated, red clover–derived isoflavone supplementation (84 mg/d) for 2 months on serum concentrations of IGF-I, IGF-II, and IGFBP-1, IGFBP-2, and IGFBP-3, as well as tissue mRNA expression of several components of the *IGF* system in postmenopausal women at increased risk of colorectal cancer that could potentially benefit most from this intervention.

Table 1. Primers and probes for real-time PCR

Gene	Exon	GenBank accession no.	Primer/probe*	Sequence
IGF-I	1, 2	NM_000618	F	5′-AGCAGTCTTCCAACCCAATTATTTA-3′
			R	5′-AGATGCGAGGAGGACATGGT-3′
			Probe	5′-TCTTCACCTTCAAGAAATCACAAAAGCAGCA-3′
IGF-II	8, 9	NM_000612	F	5′-CCGTGCTTCCGGACAACTT-3′
			R	5′-GGACTGCTTCCAGGTGTCATATT-3′
			Probe	5′-CCCAGATACCCCGTGGGCAAGTTCT-3′
IGFBP-3	2, 3	BC018962	F	5′-AGAGCACAGATACCCAGAACTTCTC-3′
			R	5′-ATTGAGGAACTTCAGGTGATTCAGT-3′
			Probe	5′-CATTTCTCTACGGCAGGGACCATATTCTGTCT-3′
IGF-IR	8, 9	X04434	F	5′-AAGGCTGTGACCCTCACCAT-3′
			R	5′-CGATGCTGAAAGAACGTCCAA-3′
			Probe	5′-TTCGCACCAATGCTTCAGTTCCTTCC-3′

*F, forward primer; R, reverse primer.

Materials and Methods

Study Population. We selected women aged 50 to 75 years with a personal history of colorectal adenomas or at least one first-degree family member with a history of colorectal cancer. All women were postmenopausal, i.e., no menstrual cycles in the past 12 months. In case of hysterectomy, postmenopausal status was confirmed on the basis of serum follicle-stimulating hormone levels. Asymptomatic women scheduled to undergo a colonoscopy for surveillance purposes were selected from the medical registries and pathology databases, and were sent an invitation letter for participation in our study. Exclusion criteria were a history of cancer, familial adenomatous polyposis syndrome, Li-Fraumeni syndrome, chronic inflammatory bowel disease, diabetes mellitus, acromegaly, significant liver or renal disease, (partial) bowel resection, nonremissive celiac disease, diverticulitis, other severe comorbidity, laxative abuse, and the use of food supplements containing isoflavones.

Participants were recruited between November 2003 and December 2005. In total, 182 women were invited to participate in this trial (Fig. 1A). Of 138 eligible women, 39 were included in the present trial and 5 were assigned to another trial (32% response). We obtained written informed consent from all participants.

The study was conducted in four hospitals in the Netherlands: the Antoni van Leeuwenhoek Hospital in Amsterdam, the Gelderse Vallei Hospital in Ede, the Slotervaart Hospital in Amsterdam, and the Sint Antonius Hospital in Nieuwegein. The study protocol was approved by the medical ethical committees of all participating centers.

Design. We conducted a randomized, placebo-controlled, double-blinded crossover study (Fig. 1B). The total duration of the study was approximately 6 months, consisting of two 8-week intervention periods, separated by an 8-week wash-out period. Surveillance colonoscopies were always planned at the end of the first intervention period. Subjects were allocated to receive isoflavone tablets in the first intervention period and placebo tablets in the second intervention period (isoflavones-placebo, the I-P group) or vice versa (placebo-isoflavones, the P-I group), according to a randomization scheme with permuted blocks. The

Table 2. General characteristics (mean ± SD) of the study population for the isoflavones-placebo (I-P) and placebo-isoflavones (P-I) groups

	I-P group (n = 15)	P-I group (n = 19)
Age (y)	59.3 ± 4.3	58.9 ± 7.2
Weight (kg)	71.4 ± 9.1	73.2 ± 12.9
BMI (kg/m^2)	25.6 ± 3.2	26.7 ± 4.3
Waist circumference (cm)	87.2 ± 9.9	90.3 ± 11.1
Smoking, n (%)		
Yes	5 (33%)	3 (16%)
Past	9 (60%)	8 (42%)
Never	1 (7%)	8 (42%)
Colorectal cancer risk factor, n (%)		
Family history and adenomas	5 (33%)	3 (16%)
Family history only	3 (20%)	7 (37%)
Adenomas only	7 (47%)	9 (47%)
Age at menopause (y)	49.7 ± 5.3	47.9 ± 3.5
Parity, n (%)		
Yes	12 (80%)	13 (68%)
No	3 (20%)	6 (32%)
Hormone replacement therapy, n (%)*		
Never	11 (73%)	10 (56%)
Past	4 (27%)	8 (44%)

*Percentage over valid values (missing, n = 1).

Table 3. Differences in serum concentrations of IGF system components after isoflavone supplementation and after placebo

	Concentration after isoflavones	Concentration after placebo	Within-person difference	
			Absolute	Relative (%)
	Mean ± SD	Mean ± SD	Mean	Mean (95% confidence interval)
Total IGF-I (μg/L)	121 ± 45	123 ± 39	−3	−2.0 (−8.0 to 3.9)
Total IGF-II (μg/L)	546 ± 92	559 ± 98	−16	−1.7 (−6.3 to 2.8)
IGFBP-1 (μg/L)*	45 (2-206)	42 (2-121)	−2	−6.2 (−55.3 to 505.9)
IGFBP-2 (μg/L)	234 ± 137	223 ± 108	19	11.0 (−0.1 to 22.1)
IGFBP-3 (mg/L)	1.96 ± 0.30	2.01 ± 0.30	−0.04	−1.8 (−4.5 to 0.9)
Estradiol (pmol/L)*	42 (22-155)	42 (22-95)	1	2.7 (−53.3 to 344.2)
SHBG (nmol/L)	47 ± 17	47 ± 18	0	2.0 (−3.6 to 7.6)

*Median and range, as IGFBP-1 and estradiol levels were not normally distributed.

isoflavone tablets (Promensil, Novogen) contained an isoflavone extract derived from red clover with 42 mg of total isoflavones (25 mg biochanin, 8 mg formononetin, 4 mg genistein, and 5 mg daidzein). Subjects were asked to take two tablets per day, one with breakfast and one with dinner (total dose, 84 mg isoflavones/d). Subjects were asked to maintain their habitual lifestyle and diet, including non–isoflavone supplement use.

The sample size calculation for this randomized controlled trial was based on a pilot study in six individuals in which we observed a within-person coefficient of variation of 11% in total IGF-I concentrations in multiple serum samples drawn 3 to 12 months apart. This resulted in a required sample size of 26 participants to detect a 10% difference in serum total IGF-I concentrations between the treatment groups with 90% power.

Data and Sample Collection. Study procedures and data collection were identical to the male counterpart of this study described elsewhere (20). In summary, subjects visited the hospital at the beginning and end of both intervention periods, when body weight, and waist and hip circumference were measured. Dietary intake on the day preceding the visit was assessed using a 24-h recall, according to a standard protocol for interviewing and coding. Habitual physical activity over the 2 months preceding each visit was assessed using a validated self-administered short questionnaire. Fasting blood samples were obtained at all four time points. Fasting serum and EDTA-plasma samples were frozen and stored at −30°C until further analysis.

A surveillance colonoscopy was scheduled at the second visit, i.e., at the end of the first intervention period, after whole-gut lavage with 4 L of macrogol [Klean-Prep (Norgine BV) or Coloforte (Ipsen Farmaceutica BV)]. Biopsies from macroscopically normal mucosa were collected from the ascending colon and the rectum, and were immediately snap-frozen in liquid nitrogen and stored at −70°C until further analysis.

During both intervention periods, subjects kept a daily notebook in which they recorded information about their health, medicine use, smoking, study tablets taken, and consumption of foods rich in isoflavones. Compliance was measured by evaluating the participants' own records and by counting the number of returned tablets.

Serum Analyses. Serum total IGF-I, total IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 concentrations were measured at the end of both intervention periods. Serum total IGF-I was measured using an immunometric technique on the Immulite 1000 analyzer (Diagnostics Products Corporation). The sensitivity established in our laboratory was 12.0 μg/L, intra-assay coefficients of variation were <4.0% at 45, 150, and 370 μg/L mean serum IGF-I, and inter-assay coefficients of variation were 7.0%, 6.5%, and 7.0% at 45, 150, and 370 μg/L mean serum IGF-I, respectively. Serum IGF-II concentrations were determined in Sep-Pak C18 extracts of serum by RIA, and serum IGFBP-1, IGFBP-2, and IGFBP-3 were

Table 4. Normal colorectal tissue mRNA expression of *IGF* system components after isoflavone or placebo supplementation (in arbitrary units)

	Isoflavones (n = 15)			Placebo (n = 19)			P*
	Mean ± SD	Median	Range	Mean ± SD	Median	Range	
Ascending colon							
IGF-I	0.40 ± 0.24	0.35	0.17-1.03	0.32 ± 0.20	0.33	0.09-0.84	0.23
IGF-II	0.14 ± 0.08	0.14	0.03-0.34	0.16 ± 0.11	0.14	0.04-0.45	0.98
IGFBP-3	0.29 ± 0.24	0.18	0.08-1.02	0.29 ± 0.17	0.25	0.04-0.66	0.99
IGF-IR	3.25 ± 2.58	2.71	0.00-10.91	2.58 ± 1.59	2.42	0.00-5.54	0.66
Rectum							
IGF-I	1.04 ± 0.94	0.73	0.17-3.14	0.80 ± 0.46	0.85	0.19-1.90	0.35
IGF-II	0.30 ± 0.24	0.23	0.07-1.09	0.26 ± 0.11	0.25	0.05-0.45	0.56
IGFBP-3	1.25 ± 0.86	1.02	0.35-3.44	1.14 ± 0.57	1.10	0.18-2.28	0.65
IGF-IR	6.07 ± 2.28	6.80	2.98-11.08	7.10 ± 2.25	6.36	4.23-12.78	0.47

*P value based on independent t test of natural log-transformed IGF-I, IGF-II, and IGFBP-3, and Mann-Whitney test for IGF-IR.

Table 5. Human intervention studies on the effect of soy protein/isoflavones on blood levels of IGF system components

Author, year	Study population	Sample size*	Study design†	Isoflavone intervention‡	Duration (mo)	Protein intake	Results
Wangen et al., 2000 (29)	Healthy women (pre + post)	31	Cross	ISP LI (65) vs. HI (130) vs. C (8)	3	Increase in all three periods	Pre: increase in IGF-I in LI (+11%, ns), not HI (vs. C) Post: no change in IGF-I in HI and LI vs. C. Increase in IGF-I in C (+18%) and LI (24%), not HI (vs. baseline). No marked change in IGFBP-3
Khalil et al., 2002 (30)	Healthy men	46	Par	IP SP (88) vs. MP	3	Increase in both groups	Increase in IGF-I after both SP (+65%) and MP (+14%, ns) Increase significantly greater after SP than MP
Adams et al., 2003 (31)	Men and women (post); high-risk colorectal cancer	150	Par	ISP HI (83) vs. C (3)	12	—	No effect on IGF-I and IGFBP-3 Increase in IGF-I (+8%) in both groups combined vs. baseline
Arjmandi et al., 2003 (32)	Healthy women (post)	42	Par	IP SP (88) vs. MP	3	Increase in both groups	Increase in IGF-I after SP (+68%) and MP (+36%) No significant difference between the two groups
Hussain et al., 2003 (33)	Male prostate cancer patients	39	Pilot	SIS (200) no control group	3 to 6	—	No effect on IGF-I and IGFBP-3
Spentzos et al., 2003 (34)	Male prostate cancer patients	17	Pilot	ISP HI (114) no control group	5	—	Increase in IGF-I (+22%) No effect on IGFBP-3
Campbell et al., 2004 (35)	Healthy women (pre + post)	23	Cross	CIS (86) vs. C (0)	1	No difference between periods	No effect on IGF-I, IGFBP-1, and IGFBP-3
Arjmandi et al., 2005 (36)	Healthy women (post)	62	Par	SF (60) vs. CF	12	Increase in both groups (higher in CF)	Increase in IGF-I after SF (+26%) and CF (+13%) Increase in IGFBP-3 after SF (+5%), not CF No significant difference between the two groups
Gann et al., 2005 (37)	Healthy women (pre)	154	Par	ISP (+Ca) HI (88) vs. C (0)	3	Increase in both groups	No effect on IGF-I and IGFBP-1 Decrease in IGFBP-3 (−2%) in both groups combined vs. baseline
Maskarinec et al., 2005 (38)	Healthy women (pre)	196	Par	SF (50) vs. RD	24	Increase in both groups (higher in SF)	No effect on IGF-I, IGFBP-3, and molar ratio Positive association between urinary isoflavone excretion and serum IGF-I
Woodside et al., 2006 (39)	Healthy women (pre)	10	Pilot	SF (80)§ no control group	0.25	—	Increase in IGF-I (+71%), IGFBP-3 (+18%), and molar ratio (+50%)
Vrieling et al., 2007 (20)	Men; high-risk colorectal cancer	37	Cross	CIS (84) vs. C (0)	2	No difference between periods	No effect on total IGF-I, free IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 Inverse association between equol and IGF-I
Dewell et al., 2007 (40)	Male cancer patients	93	Par	LP + ISP (133) vs. C (24)	12	Increase in LP + ISP vs. C	Increase in both groups in IGF-I (LP + ISP: +18%; C: +11%) and IGFBP-3 (LP + ISP: +6%; C: +7%). No significant difference between the two groups Increase in IGFBP-1 in LP + ISP (+33%), not in C (−4%) No effect on IGFBP-2
Vrieling et al., 2008 (this article)	Women (post); high-risk colorectal cancer	34	Cross	CIS (84) vs. C (0)	2	No difference between periods	No effect on total IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3

Abbreviations: ISP, isolated soy protein; HI, high isoflavones; LI, low isoflavones; C, control; IP, isolated protein; SP, soy protein; MP, milk protein; SIS, soy-derived isoflavone supplement; CIS, clover-derived isoflavone supplement; SF, soy foods; CF, control foods; RD, regular diet; LP, lifestyle program, including very-low fat vegan diet and exercise.

*Pre, premenopausal post, postmenopausal.

† Cross, randomized, crossover study; Par, randomized, parallel study; Pilot, uncontrolled pilot study.

‡ Isoflavone content in mg/d (in parentheses).

§ Total phytoestrogen content, including isoflavones.

determined by specific RIAs. All assays were done in the same laboratory. Further details have been described previously (20).

Because the biological effectiveness of isoflavones may depend on the individual ability to biotransform isoflavone metabolites to the more potent estrogenic metabolite equol (21), equol concentrations were measured in serum samples collected after isoflavone intervention (J. Lampe; Fred Hutchinson Cancer Research Center, Seattle, WA). TR-FIA kits (Labmaster) were used as described previously (22) and fluorescence was measured with a Victor 2 model 1420 spectrofluorometer (Wallac). The sensitivity was 3.3 nmol/L and the intra-assay coefficient of variation was 6.8% at a mean equol concentration of 295 nmol/L. The IGF system may also be influenced by changes in estradiol and sex hormone-binding globulin (SHBG). Therefore, we also determined estradiol and SHBG concentrations by electrochemiluminescence immunoassays using the E170 (Elecsys module) immunoanalyzer (Roche Diagnostics).

Quantitative PCR. Total RNA was extracted from the tissue samples using RNAzolB (Campro Scientific). Total RNA (2.5 μg) was reverse-transcribed to generate first-strand cDNA (total volume 50 μL) using random hexamers and Superscript II. After 10 min at room temperature for extension of the hexameric primers, the reverse transcriptase reaction was done at 42°C for 60 min, followed by heating at 98°C for 5 min. cDNA was diluted 1:1 with RNase-free water before real-time reverse transcription-PCR.

Quantitative PCR was used to assess the levels of mRNA expression of *IGF-I, IGF-II, IGFBP-3,* and *IGF-IR* in tissue biopsies obtained from the ascending colon (n = 34) and rectum (n = 34). Primers and probes for these reactions were designed using Primer Express software (Applied Biosystems, PE; Table 1). Primers were chosen in two adjacent exons, and the fluorescent-labeled probes were selected to partially encompass both exons, to avoid DNA contamination and amplification of the homologous insulin and insulin receptor genes. PCRs were carried out using the 7500 Fast Real-time PCR System (Applied Biosystems) according to the instructions of the manufacturer (50 cycles). The content of *IGF-I, IGF-II, IGFBP-3,* and *IGF-IR* transcripts was normalized to the content of the ''housekeeping gene'' β-actin. A second housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), was assessed to evaluate the validity of normalization to *β-actin* content. Because the expression levels of both genes were highly correlated (Spearman rank correlation coefficient, r = 0.91; $P < 0.001$), all results were normalized to *β-actin* (*GAPDH* data not shown). For *β-actin* and *GAPDH*, standard PCR primer and probe mixtures (Applied Biosystems) were used under the same conditions as described above. Standard curves were generated using serially diluted solutions of cDNA obtained from pooled amplified RNA from 82 breast tumors. All PCR assays were conducted in duplicate for each sample.

Statistical Analyses. The main variable of interest in our statistical analysis was the relative crossover difference in the total serum IGF-I concentration after isoflavone supplementation (see formulas below), expressed as a percentage relative to the concentration after placebo treatment. Relative crossover differences in serum concentrations of IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 were secondary end points. As the sample size of the two randomized groups differed (i.e., I-P group, n = 15; P-I group, n = 19), for both randomized groups, the mean crossover difference for each IGF system component was calculated and then pooled over the two groups, to adjust for period effects. We tested whether the pooled crossover difference significantly deviated from null with a t test using the standard error of the pooled crossover differences (23). As IGFBP-1 and estradiol were not normally distributed, we tested whether the median crossover differences significantly deviated from null using a sign test. Absolute differences between serum concentrations after isoflavones and after placebo were tested using paired t tests (total IGF-I, IGFBP-2, IGFBP-3, SHBG) or Wilcoxon signed ranks tests (IGFBP-1, estradiol).

$$\text{Relative cross-over difference } [\Delta(\mathrm{I}-\mathrm{P}) \text{ or } \Delta(\mathrm{P}-\mathrm{I})] = [\text{concentration after intervention } (C_{\mathrm{I}}) - \text{concentration after placebo } (C_P)]/C_P$$

$$\text{Pooled cross-over difference} = 1/2\ (\mathrm{mean}_{\Delta(\mathrm{I}-\mathrm{P})} + \mathrm{mean}_{\Delta(\mathrm{P}-\mathrm{I})})$$

$$\text{SE of the pooled cross-over difference} = 1/2\sqrt{[(s^2/n_{\mathrm{I}-\mathrm{P}}) + (s^2/n_{\mathrm{P}-\mathrm{I}})]}$$

$$\text{where } \mathrm{s}^2 = [(n_{\mathrm{I}-\mathrm{P}}-1)\mathrm{SD}^2_{\Delta\mathrm{I}-\mathrm{P}} + (n_{\mathrm{P}-\mathrm{I}}-1)\mathrm{SD}^2_{\Delta\mathrm{P}-\mathrm{I}}]/(n_{\mathrm{I}-\mathrm{P}} + n_{\mathrm{P}-\mathrm{I}} - 2)$$

To evaluate whether the relative crossover differences correlated with serum equol concentrations after isoflavone supplementation, a Spearman correlation coefficient was calculated.

Differences in mRNA expression of the *IGF-I, IGF-II, IGF-IR,* and *IGFBP-3* genes in normal colorectal tissue biopsies taken after the first intervention period were compared between individuals who received isoflavone intervention (I-P group) and individuals on placebo (P-I group). Natural log-transformed data were used to normalize the data, and either two-sample t tests (*IGF-I, IGF-II, IGFBP-3*) or nonparametric Mann-Whitney tests (*IGF-IR*) were done.

Descriptive characteristics were computed for both randomized groups separately. We calculated whether relevant changes occurred in dietary and lifestyle factors known to influence the IGF system, i.e., dietary intake of macronutrients, body weight, waist and hip circumference, total physical activity score, dietary intake of products relatively rich in isoflavones, during the study period for both randomized groups separately.

P values were determined by two-sided tests, and differences were considered to be statistically significant at $P < 0.05$. Statistical analyses were done using SPSS 12.0 (SPSS, Inc.).

Results

Thirty-four women finished the complete study protocol [n = 15 on isoflavones-placebo (I-P group); n = 19 on placebo-isoflavones (P-I group); Fig. 1 A]. Both groups were similar with respect to age (Table 2). However, the P-I group was slightly more overweight and consisted of more women who had never smoked. The number of participants with a family history of colorectal cancer and/or a personal history of colorectal adenomas was equally distributed among the two groups. Hormonal factors (i.e., age at menopause, parity, past use of hormone replacement therapy) also did not markedly differ between the two groups.

Isoflavone supplementation did not significantly affect serum total IGF-I and IGF-II concentrations (mean relative difference between isoflavones and placebo: IGF-I, −2.0%; 95% confidence interval, −8.0% to 3.9%; IGF-II, −1.7%; 95% confidence interval, −6.3% to 2.8%; Table 3). Additionally, the median or mean relative differences in IGFBP-1, IGFBP-2, and IGFBP-3 between isoflavones and placebo did not significantly deviate from zero. However, substantial interindividual variation in IGFBP-1 changes were observed, ranging from reductions of 50% to increases of up to 500%. Interindividual variations in IGFBP-2 changes were smaller but still substantial. No correlation was found between the change in serum IGF-I concentrations and serum equol concentrations ($r = -0.03$, $P = 0.88$). Serum estradiol and SHBG were also not significantly altered by the isoflavone intervention.

In a parallel analysis, we studied tissue mRNA expression of the *IGF-I, IGF-II, IGFBP-3,* and *IGF-IR* genes measured in normal colorectal tissue biopsies taken after the first intervention period. No statistically significant differences were observed in the mRNA levels of genes between individuals on the isoflavone intervention and individuals on placebo, neither in the ascending colon nor in the rectum (Table 4).

Based on both returned tablet counts and recordings in the daily notebooks, 91% of participants (n = 31) were compliant (≥80% of tablets taken) during both the isoflavone and the placebo intervention period. Excluding participants who were not compliant (n = 3) did not markedly affect the results (data not shown). Body weight, waist and hip circumference, total physical activity score, dietary macronutrient intake, and the number of days on which products rich in isoflavones were consumed did not materially differ between the isoflavone and the placebo intervention period (data not shown).

Discussion

In our randomized, placebo-controlled, double-blinded, crossover trial, red clover–derived isolated isoflavone supplementation of 84 mg/d for 2 months neither influenced circulating concentrations of IGF-I and IGF-II nor those of IGFBP-1, IGFBP-2, and IGFBP-3 in postmenopausal women at increased risk of colorectal cancer. In addition, colorectal tissue mRNA expression of *IGF-I, IGF-II, IGF-IR,* and *IGFBP-3* did not differ between individuals on isoflavones and individuals on placebo.

This is the first randomized controlled trial investigating the effects of isolated isoflavones on circulating IGF system components in postmenopausal women at increased risk of colorectal cancer. Populations at increased cancer risk, such as our study population, are likely to benefit most from interventions aimed at lowering circulating IGF-I levels and subsequently suppressing IGF-IR signaling. We used a crossover design, which has the important advantage that our results were not affected by the generally high between-individual variation in circulating IGF-I concentrations relative to the much lower within-individual variation.

The number of participants for our study was in concordance with the a priori design (i.e., $n \geq 26$). The dropout rate, although 20% had been anticipated, was 13% and unrelated to supplement intake. This resulted in an adequately powered trial to evaluate isoflavone effects (i.e., a 10% decrease) on serum IGF-I levels. Compliance, based on tablet counts and daily notebooks, was high (91%). Although serum genistein concentrations were not measured in the current trial, a strong increase in serum genistein concentrations was observed in the majority of men in the male counterpart of this trial after similar isoflavone intervention with comparable compliance (20). In that trial, serum genistein concentrations were within the range of those of subjects traditionally consuming a high-isoflavone soy-based diet (24). Furthermore, we assessed factors which are thought to potentially influence circulating concentrations of IGFs and IGFBPs (e.g., total energy and protein intake, consumption of products rich in isoflavones and lycopene, body weight, and physical activity). Because these factors did not materially change during the course of the study, any potential changes in various parameters of the IGF system would have been attributable to the isoflavone intervention. In principle, the 2-month duration of isoflavone supplementation as used in our study would have been sufficient to affect circulating IGF system components because previous human intervention studies on oral estrogens and selective estrogen receptor modulators have shown a decrease of ~15% to 30% in serum IGF-I levels within 2 months (5, 7). In prospective epidemiologic studies, serum IGF-I concentrations for individuals in the bottom quartiles were >50% lower than for individuals in the top quartiles who were at increased risk of developing colorectal cancer (10). Although it is unknown what individual percentage of change in serum IGF-I levels is required to ultimately decrease colorectal cancer risk, we hypothesize that smaller changes, as observed in the intervention studies discussed above, may also be relevant. In the male counterpart of this trial, we observed a negative correlation between the change in serum IGF-I concentrations and serum equol concentrations (20). These results suggest that isoflavones may lower serum IGF-I concentrations only in men who are capable of metabolizing daidzein into equol. However, our results in women do not indicate a difference between equol producers and nonproducers. This may be due to differences in hormonal background, and is confirmed by studies in mice and rats that metabolize daidzein mainly to equol (21). In male mice and rats, physiologic dietary intakes of soy decreased serum IGF-I concentrations, whereas in female mice and rats this effect was not observed (25-28).

To date, 13 intervention studies in humans have evaluated the effect of isoflavone supplementation on circulating levels of IGF system components (summarized in Table 5; refs. 20, 29-40). Besides the study described in this article, only two other randomized, crossover trials evaluated the effects of isolated, red clover–derived isoflavones (20, 35). In both studies, no overall effect of isoflavones on circulating IGF system components was observed, which is in agreement with the results in our study. In eight randomized, controlled studies, an intervention with soy foods or isolated soy protein containing isoflavones was compared with either control foods, isolated milk protein, or soy protein without isoflavones. In all of these studies, the protein intake in both the intervention group and the control group had markedly increased. Consequently, six out of eight studies observed increased blood levels of IGF-I in both groups (29-32, 36, 40), which is likely due to IGF-I increasing the effects of essential amino acids in animal and soy protein (41, 42). In only one study the increase in IGF-I levels was significantly greater for the soy protein compared with the milk protein intervention group (30). Based on this qualitative review, it can be concluded that isoflavones do not substantially increase or decrease blood levels of IGF-I.

To our knowledge, studies on the effects of isoflavone supplementation on mRNA expression of IGF system components in normal human colorectal tissue have not been previously reported. Our data reveal that mRNA expression levels of *IGF-I, IGF-II, IGF-IR,* and *IGFBP-3* in the ascending colon and the rectum do not differ between individuals on isoflavones and individuals on placebo. In contrast, *in vitro* studies using androgen-responsive prostate cancer cells showed that genistein, daidzein, and equol all consistently inhibit IGF-IR mRNA expression, even at relatively low levels of exposure (i.e., being comparable to human dietary intake; refs. 43, 44). In cultured HT-29 human colon cancer cells, genistein, but not daidzein, was found to reduce IGF-IR protein levels and inhibit IGF-IR signaling at relatively high pharmacologic concentrations (45). Although we could not confirm this finding for normal colorectal tissue *in vivo*, it must be emphasized that in our study, the effects of isoflavones were evaluated between individuals and not within individuals because only one colonoscopy was done in each participant. Substantial variability in tissue mRNA expression levels in colorectal biopsies of the same individuals sampled at different colorectal locations may occur, with a markedly higher expression level of several IGF system components in the rectum as compared with the proximal colon (46). Therefore, future studies on this subject should focus on multiple colonoscopies with multiple biopsies at fixed locations in each participant, preferably in a crossover design.

Adams et al. evaluated soy isoflavone effects on colorectal epithelial cell proliferation in a parallel trial (n = 91, men and women) comparing soy-protein powder (83 mg isoflavones/d) with ethanol-extracted soy-protein powder (containing 3 mg isoflavones/d). A colonoscopy was done before and after the 12-month intervention period. No reduction in proliferation was observed. Unexpectedly, in the sigmoid colon, an increase in cell proliferation measures was found, which was opposite to what was hypothesized (47). As mentioned previously, isoflavones as constituents of a soy protein food may have different or even opposite effects as the effects hypothesized for isolated isoflavones. Unfortunately, we were not able to study the proliferation markers in our tissue specimens because the biopsies were not oriented optimally to allow cutting slides that contained full-length crypts. The latter is required for the determination of epithelial cell proliferation (labeling index).

In conclusion, isolated isoflavones did not influence serum concentrations and tissue mRNA expression of IGF system components in our randomized, placebo-controlled, double-blind intervention study in postmenopausal women at increased risk of colorectal cancer. These results suggest that the increased serum IGF-I concentrations as observed in several previous studies investigating soy food or soy protein supplementation are most likely due to soy protein itself, and not to isoflavones. Potential effects of isoflavones on IGF-IR signaling in the colon and rectum, but also in other potential target tissues such as breast and prostate need further study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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