

Isoflavones from Phytoestrogens and Gastric Cancer Risk: A Nested Case-Control Study within the Korean Multicenter Cancer Cohort

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Abstract

Background: The role of soybean products in gastric cancer risk is not clear in epidemiologic studies due to measurement error from dietary intake questionnaires and due to different degrees of bias according to study design. To examine the association between soybean products and gastric cancer risk, we measured phytoestrogen biological markers in a nested case-control study.

Methods: The study population was composed of 131 cases and 393 matched controls within the Korean Multicenter Cancer Cohort. The concentrations of the four biomarkers in the plasma samples were measured using time-resolved fluoroimmunoassay. Conditional and unconditional logistic regression models were used to compute the odds ratio (OR) and 95% confidence intervals (CI).

Results: Median plasma concentrations of genistein (229 nmol/L for controls, 181.8 nmol/L for cases; $P = 0.07$) and daidzein (131.2 nmol/L for controls, 80.5 nmol/L for cases; $P = 0.04$) in cases were lower than in controls, whereas equol concentrations were similar. Compared with the reference group, gastric cancer risk decreased in the highest groups for genistein (OR, 0.54; 95% CI, 0.31-0.93) and daidzein (OR, 0.21; 95% CI, 0.08-0.58). Higher equol concentrations were associated with a decreased risk for gastric cancer (OR, 0.50; 95% CI, 0.27-0.90). The combination of the highest concentrations for each isoflavone category was associated with a 0.09-fold decreased risk for gastric cancer compared with the combination of the lowest concentrations for each category. There was no association between plasma lignan concentrations and gastric cancer.

Conclusions: High serum concentrations of isoflavones were associated with a decreased risk for gastric cancer.

Impact: These results suggest a beneficial effect of high soybean product intake for gastric cancer risk. *Cancer Epidemiol Biomarkers Prev*; 19(5); 1292-300. ©2010 AACR.

Introduction

Dietary modification is an important tool for cancer prevention strategies. High intake of salty foods and *N*-nitroso compounds has been suggested to increase

gastric cancer risk (1-3). In contrast, high consumption of fresh vegetables, fruits, and soy products may lower the risk of gastric cancer (4-7). In particular, soy may play a role similar to phytoestrogens that bind to estrogen receptors and therefore interfere with the action of estrogen, which is a well-established risk factor for hormone-dependent cancers such as breast and prostate cancers (8, 9). In addition, the antioxidant and anti-inflammatory effect of soy may have a protective effect for non-hormone-dependent cancers (10-12).

In epidemiologic studies, the health benefits of soy for gastric cancer are inconsistent: some studies reported that soy products, such as bean and tofu, significantly decrease the risk of gastric cancer (4, 5, 13), whereas other studies reported that soy products were not significantly associated with a decreased risk of gastric cancer (14-17). The inconsistency for dietary intake might be due to the use of food frequency questionnaires and the case-control design. Although food frequency questionnaires could measure usual dietary habits, assuming that study subjects do not change their dietary habits for long periods of time, they are also vulnerable to information bias such

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as memory decay, differential recall, and misclassification bias. The case-control design is also vulnerable to information bias (18). Because of these limitations, the health benefits of soybean products for gastric cancer risk are inconsistent (19). Thus, measurement of biological markers and the prospective cohort study design is helpful in reducing such biases (20).

Because isoflavone is one of the phytoestrogens abundant in soybeans, serum isoflavone concentrations reflect the dietary intake of soybean products (21). Although there is little epidemiologic evidence on the effect of isoflavones or phytoestrogens for gastric cancer, several experimental studies reported on the anticarcinogenic effects of isoflavones against gastric cancer (11, 22, 23). In this nested case-control study, we measured phytoestrogen biological markers to examine whether phytoestrogens are associated with the development of gastric cancer in a nested case-control design.

Materials and Methods

Study population and data collection. Eligible subjects were selected from the Korean Multicenter Cancer Cohort (KMCC). The rationale and design of the KMCC is described in detail elsewhere (24, 25). Briefly, the KMCC is a multicenter community-based prospective cohort with a biological materials bank designed to investigate the relationship between exposure to environmental, lifestyle, and genetic factors and risk of various cancers in Korea. The KMCC is composed of male and female participants aged over 30 years, recruited from 1993 to 2004 from two rural and two urban areas in Korea. Voluntary participants in the cancer screening survey served as the eligible population for the KMCC. Information on general lifestyle, physical activity, diet, reproductive factors, and pesticide exposures were obtained through structured questionnaire interviews. Blood samples and spot urine samples were also collected. Serum, plasma, and buffy coat samples were stored at -70°C . The study protocol was approved by the Institutional Review Boards of Seoul National University Hospital.

Selection of cases and controls. As of December 2003, we identified 208 gastric cancer cases through computerized record linkages to the Korea Central Cancer Registry and the National Death Certification databases. We excluded 66 subjects diagnosed before recruitment and 11 subjects with insufficient plasma ($<200\ \mu\text{L}$) for laboratory assay. There were no differences in basic characteristics between the 131 cases and 11 excluded subjects due to insufficient plasma (table not shown). We matched three controls to one cancer case based on age (± 5 years), gender, residence area, and year of recruitment. Controls were free of cancer and alive at the time of diagnosis of the matched cases. Finally, 131 cases and 393 controls were selected.

Measurement of plasma concentrations of genistein, daidzein, equol, and enterolactone. There are two major

classes of phytoestrogens—*isoflavones* and *lignans*. The main food source for isoflavones are soy products and red clover, whereas the main food source for lignans are cereals, vegetables, and fruits (26). We measured plasma concentrations of phytoestrogens: (a) isoflavones such as genistein, daidzein, and equol (a daidzein metabolite) and (b) lignans (enterolactone). The use of time-resolved fluoroimmunoassay kits (Labmaster, Finland) to measure isoflavones and lignans is a valid method because the correlation coefficient between genistein concentration from time-resolved fluoroimmunoassay and gas chromatography-mass spectrometry was 0.95 (27). For daidzein, the correlation coefficient was 0.98 (27).

The detailed measurement method for genistein is as follows: plasma samples were collected at baseline survey and frozen at -70°C until analysis; $200\ \mu\text{L}$ of acetate buffer $0.1\ \text{mol/L}$ (pH 5.0), containing $0.2\ \text{units/mL}$ of β -glucuronidase and $2\ \text{units/mL}$ of sulfatase were added to tubes containing $200\ \mu\text{L}$ of plasma. After incubating overnight at 37°C , free genistein was extracted twice with $1.5\ \text{mL}$ of diethyl ether. The combined ether phases were evaporated in a water bath at 45°C to dryness. The residue was dissolved in $200\ \mu\text{L}$ of assay buffer and $20\ \mu\text{L}$ of the sample was added to prewashed goat anti-rabbit IgG-coated microtitration wells. One hundred microliters of antigenistein antibody working solution and $100\ \mu\text{L}$ of genistein-Eu tracer working solution were added to the wells. Plates were slowly shaken at room temperature for 90 minutes. After washing the plates four times, $200\ \mu\text{L}$ of an enhancement solution was added to the wells. Plates were slowly shaken at room temperature for 5 minutes. After additional shaking, time-resolved fluoroimmunoassay was measured using the Victor 3 1420 Multilabel Counter (Perkin-Elmer). The methods used to measure daidzein, equol, and enterolactone levels were similar to that of genistein.

To verify the feasibility of the isoflavone measurements, we pretested 31 other samples before measuring isoflavones in our study subjects. As the measurement methods were similar, we pretested for only genistein and enterolactone. To assure quality control, we calculated coefficients of variation as the SD divided by the mean multiplied by 100 (%), within each batch using 39 replicated samples for genistein and enterolactone, and 70 samples for daidzein and equol. The number of samples for the replication test were different because 31 wells in the genistein and enterolactone kits were consumed for the pretest. In our study, the coefficient of variation mean was $4.70 \pm 2.87\%$ (mean \pm SD) for genistein, $3.66 \pm 2.85\%$ for daidzein, $3.68 \pm 2.60\%$ for equol, and $4.22 \pm 2.64\%$ for enterolactone. The manufacturer's manual recommended that the CV% is typically $<10\%$ over the standard curve range.

Measurement of *Helicobacter pylori* antibody. Sera were assayed using immunoblot kits (Helico Blot 2.1, MP Biomedicals Asia Pacific, Singapore) that identified IgG antibodies specific for *Helicobacter pylori* according to the instructions of the manufacturer. Sensitivity for *H. pylori* IgG antibody was 99% and specificity was

98% in the Korean population. *H. pylori* antibody had already been measured in previous studies (28, 29), and thus, we measured *H. pylori* antibody only among additional subjects.

Cutoff level of phytoestrogen biomarkers. First, we classified blood concentrations of phytoestrogen biomarkers to the 10th percentile. By increasing or decreasing gastric cancer risk [odds ratios (OR) value] according to the 10th percentile, we determined the crude cutoff level for each phytoestrogen as shown in Fig. 1. Then, we observed changes in the OR by repeated computa-

tions according to level up or level down processes by each 1-percentile from the crude cutoff levels. Second, we additionally classified phytoestrogen levels by tertiles, quartiles, and quintiles, and repeatedly computed according to level up or level down processes from the tertiles, quartiles, and quintiles. Third, we determined the final cutoff level as the level when gastric cancer risk was significantly different relative to the determined reference level of the first and second classification methods.

Statistical analysis. The χ^2 test was conducted to estimate the *P* value for the difference in proportion for

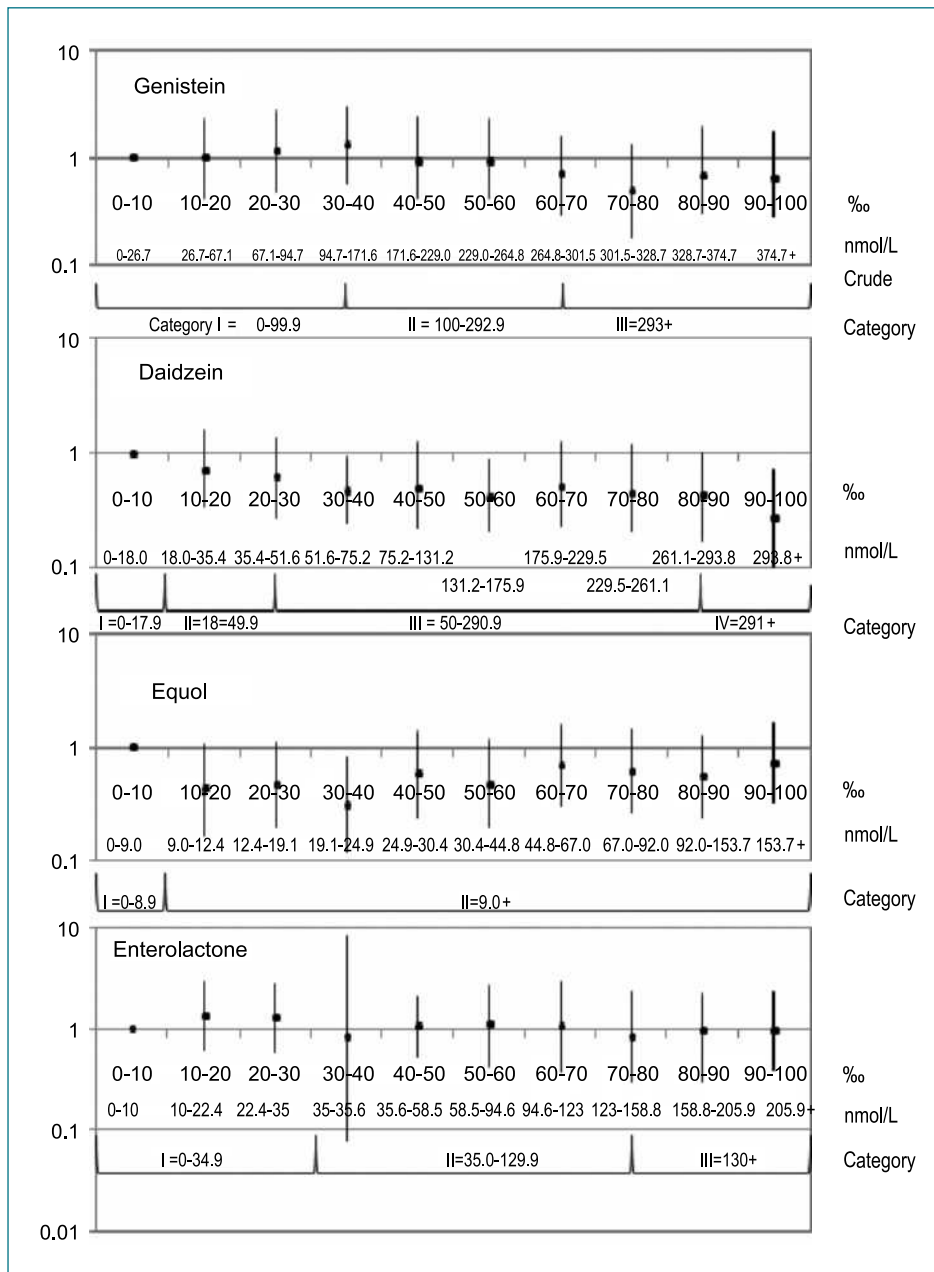


Figure 1. Gastric cancer risk of each phytoestrogen level according to the 10th percentile relative to phytoestrogen levels under the 10th percentile.

Table 1. Baseline characteristics of the subjects in a nested case-control study within the KMCC (1995-2003)

Variables	Cases (n = 131)	Controls (n = 393)	P*
Age, mean (SD)	63.7 (7.8)	62.8 (8.6)	0.28
Males, n (%)	89 (67.9)	267 (67.9)	1.00
Education over and at middle school, n (%)	94 (72.8)	268 (68.2)	0.58
Cigarette smokers, n (%)	80 (61.1)	210 (53.5)	0.28
Alcohol drinkers, n (%)	74 (51.1)	267 (51.5)	0.15
History of gastritis, n (%)	23 (16.7)	60 (11.8)	0.13
Positive <i>H. pylori</i> IgG antibody, n (%)	116 (88.6)	340 (86.5)	0.55
CagA positivity, n (%)	118 (90.1)	325 (82.7)	0.05
VagA positivity, n (%)	83 (63.4)	227 (57.8)	0.30

*P values were calculated using *t* test for age and χ^2 test for the other variables.

educational level, cigarette smoking, history of gastritis, and *H. pylori* infection between cases and controls. Using matched set strata, the Mantel-Haenszel test compared isoflavone plasma levels between cases and controls (30). ORs and 95% confidence intervals (CI) were estimated for gastric cancer risk by the determined cutoff plasma phytoestrogen levels using conditional logistic regression models adjusted for CagA and cigarette smoking, which are known risk factors for gastric cancer or effect modifiers of other risk factors. To adjust the false discovery rate of the four phytoestrogen biomarkers, we computed their corrected *P* values. Trend test for *P* value was calculated by the likelihood ratio test. Stratified analysis by *H. pylori* antibody positivity was done to examine the association between plasma phytoestrogen concentration and risk of gastric cancer using unconditional logistic regression models. Cochran's *P* values were computed to test the homogeneity of the ORs (95% CIs) before and after stratification analyses. All analyses were conducted using SAS 9.1 (SAS Institute).

Results

The demographic and lifestyle characteristics of the 131 gastric cancer cases and 393 matched controls are presented in Table 1. Sixty-eight percent of the subjects were male and 32% were female. Among the cases, 37 (28.2%) subjects were less than 60 years old, 62 (47.3%) were between the ages of 60 and 69 years, and 32 (24.4%) were more than 70 years old. There were no differences in selected characteristics including cigarette smoking history, history of gastritis, and *H. pylori* IgG antibody positivity between cases and controls. The median interval from initial blood collection to diagnosis of gastric cancer was 2.7 years (data not shown).

The median plasma concentrations of genistein (181.8 nmol/L for cases and 229 nmol/L for controls; *P* = 0.07) and daidzein (80.5 nmol/L for cases and 131.2 nmol/L for controls; *P* = 0.04) in cases were lower than those in con-

trols (Table 2). Median plasma concentrations of equol, a daidzein metabolite, were slightly but not significantly higher in cases than in controls (34.7 nmol/L for cases and 30.4 nmol/L for controls; *P* = 0.45). The median concentration of lignan was similar in cases and controls (45.0 nmol/L for cases, 45.1 nmol/L for controls; *P* = 0.92).

Compared with the reference level, the highest concentration of genistein (>293 nmol/L) was associated with a decreased risk for gastric cancer (OR, 0.54; 95% CI, 0.31-0.93; Table 3). Daidzein showed a continual decrease in risk according to level up of diadzein concentration categories (*P* = 0.0009) and a significantly decreased risk was found at concentrations over 50 nmol/L (for 50-290.9 nmol/L: OR, 0.49; 95% CI, 0.27-0.88; for the highest category over 291 nmol/L: OR, 0.21; 95% CI, 0.08-0.58). Higher equol concentrations over 9 nmol/L were associated with a decreased risk for gastric cancer (OR, 0.50; 95% CI, 0.27-0.90). There was no increased or decreased pattern of plasma concentrations of enterolactone for gastric cancer risk. After adjusting for the false discovery rate, the corrected *P* values of the three isoflavone biomarkers remained significant (*P* = 0.0461 for genistein, *P* = 0.0036 for diadzein, and *P* = 0.0456 for equol).

Table 4 shows the association between combinations of isoflavone biomarkers and gastric cancer risk. Relative to the combination with the lowest isoflavone concentration categories, an increase in the number of higher isoflavone concentration categories was associated with a lower risk for gastric cancer. The combination with the highest isoflavone concentration categories was associated with 0.09-fold (95% CI, 0.02-0.36) decreased risk for gastric cancer.

Among *H. pylori* antibody-positive subjects, the highest tertile of genistein and daidzein levels decreased the risk of gastric cancer (for genistein: OR, 0.54; 95% CI, 0.31-0.95; *P* for trend = 0.040; for daidzein: OR, 0.53; 95% CI, 0.31-0.92; *P* for trend = 0.024), whereas among *H. pylori* antibody-negative subjects (*n* = 68), these isoflavone biomarkers did not show a positive association

Table 2. Plasma concentration of isoflavones and lignans among 131 cases and 393 matched controls

Biomarkers (nmol/L)	Cases (n = 131)	Controls (n = 393)	P*
	Median (interquartile range)	Median (interquartile range)	
Isoflavones			
Genistein	181.8 (76.1-284.5)	229.0 (85.0-315.3)	0.07
Daidzein	80.5 (23.8-212.4)	131.2 (45.8-244.2)	0.04
Equol (daidzein metabolite)	34.7 (12.7-86.9)	30.4 (15.3-79.5)	0.45
Lignans			
Enterolactone	45.0 (20.6-107.1)	45.1 (20.9-116.8)	0.92

*P values were calculated using Mantel-Haenszel test with matched-set strata

with gastric cancer risk (*P* interaction for *H. pylori* antibody status: 0.49 for genistein and 0.37 for daidzein). There was no association between plasma concentrations of equol and enterolactone and gastric cancer risk regardless of *H. pylori* antibody status.

After stratification for cigarette smoking, *H. pylori*, CagA, and VagA antibodies, as well as gender, the ORs (95% CI) for gastric cancer were not heterogeneous with those before stratifications, although a decreased risk of isoflavones was found only in subjects positive for *H. pylori* and CagA antibodies (data not shown).

Discussion

Isoflavone biomarkers genistein, daidzein, and equol were associated with a decreased risk for gastric cancer in this nested case-control study in the Korean population. Phytoestrogen binds competitively to estrogen receptors but has a weak estrogenic effect of nearly 0.1% of estradiol that showed antiestrogenic effect by blocking the binding from estradiol and other estrogens (31-36). Therefore, phytoestrogens inhibit the growth and proliferation of estrogen-dependent cancer cells (32, 37). However, the

Table 3. Gastric cancer risk of the plasma concentration of phytoestrogens in a nested case-control study within the KMCC study

Phytoestrogens (nmol/L)	Cases (n = 131), no. (%)	Controls (n = 393), no. (%)	OR (95% CI)*	P value (trend test)	P value (FDR)
Isoflavones					
Genistein				0.0346	0.0461
-99.9	50 (38.2)	131 (33.3)	1.00		
100-292.9	51 (38.9)	125 (31.8)	1.11 (0.69-1.79)		
293+	30 (22.9)	137 (34.9)	0.54 (0.31-0.93)		
Daidzein				0.0009	0.0036
-17.9	24 (18.3)	39 (9.9)	1.00		
18-49.9	29 (22.1)	72 (18.3)	0.67 (0.33-1.33)		
50-290.9	71 (54.2)	237 (60.3)	0.49 (0.27-0.88)		
291+	7 (5.3)	45 (11.5)	0.21 (0.08-0.58)		
Equol (a daidzein-metabolite)				0.0228	0.0456
-8.9	22 (16.8)	38 (9.7)	1.00		
9+	109 (83.2)	355 (90.3)	0.50 (0.27-0.90)		
Lignans					
Enterolactone				0.6297	0.6297
-34.9	51 (32.8)	149 (37.9)	1.00		
35-129.9	52 (34.4)	155 (39.4)	0.92 (0.58-1.46)		
130+	28 (32.8)	89 (22.7)	0.87 (0.48-1.59)		

Abbreviation: FDR, false discovery rate.

*OR was calculated using conditional logistic regression adjusted for CagA and cigarette smoking.

Table 4. The gastric cancer risk associated with the combinations of isoflavone biomarkers

Isoflavones (nmol/L)	Cases (n = 131), no. (%)	Controls (n = 393), no. (%)	OR* (95% CI)	P value (trend test)
Lowest categories combination Genistein <100, daidzein <18, and equol <9	9 (6.9)	8 (2.0)	1.00	<0.0001
Lower categories combination (100 ≤ Genistein < 293 or 18 ≤ daidzein < 50), and equol < 9	6 (4.6)	11 (2.8)	0.54 (0.14-2.12)	
One higher categories' combination among three isoflavones [†] (Genistein ≥293, daidzein <50, and equol <9) or (Genistein <293, daidzein ≥50, and equol <9) or (Genistein <293, daidzein <50, and equol ≥9)	42 (32.1)	104 (26.5)	0.34 (0.12-1.02)	
Two higher categories' combination [†] (Genistein ≥293, daidzein ≥50, and equol <9) or (Genistein ≥293, daidzein <50, and equol ≥9) or (Genistein <293, daidzein ≥50 and equol ≥9)	47 (35.9)	140 (35.6)	0.29 (0.10-0.83)	
Three higher categories combination Genistein ≥293, 50 ≤ daidzein <291, and equol ≥9	22 (16.8)	92 (23.4)	0.20 (0.07-0.62)	
Highest categories' combination Genistein ≥293, daidzein ≥291, and equol ≥9	5 (3.8)	38 (9.7)	0.09 (0.02-0.36)	

*OR was calculated using conditional logistic regression adjusted for CagA and cigarette smoking.

[†]Cutoff level of each higher category was considered as 293 nmol/L for genistein, 50 nmol/L for daidzein, and 9 nmol/L for equol which showed significant levels in Table 3.

effect of phytoestrogens on gastric cancer is still unknown. To our knowledge, there are no published epidemiologic reports thus far that have examined the association between phytoestrogens and gastric cancer risk. Although human epidemiologic studies for soy seem to be inconsistent (4, 5, 13-17), the majority of the results show a decreased tendency for gastric cancer risk.

In our study, only the isoflavone series had a significantly adverse association with gastric cancer. The chemical structure of isoflavones is more similar to estradiol than lignans (Fig. 2). However, the protective effect of isoflavone for gastric cancer could be explained by an anti-inflammatory or antioxidative effect rather than a hormone-related effect, which is the main mechanism in estrogen-dependent cancers such as breast or prostate cancer. Several *in vitro* and animal experimental studies support a biological plausibility for this protective effect. Isoflavones, such as genistein and daidzein, directly affect gastric cancer cells (11, 22, 23). Genistein could participate in apoptosis by downregulation of apoptosis-regulated gene *Bcl2* and upregulation of apoptosis-regulated gene *Bax* (38, 39). Additionally, genistein could inhibit gastric cancer cell growth and proliferation by cell cycle arrest at G₂ and early M phase and angiogenesis and induce cell apoptosis (11, 22); daidzein could inhibit gastric cancer cell growth by cell cycle arrest at G₁ phase (22). In particular, genistein plays a role as a tyrosine kinase inhibitor

and inhibits the proliferation of gastric epithelial cells by inactivation of tyrosine kinase and ERK signal transduction cascade among *H. pylori*-infected cells (40).

Although the previous experimental results for equol were insufficient, equol can be assumed to show a similar effect as daidzein because equol is produced by metabolizing daidzein in the intestinal microflora after hydrolysis of daidzein (41).

It is well known that total daily and energy-adjusted isoflavone intake differ by race/ethnicity. Asians consume a high soy-rich diet whereas consumption is low in western populations. A negative association between isoflavone intake and gastric cancer has been shown only in studies conducted among Asians; among Western people, this may be underestimated or masked due to low levels of isoflavone intake (42).

Our study has several limitations. First, we measured plasma concentration of isoflavones only once, which was at the time of enrollment, and plasma isoflavone levels might reflect short-term rather than long-term intake. We assume that soybean product intake levels in most individuals are relatively stable over time. In the Japanese people, whose dietary habits are similar to Koreans, a validation study in the Japan Public Health Center-based prospective study showed high reliability of repeated measurements of genistein intake by food frequency questionnaire. The correlation coefficient was 0.72 for a 1-year interval and 0.61 for a 5-year interval

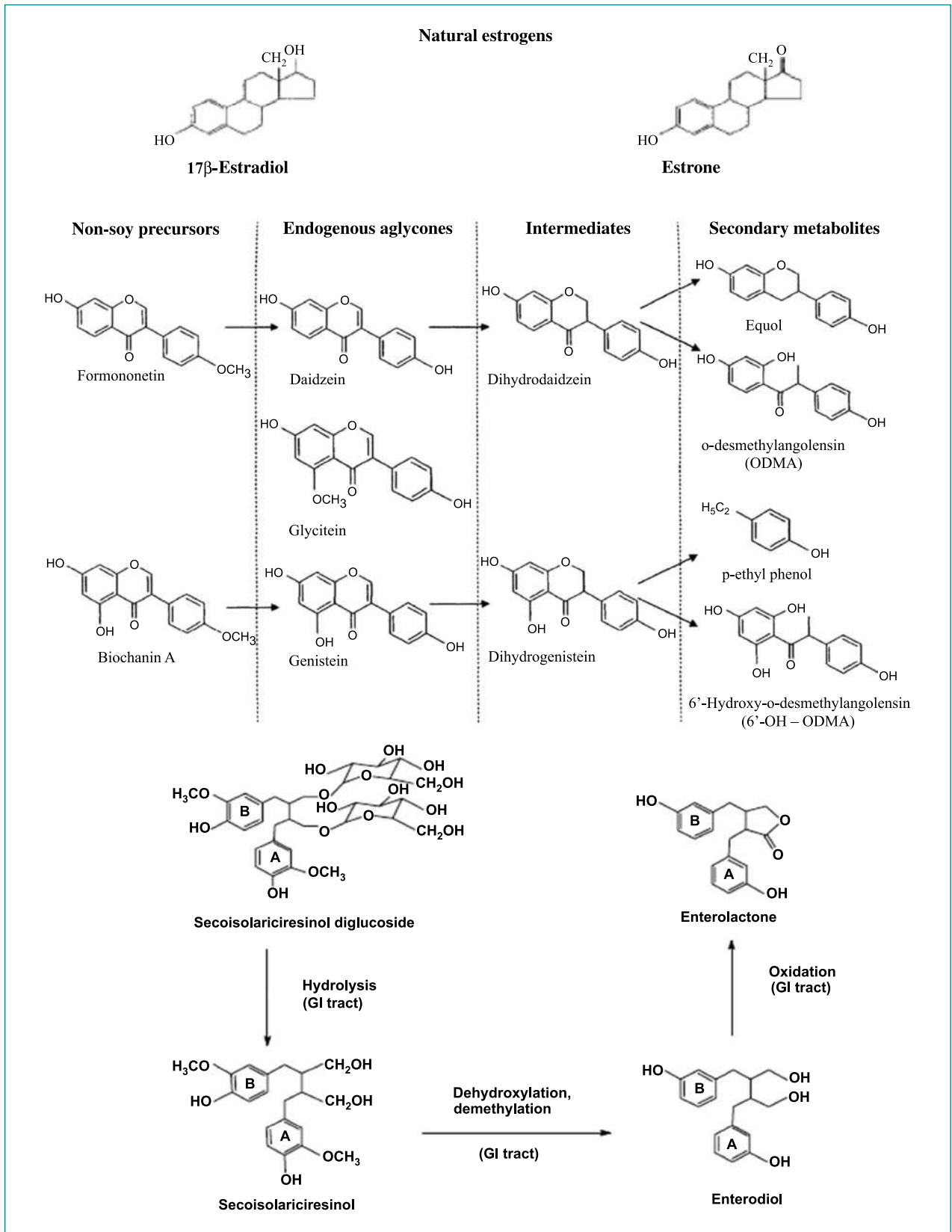


Figure 2. Chemical structures of phytoestrogens and natural estrogens (46-48).

(43, 44). Second, because isoflavones have short half-lives in blood, plasma levels are particularly affected by the time elapsed since the last meal (45). Nevertheless, we collected samples after overnight fasting so we could minimize the measurement error due to time. Third, the sample size was small and we were not able to conduct subtype analyses according to anatomic location of gastric cancer. We were not able to assess the association between isoflavone and gastric cancer among *H. pylori*-negative subjects due to the small number of cases, and thus, insufficient statistical power.

Nevertheless, our study has several strengths. First, direct measurement of plasma isoflavone concentrations provides not only an intake index but absorption and metabolism of isoflavones and blood levels may more accurately reflect relevant biological dose levels. Food frequency questionnaires as indirect measurements of dietary intake of isoflavone or soybean product are limited in measuring individual variability (18), therefore, the true association between gastric cancer risk and soybean products measured using food frequency questionnaires may be diluted. Second, using prediagnosis plasma reduces the potential recall bias for exposure among gastric cancer cases. Third, cases and controls were selected within the same cohort, thereby avoiding selection bias which is inherent in case-control studies.

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