RESEARCH ARTICLE

Urinary isoflavone phytoestrogens in German children and adolescents – A longitudinal examination in the DONALD cohort

Gisela H. Degen[1], Meinolf Blaszkewicz[1], Lijie Shi[2], Anette E. Buyken[2] and Thomas Remer[2]

[1] IfADo – Leibniz Research Centre for Working Environment and Human Factors at the TU Dortmund, Germany
[2] Research Institute of Child Nutrition (FKE), Dortmund, Germany

Received: July 13, 2010
Revised: August 26, 2010
Accepted: August 30, 2010

Scope: In light of concerns about hormonally active agents, it is important to assess human exposure to such compounds, especially in children as a susceptible subgroup. Estrogenic plant constituents are present in the human diet in varying levels, in particular the isoflavones daidzein (DAI) and genistein (GEN). We aimed to examine age-dependent and secular trends in phytoestrogen exposures and to investigate equol (EQ) excretion of German children using biomarker analysis in 24-h urine samples from a longitudinally designed study.
Methods and results: The concentrations of DAI, its metabolite EQ and GEN were determined by GC-MS analysis in 24-h urines (510 samples) collected between 1985 and 2000 in 90 (47 boys) German children (6–18 years old), who are participants in the Dortmund Nutritional and Anthropometric Longitudinally Designed study. The results from the urinary biomarker analysis indicate isoflavone exposures at quite variable levels in German children: Analyte concentrations in over 500 urine samples cover the range reported previously in adults on typical German diet and with soy intake. EQ, the DAI metabolite produced by the gastrointestinal microflora, was detected in a high fraction of all samples, with 28/90 children (31%) excreting EQ in all their urines, and 62/90 children (68%) in at least one sample. Interestingly, when multiple urines obtained from individuals at different ages (6–18 years) were analyzed, EQ formation did not appear to be a constant trait over time. When stratified by sex, DAI, EQ and GEN concentrations (ng/mL) in urines and excretion rates (μg/day) were similar in boys and girls. Total isoflavone excretion rates (μg/day) increased during childhood (6–12 years) ($p = 0.02$) and were constant during adolescence (13–18 years) ($p = 0.6$). No clear trend for changes in dietary isoflavone exposure over the total study period was seen ($p = 0.7$).
Conclusions: In conclusion, biomarkers in urine of German children and adolescents indicate a frequent, but widely variable dietary isoflavone intake and suggest no secular increase (1985–2000) in the exposure to isoflavone phytoestrogens among German children and adolescents.

Keywords:
Biomarkers / Daidzein / Equol / Genistein / Phytoestrogens

1 Introduction

Human exposure to hormonally active compounds of natural or anthropogenic origin occurs mainly with food [1, 2]. An important group of dietary estrogens is phytoestrogens [3, 4], in particular the isoflavones daidzein (DAI) and genistein (GEN). DAI and GEN are found at highest levels in soybeans and soy products, but are also present in numerous other edible plants and in fruits [3, 5]. Although estrogenic potency is rather weak by comparison to steroids [6], isoflavones may affect the endocrine system of humans when ingested in sufficient quantities and during stages of low endogenous hormone levels such as childhood and menopause [3, 4]. Because of potential health benefits (reviewed in [7, 8]), phytoestrogens have received considerable attention, also as an alternative to hormone replacement therapy. Yet, isoflavone-rich supplements due to their

Correspondence: Professor G. H. Degen, IfADo – Leibniz Research Centre for Working Environment and Human Factors at the TU Dortmund, Ardeystr. 67, D-44139 Dortmund, Germany
E-mail: degen@ifado.de
Fax: +49-231-1084-403

Abbreviations: DAI, daidzein; **DONALD, DO**rtmund **N**utritional and **A**nthropometric **L**ongitudinally **D**esigned; **EQ,** equol; **GEN,** genistein

estrogenic activity may present a risk in breast cancer survivors [9, 10], and their increased use is regarded with caution [8, 11].

Moreover, based on animal studies isoflavone phytoestrogens qualify as potential endocrine disruptors [3, 12, 13], and fetal exposure is documented in rodents [14, 15] and also in humans [16]. Of special interest are isoflavone exposures early in life, since infants on soy-based formula have a much higher intake of DAI and GEN than breast-fed infants or those receiving cow milk [8, 17]. This is also reflected in rather high blood [18], and about 500-fold higher isoflavone urine levels in infants on soy formula [19, 20]. While much attention has been paid to infants and soy isoflavone exposure in early life [11, 17], little is known about isoflavone exposure in childhood and adolescence in the general population although measures of long-term phytoestrogen exposure in relation to health outcome (*adverse* or *beneficial)* later in life are of interest.

Since the phytoestrogen metabolite equol (EQ) has a higher affinity for estrogen receptors than DAI, it has been proposed that biological effects from soy isoflavones may correlate with EQ-producing status of individuals, and that EQ excretion may serve as a biomarker for isoflavone effectiveness [21]. EQ is formed by the gut microflora from its precursor DAI, and the extent of this metabolism is apparently highly variable among populations and individuals, and also influenced by dietary components such as carbohydrates and fat [22, 23].

Overall, we know currently little about (i) age-related differences in isoflavone exposure in Western countries, and practically nil in contemporary cohorts of children or adolescents in Germany. This is the first study to examine this aspect. Moreover, since soy flour has been widely adopted in the preparation of numerous processed foods prominent in the Western diet in the past decades [24], it is also of interest to study (ii) possible long-term temporal changes in isoflavone exposure. We investigated these two longitudinal aspects by means of isoflavone biomarkers in 24-h urines of male and female German children and adolescents (age 6–18), participants of the **DO**rtmund **N**utritional and **A**nthropometric **L**ongitudinally **D**esigned (DONALD) Study, who have provided multiple samples throughout the entire study period (1985–2000). This design enabled also to address a third aspect, namely (iii) EQ excretion in our cohort over time.

2 Materials and methods

2.1 Study cohort

The present analysis is a project ancillary to the DONALD Study an ongoing, open-cohort study conducted at the Research Institute of Child Nutrition in Dortmund, Germany [25]. The DONALD study began in 1985, and has collected data on diet, growth, development and metabolism from infancy until adulthood. In brief, infants newly recruited each year, are first examined at the age of 3–6 months and from then on, detailed data on nutrition, growth, metabolism and health status are collected at regular intervals until adulthood. Moreover, from the age of 3 years on, the children are asked to collect a 24-h urine on the third day of the 3-day weighed dietary record. Body weight is assessed to the nearest 100 g with an electronic scale (Seca 753 E, Seca GmbH &KG, Hamburg, Germany) for subjects in standing position. Standing height is measured to the nearest 0.1 cm with a digital telescopic wall-mounted stadiometer. BMI was calculated using the formula weight/height^2 (kg/m^2). The study protocol has been approved by the Ethics Committee of the University of Bonn, and all examinations are performed with parental consent. Stored urine samples from this cohort were used for the present analyses.

2.2 Sample selection

The present sample was in first place selected from 84 healthy participants (age 3–18 years) of the DONALD Study with at least three complete 24-h urine samples ($n = 621$), who have previously been examined for a longitudinal study of melatonin production rates during growth [26]. Of those, only children and adolescents aged 6–18 years were included ($n = 76$, 445 urine samples). These urine samples were grouped according to the following three time periods: 1985–1989, 1990–1994 and 1995–2000. Fourteen additional DONALD participants ($n = 65$ urine samples) were selected (6 boys, 8 girls) in order to replenish the 1995–2000 time period and to assure a similar sex and age distribution across all three periods. Therefore, the current study population comprised 90 subjects (47 boys) with 510 urine samples.

2.3 Urinary collection and isoflavone measurements

Parents and children were carefully instructed on how to collect complete 24-h urine samples. All micturitions were stored immediately in preservative-free, Extran-cleaned 1 L plastic containers at $\leq -12^{\circ}$C before they were transported. At the research institute, the containers are stored at $\leq -20^{\circ}$C until analysis. For the purpose of this analysis, completeness of the 24-h urines was ascertained *via* the criteria, daily creatinine excretion rates > 0.1 mmol kg^{-1} d^{-1} [27]. Creatinine was measured by the Jaffé method with a creatinine analyzer (Beckman-2; Beckman Instruments, Fullerton, CA). Samples that were reported or found to contain incomplete micturitions were excluded from analysis. The urine samples were thawed immediately before analysis and were analyzed for the concentration of isoflavones (DAI, GEN and EQ) using a previously validated sample preparation and GC-MS method [28]. In brief,

urine aliquots (200 μL) were mixed with hydrolysis buffer and internal standards, then incubated overnight for hydrolysis of conjugates with pure β-glucuronidase and sulfatase enzyme. The aglycone analytes (DAI, EQ, GEN) were then extracted with solid-phase extraction cartridges. Eluates were evaporated and residues dissolved in derivatization reagent prior to GC-MS analysis; see [28] for all details. The limits of detection were 3.5 ng/mL for DAI, 5.3 ng/mL for GEN and 3.8 ng/mL for EQ (the gut bacterial metabolite of DAI). The 24-h excretion of isoflavones was calculated by multiplying the analyte concentrations with the 24-h urine volumes. The sum of urinary DAI, GEN and EQ excretion was used as marker of the overall daily isoflavone excretion.

2.4 Statistical analysis

All statistical tests were performed using SAS procedures (version 9.1, SAS Institute, Cary, NC). Descriptive data are given as mean ± SD or median with inter-quartile range when appropriate. Sex differences for anthropometric and urinary characteristics were tested by using unpaired *t*-test.

For the longitudinal analysis of time trends and age dependence of 24-h total isoflavone excretion, the mixed linear model PROC MIXED was used (Year of urine collection and age were both included as continuous variables). Models for age dependency were run separately in prepubertal/early pubertal (6–12 years) and pubertal children (13–18 years) adjusted for sex and year of urine collection. Models for time trends were each run for the three 5-year intervals (1985–1989, 1990–1994, 1995–2000) adjusted for sex and age. This model allowed for the inclusion of age covariates in the mean structure and repeated measurements in the same subjects. In contrast to other models, *e.g.* PROC GLM for balanced longitudinal data, the PROC MIXED model uses all the available data and not only the complete cases [29]. The level of significance was set at $p < 0.05$.

3 Results

Anthropometric and urinary characteristics of a subset of German children ($n = 90$) of the DONALD cohort are presented in Table 1. Boys included in the present analyses ($n = 47$) were 9.1 (±2.8) years old at the visit of the first urine collection. Girls included in the analyses ($n = 43$) were 9.6 (±2.7) years old at the visit of the first urine collection. Each individual in this subcohort of children and adolescents had provided at least three complete 24-h urines between 1985 and 2000. When stratified by sex, anthropometric data (weight, height, body surface, BMI and BMI-SDS) were not significantly different between boys and girls. Also urinary volume, body weight-related creatine output and isoflavone (DAI, EQ, GEN) levels were not significantly different between boys and girls. When isoflavone analyte concentrations (ng/mL) were converted into excretion rates

Table 1. Anthropometric and urinary characteristics of a sample of 90 healthy children and adolescents at the visit of the first urine collection (1985–2000) stratified by sex

	Boys $n = 47$[b)]	Girls $n = 43$[b)]	*P* for difference[a)]
Age (year)	9.1 ± 2.8[c)]	9.6 ± 2.7	NS
Year of birth	1979 ± 4	1978 ± 4	NS
Birth weight (g)	3439 ± 477	3354 ± 447	NS
Anthropometric data			
Weight (kg)	33.4 ± 14.1	34.5 ± 10.7	NS
Height (cm)	137.4 ± 18.7	140.3 ± 16.3	NS
BMI (kg/m^2)	16.9 ± 2.5	17.0 ± 2.0	NS
BMI-SDS[d)]	−0.02 ± 0.86	−0.05 ± 0.71	NS
Urinary data			
Urine volume (mL/day)	643 (448, 862)[e)]	601 (424, 844)	NS
Creatinine (mmol/kg weight)	0.19 ± 0.04	0.17 ± 0.04	NS
DAI concentration (ng/mL)	152 (71, 319)	106 (55, 202)	NS
DAI 24-h excretion (μg/day)	100 (40, 221)	61 (38, 107)	< 0.05
GEN concentration (ng/mL)	75 (42, 198)	51 (36, 97)	NS
GEN 24-hexcretion (μg/d)	51 (31, 130)	33 (26, 50)	NS
EQ concentration (ng/mL)	9.1 (5.8, 25.5)	7.4 (5.8, 10.5)	NS
EQ 24-h excretion (μg/d)	5.3 (3.5, 16.7)	4.7 (3.6, 7.6)	NS

a) Sex differences were tested with unpaired *t* test for continuous variables and chi-square test for categorical variables.
b) 18 boys and 10 girls have no measurable EQ and 3 boys and 3 girls have no measurable GEN.
c) Mean ± SD (all such values).
d) SDS, SD scores according to the German reference curve for BMI [43].
e) Median: 25th and 75th percentile in parentheses (all such values).

(μg/day) on the basis of each person's 24-h urine volume, the only significant difference was a lower DAI excretion in girls than in boys (Table 1). All subjects excreted DAI, and only 3 boys and 3 girls had no measurable GEN levels in urine. The DAI metabolite EQ was apparently formed in many but not in all children, with urines of 18 boys and 10 girls showing no measurable EQ levels.

Overall, the percentage of urines with detectable (>LOD 3.5 ng/mL) EQ levels was about 49%. EQ concentrations in urines from boys and girls of different ages at urine collection are depicted in Fig. 1, indicative of a wide range of EQ concentrations, with peak values of nearly 200 ng/mL in some samples. The longitudinal design of the DONALD study enabled us to monitor isoflavone and EQ excretion over time in individuals who had provided urines on several occasions. Examples of such profiles are given in Fig. 2, for a female with EQ in all urines collected between age 10 and 17 (Fig. 2A), a male with EQ in nearly all urines with variable levels between age 6 and 18 (Fig. 2B), and another male that is apparently a poor EQ excreter between age 6 and 11, but, a rather high EQ excreter from age 12 to 18 (Fig. 2C). Interestingly, the EQ levels were often unrelated to concurrent DAI and GEN levels reflecting high or low isoflavone intake. In other words, the extent of EQ formation was variable and apparently not only dependent on phytoestrogen precursor (DAI) ingestion.

Despite the observed intra- and inter-individual variability in EQ excretion (Figs. 1 and 2), it is obvious that this metabolite can account for an important fraction of the total urinary isoflavones. EQ as a component of total urinary biomarkers was thus included in a further analysis for possible age-related changes and secular trends of phytoestrogen isoflavone exposure in the DONALD cohort.

The results of urinary biomarker data, grouped according to age at sample collection, for children and adolescents between 6–10, 11–14 and 15–18 years are depicted in Fig. 3. There appeared to be small decline in DAI concentrations

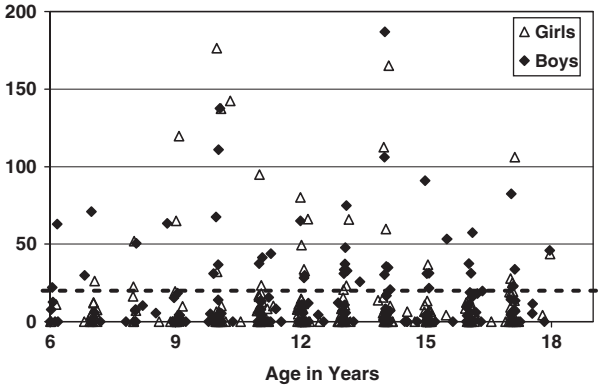

Figure 1. EQ concentrations (ng/mL) measured in 24-h urines of boys and girls from the DONALD cohort show a considerable variation, ranging from ≥3 ng/mL to nearly 200 ng/mL. The dotted line indicates the threshold of 20 ng/mL proposed to define an individual as EQ excreter [39].

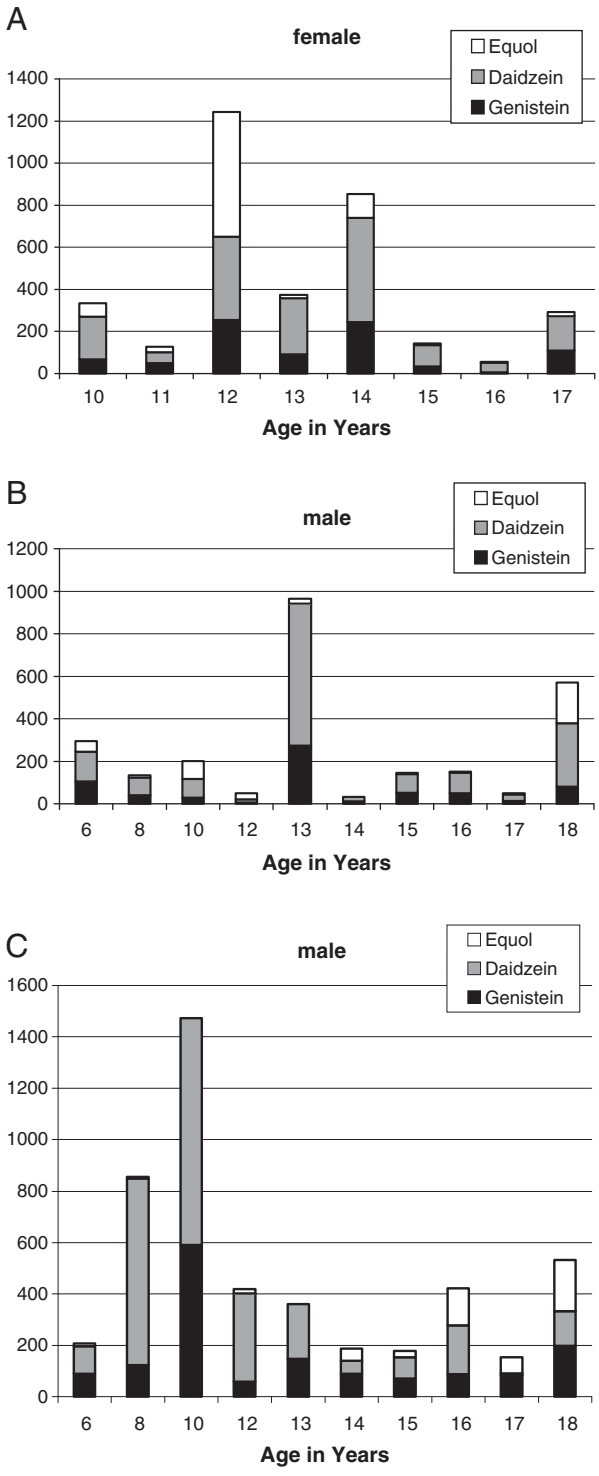

Figure 2. Urinary biomarker analysis of three individuals in different years. Profiles in 24-h urine samples from (A) a female between age 10 and 17, from (B) a male between 6 and 18, and from (C) another male between age 6 and 18 show variable excretion of EQ in different years of sampling, and apparently unrelated to the amount of its precursor DAI in that year.

with increasing age (Fig. 3A), yet a small increase in DAI daily excretion rates after adjusting analyte concentrations by 24-h urine volumes (Fig. 3B). The additional longitudinal

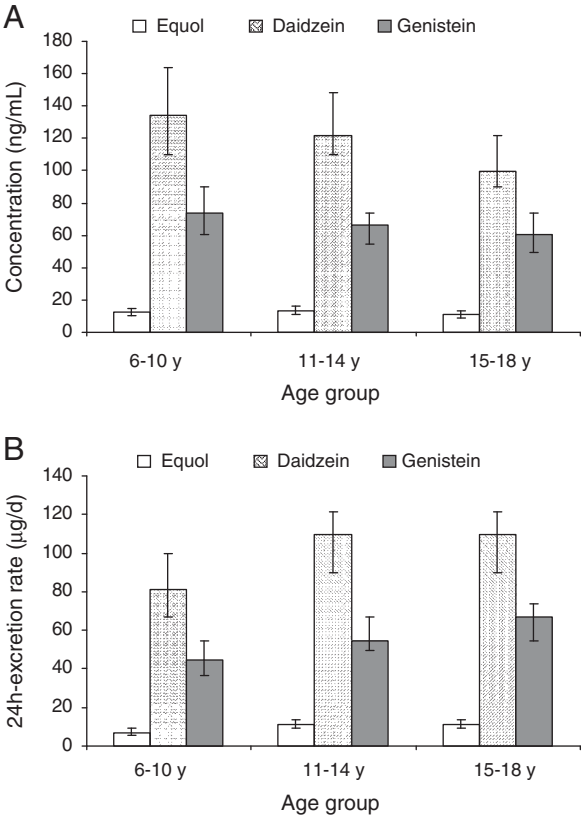

Figure 3. Isoflavone biomarkers in three age groups (6–10; 11–14 and 15–18 year) of the DONALD cohort: (A) The urinary concentrations (ng/mL) and (B) calculated excretion rates (µg/day) of DAI, EQ and GEN are given in each age group as median (95% confidence interval) of all urine samples with detectable levels of the analytes ($n = 129$, 202 and 173 for DAI; $n = 123$, 195 and 180 for GEN; $n = 92$, 155 and 142 for EQ, respectively). See text for further details.

analysis of age dependence of 24-h total isoflavone excretion (by mixed linear model PROC MIXED) revealed the following: The 24-h total isoflavone excretion increased across age among the prepubertal and early pubertal children (age 6–12 years), and was constant throughout adolescence (13–18 years) (Fig. 4). Overall, this biomarker data document frequent, albeit variable, dietary isoflavone phytoestrogen exposure in German children from infancy to adulthood.

Table 2 compiles the anthropometric and urinary characteristics of the 90 children stratified by urine collection cohort to investigate possible secular trends in phytoestrogen exposure between 1985 and 2000. As expected, the weight, height and mean urine volumes increase over the three sample collection periods (1985–1989, 1990–1994,

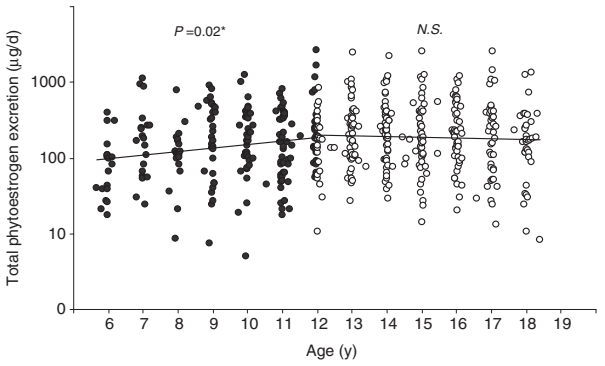

Figure 4. The total 24-h phytoestrogen isoflavonoid excretion increased across age among prepubertal and early pubertal children (age 6 to 12) and was constant throughout adolescence (age 13–18). *p refers to the significant level from PROC MIXED models adjusted for year of urine collection.

1995–2000) along with a shift in the mean age of participants (from 10 to 15 years) at urine collection. The median concentrations and 24-h excretion rates of DAI, EQ and GEN, depicted in Fig. 5, were similar in the urine samples of the three collection cohorts. Urinary isoflavone and metabolite levels (Fig. 5A) and excretion rates (Fig. 5B) showed a wide range of values among German adolescents probably related to variable dietary intake, yet no clear trend for changes in the collection cohorts between 1985 and 2000.

The additional longitudinal analysis of secular trends in total isoflavone excretion (by mixed linear model PROC MIXED) showed the following: There was a significant increase in 24-h total isoflavone phytoestrogen excretion from 1985 to 1989, no significant change between 1990 and 1994, and a significant decrease from 1995 to 2000 (Fig. 6).

4 Discussion

Our study provides novel and longitudinal data on urinary isoflavone biomarkers in German children and adolescents, participants of the DONALD study. The results demonstrate isoflavone exposures at quite variable levels in German children: Analyte concentrations in all urine samples from boys and girls cover the range reported previously in a small group of adults on typical German diet [28], and upper end concentrations resemble those of adults with soy intake [30, 31]. The predominant isoflavone excreted in children's urine was DAI, with median DAI concentrations being twice as high as those for GEN, and much higher than those for EQ (Figs. 3 and 5). This pattern, seen in all three age groups in our cohort is in accordance with results from other studies, such as spot urine analysis in adult women on Western diet, *viz* the Norfolk cohort of the European Prospective Investigation of Cancer and Nutrition, EPIC [32]. Their median urinary concentrations (DAI 88.1, GEN

Table 2. Anthropometric and urinary characteristics of the sample of 90 healthy children and adolescents (510 urine) stratified by urine collection cohort

	1985–1989	1990–1994	1995–2000
Age at measurement (ys)[a]	10.2 (8.0, 12.9)	13.0 (11.1, 15.0)	15.0 (13.0, 17.0)
n of urine measurements	164[b]	197[c]	149[d]
n of individuals (boys)	69 (35)	70 (35)	53 (28)
Year of birth	1977 (1975, 1979)	1979 (1976, 1981)	1982 (1980, 1984)
Anthropometric at urine measurements[a]			
Weight (kg)	37.8 (28.1, 49.0)	50.3 (38.0, 62.6)	56.3 (45.4, 64.1)
Height (cm)	146 (132, 161)	163 (147, 170)	169 (158, 175)
BMI (kg/m^2)	17.5 (16.0, 20.3)	19.1 (17.0, 21.6)	19.4 (17.9, 21.5)
BMI-SDS[e]	0.19 (−0.40, 0.63)	0.06 (−0.58, 0.67)	−0.05 (−0.77, 0.51)
Urinary data[a]			
Urine volume (mL/day)	660 (471, 885)	841 (637, 1140)	1040 (818, 1350)
Creatinine (mmol/kg weight)	0.17 (0.16, 0.20)	0.18 (0.16, 0.20)	0.18 (0.16, 0.21)
DAI concentration (ng/mL)	123 (59, 253)	137 (65, 270)	105 (43, 218)
DAI 24-h excretion (μg/day)	84 (42, 165)	117 (61, 218)	101 (47, 237)
GEN concentration (ng/mL)	63 (35, 150)	71 (40, 152)	54 (26, 116)
GEN 24-h excretion (μg/day)	45 (24, 96)	66 (30, 134)	53 (27, 129)
EQ concentration (ng/mL)	7.7 (5.3, 16.0)	9.0 (6.2, 21.9)	8.1 (6.1, 17.0)
EQ 24-h excretion (μg/day)[f]	4.9 (3.5, 10.9)	8.9 (5.1, 15.2)	9.0 (6.4, 16.4)

a) Median: 25th and 75th percentile in parentheses (all such values).
b) In cohort 1, EQ was not measurable in 48 urines and GEN in 12 urines.
c) In cohort 2, EQ was not measurable in 48 urines, GEN in 7 urines, and DAI in 3 urines.
d) In cohort 3, EQ was not measurable in 25 urines, GEN in 13 urines, and DAI in 5 urines.
e) SDS, SD scores according to the German reference curve for BMI [43].
f) 28 children have measurable EQ excretion in all urine samples; the other 62 children have measurable EQ excretion in at least one sample.

38.9 and EQ 2.6 ng/mL) were lower than the median urinary isoflavone concentrations in the DONALD cohort (Table 1). This probably reflects the low dietary isoflavone intake in the EPIC cohort of less than 0.5 mg/day, based on dietary records [32], while the recently calculated mean dietary isoflavone intake of German prepubertal children is 1.1 ± 4.7 mg/day [33]. The intake values reported from epidemiological studies among European omnivorous adults [34] are of similar magnitude. A recent overview [8] quotes total daily isoflavone intake in adults on typical Western diet in the range of 0.5 to ≈ 3 mg, and between 3–12 mg for vegetarians and soy consumers in European countries. By comparison, the daily isoflavone intake of adult Asians consuming traditional soy-rich food, and that of infants on soy-based formula is clearly higher, in the range of about 7–47 mg [8]. In an intervention study it was observed that isoflavones from soy food are more bioavailable in children than in adults [35]. Since this may affect comparisons of dietary intake and urinary biomarkers between adults and children, the further discussion will focus on studies in children.

A pilot study on urinary biomarkers [36] and a related cross-sectional study [37] in prepubertal girls in New York City revealed that – similar to the situation in German children – phytoestrogen exposure was common in the multiethnic US cohort. Dietary intake estimates reported for isoflavones were 1.7–2.2 mg [37], and geometric mean urinary levels for DAI, GEN and EQ in New York City girls were 112, 60.4 and 10.9 μg/L, respectively [36]. These urinary biomarker concentrations were similar to those reported for children in the NHANES 1999–2000 and 2000–2001 exposure survey [37], (U.S. Center for Disease Control and Prevention, Third National Report on Human Exposures to Environmental Chemicals, 2005, http://www.cdc.gov/exposurereport). The latter studies have all used a *cross-sectional* design to investigate isoflavone exposure in the general population.

To our knowledge, there are no *longitudinal* studies so far on isoflavone excretion in children and adolescents on Western style diet. Also, presently published data on the temporal variability of urinary isoflavone concentrations are limited, except for a study in 9-year-old girls [38] that reported a reasonable degree of temporal reliability over a short (6 months) period. Yet, most studies published so far include only a single urine sample that may or may not reflect an individual's long-term exposure level. Our results for urinary isoflavone biomarkers in the DONALD cohort shed some light on long-term phytoestrogen exposure of German children and adolescents as well as excretion of EQ in this cohort.

Measurable concentrations of EQ (≥ 3.5 ng/mL) were found in a large proportion of 24-h urines from our cohort, with no significant difference in median levels when stratified by sex (Table 1). EQ analysis in urines collected at

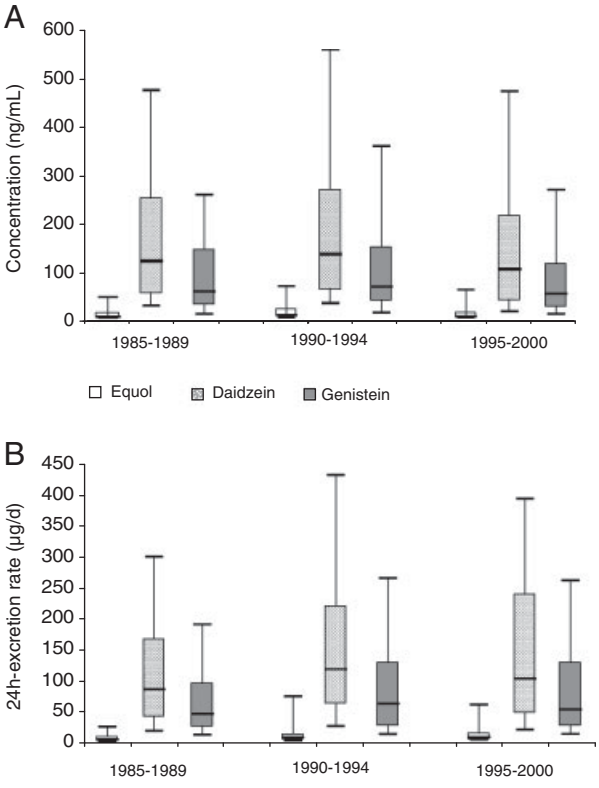

Figure 5. Isoflavone biomarkers in three sampling periods (1985–1989; 1990–1994 and 1995–2000) of the DONALD cohort: The (A) urinary concentrations (ng/mL) and (B) excretion rates (µg/day) for DAI, EQ and GEN in each period are given as median and pencentiles (5, 25, 75, 95%) of all urine samples with detectable levels of the analytes ($n = 164$, 194 and 144 for DAI; $n = 152$, 194 and 144 for GEN; $n = 116$, 149 and 124 for EQ, respectively). See text for further details.

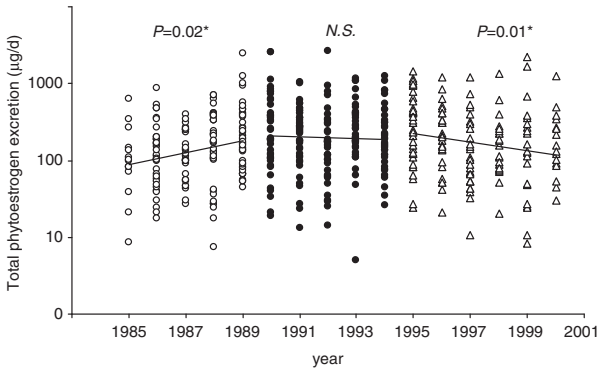

Figure 6. The 24-h total phytoestrogen isoflavonoid excretion showed a significant increase from 1985 to 1989, no significant change between 1990 and 1994, and a significant decrease from 1995 to 2000. $^*p$ refers to the significant level from PROC MIXED models adjusted for age.

different ages showed a wide range of concentrations in boys and girls (Fig. 1), and numerous samples with EQ levels ≥ 20 ng/mL, a threshold proposed to classify an individual as EQ excretor [39]. Studies with infants and young children indicate that the ability to produce EQ is not acquired until later in life. EQ excretion was rarely detected (only 5% >LOD = 3.3 ng/mL) in urine of soy formula fed US infants up to an age of about 1 year [20]. When isoflavone excretion was examined in UK infants and children from the ages of 4 months to 7 years [19], the frequency of EQ excretors across all age groups following soy challenge (about 20%) was clearly lower than reported for adults (about 35%) in the UK [22] or in the German (Berlin) cohort of the Isoheart intervention study (about 50%) [40].

In a study on isoflavone kinetics, a small UK cohort of premenopausal women received stable isotope-labeled DAI or GEN on three occasions, separated by a minimum of 2 wk. As 3 of 8 women (37.5%) who received [^{13}C]-DAI were found to excrete EQ on the first visit and the next two visits, this suggested that ''*once an equol producer, always an equol producer*'' [41]. Yet, whether this holds true for longer periods in life, and the question whether a person who is unable to produce EQ will ever be able to do so, remained unclear. In this regard long-term profiles of children and adolescents from the DONALD cohort who provided urines at different ages are of interest: The examples (Figs. 2A–C) illustrate that the excreted amount of EQ was variable, and not only dependent on isoflavone precursor (DAI) ingestion in that year. The longitudinal data indicate that efficacy of DAI conversion to EQ may vary within individuals over time. This could be due to changes in other dietary components that affect isoflavone metabolism in the gut and/or changes in their gut microflora [22, 23]. Moreover, dietary intake of EQ with milk may complicate an interpretation of biomarker results. Recently, organic skimmed Finnish cow milk was found to contain higher EQ concentrations than conventionally produced milk, with 411 ± 65 µg/L and 62 ± 16 µg/L milk, respectively [42]. Evidence that EQ ingested with milk is bioavailable and also excreted in urine comes from studies with infants whose gut microflora is not yet fully developed. EQ was present in plasma of all 4-month-old infants fed cows' milk formula, at higher levels than in soy-formula fed infants [18]. In line with this, urinary EQ was more frequently detected in US infants fed cow milk formula (22%) than in those fed soy formula (5%) [20]. In older children and adolescents, it will be more difficult to distinguish whether urinary EQ is due to dietary intake of EQ with milk and/or endogenous production from isoflavone precursor DAI in fruits and vegetables.

As both types of EQ exposure are important, the urinary EQ concentrations and excretion rates were included along with values for the other analytes to account for total isoflavone phytoestrogen biomarkers in our cohort (Figs. 4 and 6). Data for the separate analytes (DAI, EQ, GEN), when stratified by age groups (6–10, 11–14, 15–18 years; Fig. 3)

and by urine collection periods (1985–1989, 1990–1994, 1995–2000; Fig. 5), showed a wide range of values among German adolescents probably related to variable dietary intake, and a tendency for an increase with age, yet no clear trend for changes in the collection cohorts between 1985 to 2000. The urinary levels of DAI, EQ and GEN in our cohort are of similar magnitude as those reported in a large cross-sectional study with analysis of urine samples from 1999/2000 and 2000/2001 in the US population (Third National Report on Human Exposures to Environmental Chemicals, 2005, http://www.cdc.gov/exposurereport) and a pilot study in a multiethnic group of 9-year-old girls in New York City [37].

To our knowledge, this is the first longitudinal study on isoflavone exposure in children and adolescents, in Germany or elsewhere. While the relatively high socio-economic status of the DONALD Study participants compared with the German general population [25] can be regarded as a limitation of our study, it is nonetheless well suited to address the three aims stated in Section 1.

In conclusion, phytoestrogen excretion increased across age among the prepubertal and early pubertal children (age 6–12 years), and was constant throughout adolescence (13–18 years). There was an increase in the total isoflavone excretion from 1985 to 1989, no significant change between 1990 and 1994, and a significant decrease from 1995 to 2000, *i.e.* the analysis suggests no secular increase (1985–2000) in the exposure to isoflavone phytoestrogens among German children and adolescents. With regard to EQ, it is noteworthy that detection of this biomarker in urine was frequent in our cohort. Interestingly, in several individuals EQ excretion did not appear to be a constant trait over longer periods of time.

This analysis was funded by a grant from the Long-range Research Initiative (LRI) of the European Chemical Industry Council (Cefic). The DONALD Study is funded by the Ministry of Innovation, Science and Research of North Rhine Westphalia, Germany.

The authors have declared no conflict of interest.

5 References

[1] National Research Council, *Hormonally Active Agents in the Environment*. National Academy Press, Washington DC, 1999.

[2] Degen, G. H., Endokrine Disruptoren in Lebensmitteln. *Bundesgesundheitsblatt Gesundheitsschutz* 2004, *9*, 848–856.

[3] Committee-on-Toxicity, Phytoestrogens and health. London, UK: Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment, 2003, http://www.food.gov.uk/multimedia/pdfs/phytoreport0503.

[4] Cassidy, A., Potential risks and benefits of phytoestrogen-rich diets. *Int. J. Vitam. Nutr. Res.* 2003, *73*, 120–126.

[5] Ritchie, M. R., Cummings, J. H., Morton, M. S., Michael Steelm, C. *et al.*, A newly constructed and validated isoflavone database for the assessment of total genistein and daidzein intake. *Br. J. Nutr.* 2006, *95*, 204–213.

[6] Diel, P., Schmidt, S., Vollmer, G., *In vivo* test systems for the quantitative and qualitative analysis of the biological activity of phytoestrogens. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2002, *777*, 191–202.

[7] Cassidy, A., Albertazzi, P., Nielsen, I. L., Hall, W. *et al.,* Critical review of health effects of soybean phytoestrogens in post-menopausal women. *Proc. Nutr. Soc.* 2006, *65*, 1–17.

[8] Mortensen, A., Kulling, S. E., Schwarz, H., Rowland, I. *et al.*, Analytical and compositional aspects of isoflavones in food and their biologiocal effects. *Mol. Nutr. Food Res.* 2009, *53*, S266–S309.

[9] Block, K. I., Constantinou, A., Hilakivi-Clarke, L., Hughes, C. *et al.*, Point-counterpoint: soy intake for breast cancer patients. *Integr. Cancer Ther.* 2002, *1*, 90–100.

[10] Hu, S. A., Risks and benefits of soy isoflavones for breast cancer survivors. *Oncol. Nurs. Forum* 2004, *31*, 249–263.

[11] Eisenbrand, G., Isoflavones as phytoestrogens in food supplements and dietary foods for special medical purposes. Opinion of the Senate Commission on Food Safety (SKLM) of the German Research Foundation (DFG)-(shortened version). *Mol. Nutr. Food Res.* 2007, *51*, 1305–1312.

[12] Degen, G. H., Owens, J. W., in: Greim, H., Snyder, R. (Eds.), *Toxicology and Risk Assessment: A comprehensive Introduction*. Wiley, Chichester, UK, 2008, pp. 583–603.

[13] Jefferson, W. N., Padilla-Banks, E., Newbold, R. R., Disruption of the developing female reproductive system by phytoestrogens: genistein as an example. *Mol. Nutr. Food Res.* 2007, *51*, 832–844.

[14] Janning, P., Schuhmacher, U. S., Upmeier, A., Diel, P. *et al.*, Toxicokinetics of the phyto-estrogen daidzein in female DA/Han rats. *Arch. Toxicol.* 2000, *74*, 421–430.

[15] Doerge, D. R., Churchwell, M. I., Chang, H. C., Newbold, R. R., Delclos, K. B., Placental transfer of the soy isoflavone, genistein, following oral administration to Sprague–Dawley rats. *Reprod. Toxicol.* 2001, *15*, 105–110.

[16] Todaka, E., Sakurai, K., Fukata, H., Miyagawa, H. *et al.*, Fetal exposure to phytoestrogens – the difference in phytoestrogen status between mother and fetus. *Environ. Res.* 2005, *99*, 195–203.

[17] NTP Center for the Evaluation of Risks to Human Reproduction, Final CERHR Expert Panel Report on Soy Formula, January 15, 2010, http://cerhr.niehs.nih.gov/evals/genistein-soy/SoyFormulaUpdt/FinalEPReport_508.pdf.

[18] Setchell, K. D., Zimmer-Nechemias, L., Cai, J., Heubi, J. E., Exposure of infants to phyto-oestrogens from soy-based infant formula. *Lancet* 1997, *350*, 23–27.

[19] Hoey, L., Rowland, I. R., Lloyd, A. S., Clarke, D. B., Wiseman, H., Influence of soya-based infant formula consumption on isoflavone and gut microflora metabolite concentrations in

urine and on faecal microflora composition and metabolic activity in infants and children. *Br. J. Nutr.* 2004, *91*, 607–616.

[20] Cao, Y. A., Calafat, A. M., Doerge, D. R., Umbach, D. M. *et al.,* Isoflavones in urine, saliva, and blood of infants: data from a pilot study on the estrogenic activity of soy formula. *J. Expo. Sci. Environ. Epidemiol.* 2009, *19*, 223–234.

[21] Setchell, K. D., Brown, N. M., Zimmer-Nechemias, L., Brashear, W. T. *et al.*, Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am. J. Clin. Nutr.* 2002, *76*, 447–453.

[22] Rowland, I. R., Wiseman, H., Sanders, T. A., Adlercreutz, H., Bowey, E. A., 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.

[23] Yuan, J. H., Wang, J. H., Liu, X., Metabolism of dietary soy isoflavones to equol by human intestinal microflora – implications for health. *Mol. Nutr. Food Res.* 2007, *51*, 765–781.

[24] Burton, J. L., Wells, M., The effect of phytoestrogens on the female genital tract. *J. Clin. Pathol.* 2002, *55*, 401–407.

[25] Kroke, A., Manz, F., Kersting, M., Remer, T. *et al.*, The DONALD Study: history, current status and future perspectives. *J. Nutr.* 2004, *43*, 45–54.

[26] Griefahn, B., Bröde, P., Blaszkewicz, M., Remer, T., Melatonin production during childhodd and adolescence: a longitudinal study on the excretion of urinary 6-hydroxymelatonin sulfate. *J. Pineal Res.* 2003, *34*, 26–31.

[27] Remer, T., Neubert, A., Maser-Gluth, C., Anthropometry-based reference values for 24-h urinary creatinine excretion during growth and their use in endocrine and nutritional research. *Am. J. Clin. Nutr.* 2002, *75*, 561–569.

[28] Moors, S., Blaszkewicz, M., Bolt, H. M., Degen, G. H., Simultaneous determination of daidzein, equol, genistein and bisphenol A in humane urine by a fast and simple method using SPE and GC-MS. *Mol. Nutr. Food Res.* 2007, *51*, 787–798.

[29] Verbeke, G., Molenberghs, G., PROC MIXED *versus* PROC GLM. in: Verbeke G, Molenberghs G (Eds), *Linear Mixed Models for Longitudinal Data,* Springer, New York, NY 2000, pp. 119–120.

[30] Arai, Y., Uehara, M., Sato, Y., Kimira, M. *et al.,* Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. *J. Epidemiol.* 2000, *10*, 127–135.

[31] Atkinson, C., Skor, H. E., Fitzgibbons, E. D., Scholes, D. *et al.*, Overnight urinary isoflavone excretion in a population of women living in the United States, and its relationship to isoflavone intake. *Cancer Epidemiol. Biomarkers Prev.* 2002, *11*, 253–260.

[32] Grace, P. B., Taylor, J. I., Low, Y. L., Luben, R. N. *et al.*, Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European prospective investigation of cancer and nutrition – Norfolk. *Cancer Epidemiol. Biomarkers Prev.* 2004, *13*, 698–708.

[33] Cheng, G., Remer, T., Blaszkewicz, M., Degen, G. H., Buyken, A. E., Relation of isoflavones and fiber intake in childhood to the timing of puberty. *Am. J. Clin. Nutr.* 2010, *92*, 1–9.

[34] van Erp-Baart, M. A., Brants, H. A., Kiely, M., Mulligan, A. *et al.*, Isoflavone intake in four different European countries: the VENUS approach. *Br. J. Nutr.* 2003, *89*, S25–S30.

[35] Halm, B. M., Ashburn, L. A., Franke, A. A., Isoflavones from soy foods are more bioavailable in children than adults. *Br. J. Nutr.* 2007, *98*, 998–1005.

[36] Wolff, M. S., Teitelbaum, S. L., Windham, G., Pinney, S. M. *et al.*, Pilot study of urinary biomarkers of phyto-estrogens, phthalates, and phenols in girls. *Environm. Health Perspect.* 2007, *115*, 116–121.

[37] Wolff, M. S., Britton, J. A., Boguski, L., Hochman, S. *et al.*, Environmental exposures and puberty in inner-city girls. *Environ. Res.* 2008, *107*, 393–400.

[38] Teitelbaum, S. L., Britton, J. A., Calafat, A. M., Ye, X. *et al.,* Temporal variability in urinary concentrations of phthalate metabolites, phytoestrogens and phenols among minority children in the United States. *Environ. Res.* 2008, *106*, 257–269.

[39] Setchell, K. D., Cole, S. J., Method of defining equol-producer status and its frequency among vegetarians. *J. Nutr.* 2006, *136*, 2188–2193.

[40] Talbot, D. C., Ogborne, R. M., Dadd, T., Adlercreutz, H. *et al.,* Monoclonal antibody-based time-resolved fluorescence immuno-assays for daidzein, genistein, and equol in blood and urine: application to the Isoheart intervention study. *Clin. Chem.* 2007, *53*, 748–756.

[41] Setchell, K. D., Faughnan, M. S., Avades, T., Zimmer-Nechemias, L. *et al.*, Comparing the pharmacokinetics of daidzein and genistein with the use of ^{13}C-labeled tracers in premenopausal women. *Am. J. Clin. Nutr.* 2003, *77*, 411–419.

[42] Hoikkala, A., Mustonen, E., Saastamoinen, I., Jokela, T. *et al.*, High levels of equol in organic skimmed Finnish milk. *Mol. Nutr. Food Res.* 2007, *51*, 782–786.

[43] Kromeyer-Hauschild, K., Wabitsch, M., Kunze, D., Geller, F. *et al.*, Perzentile für den Body-mass-Index für das Kindes- und Jugendalter unter Heranziehung verschiedener deutscher Stichproben. *Monatsschr. Kinderheilkd.* 2001, *149*, 807–818.

