Short communication

High concordance of daidzein-metabolizing phenotypes in individuals measured 1 to 3 years apart

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Particular intestinal bacteria are capable of metabolizing the soya isoflavone daidzein to equol and/or *O*-desmethylangolensin (*O*-DMA), and the presence of these metabolites in urine after soya consumption are markers of particular intestinal bacteria profiles. Prevalences of equol producers and *O*-DMA producers are approximately 30–50 % and 80–90 %, respectively, and limited observations have suggested that these daidzein-metabolizing phenotypes are stable within individuals over time. Characterizing stability of these phenotypes is important to understand their potential as markers of long-term exposure to particular intestinal bacteria and their associations with disease risk. We evaluated concordance within an individual for the equol-producer and *O*-DMA-producer phenotypes measured at two time points (T1, T2), 1–3 years apart. Phenotypes were ascertained by analysing equol and *O*-DMA using GC-MS in a spot urine sample collected after 3 d soya (source of daidzein) supplementation. In ninety-two individuals without recent (within 3 months before phenotyping) or current antibiotics use, 41 % were equol producers at T1 and 45 % were equol producers at T2, and 90 % were *O*-DMA producers at T1 and 95 % were *O*-DMA producers at T2. The percentage agreement for the equol-producer phenotype was 82 and for the *O*-DMA-producer phenotype was 89. These results indicate that these phenotypes are stable in most individuals over time, suggesting that they provide a useful biomarker for evaluating disease risk associated with harbouring particular intestinal bacteria responsible for, or associated with, the metabolism of the soya isoflavone daidzein.

Equol: *O*-Desmethylangolensin: Soya: Daidzein

The soya isoflavone daidzein is metabolized to equol and *O*-desmethylangolensin (*O*-DMA) by particular intestinal bacteria. Although the specific bacteria responsible have not yet been definitively identified, there are several lines of evidence from *in vitro* and animal studies to indicate that intestinal bacteria, and not endogenous host metabolism, are responsible for this conversion (Chang & Nair, 1995; Blair *et al.* 2003; Bowey *et al.* 2003; Atkinson *et al.* 2004). For example, it has been observed that, *in vitro*, microbiota in faeces from equol producers can convert daidzein to equol, whereas microbiota from non-producers does not (Chang & Nair, 1995; Atkinson *et al.* 2004) and *in vivo*, that germ-free animals do not produce equol (Bowey *et al.* 2003). Equol and *O*-DMA can be absorbed from the gastrointestinal tract into host circulation and excreted in urine. Thus, urinary excretion of equol and *O*-DMA are markers of particular

intestinal bacterial profiles. Approximately 30–50 % of individuals harbour the bacteria capable of producing equol (equol producers) and 80–90 % of individuals harbour the bacteria capable of producing *O*-DMA (*O*-DMA producers; reviewed in Atkinson *et al.* 2005). Because intestinal bacteria are involved in hormone metabolism in the gut (Adlercreutz *et al.* 1976; Lombardi *et al.* 1978; Järvenpää *et al.* 1980), the variability in bacterial daidzein metabolism may be associated with hormone-related disease risk; we review this in detail elsewhere (Atkinson *et al.* 2005).

Observations from a study of Japanese men suggest that these daidzein-metabolizing phenotypes are stable in individuals over time. In forty men, 85 % retained their equol-producer phenotype when measured at two time points approximately 1·5 years apart (Akaza *et al.* 2004); the *O*-DMA-producer phenotype was not evaluated. The objective of the present study was to evaluate

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within-individual concordance of equol-producer and *O*-DMA-producer phenotypes measured at two time points, 1–3 years apart, in a population of men, women and children living in the USA. Understanding the stability of these phenotypes over time will provide insight into their importance as markers of long-term exposure to particular intestinal bacteria.

Materials and methods

Individuals who had previously participated in a family study of daidzein metabolism (Frankenfeld *et al.* 2004*a*) were approached to participate in a follow-up study. In order to achieve our target sample of 100 individuals, 182 individuals were mailed approach letters and consent forms. Urine samples were received from 112 of the 122 individuals who consented to participate in the follow-up. The Institutional Review Board at Fred Hutchinson Cancer Research Center approved all procedures and informed, written consent was obtained from all participants.

Participants followed the same protocol of soya consumption and urine collection as described in the parent study (Frankenfeld *et al.* 2004*a*). Briefly, each participant supplemented his/her usual diet with a soya food item once per d on three consecutive days. On the morning of the fourth day, each participant collected a first-void urine sample (50–80 ml). Urinary isoflavones are stable at room temperature for 14 d (Frankenfeld *et al.* 2004*a*), which enabled participants to mail urine samples along with completed questionnaires to Fred Hutchinson Cancer Research Center. When a urine sample was received in the laboratory, it was aliquoted and stored at −20°C until analysis for isoflavonoids.

Participants completed a questionnaire that included questions about current weight and height, current antibiotics use, antibiotics use in the prior 3 months, and changes to diet and antibiotics use since they had participated in the parent study.

Urinary isoflavonoids (equol, *O*-DMA, daidzein and genistein) were analysed by the same method as used in the parent study. Urine samples were extracted with diethyl ether (Heinonen *et al.* 1999) and analysed for isoflavonoids by GC-MS as described elsewhere (Frankenfeld *et al.* 2004*b*). Given the sensitivity of the assay, urine concentrations less than 182 nmol/l (44 ng/ml in urine) of equol and 170 nmol/l (44 ng/ml in urine) of *O*−DMA were considered below the level of quantification. Equol and *O*-DMA producers were defined as individuals with any concentration greater than the level of quantification of equol and *O*-DMA, respectively.

Prior to data analysis, ten individuals were excluded because daidzein and genistein concentrations were below the level of quantification suggesting non-compliance with soya consumption (*n* 4), or questionnaire information was incomplete (*n* 4), or we were unable to match the urine sample with the questionnaire because of suspected kit swapping between family members (*n* 2).

For the main analysis of phenotype concordance between the parent study (T1) and the follow-up study (T2), individuals without current or recent (within the 3 months prior to phenotyping at T2) antibiotics use were analysed. A separate analysis was conducted including all individuals. Percentage agreement was defined as the number of concordant individuals divided by the total number of individuals multiplied by 100. The κ statistic, a measure of agreement that corrects for agreement observed by chance defined as $\kappa =$ (observed − expected proportion in agreement)/ (1 − expected proportion in agreement) (Thompson & Walter, 1988), and its standard error were calculated using Stata 8.2 (Stata Corporation, College Station, TX, USA). Frequency tables were generated to explore potential factors associated with phenotype discordance.

Results

Percentage agreement between T1 and T2 for the equol-producer phenotype was 81 (κ 0·64, SE 0·10) and for the *O*-DMA-producer phenotype was 89 (κ 0·27, SE 0·10; Table 1). Including individuals with current and recent antibiotics use did not markedly alter the percentage agreement: 81 for the equol-producer phenotype (κ 0·61, SE 0·10) and 86 for the *O*-DMA-producer phenotype (κ 0·22, SE 0·09).

There was no apparent relationship between urinary equol and *O*-DMA concentrations with phenotype concordance. Equol concentrations in producers had wide variation, but were overall similar in equol-producing concordant individuals (T1: mean 4·3 µg/mg creatinine (Cr) (SD 3·6), T2: mean 3·7 µg/mg Cr (SD 2·2)) and discordant individuals who were producers at T1 but not T2 (T1: mean 2·3 µg/mg Cr (SD 4·0)) and discordant individuals who were producers at T2 but not T1 (T2: mean 5·8 µg/mg Cr (SD 5·9)). *O*-DMA concentrations were also similar in *O*-DMA-producing concordant individuals (T1: mean 2·1 µg/

Table 1. Equol-producer and *O*-desmethylangolensin (*O*-DMA)-producer phenotypes in ninety-two individuals measured at two time points (T1 and T2), 1–3 years apart, who were not taking antibiotics or had not used antibiotics in the 3 months prior to phenotyping at either measurement

(Values are *n* with % of the total in parentheses)

		Phenotype at T2		
		Equol producer	Equol non-producer	Totals
Equol-producer phenotype				
Phenotype at T1	Equol producer	31 (34)	10 (11)	41 (45)
	Equol non-producer	7 (8)	44 (47)	51 (55)
		38 (41)	54 (59)	92 (100)
		O-DMA producer	*O*-DMA non-producer	
O-DMA-producer phenotype				
Phenotype at T1	*O*-DMA producer	80 (87)	7 (8)	87 (95)
	O-DMA non-producer	3 (3)	2 (2)	5 (5)
		83 (90)	9 (10)	92 (100)

μg Cr (SD 2·3), T2: 3·6 μg/mg Cr (SD 3·1)) and discordant individuals who were producers at T1 but not T2 (T1: mean 1·8 μg/mg Cr (SD 2·1)) and discordant individuals who were producers at T2 but not T1 (T2: mean 2·7 μg/mg Cr (SD 3·1)).

Sex, race, age, change in weight, antibiotics use since participating in the parent study, dietary change and time between phenotyping measurements did not appear to be strongly associated with concordance for either phenotype (data not shown).

Among two individuals with current antibiotics use at T2, one was concordant for equol-producer phenotype and one was concordant for *O*-DMA-producer phenotype. Among eight individuals who reported antibiotics use in the 3 months prior to T2 phenotyping, four were concordant for equol-producer phenotype and six were concordant for *O*-DMA-producer phenotype. Among seventeen individuals who reported antibiotics use since participation in the parent study but not within the 3 months prior to phenotyping, twelve were concordant for equol-producer phenotype and thirteen were concordant for *O*-DMA-producer phenotype.

Discussion

We observed a high degree of agreement between equol-producer and *O*-DMA-producer phenotypes within an individual over a 1–3-year period. While we know of no studies that have measured the stability of the *O*-DMA-producer phenotype over time, our observations for the equol-producer phenotype are similar to those of Akaza *et al.* (2004). In their study of forty Japanese men, 85 % of the men retained their equol-producer phenotype, based on serum equol concentrations, which were measured twice between 144 and 616 d apart. Our observations are also consistent with several small studies of intestinal bacterial profiles in adults and in infants post-weaning, in which the presence of particular bacterial profiles, as characterized by molecular techniques, appear relatively stable over time (Zoetendal *et al.* 1998; Favier *et al.* 2002).

The stability of these phenotypes over time is an important consideration for evaluating associations between these daidzein-metabolizing phenotypes and disease risk and for evaluating these phenotypes as effect modifiers of associations between soya intake and disease risk. Because concordance is not perfect over time, a degree of misclassification is expected when phenotypes are ascertained at only one time point. Based on assumptions of non-differential misclassification and that the phenotypes have been measured at T1 and T2 with equal sensitivity, equal specificity and have independent error probabilities, we can roughly calculate the expected magnitude of bias towards the null of the risk estimate. Using κ, an approximation of the odds ratio attenuation can be calculated using the formula $OR_O = (OR_T - 1)\kappa + 1$, as described by Thompson & Walter (1988), where OR_O is the risk estimate that would be observed in the population given the true risk (OR_T) and degree of misclassification (κ). If we assume, for example, that the true risk of disease in equol and *O*-DMA non-producers is 50 % greater than the risk in equol and *O*-DMA producers ($OR_T = 1.50$), we would expect to observe $OR_O = 1.32$ for the equol-producer phenotype and $OR_O = 1.14$ for *O*-DMA-producer phenotype. These estimates suggest that associations of these phenotypes and disease risk are detectable despite the potential for some misclassification. However, as illustrated by this odds ratio attenuation, detection of association of the *O*-DMA-producer phenotype with disease

may be more difficult because most individuals are *O*-DMA producers. Potential misclassification should be considered when estimating the number of study participants needed.

We observed that some individuals were phenotype-discordant between the two phenotyping measurements. Discordance between the two phenotyping measurements did not appear to be associated with demographic factors, diet, urinary equol and *O*-DMA concentrations or antibiotics use. In an *in vitro* study (Atkinson *et al.* 2004), we observed that antibiotics altered the equol-producing and *O*-DMA-producing capabilities in faecal samples from some individuals but not all individuals, suggesting that the bacteria responsible for these phenotypes vary between individuals. It is possible that individuals with particular daidzein-metabolizing bacteria are more likely to become discordant over time, but this is not possible to evaluate until the bacteria responsible for the phenotypes are definitively identified.

There are two notable limitations of the present study. One, the time interval between the two measurements was relatively short; phenotype concordance over longer periods of time should be evaluated in future studies. Two, the two urine samples for each individual were analysed by GC-MS at different times. For the comparisons presented in this study, although inter-batch variation is generally larger than within-batch variation, we do not believe that analysing an individual's two samples separately biased the results, because GC-MS analyses were conducted using the same technician and protocol at both time points.

The high degree of concordance observed in this study suggests that these phenotypes are useful as biomarkers of exposure to intestinal bacteria responsible for, or associated with, daidzein metabolism. Further work is needed to evaluate concordance over longer periods of time and to characterize factors associated with discordance over time.

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