

Letters to the Editor

Correspondence re: L. R. Kidd *et al.*, Urinary Excretion of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in White, African-American, and Asian-American Men in Los Angeles County. *Cancer Epidemiol. Biomark. Prev.*, 8: 439–445, 1999

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Dietary meat intake has been associated with an increase of cancer risk for a number of organ sites including colon, breast, and pancreas. Recent studies point towards the possible role of HAAs² formed upon heating of meat. These have been shown to be genotoxic *in vitro* and carcinogenic in rodents albeit at rather high concentrations.

To estimate the role of these HAAs, Kidd *et al.* (1) and others (2–4) published biomonitoring studies evaluating the quantitative analysis of urinary levels of HAAs or their respective metabolites as a measure of the intake of HAAs. A more indirect approach has been the measurement of urinary mutagenic activity (5, 6).

As for most urinary biomarkers in toxicology and pharmacology, the measurement of absolute values in (repeated) 24 h urine samples appears to be the most reliable method. It has been shown (2, 4) that renal excretion of HAAs is complete with 12 h after ingestion.

Because collection of 24 or 12 h urinary samples is difficult, and these are often unavailable, normalizing of urine for creatinine has been used to allow comparison of individuals, thus excluding variations in urinary flow. Levels of creatinine excretion are regarded as a measure of renal function, and creatine catabolism and creatinine clearance are relatively constant (7).

Kidd *et al.* (1) analyzed urinary samples from different ethnic groups and were unable to observe a correlation of data from a dietary questionnaire with urinary levels of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine normalized for creatinine.

However, considerable amounts of creatinine are ingested with a meal of meat or fish that may contain 2–4 mg creatine/g wet weight, with creatinine being produced from creatine when meat is cooked. A postprandial increase in creatinine plasma levels of up to 65% is observed. Similarly, urinary creatinine levels are dependent on dietary intake of meat (8, 9).

Furthermore, creatinine has been shown to be a precursor of the formation of mutagenic HAAs with an aminoimidazo moiety such as 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoline, 2-amino-3,8-dimethyl-3H-imidazo[4,5-*f*]quinoline, or 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. Indeed, HAA formation is dependent on creatinine concentrations in model systems (10). It has been shown that HAA levels in fried meat increase with cooking time and cooking temperature (11).

Therefore, systematic errors would be introduced in a

study comparing patients with low intake of heavily cooked meat and patients with high intake of lightly browned meat: both groups may have similar HAA intake. If urinary levels of HAAs (and metabolites) or excreted mutagenicity is considered on a 24-h basis, both would be similar. If HAA levels are normalized for creatinine, patients consuming high amounts of lightly cooked meat (and thus with higher urinary creatinine levels) will appear to have a lower intake of HAA.

This effect may be even more pronounced when limited urine samples are collected, which may be within a few hours after a meat meal, rather than 24 h urine samples.

Similarly, in a study comparing urinary levels of HAAs (or mutagenicity) before and after a meal of fried meat, normalizing for creatinine content will diminish a possible postprandial increase of these biomarkers.

Therefore, one has to be cautious to normalize urinary biomarkers to creatinine levels in urine. It appears to be inappropriate to normalize excretion of HAAs to creatinine levels because this calculation may lead to spuriously low values.

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² The abbreviation used is: HAA, heterocyclic aromatic amine.

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Reply

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Division of Bioengineering and Environmental Health, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 [L. R. K., W. G. S., J. S. W., P. L. S., S. R. T.], and Kenneth Norris, Jr., Comprehensive Cancer Center, University of Southern California, Los Angeles, California 90033 [M. C. Y., R. K. R., B. E. H.]

In two studies of HAA² exposure in humans (1, 2), we expressed urinary HAA levels in units of creatinine due to the fact that 8 h urine samples were being analyzed. In his letter, Dr. W. Pfau takes issue with this approach, arguing that individual creatinine values are influenced by recent meat consumption. He presents a hypothetical example of two individuals with similar levels of HAA exposure: one (subject A) with relatively low intake of heavily cooked meat; and (b) the other (subject B) with a higher intake of lightly browned meat. Our method of adjustment would create a spurious difference in HAA excretion level between the two subjects in that subject B will show a lower HAA value than subject A.

We have reanalyzed the data sets in the studies cited above using the actual values of urinary HAA excretion (*i.e.*, without creatinine adjustment; all units in pg/ml). Our results show that among whites and Asian Americans, PhIP values, with or without adjustment, are quite similar; in whites, the unadjusted mean is 1.14, whereas the adjusted mean is 1.18. In Asian Americans, the unadjusted mean is 3.12, whereas the adjusted mean is 3.33. This is not the case in the African Americans; the unadjusted mean is 28% higher than the adjusted mean (4.29 *versus* 3.36). The same holds true for MeIQx; the unadjusted

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²The abbreviations used are: HAA, heterocyclic amine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline.

Table 1 Heterocyclic aromatic amines in the study population

HAA means	African Americans	Asian Americans	Whites
PhIP	4.29	3.12	1.14
MeIQx	5.07	2.70	1.21
PhIP + MeIQx	10.55	6.70	2.60

mean in African Americans is 34% higher than the adjusted mean (5.07 *versus* 3.78). Based on unadjusted means, African Americans clearly possess the highest levels of HAAs, with intermediate and lowest values in Asian Americans and whites, respectively. These data of the unadjusted HAA means among the three groups are given in Table 1. Our results show that with or without adjustment, HAA values are statistically different across the three racial/ethnic groups.

Adjustment has relatively little influence on the interrelationship between PhIP and MeIQx values within individuals. With adjustment, we found perfect concordance among the subjects to be 47% (Table 3 in Ref. 2). Without adjustment, the degree of perfect concordance is slightly lower (41%). However, extreme discordance also decreased from 8% based on adjusted values to 5% using unadjusted values.

With or without adjustment, there is no association between urinary PhIP level and intake frequencies of various meats (Table 4 in Ref. 2). Also, with or without adjustment, there are no associations between urinary PhIP level and NAT2 phenotype, cigarette smoking, and intake of selected types of vegetables. Without adjustment, the association between MeIQx excretion and intake frequencies of various meats is in accord with the earlier study (Table 2 in Ref. 1), showing a relatively higher MeIQx exposure in frequent consumers of pork (including bacon), a lower MeIQx exposure in frequent consumers of pork (including bacon), and a lower MeIQx exposure in frequent consumers of chicken.

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Correspondence re: S. A. Khan *et al.*, The Normal Breast Epithelium of Women with Breast Cancer Displays an Aberrant Response to Estradiol. *Cancer Epidemiol. Biomark. Prev.*, 8: 867–872, 1999

Letter

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S. A. Khan *et al.* (1) in their case-control study on serum estradiol and progesterone levels and expression of estrogen and progesterone receptors and epithelial proliferation observed an inverse correlation of serum estradiol with estrogen receptor only in control women and a direct association of estradiol with progesterone receptor only in cases. Furthermore, they did not find association of both serum estradiol and progesterone with epithelial proliferation in both cases and controls.

The report addresses a very relevant issue for epidemiological and clinical studies linking hormones to breast cancer etiology; however, the authors did not consider important methodological issues related to control of biological hormone variability that may explain such controversial results.

The first issue concerns the control of two relevant sources of hormone biological variability: (a) hormone fluctuations during the menstrual cycle; and (b) circadian rhythm. In the authors' study, blood samples were collected before surgery or during the procedure; however, there is no mention if and/or how the two sources of variability were taken into consideration. Production of hormones is characterized by strong fluctuations during the menstrual cycle (in premenopausal women). Relevant circadian variation in serum hormone concentration has been detected in premenopausal and postmenopausal women between early, late morning, and early afternoon (2). Adjustment for phase of menstrual cycle and exact time at which blood was drawn (not mentioned in the authors' paper) could help in controlling for this possible source of error (3). Furthermore, hormone fluctuation according to ovarian cycle may also potentially affect hormone receptor expression.

The second issue concerns the technical variability in hormone, hormone receptor, and epithelial proliferation determinations. The authors did not mention the size of the error in measurement for all biomarkers, and they did not address how it was taken into consideration in the study design or in the study analysis (4).

In conclusion, study of the relation between hormones and diseases is made particularly difficult by numerous sources of variability. Careful attention should be paid to these before conclusive statements can be made regarding presence or absence of potential associations.

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Reply

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Dr. P. Muti draws attention to several important features relating to studies of hormones and disease etiology. Although it is true that diurnal and menstrual cycle fluctuations exist in the levels of many hormones, our study was directed at the relationships between the levels of hormones and receptor expression in breast epithelium. Our major concern therefore was that the blood samples for hormone assays be obtained as close as possible to the time of acquisition of the breast tissue samples. All samples were drawn either in the operating room or within 1 h prior to the patient entering the operating room (generally between 9 a.m. and 2 p.m.). We did not attempt to compare hormone levels between groups of patients and therefore do not feel that diurnal fluctuations would be relevant to the major point of analysis in our study (*i.e.*, a comparison of hormones with simultaneously obtained tissue samples).

With regard to menstrual cycle variations in the levels of estradiol and progesterone, there is undoubtedly large variation in the levels of these hormones through the menstrual cycle. In previous studies of breast epithelial hormone receptor expression and proliferation, menstrual cycle phase has been used as a surrogate for hormone levels. However, there is considerable interindividual variation in estradiol and progesterone levels within the same phase of the menstrual cycle. For example, in our population, the mean serum estradiol level was 135 pg/ml in women who were in the second week of a standardized 28-day cycle, but the range was 44–310 pg/ml. Because our study was designed to directly measure hormone levels in each individual rather than use menstrual cycle phase as a surrogate

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for expected hormone levels within each phase, we felt that further adjustment for menstrual cycle phase was not necessary.

Lastly, Dr. P. Muti raises the very germane issue of variability in hormone and receptor determinations. Hormone assays were performed in batches as they were acquired chronologically. Duplicate serum samples were included in different runs, and the variation between duplicates was less than 10%. Immunohistochemical assays for receptors and proliferation were not repeated, but repeat counts were performed by at least

two observers. Although there was some variation between observers (up to 20%), the rank correlation between observers was good.

We would like to thank Dr. P. Muti for her comments and again point out that our main purpose was to explore case-control differences in the *relationships* between parameters (*e.g.*, hormones and receptors) rather than differences in individual parameters, as has been done in earlier studies. We feel that additional such studies of organ responsiveness will yield further insights into disease etiology.