

# 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<sup>1</sup>

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## Abstract

Meats, such as beef, pork, poultry, and fish, cooked at high temperatures produce heterocyclic aromatic amines, which have been implicated indirectly as etiological agents involved in colorectal and other cancers in humans. This study examined the urinary excretion of a mutagenic/carcinogenic heterocyclic aromatic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), among 45 African-American, 42 Asian-American (Chinese or Japanese), and 42 non-Hispanic white male residents of Los Angeles who consumed an unrestricted diet. Total PhIP (free and conjugated) was isolated from overnight urine collections, purified by immunoaffinity chromatography, and then quantified by high-pressure liquid chromatography combined with electrospray ionization mass spectrometry. Geometric mean levels of PhIP in Asian-Americans and African-Americans were approximately 2.8-fold higher than in whites. The urinary excretion levels of PhIP were not associated with intake frequencies of any cooked meat based on a self-administered dietary questionnaire, in contrast to our earlier finding (Ji *et al.*, *Cancer Epidemiol. Biomark. Prev.*, 3: 407–411, 1994) of a positive and statistically significant association between bacon intake and the urinary level of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) among this same group of study subjects. Although there is a statistically significant association between urinary levels of PhIP and MeIQx (2-sided  $P = 0.001$ ), 10 subjects (8%) displayed extreme discordance between urinary PhIP and MeIQx levels.

Several factors, including variable contents of heterocyclic aromatic amines in food, enzymic and interindividual metabolic differences, and analytical methodology determine the degree of concordance between the urinary excretion levels of PhIP and MeIQx. Accordingly, urinary excretion levels of a single heterocyclic aromatic amine can only serve as an approximate measure of another in estimating exposure to these compounds in humans consuming unrestricted diets.

## Introduction

Humans are exposed to a number of mutagenic and carcinogenic HAAs<sup>3</sup> formed in the low parts per billion in meats prepared under normal heating conditions (1–3). The most abundant HAA detected in the browned surfaces of cooked meat (*i.e.*, beef, poultry, fish, and pork) is PhIP. Cooking methods such as frying, grilling, and broiling yield concentrations of PhIP ranging from 0.56–69.2 ng/g of cooked meat (2). In addition, significant amounts of HAAs including PhIP are found in the corresponding pan residues obtained from fried/grilled meats (4, 5). Style of preparation and variation of cooking methods will lead to differing levels of HAAs. For example, appreciably high levels of PhIP compared with other HAAs are measured in high-temperature cooked chicken (6), whereas reduced amounts of PhIP but not MeIQx are produced if meats are marinated before cooking (7). Thus, daily human exposure to HAAs can vary greatly and is dependent on various factors, including food preferences and cooking practices. It has been estimated that the total dietary intake of HAAs per person can range up to tens of micrograms per day (8).

Unlike other HAAs, PhIP is not a hepatocarcinogen in rodents but does induce carcinomas of the mammary gland, large intestine, and prostate in F344 rats (9, 10) and lymphomas in CDF1 mice (11). It is presently under examination for carcinogenicity in nonhuman primates (12). Layton *et al.* (13), in assessment studies based on rodent carcinogenicity data and market-based meat surveys, estimated the percentages of total incremental cancer risk associated with dietary consumption of five principal HAAs among North Americans and suggested that PhIP may play a vital role in human carcinogenesis.

A number of epidemiological studies have implicated foods containing HAAs in human colorectal carcinogenesis (14). Schiffman and Felton (15) postulated that the degree of

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<sup>3</sup> The abbreviations used are: HAA, heterocyclic aromatic amine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; NAT2, *N*-acetyltransferase; ESI, electrospray ionization; MS, mass spectrometry; LC-MS, liquid chromatography-MS; GC-MS, gas chromatography-MS; ESI-MS/MS, ESI tandem MS; HPLC, high-performance liquid chromatography.

doneness of cooked meats is an important determinant in HAA intake. They performed a preliminary case-control study of colorectal cancer ( $n = 50$  cases) that revealed a 3.5-fold increase in risk among individuals who consumed well-done cooked meat compared with subjects who consumed rare to medium-rare red meat. In a Swedish study, Gerhardtsson De Verdier *et al.* (16) demonstrated an increase in risk for colorectal cancer in those people having a dietary preference for heavily browned meat. Lang *et al.* (17) showed that a preference for meat that was cooked well done combined with rapid metabolic phenotypes for cytochrome P4501A2 and acetyltransferase (NAT2) is more prevalent among the colorectal polyp/cancer patients than among control participants (35% versus 16%;  $P = 0.002$ ). Furthermore, they found that the use of cooking oil (which results in increased cooking temperatures when compared with other cooking methods) occurred more frequently in the cases compared with the controls. However, other studies have failed to observe an association between consumption of cooked meats containing high levels of HAAs and human colorectal cancer (18, 19). Discrepancies in associating HAAs in the diet and the risk of colorectal cancer may be due to the use of poor surrogates in evaluating HAA intake through questionnaires assessing cooking methods, degree of doneness, and frequency of meat consumption.

Recently, a number of studies have been conducted in which quantitative analysis of urinary MeIQx and PhIP was used to assess human exposure to these HAAs. In a dietary investigation, Murray *et al.* (20) reported an average excretion of 2% of the ingested dose of MeIQx as the unchanged amine in urine. In a later study Lynch *et al.* (21) found that an average of 1.1% of the oral dose of PhIP was excreted unchanged in urine. This work also evaluated the intra- and interindividual variation in systemic exposure of MeIQx and PhIP in humans and showed that the urinary excretion of unchanged MeIQx and PhIP (expressed as percentage of dose) remained relatively constant for each individual, but that intersubject variation was greater. An estimation of human exposure to HAAs in Japanese subjects eating an unrestricted diet was carried out by Ushiyama *et al.* (22). The excretion of MeIQx ranged between 11 and 47 ng in the 24-h urine, whereas excreted PhIP ranged between 0.12 and 1.97 ng. Several laboratories have investigated the human metabolism of PhIP and MeIQx. In addition to unchanged MeIQx, the  $N^2$ -sulfamate and  $N^2$ -glucuronide metabolites of MeIQx were characterized as important elimination products of MeIQx in human urine (23). A recent investigation (24) of PhIP metabolism in human subjects reported that the levels of total PhIP (unchanged plus acid-labile conjugates) in 24-h urine ranged from 2 to 8.5% of the ingested dose; moreover, 2-amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine was identified as a urinary metabolite. A collaborative study (25) was carried out involving 66 residents of Maryland who consumed a uniform meat-based diet. It was found that the excretion of total (free and amine-conjugated) MeIQx in 0–12-h urine collections ranged from 3.2 to 22.7% of the ingested dose, whereas PhIP (free and acid-labile conjugates) excretion in urine ranged from 1.9 to 9.8% of the dose. A comparison of the amounts of MeIQx and PhIP excreted in urine, expressed as percentages of the ingested doses, showed a statistically significant but moderate correlation ( $r = 0.37$ ;  $P = 0.005$ ). Furthermore, this investigation and work by others (26) found that in humans the enzymatic activity of hepatic cytochrome P4501A2 more strongly influenced the metabolism of MeIQx than that of PhIP.

The population of Los Angeles County is ethnically diverse and includes large numbers of Asian-American, African-

American, and Hispanic and non-Hispanic white (white) residents. There also is a population-based cancer surveillance program in place that has characterized, since 1972, the incidence rates of different types of cancers among the various racial-ethnic groups (27). This combination of factors make the populace of Los Angeles County a suitable one in which to investigate the effect of culturally/ethnically mediated differences in exposure to or metabolism of suspected carcinogens on cancer outcome. As a means of assessing human exposure to MeIQx in a multi-ethnic study, we previously examined the urinary excretion of total (free plus amine-conjugated) MeIQx among 131 Los Angeles male residents (47 African-Americans, 41 Asian-Americans (Japanese or Chinese), and 43 whites) who consumed an unrestricted diet (28). Statistically significant differences in levels of urinary MeIQx were detected among the three racial groups; the geometric mean level of MeIQx in African-Americans was 1.3- and 3.0-fold higher than that in Asian-Americans and whites, respectively. The data further showed a positive and statistically significant association between intake frequency of bacon and urinary MeIQx level in all of the subjects, consistent with the observation that blacks had the highest consumption rate of bacon among the three racial-ethnic groups. Intake frequencies of other pork products asked in the dietary questionnaire (pork/ham, sausage/luncheon meat) also exhibited positive but statistically nonsignificant associations with the urinary MeIQx level (28). We have extended this prior study to include the measurement of urinary PhIP levels in all of the study subjects. In the present study, we (a) compared the levels of urinary PhIP across the three racial-ethnic groups; (b) compared levels of urinary PhIP and MeIQx within study subjects; (c) examined the relationship between urinary PhIP level and intake frequencies of six meat items that have the potential of generating appreciable amounts of HAA during cooking; and (d) examined the association of NAT2 phenotype, cigarette smoking, and intake frequencies of selected vegetables—factors that have the potential to influence HAA metabolism—on urinary PhIP excretion.

## Materials and Methods

**Chemicals.** PhIP was purchased from Toronto Research Chemicals (Ontario, Canada). PhIP- $d_5$ -phenyl was kindly provided by Dr. F. F. Kadlubar (National Center for Toxicological Research, Jefferson, AR). Isotopic purity of the deuterated standard was determined by LC-MS to be 98.6%. Stock solutions of deuterated PhIP were prepared in methanol and quantified with a Hewlett-Packard 8452A spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) using an extinction coefficient value of  $18,133 \text{ m}^{-1} \text{ cm}^{-1}$  for PhIP at 316 nm (29). Stock standard solutions were prepared as needed and not stored for future use. Methanol and ethyl acetate were distilled-in-glass grade (Omnisolve, EM Science, Gibbstown, NJ). High-purity water was used to prepare buffers and acid solutions. Monoclonal antibodies raised against PhIP were used in the preparation of immunoaffinity columns (30). The antibodies were immobilized on CNBr-activated Sepharose 4B (Sigma Chem. Co., St. Louis, MO) at a concentration of 3–4 mg of protein/ml of gel. Blocked gel for precolumns was prepared by treatment of CNBr-activated Sepharose 4B with 0.2 M Tris-HCl (pH 8.0). Small cartridges of the antibody-gel were prepared using 2 ml of the gel preparation. The antibody gel preparations were cleaned before use by washing with 1 N acetic acid in water (25 ml), followed by 10 ml of sodium bicarbonate buffer [0.1 M containing 0.5 M NaCl (pH 8.3)], and then stored in PBS containing 4 mM sodium azide at 4°C.

**Subject Profile.** Detailed characteristics of subjects and informed consent approval have been described by Yu *et al.* (31). Briefly, the subjects included 129 males who were above the age of 35 years and were African-American ( $n = 45$ ), Asian-American ( $n = 42$ ), or white ( $n = 42$ ). By design, approximately one-half ( $n = 68$ ) of the subjects were lifelong non-smokers; the remaining 61 subjects were current cigarette smokers of varying intensity. The majority of subjects ( $n = 75$ ) were participants of a cross-sectional survey among African-American, Asian-American, and white male residents of Los Angeles County, in which current dietary information was collected through a self-administered questionnaire (28). The remaining subjects ( $n = 54$ ) were primarily employees of the University of Southern California. All of the subjects were phenotyped for *N*-acetyltransferase 2 (NAT2) using caffeine as substrate (31). No caffeine metabolites were detected in the urine of two white subjects (presumably they did not consume the coffee packet as instructed) and they were excluded from the analysis involving NAT2 phenotype. The urine samples were collected over a period beginning at bedtime and ending with the first morning void. The samples were preserved by acidification with ascorbic acid and stored at  $-20^{\circ}\text{C}$  shortly after collection. Creatinine levels in the urine samples were measured to establish a basis for the comparison of individuals.

**Dietary Questionnaire.** The food frequency questionnaire asked subjects to indicate their usual dietary habits during the preceding year. Seven frequency categories ranging from never to three or more times a day were given, and the subjects were asked to identify the category that best described his intake frequency of a particular food or beverage item. Over 100 food and beverage items were listed on the questionnaire. There were six specific meat items that were judged to possess the potential of generating high levels of HAAs during cooking. They were:

- (a) "beef, lamb, veal, or goat (such as steak, roast, corned beef, ground beef, shortribs, teriyaki, bulgogi, etc.)" (beef);
- (b) "pork or ham (such as chops, roast, spareribs, ham hocks, chitterlings, kalua pig, pork adobo, lechon, pig's feet, etc.)" (pork);
- (c) "chicken, turkey, or duck (such as fried, roasted, nuggets, adobo, inihaw, etc.)" (chicken);
- (d) "fresh or frozen fish" (fish);
- (e) "sausages or luncheon meats (such as hot dogs, salami, Spam, bologna, lup chong, gone chong, etc.—includes pork, beef, chicken and turkey kinds)" (sausage); and
- (f) "bacon (includes Canadian bacon, lup yuk, lup arp, etc.)" (bacon).

The questionnaire did not ask about preferred methods of cooking these selected meats, nor did it ask about how well-done these meats were usually cooked.

The questionnaire also listed five specific vegetable groups:

- (a) "green lettuce (such as iceberg or head lettuce, romaine, red, butter or Manoa lettuce, or endive)" (salad greens);
- (b) "tomatoes" (tomatoes);
- (c) "green vegetables—not in soups or mixed dishes (such as broccoli, green peas or beans, bok choy, spinach, won nga bok or Napa cabbage, leafy greens, etc.)" (green vegetables);
- (d) "yellow or orange vegetables—not in soups or mixed dishes (such as carrots, corn, acorn or yellow squash, sweet potatoes, yams, etc.)" (yellow or orange vegetables); and
- (e) "other vegetables—not in soups or mixed dishes (such as head cabbage, zucchini, white squash, cauliflower, beets, eggplant, okra, etc.)" (white or other vegetable).

**Determination of PhIP in Urine.** The isolation of total (free and acid-labile conjugates) PhIP from human urine samples has been described previously (25). In brief, urine samples (10 ml) were placed in 50-ml glass-stoppered glass tubes, spiked with the internal standard (typically 5 ng of  $d_5$ -PhIP), acidified with 6 N HCl (2 ml) and incubated at  $70^{\circ}\text{C}$  for 4 h. After the hydrolysis period, the samples were allowed to cool to room temperature and neutralized with 6 N NaOH (2 ml). The samples were basified by the addition of sodium carbonate (0.5 g) and were extracted twice with two volumes of ethyl acetate. The organic phase was placed in a  $-16^{\circ}\text{C}$  freezer for 30 min to freeze out residual aqueous phase and then decanted into a clean glass centrifuge tube containing 2 ml of 0.1 N HCl. After vortexing the solutions, the acidic layer was removed, and a second extraction was performed with 1.0 ml of 0.1 N HCl. The acidic extracts were combined and placed into 7-ml vials and taken to dryness by vacuum centrifugal concentration (Savant Instruments, Inc., Farmingdale, NY). The dried samples were stored at  $-16^{\circ}\text{C}$  until further work-up.

**Purification by Immunoaffinity Chromatography.** Dried samples were reconstituted in 3 ml of PBS solution. After pH adjustment to 8.0 by the addition of 0.2–0.4 ml of 0.1 N NaOH, the urine extracts were applied to small precolumns (1 ml of Tris-blocked cyanogen bromide-activated Sepharose 4B) followed by monoclonal antibody columns (1.5 ml). The columns were washed with 20 ml of PBS solution, the precolumns were removed, and an additional 20 ml of PBS solution was applied to the antibody columns. The columns were then washed with an additional 35 ml of water. The bound-PhIP fraction was eluted from the antibody columns with 4 ml of 1 N acetic acid. The samples were dried by vacuum centrifugation and then reconstituted with 0.5 M sodium carbonate (0.3 ml) and extracted with ethyl acetate ( $2 \times 1$  ml). The organic layer was chilled at  $-16^{\circ}\text{C}$  for 30 min, transferred to clean vials, and dried by evaporation under  $\text{N}_2$ . The extracts were then transferred with a small volume of ethyl acetate ( $2 \times 100 \mu\text{l}$ ) to micro-inserts positioned in screw-cap vials (DP-Target, Hewlett-Packard Co.). The samples were dried under a stream of  $\text{N}_2$  and stored at  $-16^{\circ}\text{C}$  until analysis by LC-ESI-MS.

**Quantitative Analysis by ESI/LC-MS and ESI/MS.** The purified samples were analyzed by one of two methods. In one procedure, 85 samples were analyzed using a Hewlett-Packard HPLC 1090 series instrument coupled to a Hewlett-Packard single-stage quadrupole mass spectrometer equipped with an ESI source. Separations were carried out on a micro-bore C-18 reverse-phase column ( $150 \times 1.0$  mm,  $5 \mu\text{m}$ ; Vydac, Rancho Cordova, CA). The mobile phase consisted of (solvent A) 40  $\mu\text{M}$  ammonium acetate in water (pH 4.0) containing 10% methanol and (solvent B) methanol:40  $\mu\text{M}$  ammonium acetate (98:2, v/v). A linear gradient was used (20 min) with a flow rate of 50  $\mu\text{l}/\text{min}$ . The mass spectrometer was operated in positive ion mode, and the samples were analyzed by selected ion monitoring of the protonated molecular ions of PhIP and its deuterated ( $d_5$ -phenyl) internal standard at  $m/z$  225 and  $m/z$  230, respectively.

Because of mass spectrometer (Hewlett-Packard) unavailability, the remaining 44 samples were analyzed by a LC-MS/MS method (25) using a Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with an ESI source. In this case, the HPLC was the same as described above; however, a narrow-bore column ( $150 \times 2.1$  mm,  $5 \mu\text{m}$ ) was used and the mobile phase consisted of methanol:water (50:50) with 0.1% formic acid at a flow rate of 200  $\mu\text{l}/\text{min}$ . PhIP and  $d_5$ -PhIP were analyzed in

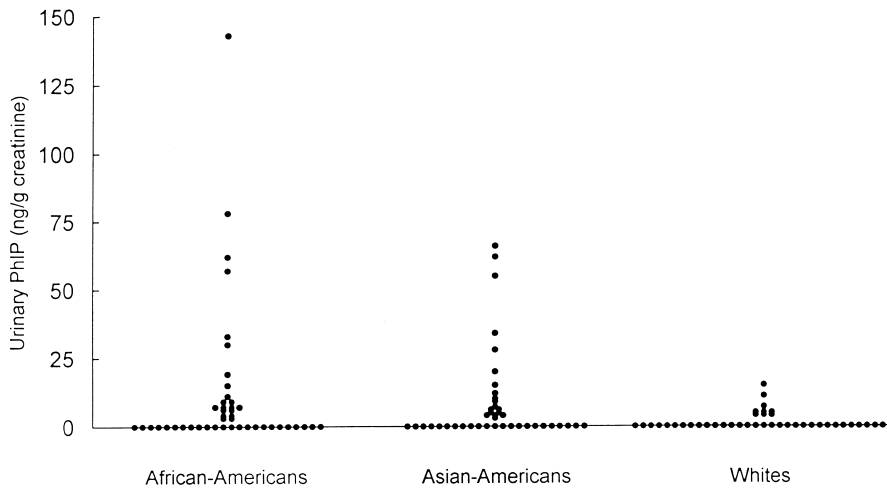


Fig. 1. Concentration of PhIP in urine of subjects of different racial/ethnic origins. Points, single determinations for single subjects consuming their usual free-choice diet.

positive ion mode using a selected ion-reaction monitoring procedure, which monitors collision-induced dissociation of a precursor ion to its corresponding product ion. With the collision voltage and gas (argon) set at  $-32$  eV and 3.2 mTorr, respectively, the protonated molecular ions were fragmented to their associated product ions. The Finnigan data system monitored the transition of the protonated molecular ions of PhIP at  $m/z$  225 and  $d_5$ -PhIP at  $m/z$  230 to their respective product ions at  $m/z$  210 and  $m/z$  215. Instrumental parameters were optimized before use by adjusting the capillary and tube lens voltages to ensure maximum sensitivity. The heated capillary was set at  $250^\circ\text{C}$ , and the nitrogen sheath gas was held at 80 psi and the auxiliary nitrogen set at 18 on the dial. Unextracted standards were analyzed under the same conditions and used to correct for background contribution of nondeuterated PhIP present in the deuterated PhIP. In addition, blank urine samples were analyzed under the corresponding conditions to determine the presence of background interference. Analyses were conducted periodically of representative standards in the range expected of actual samples. Calibration plots of  $d_0/d_5$ -PhIP using standard compounds were prepared and showed good linearity over the low picogram to nanogram range.

The limit of quantification for PhIP was similar for the two ESI procedures, *i.e.*, 4 pg/ml of urine. The amount of PhIP in the samples was determined by multiplying the peak area ratio of the product ions of  $d_0$ -PhIP and  $d_5$ -PhIP by the amount of internal standard and applying the appropriate correction factor. Recovery rates were determined by comparing the response of the internal standard with unextracted standards analyzed under the same conditions.

**Statistical Analysis.** The distribution of the PhIP:creatinine ratio was markedly skewed in our study population; therefore, formal statistical testings were performed on logarithmically transformed values of the ratio, and geometric (as opposed to arithmetic) mean values are presented. We used the ANOVA method to compare log values of the PhIP:creatinine ratio across the three racial-ethnic groups (32). In addition, we used the contingency table analysis method to compare values of the PhIP:creatinine ratio (*a*) across the three racial-ethnic groups; (*b*) by NAT2 phenotype, current smoking status, and intake frequencies of five vegetable items (salad greens, tomatoes, green vegetables, yellow or orange vegetables, and white or other vegetables) after adjustment for race-ethnicity; and (*c*) to determine the degree of concordance between urinary excretion

levels of PhIP and MeIQx within subjects (32). Finally, we used the logistic regression method (33) to examine the association between urinary PhIP level (negative, positive) and intake frequencies of six meat items after adjustment for race-ethnicity. The strengths of the associations were estimated by the odds ratios and their corresponding 95% confidence intervals and significance tests. All of the *P*s cited were two-sided.

## Results

Because urine specimens were subjected to acid treatment, the values of our measurements represent the sum of unmetabolized and conjugated PhIP. These values ranged from nondetectable ( $<4$  pg/ml of urine) to 115 pg/ml of urine. The overall recovery of PhIP through the procedure was 20% or greater based on the analysis of unextracted deuterated standards. The analysis of blank urine samples showed no detectable amounts of PhIP.

Fig. 1 depicts the individual values of urinary PhIP expressed in nanograms of PhIP/gram of creatinine for the study subjects by race. Detectable levels of urinary PhIP ranged from 3.0 to 142.5 ng/g creatinine in African-Americans, 3.2 to 66.1 ng/g creatinine in Asian-Americans, and 3.8 to 14.7 ng/g creatinine in whites. Table 1 shows the geometric mean levels of urinary PhIP, which were lowest in whites (1.18) when compared with African-Americans (3.36) and Asian-Americans (3.33). This difference was statistically significant ( $P = 0.007$ ) and was primarily due to the high PhIP values in African-Americans and Asian-Americans relative to whites. There was no statistical difference in mean PhIP values between black and Asian subjects ( $P = 0.98$ ). Table 1 also lists the distribution of PhIP levels among the three ethnic groups. The two cutpoints, 6.0 and 13.0 ng/g creatinine, were selected to separate the positive subjects into approximate tertiles. There was a statistically significant difference in distributions across race ( $P = 0.01$ ). Forty-nine % of African-Americans and 55% of Asian-Americans had nondetectable levels of PhIP in comparison with 79% of white subjects. Whereas 22% of African-Americans and 12% of Asian-Americans had levels of urinary PhIP between 6 and 13 ng/g creatinine, only 5% of white subjects had amounts in this range. The difference between nonwhites and whites was most dramatic in the third tertile: 18 and 19% of the nonwhites (African- and Asian-Americans, respectively) ex-



Table 1 Levels of urinary PhIP<sup>a</sup> among study subjects<sup>b</sup>

|                           | African-American     | Asian-American | White   | Total |
|---------------------------|----------------------|----------------|---------|-------|
| ND <sup>c</sup>           | 22 (49) <sup>d</sup> | 23 (55)        | 33 (79) | 78    |
| ND < R <sup>a</sup> ≤ 6.0 | 5 (11)               | 6 (14)         | 6 (14)  | 17    |
| 6.0 < R ≤ 13.0            | 10 (22)              | 5 (12)         | 2 (5)   | 17    |
| 13.0 ≤ R                  | 8 (18)               | 8 (19)         | 1 (2)   | 17    |
| Total                     | 45                   | 42             | 42      | 129   |
| Geometric mean level      | 3.36                 | 3.33           | 1.18    | 2.47  |

<sup>a</sup> Expressed as ng/g creatinine.

<sup>b</sup>  $\chi^2$  (6 df) = 16.2,  $P$  = 0.01.

<sup>c</sup> ND, not detectable.

<sup>d</sup> Table entries are number of subjects with percentages in parentheses.

creted greater than 13 ng/g creatinine of urinary PhIP, whereas only 2% of the whites had levels exceeding this amount.

When urinary levels of PhIP and MeIQx were summed (Table 2), the observed ethnic pattern was similar to the distribution of urinary PhIP alone among the three ethnic groups.

To determine whether the urinary excretion levels of MeIQx (values obtained in the previous study; Ref. 28) can be used as an index for the urinary levels of PhIP, a 3 × 3 contingency table was generated (Table 3). A  $\chi^2$  test of association revealed that the distribution of MeIQx and PhIP was significantly correlated among the subjects ( $P$  = 0.001, contingency coefficient = 0.47). Perfect concordance was observed in 47% ( $n$  = 60) of the subjects, and this rate of concordance was similar across the three levels of PhIP. However, we noted that a number of subjects (8% of the total sample) displayed extreme discordance between PhIP and MeIQx levels. Five (4%) subjects who had undetectable levels of PhIP showed high levels (>7 ng/g creatinine) of MeIQx, and conversely 5 (4%) subjects with PhIP levels above 7 ng/g creatinine had no detectable levels of MeIQx. Overall, 34% of subjects had no detectable urinary PhIP but exhibited MeIQx in their urine, and another 13% of subjects had no detectable urinary MeIQx but had detectable PhIP in their urine (Table 3).

Table 4 compares, between subjects exhibiting negative (nondetectable) versus positive (detectable) levels of urinary PhIP, the intake frequencies of selected meats (*i.e.*, beef, pork, chicken, fish, sausage, and bacon) that have the potential of generating high levels of HAAs during cooking. There was no association between the intake frequencies of meat based on self-administered questionnaires and the levels of urinary PhIP within the subjects.

We also examined the possible associations between urinary excretion of total PhIP (unmetabolized plus conjugated) and variables that have the potential of modifying HAA metabolism including the NAT2 phenotype, cigarette smoking, and intake of selected types of vegetables (salad greens, tomatoes, green vegetables, yellow or orange vegetables, and white or other vegetables). There was no difference in the prevalence of positive PhIP levels between cigarette smokers and non-smokers after adjustment for race-ethnicity ( $P$  = 0.41). Also, there was no difference in the prevalence of positive PhIP levels between slow and rapid NAT2 acetylators after adjustment for race-ethnicity ( $P$  = 0.97). For the five types of vegetables asked in the dietary questionnaire, none showed an association with urinary PhIP level: salad greens ( $P$  = 0.60), tomatoes ( $P$  = 0.89), green vegetables ( $P$  = 0.16), yellow or orange vegetables ( $P$  = 0.81), and white or other vegetables ( $P$  = 0.14).

Table 2 Levels of urinary PhIP<sup>a</sup> plus MeIQx<sup>a</sup> among study subjects<sup>b</sup>

|                           | PhIP plus MeIQx | African-American    | Asian-American | White   | Total |
|---------------------------|-----------------|---------------------|----------------|---------|-------|
| ND <sup>c</sup>           |                 | 5 (11) <sup>d</sup> | 8 (19)         | 21 (50) | 34    |
| ND < R <sup>a</sup> ≤ 4.0 |                 | 13 (29)             | 11 (27)        | 12 (28) | 36    |
| 4.0 < R ≤ 10.0            |                 | 12 (27)             | 9 (22)         | 7 (17)  | 28    |
| 10.0 ≤ R                  |                 | 15 (33)             | 13 (32)        | 2 (5)   | 30    |
| Total                     |                 | 45                  | 41             | 42      | 128   |
| Geometric mean level      |                 | 7.7                 | 7.1            | 2.6     | 5.4   |

<sup>a</sup> Expressed as ng/g creatinine.

<sup>b</sup>  $\chi^2$  (6 df) = 26.0,  $P$  = 0.001.

<sup>c</sup> ND, not detectable.

<sup>d</sup> Table entries are number of subjects with percentages in parentheses.

## Discussion

These reported values for PhIP can be compared with a prior study, although not directly. Our laboratory measured the sum of free PhIP and its acid-labile conjugate(s) and expressed it in terms of nanograms of total PhIP per gram of creatinine. An investigation by Ushiyama *et al.* (22) measured unmetabolized PhIP and expressed it as nanograms of PhIP per 24-h urine collection period. They reported that unmetabolized PhIP excreted in 24-h urine samples, collected from ten healthy Japanese men (natives of Tokyo, Japan) consuming an unrestricted diet, ranged from 0.12 to 1.97 ng. The Japanese in our study excreted levels of PhIP ranging from nondetectable to 66 ng/g creatinine with an arithmetic mean of 11.0. On a 24-h rather than a per gram of creatinine basis, the numbers would be 50 to 100% higher. After adjusting for the free PhIP:conjugate ratio, our values are 2–40 times higher than Ushiyama's. Assuming urinary excretion of total PhIP expressed in terms of the percentage of the ingested dose is a marker of HAA exposure, 54% of the Japanese in our study may have consumed a recent meal containing 0.1 to 2  $\mu$ g of PhIP. Although the Japanese in Ushiyama's study were exposed to dietary PhIP, the amounts presumably did not exceed 0.3  $\mu$ g of PhIP (34).

A more direct comparison may be made with a prior investigation in which urinary PhIP (free plus conjugated) was analyzed among individuals in a controlled dietary study (25). We found that subjects excreted an average of 351 ng of PhIP in their 12-h urine after the consumption of cooked beef containing an average of 8.1  $\mu$ g of PhIP. After adjusting for an average total volume of 1000 ml for a 12-h period, the positive values (*i.e.*, 4–115 pg/ml of urine) within our three ethnic groups would range from 4–115 ng in African- and Asian-Americans, and 4–12 ng in whites. These values are approximately 3- to 90-fold lower than those reported above. Few if any of our subjects in the present study would be expected to have had a recent meal with a PhIP content of 8  $\mu$ g.

Preceding this study, our laboratory examined the urinary excretion of MeIQx among the same individuals described in the current investigation (28). We found marked differences in the excreted values of MeIQx between whites, Asian-, and African-Americans. The urinary levels of MeIQx/gram of creatinine were highest in African-Americans, intermediate in Asian-Americans, and lowest in whites. A comparison of the excreted values of urinary PhIP among the three groups in the present study also showed that whites had the lowest values for PhIP, whereas the Asian- and African-Americans had values approximately 2.8-fold higher. There was a moderate but significant association between the urinary levels of MeIQx and PhIP within study subjects as demonstrated by a contingency coefficient of 0.47. A comparison of the positive versus negative HAA values for all of the subjects resulted in the following

Table 3 Two-way contingency table of urinary PhIP<sup>a</sup> versus MeIQx<sup>a,b</sup>

| PhIP            | MeIQx           |                           |         | Total |
|-----------------|-----------------|---------------------------|---------|-------|
|                 | ND              | ND < R <sup>c</sup> ≤ 7.0 | 7.0 < R |       |
| ND <sup>c</sup> | 34 <sup>d</sup> | 38                        | 5       | 77    |
| ND < R ≤ 7.0    | 11              | 11                        | 2       | 24    |
| 7.0 < R         | 5               | 7                         | 15      | 27    |
| Total           | 50              | 56                        | 22      | 128   |

<sup>a</sup> Expressed as ng/g creatinine.

<sup>b</sup> Chi-square (4 df) = 29.8, *P* = 0.001.

<sup>c</sup> ND, not detectable.

<sup>d</sup> Table entries are number of subjects.

observations: (a) 13% of the subjects excreted only PhIP; (b) 34% excreted only MeIQx; (c) 27% excreted both PhIP and MeIQx; and (d) 27% did not excrete either PhIP or MeIQx. The degree of concordance between these two HAAs is dependent on multiple factors, including the enzymic and interindividual differences in HAA metabolism and disposition discussed earlier (21, 25). In addition, the style of preparation, cooking time, heating temperature, and degree of doneness can greatly influence the relative proportions of HAAs formed in cooked meats (5, 6). A combination of these variables will, in turn, affect the profile of urinary HAAs. Accordingly, in determining exposure assessments of these compounds in humans, the measurement of several HAAs may be necessary because no individual HAA can be used as a precise indicator of exposure to another HAA.

The degree of concordance between the urinary excretion levels of PhIP and MeIQx also may be influenced by the analytical methodology used for the measurement of these two HAAs in urine extracts. MeIQx and PhIP were assayed by two different procedures: a GC/MS methodology was used in the previous MeIQx study, whereas a LC-MS procedure was used for the analysis of PhIP. The GC-MS assay allowed for a minimum quantification limit of 1 pg of MeIQx per ml urine. However, this GC-MS method was not adaptable for use in the analysis of PhIP. Instead, a LC-MS procedure was used that allowed a quantification limit of 4 pg of PhIP per ml of urine. This relatively higher limit of quantification may affect the number of subjects in the present study having nondetectable levels of PhIP in urine versus nondetectable levels of MeIQx. In the present study, two different modes of detection were used in the analysis of PhIP, *i.e.*, selective ion monitoring and selective reaction monitoring (MS/MS). The MS/MS assay is more selective; however, detection by selective ion monitoring showed good sensitivity and specificity using a micro-bore HPLC column with a gradient solvent program. Quantification of HAAs in biological extracts using either selective ion monitoring or MS/MS detection requires a high degree of column and MS maintenance, which was attained in these studies. The determination of PhIP by the ESI-LC-MS/MS method previously reported (25) showed a high relative SD when replicate urine samples were analyzed over a period of 4 months. This may be due to several factors, including accuracy in measurements of LC-MS peak areas, which are generally broad in comparison with GC-MS. In addition, ion ratio precision using MS/MS may be less than that obtained by selective ion monitoring (35).

To estimate exposure to dietary HAAs, it is important to determine whether meat intakes based on food frequency questionnaires are associated with urinary HAA levels. In contrast to our earlier finding (28) of a strong association between bacon intake and urinary MeIQx level in the same study subjects, the

Table 4 Frequencies of meat intake according to levels of urinary PhIP among study subjects

Seventy-five of the 129 subjects participated in a survey in which current dietary information was collected through self-administered questionnaires.

| Meat item | Urinary PhIP, no. of subjects (percentage) |          | OR <sup>b</sup> (95% CI) |
|-----------|--|----------|--------------------------|
|           | Negative <sup>a</sup>                      | Positive |                          |
| Beef      |  |          |                          |
| <wkly     | 18 (38)                                    | 14 (52)  | 1.0                      |
| wkly      | 30 (62)                                    | 13 (48)  | 0.6 (0.2–1.6)            |
| Pork      |  |          |                          |
| <wkly     | 35 (73)                                    | 18 (67)  | 1.0                      |
| wkly      | 13 (27)                                    | 9 (33)   | 1.1 (0.4–3.2)            |
| Chicken   |  |          |                          |
| <wkly     | 20 (42)                                    | 14 (52)  | 1.0                      |
| wkly      | 28 (58)                                    | 13 (48)  | 0.8 (0.3–2.2)            |
| Fish      |  |          |                          |
| <wkly     | 36 (78)                                    | 20 (74)  | 1.0                      |
| wkly      | 10 (22)                                    | 7 (26)   | 1.0 (0.3–3.3)            |
| Sausage   |  |          |                          |
| <wkly     | 30 (62)                                    | 21 (78)  | 1.0                      |
| wkly      | 18 (38)                                    | 6 (22)   | 0.5 (0.2–1.6)            |
| Bacon     |  |          |                          |
| <wkly     | 34 (72)                                    | 22 (77)  | 1.0                      |
| wkly      | 13 (28)                                    | 6 (23)   | 1.0 (0.3–3.5)            |

<sup>a</sup> Negative, undetectable levels of PhIP; Positive, detectable levels of PhIP; OR, odds ratio; CI, confidence interval.

<sup>b</sup> The odds of weekly consumers of the given meat item possessing detectable levels of PhIP in urine relative to those consuming the meat less frequently, after adjustment for race-ethnicity.

present study showed that urinary PhIP level was unrelated to intake frequencies of the six meat items asked in the dietary questionnaire. This suggests that PhIP intake is more difficult to assess than MeIQx via a food frequency questionnaire that did not ask about methods of food preparation.

Some cancers occur more frequently among African-American males than among whites or Asian-Americans in Los Angeles County (27). Dietary factors have long been associated with increasing risks of colon, prostate, and pancreatic cancers (36). At present, there are suggestive data linking HAA exposure to the development of colorectal cancer in humans. This study extends our previously reported data on MeIQx (28) and demonstrates that African-Americans as well as Asian-Americans excreted higher levels of PhIP, the most common HAA exposure in humans, compared with whites. On the basis of these findings, the daily exposure of African- and Asian-Americans to these HAAs through the diet is higher than that for whites. The reason for the lower levels of HAAs in diets of whites may be due to a dietary preference toward meats prepared under low to moderate heating temperatures. The results presented here indicate that self-administered dietary questionnaires may not adequately assess cooked meat preferences and frequency intakes. To achieve a direct comparison of the extent of human exposure to these HAAs among different ethnic groups, it may be necessary to obtain more comprehensive data on food cooking techniques including the style of meat preparation as well as choices toward done versus well-done cooked meat and lightly browned versus heavily browned meat (*i.e.*, other surrogates of HAA exposure). This information should provide a more complete estimation of exposure level and, thereby, allow a better assessment of cancer risk attributable to dietary HAAs.

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# BLOOD CANCER DISCOVERY

## 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

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