Biomonitoring the Cooked Meat Carcinogen 2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]Pyridine in Hair: Impact of Exposure, Hair Pigmentation, and Cytochrome P450 1A2 Phenotype

Robert J. Turesky1, Lin Liu1, Dan Gu1, Kim M. Yonemori2, Kami K. White2, Lynne R. Wilkens2, and Loïc Le Marchand2

Abstract

Background: Hair is a promising tissue to assess exposure to 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), a carcinogen formed in cooked meats. However, an understanding of how dietary exposure to PhIP, cytochrome P450 1A2 activity—a key enzyme involved in PhIP metabolism, and hair pigmentation affect the level of PhIP accrued in hair is required to determine the reliability of the PhIP hair level as a biomarker of exposure to this carcinogen.

Methods: We examined the impact of PhIP exposure, cytochrome P450 1A2 activity, and hair pigmentation on the levels of PhIP accumulated in the hair of volunteers on a 4-week semicontrolled diet of cooked meat containing known quantities of PhIP.

Results: The amount of PhIP in hair increased, on average, 15-fold in light- and dark-haired individuals during consumption of cooked meat. PhIP levels in hair were correlated to PhIP intake ($r = 0.53; P < 0.001$), and the relationship was strengthened when PhIP levels were normalized for the melanin content of hair ($r = 0.71; P < 0.001$). However, PhIP accrual in hair was not correlated to cytochrome P450 1A2 activity, as assessed by the caffeine test, or to the levels of unmetabolized PhIP in urine or to the metabolic ratio of the major urinary metabolite $N^2$-(β-1-glucosiduronyl-2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5-b]pyridine to unmetabolized PhIP.

Conclusions: The use of the PhIP hair biomarker should take hair pigmentation into account for accurate exposure assessment of PhIP.

Impact: PhIP hair levels can serve as a biomarker in epidemiologic studies investigating the association of heterocyclic aromatic amine (HAA), cooked meat, and cancer risk. Cancer Epidemiol Biomarkers Prev; 22(3); 356–64. ©2013 AACR.

Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is a carcinogenic heterocyclic aromatic amine (HAA) formed in well-done cooked meats (1) and comprises about 70% of the daily mean intake of HAAs in the United States (2). PhIP is a multisite carcinogen in rodents: it induces colorectal, pancreatic, prostate, and mammary gland tumors (3). Therefore, PhIP may have an etiologic role in these cancers in humans (3). Indeed, some epidemiologic studies have reported that the frequent consumption of well-done cooked meats containing HAAs increases the risk of developing cancers at these sites; however, other studies have not observed an association between consumption of well-done meats and the risk of cancer (4–9).

The accurate assessment of exposure is a major limitation in epidemiologic studies addressing diet and cancer risk. The amount of PhIP formed is highly dependent on the type of meat cooked, as well as the method, temperature, and duration of cooking, which can lead to differences in the levels of PhIP by more than 100-fold (1, 10). As a result, inferences based on PhIP intake from food frequency questionnaires (FFQ) may be inaccurate (11); indeed, the mean intake of PhIP from an FFQ was not associated with the levels of urinary PhIP (12). The inconsistent findings in the literature on the association of HAA intake and cancer is probably because of the difficulty in assessing exposure and differences in meat cooking methods across populations.

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Analytical methods have been established to measure PhIP and its metabolites in urine, following meat consumption (13–15); however, the occurrence of urinary biomarkers is transient, and the measured level only captures the preceding 24 hours of exposure. For individuals who chronically but intermittently consume cooked meats, urinary biomarkers of PhIP can be at undetectable levels and these individuals can be misclassified. There are 2 reports on blood protein adducts of PhIP (16, 17), but the identities of the adducts have yet to be determined, and information on the kinetics of adduct formation and persistence are lacking. Putative DNA adducts of PhIP have been characterized in human tissues (16, 18, 19), but these measurements are often precluded by the unavailability of biopsy samples in large-scale human studies. There is a need to establish stable, long-term biomarkers of PhIP, which can be implemented in molecular epidemiology studies to evaluate the health risk of this genotoxicant.

Human hair has served as a matrix to biomonitor various drugs and contaminants, such as nicotine, narcotics, and hormones (20–22). Alexander and colleagues showed that a small portion of the dose of PhIP becomes entrapped within the melanin-rich tissues of rodents, including the hair follicle, and PhIP becomes incorporated in the cortex of the newly grown hair shafts (23). Subsequently, gas chromatography–negative ion chemical ionization mass spectrometry (24) and liquid chromatography/mass spectrometry (LC/MS) methods have been established to quantitate PhIP in human hair (25–27). The levels of PhIP in hair vary widely among individuals (24–27). The main factors expected to influence the level of PhIP in hair are: (i) the different concentrations of PhIP in the diet; (ii) interindividual differences in the pharmacokinetics and metabolism of PhIP; and (iii) hair pigmentation. The level of cytochrome P450 1A2, a major hepatic enzyme involved in PhIP metabolism (28), can vary by more than 50-fold in humans (29) and may affect the level of PhIP that reaches the hair follicle, following first-pass metabolism. PhIP has a high binding affinity for eumelanin, a pigment that is more predominant in black hair than in lighter-colored hair (23, 24). Thus, pigmentation may influence the sequestering of PhIP in hair.

In this study, we have examined the influence of PhIP exposure, P450 1A2 phenotype, and hair pigmentation on the level of PhIP accrual in hair of subjects put on a semiconrolled diet of well-done cooked meat containing known quantities of PhIP. This research is necessary to accurately gauge the use of hair as a reliable tissue to assess exposure to PhIP and the practicality of using PhIP hair level as a biomarker in large epidemiologic studies investigating the association of HAAs with disease risk. The biomarkers of PhIP used for study are shown in Fig. 1.

**Materials and Methods**

**Study subjects**

A group of \( N = 44 \) (82% males, 18% females, and 45% white, 44% Asian/Pacific Islander, 9% African American, 2% Hispanic Non-white) healthy, nonsmoking volunteers were recruited among University of Hawaii (Honolulu, HI) students and staff on the Manoa campus. Inclusion criteria included regular beef eater, age \( > 18 \), no use of hair dyes, taking no prescribed or over-the-counter medication except an occasional analgesic, no history of gastrointestinal tract disorders, having a weight not less than 90% or greater than 130% of 1983 Metropolitan Life Insurance desirable weights, no weight change of more than 10 pounds in the past 12 months, no special diet (e.g., vegetarian, macrobiotic, weight loss, diabetic, etc.), fruit and vegetable intake \( \leq 7 \) servings/d, fiber intake \( < 22 \) g/d, alcohol intake no greater than 2 drinks/d, and caffeine intake no greater than 2 caffeinated drinks/d.
Recruitment was carried out through advertising on the University of Hawaii Manoa campus in Honolulu, HI. The feeding study was divided into 3 phases. In phase I, a 3-week prefeeding period, subjects refrained from eating pan-fried, grilled, or oven-broiled meats, poultry, or fish, cooked well-done. Thereafter, in phase II, volunteers ate dinner, 5 d/wk, for 4 weeks at a study site on the University of Hawaii Manoa campus. As part of this meal, subjects were fed a ground beef patty (150 or 200 g) grilled well-done. The rest of the meal was varied on a 5-day rotating basis but was low in dietary fiber and included a starch (rice, potato, or pasta), a vegetable, a fruit, dessert, and a drink. For the remainder of their daily meals and on Saturdays and Sundays, the participants followed their normal diet, except that they were asked to avoid eating any well-done meat or fish. After this 4-week feeding period, in phase III, the volunteers went back to their regular diet but refrained from eating meats that were grilled well done for 4 weeks. This study was run in groups of 10 to 20 subjects. All subjects provided informed consent, and the study was approved by the Institutional Review Boards at the Wadsworth Center (Albany, NY) and the University of Hawaii.

Preparation of cooked meat
Ground beef (15% fat) was cooked as half pound patties on a commercial flat top griddle for an average of 10 min/side. The surface temperature of the griddle was monitored and ranged from 440 to 600°F. The patties were flipped only once. After cooking, the patties were refrigerated overnight. They were then minced and homogenized with a food processor and frozen until needed. On each day of feeding, the meat was reheated in a warmer so no additional HAAs would be formed. Several samples of the cooked meat were analyzed for PhIP to estimate the dose provided to each study group.

Biological specimens
Urine was collected for the 12-hour period immediately after dinner time until rising the following morning and kept on blue ice in a cooler until aliquoting and kept on blue ice for 1 hour. Thereafter, PhIP was isolated by tandem solvent SPE (27).

Isolation of PhIP from cooked meat
PhIP was isolated from meat by tandem solid-phase extraction (SPE; ref. 10). Quantitative mass spectrometric measurements were done as described below.

Isolation of PhIP from hair
Minced hair (25–50 mg) spiked with 1-[2H3C]-PhIP (100 pg per 50 mg hair) was digested in 1N NaOH (1 mL) at 80°C for 1 hour. Thereafter, PhIP was isolated by tandem solvent SPE (27).

Spectrophotometric characterization of melanin in hair
Hair (5 mg) was digested in Soluene 350:H2O (9:1 v/v, 1 mL) by heating at 95°C for 1 hour. Spectra were acquired with an Agilent 8453 model UV/VIS spectrophotometer. The absorbance at 500 nm was used to estimate the total amount of melanin (31).

Isolation of PhIP and metabolites in urine
PhIP and its metabolites were isolated by SPE (15, 30). 1-[3H]-PhIP and 1-[1H3]-HON-PhIP-N3-Gl metabolites were added, respectively, at a concentration of 100 and 1000 pg/mL urine (15).

Ultraperformance liquid chromatography-electrospray ionization/tandem MS measurements of PhIP in hair and cooked meat and metabolites of PhIP from urine
The quantification of PhIP analytes was conducted with a NanoAcquity UPLC (Waters Corporation) equipped with a Michrom C18 AQ column (0.3 × 150 mm, 3-µm particle size; Michrom Bioreources Inc.) and a Michrom Captive-Spray source interfaced to a Finnigan Quantum Ultra mass spectrometer (Thermo Fisher). The chromatographic conditions and instrument tune parameters were reported previously (15). Analyses were conducted in the positive ionization mode using the following transitions: PhIP: 225.1 → 210.1; and 1-[2H3C]-PhIP: 228.1 → 210.1 lov, isomeric HON-PhIP-N3-Gl: 417.1 → 225.1, 224.1, and 223.1; [3H3]-HON-PhIP-N3-Gl: 420.1 → 228.1, 227.1 and 225.1.

Caffeine phenotyping for P450 1A2 activity
Subjects fasted for 10 hours, then consumed 2 cups of coffee (about 100 mg of caffeine) upon rising, maintained fasting for another 2 hours, abstained from other caffeine consumption, and collected their urine during the fifth hour after dosage. Caffeine metabolites (17U: 1,7-dimethyluric acid; 17X: 1,7-dimethylxanthine; and 137X: caffeine) were analyzed by high-performance liquid chromatography (HPLC) with a photodiode array detector to assess metabolic phenotypes (32). The coefficient of variation (CV) for the caffeine metabolite ratio based on 69 blind duplicate pairs analyzed with the study samples.

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was 2.6%. The caffeine tests were carried out twice in each phase, a week apart. All samples from each subject were analyzed in the same analytical batch.

Statistical analyses

The PhIP values in hair and the urine and caffeine metabolic phenotypes were analyzed using linear mixed regression models, with person included as a random effect and study day as a set of fixed-effect indicators. The PhIP values were log-transformed as ln(x + 1) to meet model assumptions. Geometric means and 95% confidence intervals (CI) were computed across study days as the antilog of the covariate-adjusted means and their 95% CIs. Spearman rank correlation coefficients (ρ) were used to assess associations between PhIP levels in hair with dietary intake of PhIP, P450 1A2 caffeine metabolic phenotype, and PhIP and its metabolite levels in urine. Additional linear regression models for PhIP accrued in hair at day 49, with and without normalization for melanin, were conducted to examine its associations with dietary exposure to PhIP (µg) and melanin. All analyses were conducted using SAS, version 9.2 (SAS Institute, Inc.). P values were 2-sided, and P < 0.05 is considered statistically significant.

Results

PhIP content in well-done cooked meats and dietary exposure in semiconrolled feeding study

The concentration of PhIP in cooked meat ranged from 6.2 to 81 ng/g. The meat portion size was adjusted (150 or 200 g) so that the amounts of PhIP given per serving to the subjects were on average (µg PhIP/serving): 1.2 (study group 1), 3.0 (study group 2), 11.7 (study group 3), and 8.0 (study group 4).

PhIP biomarkers in urine and hair

The analytic methods for measurement of PhIP and its metabolites in urine and PhIP in hair resulted in within-day and between-day CVs (%) ≤ 10% (14, 15, 27). The mass chromatograms of PhIP and its metabolites in urine collected from a typical subject during the study are shown in Fig. 2. There was no evidence of urinary biomarkers of PhIP in the pre-dose urine specimen, whereas PhIP and its metabolites were readily identified in the urine specimen during the feeding phase. The unmetabolized PhIP present in urine 12 hours following consumption of cooked meat ranged from undetectable to 0.39% (day 23); undetectable to 0.81% (day 35); and undetectable to 0.51% (day 49) of the ingested dose.
Consistent with previous studies (13, 15, 30, 33), HON-PhIP-N2-Gl, a conjugate of the genotoxic metabolite 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine (HONH-PhIP), was the predominant urinary metabolite and accounted for 6.1% to 80% (day 23); 8.6% to 75% (day 35); and 2.4% to 91% (day 49) of the ingested dose, HON-PhIP-N3-Gl accounted for 0.3% to 7.2% (day 23); 0.4% to 6.4% (day 35); and 0.2% to 6.5% (day 49) of the ingested dose. The analysis of PhIP accrued in newly grown hair collected from one typical subject during the predose and immediately following the 4-week controlled meat feeding phase is shown in Fig. 2. The product ion spectra of the analyte corroborated its identity as PhIP.

The geometric mean levels of PhIP in hair are summarized by study day in Table 1. The mean levels of PhIP are expressed in 3 ways: per pg/g of hair, unadjusted and adjusted for melanin content in the regression model, and expressed as ng/g melanin to account for the influence of pigmentation on the accrual of PhIP in human hair (24, 34). The prefeeding period average levels of PhIP in hair of subjects before consuming well-done cooked meat diet (days 0 and 21) ranged between 79 and 93 pg/g hair; these values are about 2-fold above the limit of quantification (LOQ). During this period, the levels of PhIP in hair of subjects before consuming well-done red meat induced P450 1A2 activity (Table 2).

The positive effect of grilled meat consumption on the induction of P450 1A2 activity (Table 2). The average level of P450 1A2, as assessed by the urinary ratio (17U + 17X)/137X, varied from 6.3 to 7.1 before meat consumption, from 9.6 to 10.4 during the meat feeding period and from 7.1 to 7.2 during the postfeeding period; the difference in means was significantly different between feeding and nonfeeding periods (P = 0.0001). The positive effect of grilled meat consumption on the induction of P450 1A2 activity is consistent with findings from a previous study (35).

### Table 1. Mean PhIP content in hair and PhIP content in hair by melanin levels of subjects on a semicontrolled well-done meat diet

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Melanin level*</th>
<th>Day 0 (n = 43)</th>
<th>Day 21 (n = 33)</th>
<th>Day 49 (n = 44)</th>
<th>Day 77 (n = 44)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhIP (pg/g hair)</td>
<td>ALL</td>
<td>93 (69-126)</td>
<td>79 (60-104)</td>
<td>1,077 (809-1,432)</td>
<td>1,492 (1,120-1,989)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Quartile 1</td>
<td>36 (22-61)</td>
<td>41 (24-72)</td>
<td>492 (297-815)</td>
<td>641 (394-1,041)</td>
<td>900 (552-1,500)</td>
<td></td>
</tr>
<tr>
<td>Quartile 2</td>
<td>116 (70-193)</td>
<td>94 (57-155)</td>
<td>661 (390-1,120)</td>
<td>910 (552-1,500)</td>
<td>910 (552-1,500)</td>
<td></td>
</tr>
<tr>
<td>Quartile 3</td>
<td>165 (99-274)</td>
<td>99 (58-167)</td>
<td>1,865 (1,120-3,107)</td>
<td>2,281 (1,400-3,714)</td>
<td>2,487 (1,536-4,026)</td>
<td></td>
</tr>
<tr>
<td>Quartile 4</td>
<td>98 (58-165)</td>
<td>97 (57-164)</td>
<td>1,527 (924-2,522)</td>
<td>2,487 (1,536-4,026)</td>
<td>2,487 (1,536-4,026)</td>
<td></td>
</tr>
<tr>
<td>PhIP (pg/g hair)</td>
<td>ALL</td>
<td>91 (69-120)</td>
<td>76 (58-98)</td>
<td>1,027 (801-1,316)</td>
<td>1,427 (1,121-1,817)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>adjusted for melanin (mg/g hair)*</td>
<td>Quartile 1</td>
<td>50 (21-119)</td>
<td>58 (24-141)</td>
<td>677 (292-1,566)</td>
<td>871 (393-1,930)</td>
<td></td>
</tr>
<tr>
<td>Quartile 2</td>
<td>120 (71-202)</td>
<td>96 (58-160)</td>
<td>683 (402-1,158)</td>
<td>951 (572-1,578)</td>
<td>951 (572-1,578)</td>
<td></td>
</tr>
<tr>
<td>Quartile 3</td>
<td>149 (86-258)</td>
<td>87 (48-157)</td>
<td>1,619 (804-2,900)</td>
<td>2,014 (1,163-3,488)</td>
<td>2,014 (1,163-3,488)</td>
<td></td>
</tr>
<tr>
<td>Quartile 4</td>
<td>77 (38-157)</td>
<td>77 (35-156)</td>
<td>1,191 (588-2,419)</td>
<td>1,944 (989-3,900)</td>
<td>1,944 (989-3,900)</td>
<td></td>
</tr>
<tr>
<td>PhIP (ng/g melanin)</td>
<td>ALL</td>
<td>5.8 (4.4-7.5)</td>
<td>4.8 (3.7-6.3)</td>
<td>64 (50-80)</td>
<td>87 (69-109)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Quartile 1</td>
<td>5.0 (2.9-8.2)</td>
<td>6.0 (3.3-10)</td>
<td>68 (42-108)</td>
<td>83 (52-122)</td>
<td>83 (52-122)</td>
<td></td>
</tr>
<tr>
<td>Quartile 2</td>
<td>7.8 (4.7-13)</td>
<td>6.2 (3.7-10)</td>
<td>45 (27-74)</td>
<td>62 (38-101)</td>
<td>62 (38-101)</td>
<td></td>
</tr>
<tr>
<td>Quartile 3</td>
<td>7.9 (4.8-13)</td>
<td>4.6 (2.6-7.9)</td>
<td>80 (49-128)</td>
<td>101 (63-161)</td>
<td>101 (63-161)</td>
<td></td>
</tr>
<tr>
<td>Quartile 4</td>
<td>3.7 (2.0-6.3)</td>
<td>3.6 (1.9-6.3)</td>
<td>52 (32-83)</td>
<td>84 (53-134)</td>
<td>84 (53-134)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Feeding with well-done meat commenced on day 22 and ceased on day 49. Geometric mean and 95% CI based on mixed linear model.

*Melanin levels by quartiles: <11.7, 11.7-20.8, 20.9-25.5, >25.5 mg/g.

*Comparing the values of days 0 and 21 to values of days 49 and 77 based on an F test with (1, 43) degrees of freedom.

*Melanin was included as an adjustment factor in the linear model.
PhIP intake and levels of PhIP accumulated in hair (scale) versus PhIP intake are presented in Fig. 3A and B. There was a significant correlation between amount of PhIP accrued in hair and the factors potentially affecting PhIP hair levels. Correlations are summarized in Table 3, and plots of PhIP levels in hair, expressed either as pg/g hair or ng/g melanin (on a logarithmic model), when the melanin content of hair was taken into account (r = 0.53, P < 0.001), and the correlation was strengthened when the melanin content of hair was taken into account (r = 0.71, P < 0.001). Subjects with dark hair and higher melanin content sequestered more PhIP in their hair than subjects with lighter hair pigmentation, with r = 0.48 (P = 0.001) between PhIP accrual in hair and melanin content (Table 3). We further explored this relationship in Fig. 3C and D. The correlation is poor when the level of PhIP in hair is further adjusted for melanin content; r = −0.04 (P = 0.79) for PhIP (ng/g melanin)/PhIP intake (µg) versus melanin, thereby showing that melanin pigmentation is a major factor in PhIP binding to hair.

The amount of PhIP present in urine was not correlated to the amount of PhIP accrued in hair, irrespective of adjustment for the melanin content of hair (unadjusted r = −0.06, adjusted r = −0.11, P ≥ 0.47). The levels of PhIP in urine were also not strongly correlated to rapid P450 1A2 phenotype, based upon caffeine metabolic ratios (r = −0.20, P = 0.20). Moreover, a significant inverse correlation was not observed between the levels of PhIP in hair, normalized for melanin content, and rapid phenotype of PhIP metabolism, using the metabolic ratio of the 2 major urinary metabolites of PhIP, HON-PhIP-N²-Gl/PhIP (r = −0.23, P = 0.14) or HON-PhIP-N3-Gl/PhIP (r = −0.07, P = 0.63).

**Correlations associating PhIP levels in hair with dietary exposure to PhIP, P450 1A2 metabolic phenotype, PhIP metabolic phenotype and hair pigmentation**

We examined the interrelationships among PhIP accrued in hair and the factors potentially affecting PhIP hair levels. Correlations are summarized in Table 3, and plots of PhIP levels in hair, expressed either as pg/g hair or ng/g melanin (on a logarithmic scale) versus PhIP intake are presented in Fig. 3A and B. There was a significant correlation between amount of PhIP intake and levels of PhIP accumulated in hair (r = 0.53, P < 0.001), and the correlation was strengthened when the melanin content of hair was taken into account (r = 0.71, P < 0.001). Subjects with dark hair and higher melanin content sequestered more PhIP in their hair than subjects with lighter hair pigmentation, with r = 0.48 (P = 0.001) between PhIP accrual in hair and melanin content (Table 3). We further explored this relationship in Fig. 3C and D. The correlation is strengthened after normalization for PhIP intake: r = 0.63 (P < 0.001) for PhIP (pg/g hair)/PhIP intake (µg) versus melanin content (mg/g hair). However, the correlation is poor when the level of PhIP in hair is further adjusted for melanin content: r = −0.04 (P = 0.79) for PhIP (ng/g melanin)/PhIP intake (µg) versus melanin, thereby showing that melanin pigmentation is a major factor in PhIP binding to hair.

The amount of PhIP present in urine was not correlated to the amount of PhIP accrued in hair, irrespective of adjustment for the melanin content of hair (unadjusted r = −0.06, adjusted r = −0.11, P ≥ 0.47). The levels of PhIP in urine were also not strongly correlated to rapid P450 1A2 phenotype, based upon caffeine metabolic ratios (r = −0.20, P = 0.20). Moreover, a significant inverse correlation was not observed between the levels of PhIP in hair, normalized for melanin content, and rapid phenotype of PhIP metabolism, using the metabolic ratio of the 2 major urinary metabolites of PhIP, HON-PhIP-N²-Gl/PhIP (r = −0.23, P = 0.14) or HON-PhIP-N3-Gl/PhIP (r = −0.07, P = 0.63).

**Discussion**

PhIP is present in the hair of omnivores (24, 26, 27), whereas the levels of PhIP are negligible in the hair of vegetarians (27). Moreover, the levels of PhIP in hair samples from individual omnivores were reported to vary by less than 24% over a 6-month interval, signifying that the exposure to PhIP is relatively constant over time (27). These promising findings suggest that the measurement of PhIP in hair tissue can be used as a reliable, long-lived biomarker to assess exposure to this dietary carcinogen.

### Table 2. Mean P450 1A2 caffeine metabolic phenotype of subjects on a semicontrolled well-done meat diet

<table>
<thead>
<tr>
<th>Study day</th>
<th>N</th>
<th>S1 + S2</th>
<th>N</th>
<th>S3 + S4</th>
<th>N</th>
<th>All *</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>21</td>
<td>6.4 (4.7–8.7)</td>
<td>23</td>
<td>7.7 (5.7–10.2)</td>
<td>44</td>
<td>7.1 (5.7–8.7)</td>
</tr>
<tr>
<td>21</td>
<td>21</td>
<td>6.3 (4.3–8.9)</td>
<td>22</td>
<td>6.4 (4.4–9.0)</td>
<td>43</td>
<td>6.3 (4.9–8.1)</td>
</tr>
<tr>
<td>35</td>
<td>21</td>
<td>10.2 (7.5–13.9)</td>
<td>23</td>
<td>10.5 (7.8–14.1)</td>
<td>44</td>
<td>10.4 (8.4–12.8)</td>
</tr>
<tr>
<td>49</td>
<td>21</td>
<td>9.8 (7.2–13.4)</td>
<td>23</td>
<td>9.2 (6.8–12.4)</td>
<td>44</td>
<td>9.6 (7.7–11.8)</td>
</tr>
<tr>
<td>70</td>
<td>21</td>
<td>6.4 (4.5–9.2)</td>
<td>23</td>
<td>7.7 (5.35–10.8)</td>
<td>44</td>
<td>7.1 (5.6–9.0)</td>
</tr>
<tr>
<td>77</td>
<td>20</td>
<td>7.6 (5.4–10.5)</td>
<td>23</td>
<td>6.8 (4.9–9.3)</td>
<td>43</td>
<td>7.2 (5.7–9.0)</td>
</tr>
</tbody>
</table>

NOTE: Feeding with well-done meat commenced on day 22 and ceased on day 49. Geometric mean and 95% CI based on mixed linear model.

*P = 0.0001 comparing the values of days 14, 21, 70, and 77 to values of days 35 and 49 based on an F test with (1, 43) degrees of freedom.

### Table 3. Spearman correlations between PhIP in hair and other analytes at day 49

<table>
<thead>
<tr>
<th></th>
<th>PhIP (pg/g hair)</th>
<th>PhIP (ng/g melanin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>P</td>
</tr>
<tr>
<td>PhIP intake, ng</td>
<td>0.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Melanin (mg/g hair)</td>
<td>0.48</td>
<td>0.001</td>
</tr>
<tr>
<td>P450 1A2 (metabolic ratio, based on 12-hour urine)</td>
<td>−0.21</td>
<td>0.18</td>
</tr>
<tr>
<td>% dose PhIP</td>
<td>−0.06</td>
<td>0.71</td>
</tr>
<tr>
<td>% dose HONH-PhIP-N²-Gl</td>
<td>−0.21</td>
<td>0.17</td>
</tr>
<tr>
<td>% dose HONH-PhIP-N3-Gl</td>
<td>−0.13</td>
<td>0.38</td>
</tr>
<tr>
<td>Metabolic ratio:HONH-PhIP-N²-Gl/PhIP</td>
<td>−0.18</td>
<td>0.24</td>
</tr>
<tr>
<td>Metabolic ratio:HONH-PhIP-N3-Gl/PhIP</td>
<td>−0.11</td>
<td>0.47</td>
</tr>
</tbody>
</table>
There is a large interindividual variation in the levels of PhIP accrued in hair of subjects on unrestricted diets (24, 26, 27). A feeding study with known exposure to PhIP is essential to understand the interrelationship among PhIP hair levels, dietary intake of PhIP, phenotypes associated with PhIP metabolism, and hair pigmentation; all of these interactive factors are expected to influence the accrual of PhIP in hair.

In our meat-feeding study, the level of PhIP intake and its biomarkers in urine and hair were carefully monitored during the predose, the 4-week meat-feeding, and postdose phases. Hair from the vertex posterior position of the scalp is often used for drug testing of chemicals 4 to 5 weeks after exposure (36). However, we selected newly grown hair from the nape of the neck because multiple collections of hair were required to measure the kinetics of PhIP uptake in hair within short time intervals. The mean levels of PhIP substantially increased after initiation of the well-done meat diet and continued to increase in newly grown hair 4 weeks after termination of the meat diet. An increase was observed in individuals with lighter and darker hair. In contrast to the findings of the hair biomarker, PhIP and its metabolites were not detected in urine samples, during this washout phase. The levels of PhIP in hair returned to basal levels at 12 weeks after the meat-feeding phase. Thus, the time interval between the absorption of PhIP into the hair follicle and the outgrowth of the corresponding hair shaft through the subjects’ skin surface is at least 4 weeks.

The consumption of PhIP in our semicontrolled feeding study corresponds to a dosage range from 17 to 166 ng of PhIP/kg body weight, assuming an average body weight of 70 kg. These amounts of PhIP are 2- to 13-fold higher than the average intake estimated between 9.2 and 12.6 ng of PhIP/kg body weight in U.S. males (2). The geometric mean for hair level of PhIP, expressed as ng/g melanin, peaked at 87 (95% CI, 69–109) on day 77. When the data are stratified by study group, the 9 volunteers who consumed the smallest dose of 1.2 mg PhIP per day had a mean level of PhIP in hair of 27 ng/g melanin (95% CI, 18–39), and the 16 subjects who consumed the highest dose of 11.7 mg PhIP per day had a mean level of 133 ng/g melanin (95% CI, 106–165). In comparison, in an independent study, the geometric means of PhIP in hair were estimated at 32 ng/g melanin (95% CI, 15–70) for 5 subjects from Albany, NY, when measured by the same analytical method (27). In comparison, a mean of 16.6 ng PhIP/g melanin (95% CI, 9–18) was determined for subjects (n = 20) on a free-choice diet in Japan, where a different analytic method was used to estimate the level of PhIP in hair.

P450 1A2 is the principal cytochrome P450 enzyme involved in the metabolism of PhIP (29, 37, 38), and it accounts for approximately 70% of the clearance of PhIP in hair.

There is a wide range of PhIP in hair levels versus dietary exposure to PhIP, with a strong correlation (Adjusted $R^2 = 0.52$). Similarly, there is a strong correlation in PhIP hair levels normalized for dietary intake of PhIP and melanin hair content (Adjusted $R^2 = 0.31$). The relationship between PhIP intake and hair levels is weakly positively correlated (Pearson’s $\rho = 0.63$, $P < 0.0001$), while the relationship between PhIP intake and melanin hair content is weakly negatively correlated (Pearson’s $\rho = -0.04$, $P = 0.79$).
The interindividual difference in hepatic P450 1A2 protein content can vary by more than 50-fold (29), and similar variations in P450 1A2 activity are observed in vivo, when using caffeine as the metabolic probe (39). The amount of unmetabolized PhIP in the bloodstream that reaches the hair follicle, following first-pass metabolism by hepatic P450 1A2, was expected to differ among individuals, and the phenotypic activity of P450 1A2 was considered to be an important factor influencing the level of PhIP accrued in hair: subjects with rapid P450 1A2 phenotypes were expected to have the lowest levels of PhIP. However, a significant inverse correlation was not observed between the levels of PhIP in hair and rapid P450 1A2 activity, when using caffeine as the metabolic probe. The range in levels of this measure of P450 1A2 activity among the volunteers in our feeding study showed an 18-fold difference, which may have not been sufficient to impact the levels of PhIP binding to hair. Earlier studies examining urinary caffeine metabolic ratios also did not detect an inverse relationship between P450 1A2 activity and the levels of PhIP excreted in urine (40). Hepatic P450 1A2 expression, even in subjects with slow P450 1A2 phenotypes (41), may be at levels sufficient to significantly reduce the concentration of PhIP during first-pass metabolism before it reaches systemic circulation.

Rapid metabolizers of PhIP, based on the urinary metabolic ratio HONH-N²-PhIP-Glu/PhIP, also did not have significantly lower levels of PhIP in their hair than the slow metabolizers of PhIP. However, the metabolic ratio HONH-N²-PhIP-Glu/PhIP for many subjects showed significant intra-individual variation during study days when urine was collected. This short-term urinary biomarker represents a “snap-shot” of the metabolic phenotype and may not accurately reflect the overall phenotype during the 4-week feeding study and thus may not be expected to correlate to the long-term hair biomarker.

Pigmentation, and melanin in particular, has a pronounced effect on the binding of PhIP to fur of animals (34, 42) and also human hair (23, 26, 27). Our data show that the binding of PhIP to hair should be adjusted for melanin content to standardize the accrual of PhIP in hair, particularly for subjects with red, blonde, or gray hair where the melanin content is low (31). The role of melanin content in PhIP uptake in light-haired subjects merits further study. The intake of PhIP in our semicontrolled feeding study was up to 15-fold higher than the average intake levels of PhIP estimated in the United States, and the meat was cooked very well done. Ongoing feeding studies with a lesser intake of PhIP from meat cooked at lower temperatures than our current study will enable us to further our understanding about PhIP accrual in hair and its use as a biomarker. The use of hair containing PhIP represents the first long-term biomarker of HAAAs, which are potential human carcinogens. The analysis of PhIP levels in hair may be used in molecular epidemiology studies to estimate exposure and assess the risk of disease by consumption of this HAA formed in well-done cooked meats.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
Biomonitoring the Cooked Meat Carcinogen 2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]Pyridine in Hair: Impact of Exposure, Hair Pigmentation, and Cytochrome P450 1A2 Phenotype

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