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Abstract

Epidemiologic evidence indicates that exposure to heterocyclic amines (HAs) in the diet is an important risk factor for the development of colon cancer. Well-done cooked meats contain significant levels of HAs which have been shown to cause cancer in laboratory animals. To better understand the mechanisms of HA bioactivation in humans, the most mass abundant HA, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), was used to assess the relationship between PhIP metabolism and DNA adduct formation. Ten human volunteers where administered a dietary relevant dose of [^{14}C]PhIP 48-72 h prior to surgery to remove colon tumors. Urine was collected for 24 h after dosing for metabolite analysis, and DNA was extracted from colon tissue and analyzed by accelerator mass spectrometry for DNA adducts. All ten subjects were phenotyped for CYP1A2, NAT2, and SULT1A1 enzyme activity. Twelve PhIP metabolites were detected in the urine samples. The most abundant metabolite in all volunteers was N-hydroxy-PhIP-N^2-glucuronide. Metabolite levels varied sifnificantly between the volunteers. Interindividual differences in colon DNA adducts levels were observed between each individual. The data showed that individuals with a rapid CYP1A2 phenotype and high levels of urinary N-hydroxy-PhIP-N^2-glucuronide, had the lowest level of colon PhIP-DNA adducts. This suggests that glucuronidation plays a significant role in detoxifying N-hydroxy-PhIP. The levels of urinary N-hydroxy-PhIP-N^2-glucuronide were negatively correlated to colon DNA adduct levels. Although it is difficult to make definite conclusions from a small data set, the results from this pilot study have encouraged further investigations using a much larger study group.

Introduction

Humans are constantly exposed to a wide range of chemical carcinogens in their environment. One of the most significant sources of environmental exposure is through the diet (1). Heterocyclic amines (HAs), formed during the cooking of muscle meats have been determined to be mutagenic in standard test systems and carcinogenic in animal models (2). These compounds are found in concentrations of up to 500 ppb in foods commonly consumed in a typical Western diet. 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most mass abundant HA found in well-done cooked chicken and beef (3, 4), has been shown to cause colon, mammary, and prostate tumors in rats, lymphomas in mice and hepatic adenomas in neonatal mice (5-8). In humans, epidemiology studies have indicated an increased risk of colon and breast tumors associated with PhIP exposure from well-done red meat consumption (9, 10). PhIP has also been designated as "reasonably anticipated to be a human carcinogen" by the National Toxicology Program. These findings, together with the relative abundance of PhIP in cooked foods, suggests that exposure to PhIP is a significant cancer risk factor for humans.

Heterocyclic amines require metabolic activation to a bio-reactive species before they can interact with DNA to form adducts. The bioactivation of PhIP to its carcinogenic species is highly dependent on the cytochrome P4501A2-mediated hydroxylation of the exocyclic amine group to form the corresponding 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-hydroxy-PhIP) (11, 12). Subsequent esterification by sulfotransferases and/or acetyltransferases generates the highly electrophilic *O*-sulfonyl and *O*-acetyl esters, respectively. These esters are capable of covalently binding DNA (13, 14). N-Hydroxy-PhIP can also form the less reactive glucuronide conjugates at the N^2 amine nitrogen or the N3 ring nitrogen which can be excreted through the urine or bile, or be transported to other tissue were further metabolism can occur (15, 16). This pathway is a predominant step in the biotransformation of PhIP in humans (17).

Studies have shown that the rate of HA metabolism can be influenced by enzyme phenotype and/or genotype. A correlation between cytochrome P4501A2 (CYP1A2) activity levels and urinary excretion of HA metabolites was observed in human

populations fed well-done cooked meat. High levels of CYP1A2 activity was associated with reduced levels of unmetabolized PhIP in the urine, indicating more PhIP is being converted to the bioactive N-hydroxy derivative compared to urinary HA levels from individuals with low CYP1A2 activity (18-20). In addition, polymorphisms in the UDP-glucuronosyltransferase 1A1 (*UGT1A1*) gene was associated with a decreased ability to detoxify N-hydroxy-PhIP via glucuronidation in human liver samples due to reduced UGT1A1 activity (21).

Enzyme polymorphisms can lead to interindividual variation in DNA adduct formation as well. A positive correlation was reported between the level of DNA adducts and the level of CYP1A2 activity in human liver microsomes incubated with PhIP. As CYP1A2 activity increased there was a concomitant increase in PhIP-DNA adduct levels. These results indicate that DNA adducts can be used as an indicator of interindividual variability in the metabolic activation of HAs (22).

Increases in bioactivation and DNA adduct formation due to enzyme polymorphisms have been implicated as increased cancer risk factors associated with HA exposure. For example, phenotypic differences in CYP1A2 and N-acetyltransferase 2 (NAT2) activity have been linked to colon cancer risk. People who possess the rapid phenotype for both enzymes and consume high levels of dietary HAs have been shown to be at a greater risk to develop colon cancer than people who possess the slow phenotype (23). Therefore, susceptibility to the carcinogenic risks associated with HA exposure can depend on exposure levels, as well as, specific enzyme phenotypes that can effect the bioactivating capacity of certain metabolizing enzymes.

The relationship between PhIP-DNA adduct formation, metabolism, and exposure has been primarily established at high PhIP doses using animal models, mostly due to limitations in assay sensitivity and the difficulties associated with human *in vivo* studies. In the present study these limitations have been overcome by using accelerator mass spectrometry (AMS), which is capable of accurately measuring attomole (10^{-18}) quantities of radiolabeled compound (24, 25). The goal of the current study is to determine the relationship between PhIP metabolism, DNA adduct levels, and enzyme phenotype in humans, at a dietary relevant dose of PhIP, and to determine if metabolite

levels and/or enzyme phenotype can predict interindividual susceptibility to DNA adduct formation.

Accelerator mass spectrometry was used to measure colon DNA adducts in a human population exposed to a dietary equivalent dose of PhIP, labeled with a very low level of 14-carbon. The 0.011mSv radioactive dose each subject received is equivalent to 1/29 of the energy received from an average chest x-ray. Urinary PhIP metabolite levels and selected enzyme phenotypes were also assessed in an effort to establish a link between metabolite levels, enzyme phenotype, and DNA adducts. Data is presented that suggests urinary PhIP metabolite levels can serve as a biomarker to predict interindividual differences in DNA adduct levels at dose levels that are typical of human exposure conditions. This pilot study should provide a better understanding of the critical role each metabolic step has in the bioactivation and detoxification of this carcinogen. Ultimately, an assessment of individual susceptibility to the potential cancer risks from PhIP exposure should be possible.

Materials and Methods

Chemicals. [2-^{14}C]PhIP was obtained from Toronto Research Chemicals (North York, Ontario, Canada). After repurification the chemical purity was > 99% and the radiopurity was > 95%. Qiagen tip-500 anion exchange columns for DNA extractions were obtained from Qiagen (Valencia, CA). Proteinase K, Rnase A and Rnase T_1 were purchased form Sigma Chemical Co. (St. Louis O). All other reagents were of analytical grade or better.

Human Study. The human study protocol was independently reviewed and approved by the Institutional Review Boards for Human Subjects at the Lawrence Livermore National Laboratory, the University of Arkansas Medical School Hospital, and the J.L. McClellan Memorial Veterans Administration Medical Center in Little Rock AR. Details of the study protocol are reported elsewhere (26). Briefly, ten human volunteers were recruited for the study. All volunteers were undergoing surgery to remove colon carcinoma(s) and gave informed consent prior to enrollment. The study population consisted of 9 Caucasian males and 1 Caucasian female ranging in age from 44-82 yrs. [^{14}C]PhIP was administered orally in a gelatin capsule prior to surgery. Subjects 1 and 2 received a dose

of 70 µg [^{14}C]PhIP per person (specific activity, 56 mCi/mmol), and subjects 3-10 received a dose of 84 µg [^{14}C]PhIP (specific activity 41.8 mCI/mmol). The differences in the amounts and the specific activity of [^{14}C]PhIP were a result of preparing the PhIP capsules on two different occasions from two batches of [^{14}C]PhIP.

Urinary Metabolite Characterization. Urine was collected at various time points up to 24 h after [^{14}C]PhIP exposure and then frozen at –20°C until processing for metabolite analysis. Details of the urinary metabolite analysis have been previously reported (17). Briefly, an aliquot of each urine sample containing approximately 6000-8000 DPM was analyzed by HPLC for PhIP and PhIP metabolites. Each sample was directly injected into a Rainin HPLC system (Varian, Walnut Creek, CA) equipped with a 5 µm, 4.6 x 220 mm TSK-GEL ODS-80 TM column (TosoHaas, Montgomeryville, PA), and monitored at 315 nm. The column eluate was collected at 1-minute intervals and radioactivity was quantified by scintillation counting (Wallic, Gaithersburg, MD). Metabolites were identified by co-elution with authentic PhIP metabolite standards and by mass spectral characterization (17). For mass spectral analysis each isolated metabolite was directly injected into a Michrom µLC system (Michrom Bioresources Inc., Auburn, CA) equipped with a Zorbax C18 SB column (0.2 x 150 mm) (Michrom Bioresources Inc., Auburn, CA) and coupled to a Finnigan MAT TSQ-700 triple quadrupole mass spectrometer through a Finnigan electrospray interface (Finnigan MAT, San Jose, CA). Full scan and collision induced dissociation (CID) mass spectra were obtained for each metabolite, using previously described parameters (17).

Tissue Collection and DNA Extraction. Forty-eight to seventy-two hours after dosing the volunteers underwent surgery for a partial colonectomy. Colon tissue not required for diagnosis or staging was collected and frozen at –80°C until analysis. DNA and protein was extracted from the colon tissue and analyzed by AMS for PhIP-adduct formation using previously reported methods (26, 27).

Enzyme Phenotyping. Blood was collected prior to [^{14}C]PhIP dosing for phenotyping sulfotransferase 1A1 (SULT1A1) activity. Separation of platelets and preparation of

cytosol for SULT1A1 phenotyping has been described previously (28). Phenotyping for CYP1A2 and N-acetyltransferae 2 (NAT2) was done pre- or post-operatively using the caffeine phenotyping assay (29, 30).

Statistical Analysis. Two statistical approaches were used to analyze the data. The first utilized univariate linear modeling, and stepwise variable selection using the Akaike Information Criterion (AIK), method. These parametric methods were used because of the small number of degrees of freedom (df = 9) available in this study. Since there were 26 variables (Table 5) but only ten observations in the study, and the degrees of freedom in a linear model is df = n - p, where n is the number of observations, and p is the number of parameters or variables, a model including all 26 variables could not be fitted.

The second approach, used the nonparametric Random Forest Regression analysis, developed by Breiman and Cutler (http://www.stat.berkeley.edu/users/breiman/RandomForest/), which is a test to determine the importance of one variable in relation to another. This method ranks all variables in a study in the order of importance. The importance measure, which is the percent increase in Mean Squared Error (MSE), is calculated for each variable. Each variable is then randomly shuffled across observations, while all other variables are left as is. The new MSE is calculated using the Random Forest Regression algorithm. If the variable in question is an important predictor of the outcome (i.e. DNA adducts), the new calculated MSE will be higher than the original MSE. The larger the percent increase in MSE, the more important the variable is as a predictor of outcome.

Results

Urinary Metabolite Analysis. The total amount of radioactivity recovered in the urine over 24 h varied among the ten individuals, ranging between 32-85% of the administered dose (Figure 1). Part of this variation may be due to inconsistencies in the sample labeling from subjects 2 and 9, non-continuous sampling from subject 3, and fecal contamination in the urine from subject 8. Therefore, no further conclusions were made from these observations. However, the amount of recovered urinary radioactivity also varied over time. In subjects 1, 4, 7, 8 and 10 over 45% of the total amount of recovered

radioactivity was excreted in the first 4 h after [^{14}C]PhIP dosing. In subjects 2, 3, 5, 6, and 9, the majority of radioactivity was recovered in the later time periods (Figure 2).

All ten volunteers produced 12 radioactive metabolite peaks associated with PhIP from the first 24 h of collected urine. Identification of the major urinary PhIP metabolites from a subset of these individuals has been previously reported (17), and is presented in Table 1. Six of the twelve metabolites were identified as PhIP glucuronide conjugates. In all ten subjects the amount of PhIP-glucuronide metabolites accounted for 60-85% (Table 2) of the total metabolites indicating that in humans, glucuronidation is a major pathway in the biotransformation of PhIP.

The most abundant PhIP metabolite in all ten volunteers was determined to be N-hydroxy-PhIP-N^2-glucuronide. The amount of this metabolite varied greatly between individuals, accounting for 37-62% of the total amount of recovered urinary metabolites (Table 2). Excretion rates for this metabolite also varied significantly between each subject (Figure 3). In five out of the ten subjects over 45% of N-hydroxy-PhIP-N^2-glucuronide was excreted in the first four hours after PhIP dosing, with subject 4 being the highest at 63% excreted in 4 h. Subject 2 had the lowest at 19% excreted in 4 h. Metabolites 1, 2, and 3 (all of which have been tentatively identified as PhIP-glucuronide conjugates) displayed a wide range of variability between the ten subjects as well, each accounting for 0.6-8% of the total amount of recovered metabolites. No other metabolites displayed such a wide range of variability between the subjects. Interestingly, in subjects where N-hydroxy-PhIP-N^2-glucuronide was present at greater than 50% of the total recovered metabolites, the cumulative total of metabolites 1, 2, and 3 was less than 7% of the total recovered metabolites. Whereas, when N-hydroxy-PhIP-N^2-glucuronide levels were below 50% of the total recovered metabolites, the cumulative total of metabolites 1, 2, and 3 ranged between 8-20% (Table 2). These results suggest that the enzymes involved in catalyzing the formation of these metabolites may be in competition with each other for substrate.

DNA and Protein Adduct Analysis. The levels of covalently bound [^{14}C]PhIP in colon DNA and protein, and total [^{14}C] levels in whole tissue are presented in Table 3. Interindividual differences were evident in the amount of PhIP bound to all three tissue

fractions. There was no correlation, however, between the binding levels of PhIP in DNA and the levels in the protein or tissue within each individual. Only subject six displayed a consistent binding pattern, having the highest level of [^{14}C]PhIP in DNA, total protein, and whole tissue compared to the rest of the study group. Blood albumin was also collected and assessed for covalent binding of [^{14}C]-PhIP. There was no correlation between albumin adducts and the colon DNA, protein, or tissue adducts (data not shown).

Phenotype Analysis. Each subject was phenotyped for CYP1A2, NAT2, and SULT1A1 enzyme activity. All of which are known to be involved in PhIP metabolism. Table 4 shows the phenotypic activity levels for CYP1A2, NAT2, and SULT1A1 for the ten subjects. The CYP1A2 and NAT2 activities, estimated from the caffeine metabolism phenotyping assay, showed a dichotomized distribution. CYP1A2 activity levels greater that 10 were categorized as having a rapid CYP1A2 phenotype, and values less than 10 were considered a slow metabolizer, based on the analysis of the caffeine metabolite ratios (31). Subject 4 had the highest CYP1A2 activity with a value nearly three times higher then the next highest value in subject 1. The NAT2 values were also divided into 2 groups: a slow group with caffeine metabolite ratios less than 0.66 and a fast group with ratios greater than O.66 (31). Based on this criterion, subjects 1, 4, 5, 9, and 10 were labeled as having a rapid NAT2 phenotype. Only two subjects, (subject 6 and subject 8) were considered to have fast SULT1A1 activity levels when the phenotype values were dichotomized at 1.0 nmol/min/mg protein (32).

Statistical Analysis. Data obtained from the metabolite analysis, enzyme phenotype, and PhIP-adduct data sets were analyzed by various statistical methods to determine if any of the measured variables were correlated to colon DNA adducts. There were twenty-six variables that were considered for this analysis. Both univariate and non-parametric models were used to evaluate the data. Since there were 26 variables but only 9 degrees of freedom it was not possible to fit a full univariate linear model to include all the variables. Therefore, a univariate linear analysis was done for each variable and the p-values were compared from each analysis. Table 5 shows the univariate linear model results for all the variables. N-Hydroxy-PhIP-N^2-glucuronide, metabolite 7, and CYP1A2

activity had the lowest p-values indicating these variables are the most significantly ($p < 0.05$) correlated to colon DNA adducts. However, the low level, and the lack of significant variation in metabolite 7 between each subject deemed this metabolite not useful for predicting DNA adducts. N-Hydroxy-PhIP-N^2-glucuronide was determined to be the best predictor of colon DNA adducts. Because of the low degrees of freedom due to the small sample set, the data was independently re-evaluated using the non-parametric Random Forrest analysis. Although this is a less robust test compared to the univariate analysis, the two independent tests provided the same apparent conclusion that N-hydroxy-PhIP-N^2-glucuronide was the most important variable in predicting the level of PhIP-DNA adducts in the colon.

Linear regression analysis showed that both N-hydroxy-PhIP-N^2-glucuronide levels in the urine and CYP1A2 activity were negatively correlated with the level of PhIP-DNA adducts in the colon. A higher level of N-hydroxy-PhIP-N^2-glucuronide in the urine and a rapid CYP1A2 phenotype was associated with lower levels of colon PhIP-DNA adducts (Table 6).

Discussion

This study has allowed, for the first time, a comprehensive assessment of PhIP metabolism and DNA adduct formation in humans at a dietary relevant dose of PhIP. The use of radiolabeled PhIP and AMS technology allowed for the quantification of urinary metabolites and colon DNA adducts at levels that are consistent with a typical dietary exposure. The findings from this pilot study is consistent with earlier data showing that PhIP urinary metabolites and DNA adducts are formed in significant quantities even from a low dose exposure of PhIP (17, 26). The present study reports on variations in PhIP metabolite excretion and DNA adduct formation within a small study population of ten human volunteers. The data suggests there is a significant correlation between urinary metabolite levels and colon DNA adducts.

Interpretation of the results from this study must take into account that the human subject volunteers were elderly cancer patients undergoing colorectal cancer surgery. Each subject was taking numerous medications prior to surgery for a number of conditions. Furthermore, the PhIP dose they received was administered in capsule form

and was not part of the diet. These factors could have influenced the disposition of PhIP in these subjects.

Six of the twelve PhIP metabolites were identified as glucuronide conjugates indicating that glucuronidation plays a significant role in the metabolism of PhIP. The identification of N-hydroxy-PhIP-N^2-glucuronide as the major urinary metabolite in all ten subjects is consistent with earlier studies showing UGT1A1-mediated glucuronidation as being a major pathway in the biotransformation of PhIP (33). The presence of this metabolite also serves as an indirect indicator of PhIP bioactivation, since formation of the CYP1A2-mediated reactive intermediate N-hydroxy-PhIP is a prerequisite step to forming N-hydroxy-PhIP-N^2-glucuronide (15, 16). These results are contrary to what has been reported for rodents. In both rats and mice N-hydroxy-PhIP-N^2-glucuronide is a minor urinary metabolite. The major metabolite in the rodent is 4'-PhIP-sulfate indicating that hydroxylation at the 4' ring position (a detoxification step) followed by conjugation by sulfotransferase is more prevalent in rodents than in humans (34, 35). This could be due to differences in substrate selectivity and oxidation rates of the different CYP450 isozymes between humans and the rodent model. Consequently, these differences will make it difficult to make accurate extrapolations from rodent data to humans.

The finding that glucuronidation is a major detoxification pathway in the biotransformation of PhIP is significant because the capacity to glucuronidate N-hydroxy-PhIP can vary greatly among individuals due to genetic polymorphisms in the *UGT1A1* gene. The most notable polymorphisms are variants in the *UGT1A1* gene that result in significant down regulation of UGT1A1 activity. The most prevalent polymorphism is characterized by an allelic variant in the *UGT1A1* gene which contains an additional (TA) dinucleotide repeat in the "$A(TA)_nTAA$" box region of the promoter (36). Wild-type UGT1A1 activity is associated with six repeats whereas the variant allele contains seven TA repeats (*UGT1A1*28*). Significant down regulation of UGT1A1 activity results when this variant is present in both alleles (homozygous). The frequency of occurrence is relatively high at 10-12% of the general population. There is evidence to suggest that individuals with the *UGT1A1*28* genotype may be at greater risk for cancer from exposure to HAs that are conjugated by UGT1A1 because their ability to detoxify these compounds would be diminished (37). For example, a recent study has reported a

correlation between the *UGT1A1*28* polymorphism and a decreased ability to glucuronidate and detoxify N-hydroxy-PhIP in human liver microsomes (21). Samples derived from subjects who were homozygous for the *UGT1A1*28* allelic variant showed significant decreases in UGT1A1 protein expression and N-hydroxy-PhIP glucuronidation activity in liver microsomes when compared to samples derived from subjects having the wild-type *UGT1A1*1* genotype.

To determine if the observed variations in metabolism and DNA adducts from the current study population were due to variations in UGT1A1 expression, each individual was genotyped for the *UGT1A1*28* polymorphism. Seven out of the nine individuals tested were heterozygous (6/7) for the "$A(TA)_nTAA$" repeat. The remaining two subjects were homozygous wild-type (6/6). Previous studies have shown that there is no significant difference in UGT1A1 activity between the wild-type and the heterozygous variant (38, 39). Only individuals possessing the homozygous variant (7/7) display significant down regulation of UGT1A1 activity. Therefore, due to the lack of significant UGT1A1 genotype variation, and the homogeneity of the study population, UGT1A1 genotype would not be expected to influence the metabolism or the DNA adduct profiles of these individuals. Additional experiments, using a larger study population, are needed to determine the role of UGT1A1 activity on PhIP-DNA adduct formation.

The differences in urinary PhIP metabolite levels, metabolite excretion rates, and DNA and protein adducts between the study subjects suggests that interindividual phenotypic variation of specific enzymes associated with PhIP biotransformation may be contributing to these observed variations. Although it is difficult to make any definite conclusions from the data due to the small sample size, several trends in metabolite excretion rates, DNA adduct levels and enzyme phenotypes were observed. Subjects 1, 4, and 7, who were categorized as having a rapid CYP1A2 phenotype, had the lowest level of [^{14}C]PhIP bound to colon DNA. These subjects also had the fastest excretion rate and the highest levels of urinary N-hydroxy-PhIP-N^2-glucuronide after 24 h of collection, suggesting detoxification of N-hydroxy-PhIP predominated over bioactivation. These observations are in agreement with the linear regression analysis that showed both N-hydroxy-PhIP-N^2-glucuronide and CYP1A2 activity were negatively correlated to colon DNA adducts. Furthermore, the univariate analysis showed that N-hydroxy-PhIP-N^2-

glucuronide levels and CYP1A2 phenotype were also the most important variables in predicting colon DNA adduct levels.

Subjects 8 and 10 also had rapid excretion rates of N-hydroxy-PhIP-N^2-glucuronide, although, these individuals were categorized as having a slow CYP1A2 phenotype. These two subjects, however, did have a *UGT1A3*3* genotype (unpublished data), which is associated with increased expression of UGT1A3 (40). The increased expression of UGT1A3 could have contributed to an increase in the formation of N-hydroxy-PhIP-N^2-glucuronide (33). In another observation subject 6 had the highest level of PhIP macromolecular adducts in all three endpoints (DNA, total protein and whole tissue), and was the only subject with both NAT2 and SULT1A1 rapid phenotypes. Statistical analysis however, indicated there was no correlation ($p < 0.05$) between the NAT2 or SULT1A1 phenotype and DNA adduct levels. Furthermore, the differences among each individual in the time the colon tissue was harvested after [^{14}C]PhIP exposure and the relative rate of DNA repair could have influenced DNA adduct levels. Further study is needed to determine if these observations are relevant to human cancer risk. Other variables that were excluded from analysis, due to the lack of relevance to PhIP metabolite levels and DNA adducts, were CYP1A1, NAT1 and glutathione-S-transferase genotypes (data not shown).

The finding that colon DNA adducts were lower in subjects with a rapid CYP1A2 phenotype and higher urinary N-hydroxy-PhIP-N^2-glucuronide levels suggests that detoxification by glucuronidation predominated over bioactivation in these individuals. This was somewhat unexpected since Lang *et al*. reported a correlation between rapid CYP1A2 and rapid NAT2 activity with a higher incidence of colon cancer among individuals who consume heterocyclic amines in their diet (23). However, Sachse et al. reported that CYP1A2 activity was lower in colon cancer patients compared to controls (41). These findings suggests that DNA adducts alone may not be good predictors of cancer incidence. The low levels of adducts and high N-hydroxy-PhIP-N^2-glucuronide levels observed in rapid CYP1A2 individuals seen in this present study could partially be explained by the differences in the K_m and V_{max} values for CYP1A2 and UGT1A1, as well as the phase II activation enzymes (i.e. acetyltransferase and/or sulfotransferase). High levels of N-hydroxy-PhIP-N^2-glucuronide indicates that the reactive intermediate,

N-hydroxy-PhIP, was detoxified by glucuronidation at a faster rate compared to conjugation by acetyltransferase and/or sulfotransferase, which results in further activation to the DNA binding species. Therefore, it can be postulated that individuals with compromised UGT activity may be at greater risk from exposure to PhIP than individuals with normal or elevated levels of UGT because their ability to detoxify PhIP will be diminished. These results are consistent with the present understanding of PhIP metabolism (42).

Based on the above observations, and from the statistical analysis of the data it was determined that out of all the variables investigated, N-hydroxy-PhIP-N^2-glucuronide was the best predictor of colon DNA adducts. The subjects with high levels of N-hydroxy-PhIP-N^2-glucuronide in their urine had the lowest level of PhIP-DNA adducts in their colon. This is the first time a urinary biomarker has been linked to a genotoxic endpoint. These conclusions must be made with caution, however, since they are based on a small study group that may not be representative of the general population. Nevertheless, this pilot study has provided some insight into the disposition of PhIP in a human population at a dietary relevant dose, and has served as a basis for continual study to determine if N-hydroxy-N^2-glucuronide can serve as a reliable urinary biomarker to predict an individual's propensity to form colon DNA adducts.

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Table 1. Identity of Urinary Radioactive HPLC Peaks Associated with PhIP

Peak no.	HPLC rt (min)	*m/z* $[M+H]^+$ *	Metabolite peak assignment
1	20	417.1/241/225	PhIP-glucuronide†
2	22	417.1/241/225	OH-PhIP-glucuronide†
3	27	417.1/241/225	OH-PhIP-glucuronide†
4	32	n/a	Unknown
5	36	n/a	Unknown
6	41	320.3/240.9/224.9	4'-SO_4-PhIP
7	45	n/a	Unknown
8	50	401.3/224.9	PhIP-N^2-glucuornide
9	52	417/241/225.1	N^2-OH-PhIP-N^2-glucuronide
10	57	417/225.1	N^2-OH-PhIP-N3-glucuornide
11	62	n/a	N^2-OH-PhIP†
12	65	225	PhIP

*LC/MS/MS analysis showing parent and fragment ions; n/a=not available
†Tentative identification

Table 2. PhIP metabolites recovered from human urine in 24 h after a ^{14}C-PhIP dose*

metab. no.†	Subject Number									
	1	2	3	4	5	6	7	8	9	10
1	0.0	6.3	1.8	1.1	3.1	3.4	0.8	2.7	1.2	1.3
2	0.6	5.6	2.7	3.1	6.2	7.9	1.6	4.6	4.8	2.1
3	0.0	7.9	3.5	2.8	7.2	6.0	1.8	5.2	5.6	2.4
4	0.7	0.0	1.2	1.4	1.0	0.7	1.9	1.0	1.4	1.4
5	0.0	3.7	1.0	0.7	1.0	1.8	0.9	0.8	1.6	0.9
6	7.9	6.1	10.0	7.3	10.3	5.5	6.5	7.6	7.4	4.0
7	1.2	2.0	2.3	1.1	2.1	1.8	1.1	1.7	1.8	1.2
8	6.5	9.0	3.8	4.3	7.0	10.7	4.1	8.0	7.4	7.2
9	61.4	47.3	37.2	56.8	45.7	47.7	62.3	48.3	47.4	59.7
10	8.6	8.1	9.6	7.5	7.9	9.6	8.4	9.6	6.4	11.0
11	7.3	2.8	7.6	6.2	5.5	2.4	6.8	5.8	9.3	5.2
12	0.7	1.2	0.6	0.4	0.6	0.5	0.4	0.6	0.7	0.3

*Data is expressed as percent of recovered urinary radioactivity in 24 h
†See Table 2 for metabolite identity

Table 3. Covalent binding of [^{14}C]PhIP to colon DNA and protein, and total [^{14}C] levels in whole tissue*

Subject no.	DNA	Total protein	Whole tissue
1	30.25 ± 2.3	67.44 ± 1.4	41.97 ± 7.9
2	49.99 ± 2.6	107.73 ± 1.8	133.95 ± 27.4
3	97.24 ± 7.3	98.32 ± 1.2	77.22 ± 7.3
4	26.14 ± 3.9	183.09 ± 48.9	92.39 ± 35.0
5	50.96 ± 11.0	66.45 ± 0.8	58.09 ± 19.0
6	119.34 ± 2.5	398.85 ± 86.2	279.29 ± 68.9
7	34.55 ± 4.2	266.51 ± 87.5	42.76 ± 15.3
8	71.19 ± 1.8	223.57 ± 6.9	259.99 ± 72.4
9	100.85 ± 1.0	247.84 ± 4.2	169.84 ± 1.2
10	45.25 ± 2.7	138.16 ± 2.5	67.09 ± 40.1

*Data are expressed as pg PhIP/g DNA, protein or whole tissue ± the standard deviation of at least 3 measurements.

Table 4. CYP1A2, NAT2, and SULT1A1 Phenotype data

Subject no.	CYP1A2*	NAT2†	SULT1A1‡
1	17.0	0.77	0.71
2	2.27	0.62	0.36
3	7.49	0.62	0.56
4	48.92	3.12	0.87
5	6.11	2.25	0.64
6	1.99	2.56	3.52
7	12.67	0.28	0.31
8	4.48	0.28	1.83
9	2.85	2.48	0.83
10	2.04	2.41	0.46

*CYP1A2 activity is the urinary molar ratio of [17U + 17X]/137X, where 137X [caffeine] is 1,3,7-trimethylxanthene, [17U] is 1,7-dimethyluric acid, and [17X] is 1,7-dimethylxanthene.

†NAT2 activity is the urinary molar ratio of AFMU/1X, where AFMU is 5-acetylamino-6-formylamino-3-methyluracil and 1X is 1-methylxanthine.

‡SULT1A1 activity is expressed as nmol/min per mg protein.

Table 5. Univariate linear model results from all variables for outcome related to DNA adduct levels. Variables are listed in order of decreasing importance in relation to DNA adduct levels.

Variable	Estimate	Std. Error	t value	p value	FDR adjusted p value*
N-OH-PhIP-N^2-gluc.	-2.75	0.94	-2.93	0.02	0.27
metabolite 7	48.47	17.97	2.70	0.03	0.27
CYP1A2 activity	-46.09	17.68	-2.61	0.03	0.27
albumin adducts T.02 h	-0.11	0.05	-2.29	0.05	0.34
subject age delta	1.74	0.85	2.05	0.07	0.35
albumin adducts T.04 h	-0.23	0.12	-1.90	0.09	0.35
metabolite 2	7.50	3.92	1.91	0.09	0.35
SULT1A1 activity	40.86	23.46	1.74	0.12	0.4
albumin adducts T.005 h	-0.07	0.05	-1.39	0.2	0.49
albumin adducts T.01 h	-0.04	0.03	-1.39	0.2	0.49
metabolite 3	5.87	4.02	1.46	0.18	0.49
Area under curve	-0.02	0.01	-1.24	0.25	0.52
metabolite 5	15.78	13.36	1.18	0.27	0.52
PhIP-N^2-gluc.	5.57	4.91	1.14	0.29	0.52
time to surgery 3	-30.50	24.64	-1.24	0.26	0.52
albumin adducts T.24 h	0.06	0.06	0.87	0.41	0.69
metabolite 1	4.24	6.25	0.68	0.52	0.83
albumin adducts T.08 h	0.07	0.12	0.60	0.57	0.86
albumin adducts T.12 h	0.02	0.08	0.25	0.81	0.95
metabolite 4	-8.51	21.72	-0.39	0.71	0.95
N-OH-PhIP-N3-gluc.	2.21	8.69	0.25	0.81	0.95
metabolite 11	-1.73	5.42	-0.32	0.76	0.95
time to surgery 2	-12.55	28.23	-0.44	0.67	0.95
4'-PhIP-sulfate	1.03	6.52	0.16	0.88	0.96
PhIP	4.27	47.82	0.09	0.93	0.96
NAT2 activity	-1.11	22.49	-0.05	0.96	0.96
subject weight	-0.01	0.19	-0.05	0.96	0.96

*False discovery rate adjusted p value

Table 6. Relationship between CYP1A2 phenotype, urinary N-OH-PhIP-N2-glucuronide levels, and colon DNA adduct levels

	Subject number									
	1	2	3	4	5	6	7	8	9	10
CYP1A2 phenotype	Fast	Slow	Slow	Fast	Slow	Slow	Fast	Slow	Slow	Slow
N-OH-PhIP-N^2-gluc.*	61.3	47.3	37.2	56.7	45.7	47.6	62.3	48.2	47.3	59.6
DNA adducts†	30.2	49.9	97.2	26.1	50.9	119.3	34.5	71.2	100.8	45.25

*Data is expressed as percent of recovered radioactivity

†Data is expressed as pg PhIP/g DNA

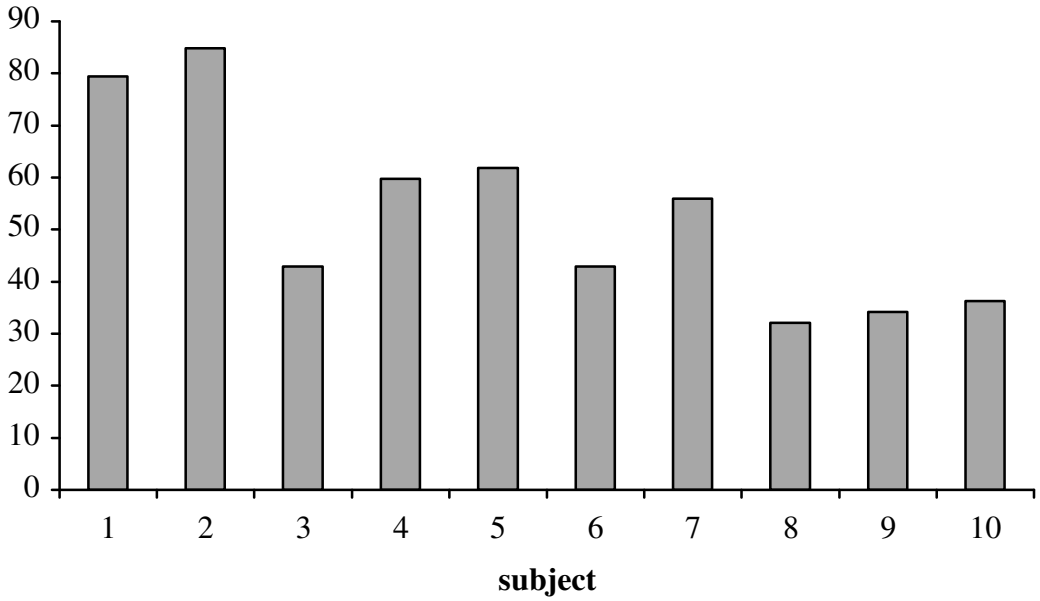
Figure Legends

Figure 1. The percent of radioactivity recovered in the urine of each subject in 24 h after an oral [^{14}C]PhIP dose. Data is expressed as percent of administered dose.

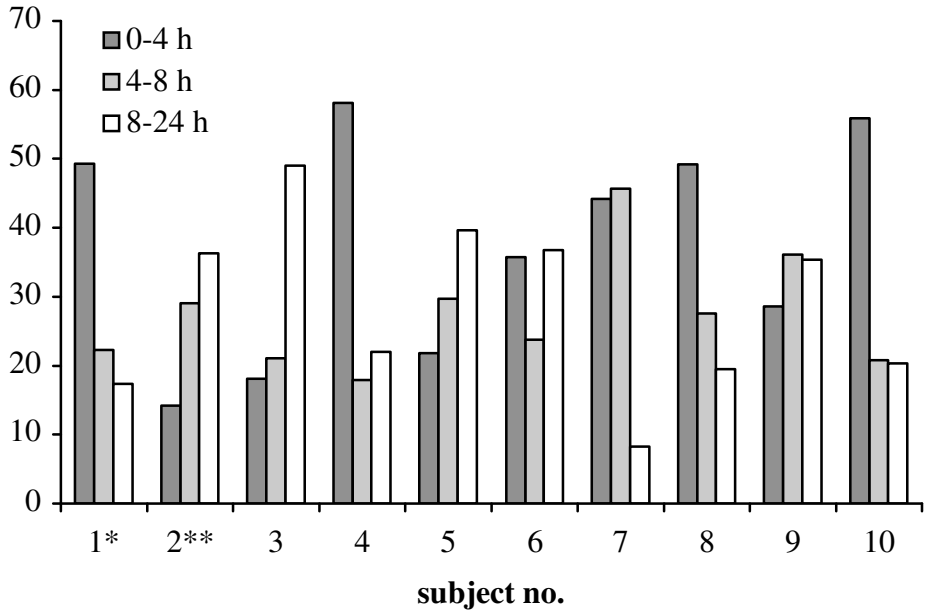
Figure 2. Radioactivity levels in human urine over time after an oral [^{14}C]PhIP dose. Data is expressed as percent of excreted radioactivity in 24 h.
*collection time was 0-4 h, 4-12 h, 12-24 h; **collection time was 0-4 h, 4-17.5 h, 17.5-33 h.

Figure 3. Rate of N-hydroxy-PhIP-N^2-glucuronide excreted in human urine after an oral [^{14}C]PhIP dose. Data is expressed as percent of N-hydroxy-PhIP-N^2-glucuronide excreted over time ± the standard deviation. Each subject was analyzed three times.

Malfatti et al. Figure 1



Malfatti et al. Figure 2



Malfatti et al. Figure 3