Genotyping Cytochrome P450 1B1*3(Phase I) and Glutathione S-transferase Mu 1 null (Phase II), Metabolizing Enzymes in a [^{14}C]Benzo[a]pyrene Human Micro Dosing Study

By Yun Li Luo

An Undergraduate Thesis Submitted to

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In partial fulfillment of
the requirements for the
degree of
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Polycyclic Aromatic Hydrocarbons (PAHs) have been studied for their carcinogenic toxicity. PAHs are formed by incomplete carbon combustion. Benzo[a]pyrene (BaP) is an IARC classified Class 1 Human carcinogenic PAH. New studies are conducted to gain a better understanding of PAHs and how to reduce exposure. Glutathione S-transferase M 1 (GSTM1 null) and Cytochrome P4501B1*1/*3 (CYP1B1*1/*3) polymorphisms have been implicated for their increased cancer risk through PAH oxidative metabolism. In this study, DNA was extracted from blood samples taken from study participants given one dose of ^{14}C-BaP. DNA was genotyped for GSTM1 null and CYP1B1*1/*3 in order to compare to the same individual's BaP metabolic profiles measured on accelerator mass spectrometry UPLC/AMS). Identifying major metabolites associated with specified SNPs can help in estimating carcinogenic risk. Of the ten participants, BaP021 and BaP023 are both positive for GSTM1. The rest of the eight participants are GSTM1 nulls. BaP21, 27, and 31 are wild type alleles while participants BaP23, 24, 25, 41 are heterozygotes, and BaP22 is homozygous. There were no significant trend indicating that participants with CYP1B1*1/*1 allele have increased CYP1B1 expression. The data did not demonstrate that participants with CYP1B1*1/*1 allele detoxified B[a]P more efficiently.

Figure 5 shows a graph containing the 7,8-dihydrodiol levels in the time intervals we sampled. 7,8-dihydrodiol is a procarcinogen. The 7,8-dihydrodiol levels were not too different from the other two participants despite having the homozygous polymorphism. It actually has lower 7,8-dihydrodiol levels contrary to our assumptions.

Key Words: Polycyclic Aromatic Hydrocarbons, Benzo[a]pyrene, Cytochrome P450 1B1, Glutathione S-transferase Mu 1

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Yun Li Luo, Author

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Introduction

Polycyclic Aromatic Hydrocarbons

Polycyclic Aromatic Hydrocarbons (PAHs) belong to a class of environmental contaminants formed through the incomplete combustion of carbon-containing materials including fossil fuels. Exposure to PAHs is common, as they are abundant in the environment and can be absorbed dermally, ingested, or inhaled.

The leading source of PAH exposure in humans is through dietary intake. Inhalation and dermal means are other prominent routes of exposure (Duan et al., 2015). PAHs are present in most food due to environmental pollution of the air, water, and soil (Karyab et al., 2013). The process of cooking and preparation of food can increase the quantity of dietary PAHs present. Barbequed, charcoal grilled, or smoked meats are associated with increased PAH levels (Parada et al., 2017). Urban runoffs and rainwater contribute to waters contaminated with PAH (Karyab et al., 2013). Inhalation of vehicular exhaust and industrial emissions is a major source of PAH exposure. The low molecular weight and volatile allow PAHs to be gaseous, while PAHs with higher molecular weight may be present in a particulate phase (Samburova et al., 2017).

While some PAHs are considered benign, some are known carcinogens. These chemicals have been studied extensively in recent years to determine the toxic nature of specific PAHs individually, of PAH mixtures, and PAH metabolites.

Benzo[a]pyrene

Benzo[a]pyrene(B[a]P) is considered the prototypical PAH. The carcinogenicity and mutagenicity of B[a]P is well-documented and used in risk assessment of PAH mixtures. B[a]P is categorized as a class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 2010).

Figure 1 shows the pathway by which B[a]P is metabolized and converted into DNA adducts. Cytochrome P450 (CYP) genes are responsible for the phase I conversion of B[a]P into metabolites such as the B[a]P-9,10-oxide, B[a]P-7,8-oxide, or 3-OH-B[a]P. When CYP forms an epoxide at the 3 location and becomes 3-OH-B[a]P, it can undergo glucuronidation to form the noncarcinogenic 3-OH-B[a]P-glucuronide. Alternatively, B[a]P can be metabolized by CYP1A1 and CYP1B1 into two stereoisomers, B[a]P-9,10-oxide or B[a]P-7,8-oxide. B[a]P-9,10-oxide and B[a]P-7,8-oxide can be hydrolyzed by epoxide hydrolase producing two trans-dihydrodiols, B[a]P-9,10-diol or B[a]P-7,8-diol, which can be metabolized by UDP-glucuronosyltransferase into glucuronides. B[a]P-7,8-oxide can undergo a secondary epoxygenation to produce four enantiomers of the dihydrodiol-epoxide ((+)-*syn*-, (-)-*syn*-, (-)-*anti*, or (+)-*anti*-B[a]P-7,8-dihydrodiol-9,10-epoxide) via CYP enzymes. The resulting epoxides can be detoxified by glutathione conjugation by the phase II enzyme glutathione S-transferase, hydrolyzed to become water soluble tetraols, or attached to guanine in DNA. The orientation of DNA attachment can affect the probability of DNA damage. The N_2-dG adduct in the cis-formation can be corrected by the nucleotide excision repair (NER) enzymes. The trans-N_2-dG adducts is resistant to the NER which

causes a single nucleotide polymorphism (SNP) in which case dG becomes dT (Marinković, et al., 2013).

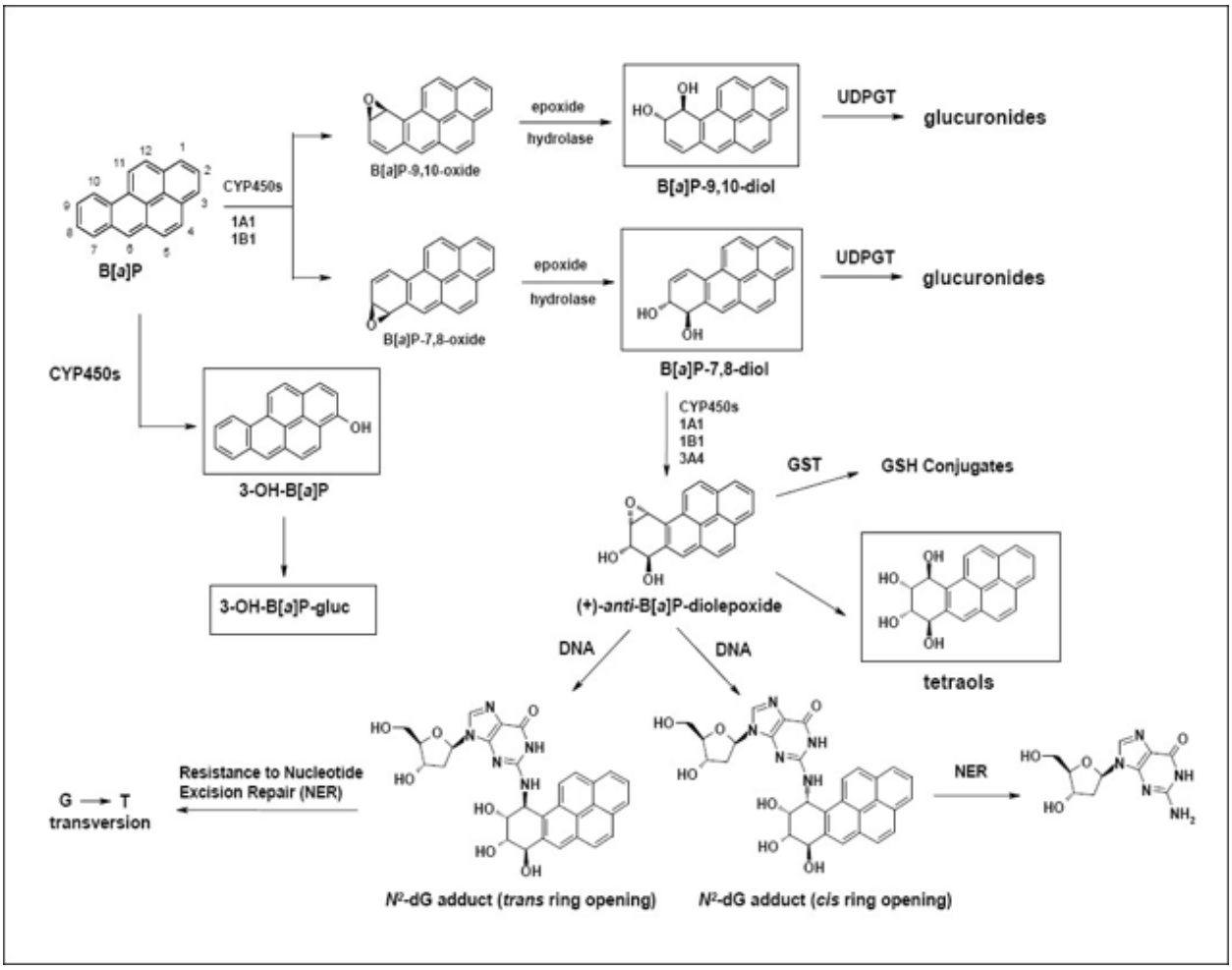

Figure 1. The activation pathway of B[a]P metabolism into DNA adducts (Trushin et al., 2012).

CYP 1B1 and Glutathione S-Transferase Mu 1 Metabolizing Enzymes

CYP family 1, subfamily B, member 1 (CYP1B1) is an enzyme from the CYP superfamily critical in the metabolism of PAHs. In the B[a]P metabolic pathway, CYP1B1 acts as a phase I enzyme in the bioactivation of B[a]P. CYP1B1 is associated with the formation of 7,8-dihydrodiols. There are many known single nucleotide polymorphism

(SNP) changes in the *CYP1B1* gene, four of which causes amino acid changes (Chang et al., 2013). A prevalent *CYP1B1* SNP associated with increased cancer incidence is the *CYP1B1*3* SNP investigated in this study. The common allele is denoted as CYP1B1*1 (Leu432) has a cytosine nucleotide in the codon for amino acid leucine. The heterozygous genotype has one copy of each allele (*CYP1B1*1/*3*). The homozygous mutant (*CYP1B1*3/*3* Leu432Val; rs1056836) genotype results in both alleles with a SNP change in which the nucleotide cytosine is replaced by guanine resulting in the amino acid change from leucine to valine at position 432. Previous studies demonstrated an increase of CYP1B1 expression in participants with the *1/*1 allele compared to *3/*3 (Rylander-Rudqvist et al., 2003). Genotyping *CYP1B1* alleles can provide an insight into the predisposition of individuals to cancer and estrogen (also a good CYP1B1 substrate) metabolism.

Glutathione S-transferase Mu 1 (*GSTM1)*, located on 1p13.3, is part of the GST supergene family. GSTM1 belongs to the mu class and is recognized for its involvement in the metabolism and detoxification of xenobiotics (Yu et al., 2016). Epidemiological studies demonstrate the ability of GSTM1 to decrease chromosomal damage when compared to individuals without the *GSTM1* gene (Minina et al., 2017). Determining the presence of GSTM1 genotype can predict the efficacy of B[a]P detoxification.

Hypothesis

Glutathione S-transferase Mu 1 null (*GSTM1*-null) and CYP1B1 (*CYP1B1*3*) allelic variants have been implicated in increased cancer risk. In this study, DNA was taken from participants microdosed with [$_{14}$C]-benzo[a]pyrene. The DNA was isolated from peripheral blood mononuclear cells (PBMCs), genotyped for *GSTM1* and *CYP1B1*, and compared to the

B[a]P metabolic profiles measured by accelerator mass spectrometry (UPLC/AMS). Our hypothesis was that participants with the *CYP1B1*3* and/or *GSTM1* null allelic variants would exhibit different metabolic profiles than their more prevalent counterparts with a higher proclivity to cancer.

Materials and Methods

[^{14}C]-BaP Human Micro Dosing Sample Collection and DNA Isolation

All protocols and procedures, including plans for recruitment and volunteer informed consent documents, were approved by the Oregon State University Institutional Review Board (protocols #8223, #8554, #8789) and an IND (#117175) from the FDA. The study consisted of 10 healthy, nonsmoking, individuals between the ages of 21 to 65. Both male and female participants were recruited. Females were either sterile or menopausal. Participants were told to comply with strict dietary guidelines, including the exclusion of smoked or charcoal grilled foods, to reduce PAH exposure. An IRB approved food diary was kept by each participant to monitor potential PAH consumption.

Participants fasted overnight prior to each cycle. At the start of each cycle a time zero 10 ml blood draw and spot urine sample were collected. Participants were then given a capsule containing 25, 50, 100 or 250 ng of [^{14}C]-BaP (2.65, 5.30, 10.60, or 26.50 nCi ^{14}C). Ten ml of blood were drawn at 0.25, 0.5, 1.0, 1.5, 2.0, 3, and 4, hours with an indwelling intravenous catheter with an indwelling intravenous catheter at 8, 24 and 48 hours. There was a 3 week “washout” period in between dosing cycles. Plasma was immediately separated by centrifugation at 3000 rpm in a clinical centrifuge. Plasma was removed within 3 hours of initial blood draw and the buffy coat containing PBMCs were transferred

to a 50 ml conical tube. DNA was extracted using a DNA Isolation Kit for Mammalian Blood (Sigma-Aldrich #11667327001) following the manufacturer's protocol. DNA was quantified using a Synergy HTX plate reader/Take 3 (BioTek, Winooski, VT).

Genotyping for GSTM1 and CYP1B1 Polymorphisms

*GSTM1*1* and *GSTM1*0* alleles:

PCR reactions (20 µL) contained 4.0 µL SuperFi buffer, 2 µL each of 2.5 µM *GSTM1* forward and reverse primers (table 1), 2 µL each of 2.5 µM human hemoglobin β chain (HBB) forward and reverse primers (table 1), used for a PCR positive control, 0.4 µL of 10 mM dNTPs, 0.2 µL Platinum™ SuperFi DNA polymerase (Thermo Fisher Scientific), 5.4 µL deionized water, and 2 µL (200 ng) DNA. Products were placed in a Veriti™Thermal Cycler (ThermoFisher Scientific) 1 cycle for 3 minutes at 95°C, 40 cycles at 15 seconds for 95°C and 30 seconds at 60°C, elongation for 5 minutes at 60°C. The samples were stored at 6°C until removed. The products were separated on a 10% TBE gel and separated the XCell SureLock Mini-Cell Electrophoresis System (Thermo Fisher Scientific). The gel was removed from the casing and stained with GelRed Nucleic Acid Gel Stain for 2 minutes. PCR products were visualized on a ChemiDoc Imaging System (BioRad, Hercules, CA.).

*CYP1B1*1*1, *1/*3*, and **3/*3* genotypes:

PCR products were amplified in a 20 µL reaction as described above using *CYP1B1* primers (Table 1). The ThermalCycler protocol was repeated. The DNA products were cleaned with the Zymo Research DNA Clean & Concentrator™ Kit -25 (Zymo Research, Irvine, CA) per manufacturer instructions and sequenced at the Oregon State University

Center for Genome Research and Biocomputing (CGRB) with Sanger Sequencing. The specific alleles were visualized with FinchTV Version 1.5.

Table I. PCR Primer Sequences Used to Identify the Genes GSTM1, CYP1B1, and HBB

Gene	Primer Sequence	Product Size
GSTM1[1]	Forward: 5'-GGA GAA GAT TCG TGT GGA CA-3' Reverse: 5'-CTG GAT TGT AGC AGA TCA TAC-3'	80 bp
HBB[1]	Forward: 5'-GTG CAC CTG ACT CCT GAG GAG A-3' Reverse: 5'-CCT TGA TAC CAA CCT GCC CAG-3'	102 bp
CYP1B1[2]	Forward: 5'-CAC TGC CAA CAC CTC TGT CTT G-3' Reverse: 5'-AAG AAT CGA GCT GGA TCA AAG TTC-3'	129 bp

([1]Cote et al., 2005, and [2]Wenzlaff et al., 2005)

UPLC-AMS Analysis of [^{14}C]-BaP and Metabolites

Plasma samples (1.5 mL) were transferred to glass tubes containing 1.5 g K_2SO_4 , 1.5 mL H_2SO_4 and 50 µL (10 attomole) [^{14}C]-Dibenzo[*def,p*]chrysene, as an internal standard was added, and samples mixed by vortex. Plasma was then extracted 3X with 1.5 ml ethyl acetate. The pooled ethyl acetate was evaporated with a N_2 stream, and samples resolubilized in 100 µl fresh ethyl acetate. Samples were shipped to Lawrence Livermore National Laboratory for analysis using UPLC-AMS(ultra pressure liquid chromatography - accelerator mass spectrometry)

Results & Discussions

GSTM1 Allele Determination

*GSTM1*0* and *CYP1B1*3* polymorphisms are known to elevate cancer risk through PAH metabolic activation (Zhang et al., 2013 & Yu et al., 2016). GSTM1 assists in detoxication of foreign xenobiotics via conjugation of electrophilic metabolites with glutathione. *GSTM1*0*0* individuals are unable to detoxify chemicals as efficiently. CYP1B1 is an enzyme involved in the bioactivation pathway of B[a]P. CYP1B1 polymorphisms are implicit in increasing formation of the toxic bay region dihydrodiol-epoxide. Ten participants were genotyped for *GSTM1* and *CYP1B1* alleles to determine their genotypic variation and as an indicator of risk. The metabolic profiles were generated by UPLC-AMS to evaluate the major metabolites from B[a]P metabolism.

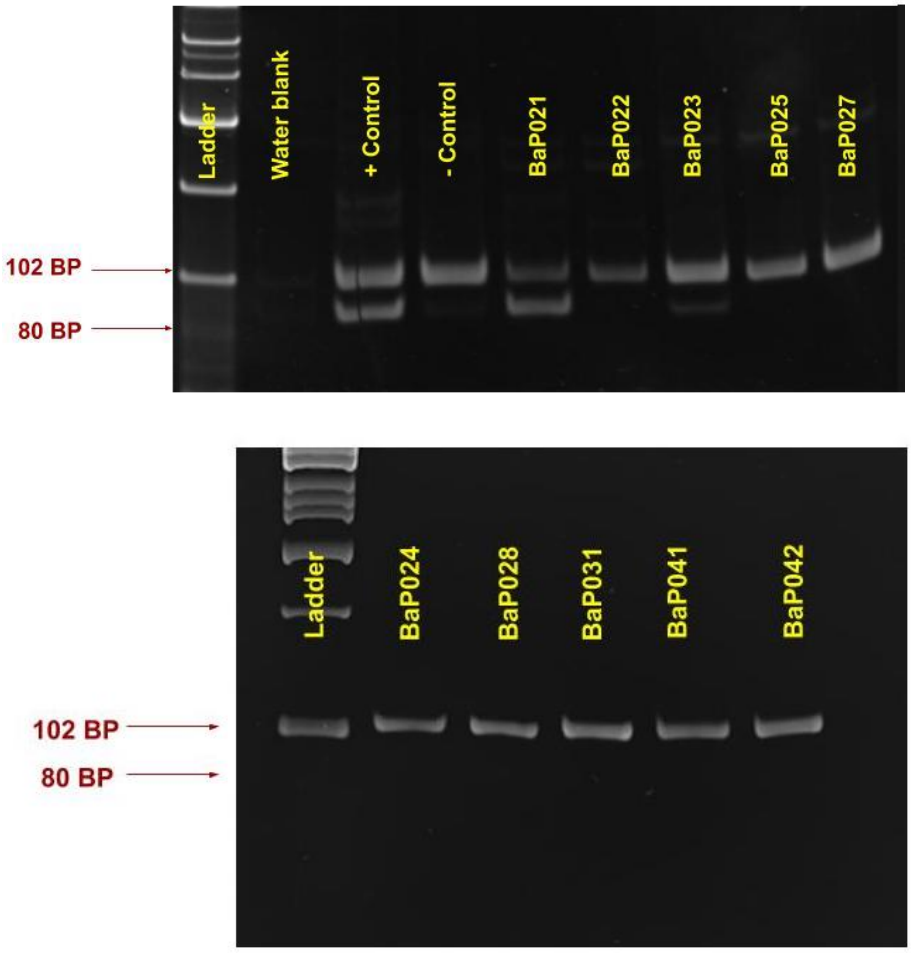

Figure I. *GSTM1* product separated on a 10% TBE gel. The 102 bp band is the HBB control. Except for BaP021 and BaP023, all participants do not have the 80 *GSTM1* band and are *GSTM1*0*0*

CYP1B1 Allele Determination

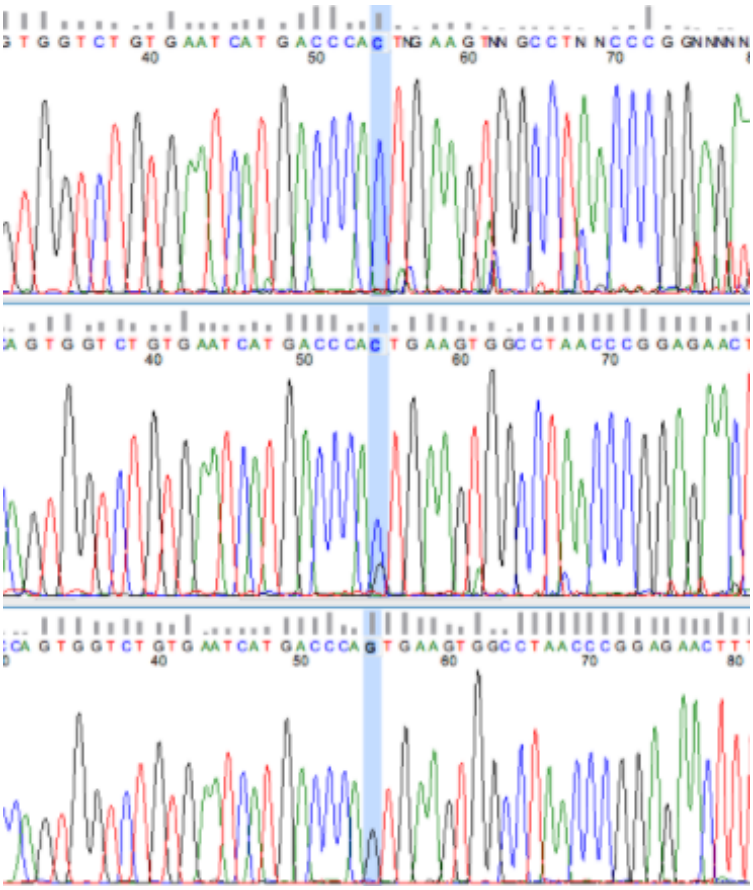

Figure II. Three genotypic variants of *CYP1B1* visualized on FinchTV. Top graph is the common (*CYP1B1*1/*1*), middle graph is the heterozygous (*CYP1B1*1/*3*, and the bottom graph is homozygous (*CYP1B1*3/*3*).

Table II. Distribution of GSTM1 Genotypes and *CYP1B1* Alleles

Participants	GSTM1*1	CYP1B1 Alleles
BaP021	+	*1/*1
BaP022	-	*3/*3
BaP023	+	*1/*3
BaP024	-	*1/*3
BaP025	-	*1/*3
BaP027	-	*1/*1
BaP028	-	*1/*1
BaP031	-	*1/*1
BaP041	-	*1/*3
BaP042	-	*1/*1

Of the ten participants, all but two (BaP21 and 23) were *GSTM1*-nulls (Figure I and II). In previous studies, it was documented that approximately 50% of the Caucasian population are *GSTM1*-nulls (Alexandrov et al., 2002). The genotypic disparity between the established literature and the genotypes acquired in the study may be the result of the small sample size. Five participants (BaP021, 27, 28, 31, and 42) possessed two *CYP1B1*1/*1* alleles, four participants (BaP023, 24, 25, and 41) are heterozygous with both *CYP1B1*1/*3* alleles and one participant possesses both *CYP1B1*3/*3* alleles (Figure II). The CYP1B1*1/*3 genotype is found in roughly 48% of the population (Wu et al., 2012) while CYP1B1*3/3 is found in roughly 34% (Aklillu et al., 2005).

Table III. BaP022 Select compounds of interest from the metabolic profile of participant in fg/ml plasma following oral dosing with 100 ng [14 C]-benzo[a]pyrene using UPLC/AMS

Time(h)	0.25	0.5	1	1.5	2	3	4	8	24	48
Compounds										
Tetrols	2.84	10.19	12.22	11.97	6.51	7.04	5.58	4.51	2.31	1.06
9,10-dihydrodiol	1.26	5.36	6.04	4.96	3.22	2.93	2.81	2.01	1.05	
7,8-dihydrodiol	1.69	5.56	5.22	4.10	4.06	2.53	2.39	1.42	1.08	0.60
3-OH		1.44	1.23	0.93	0.98	0.91				
BaP	0.72	0.76								
DBC	1.82	1.75	1.54	0.65	1.75	1.03	1.08	1.54	1.09	0.90

Table IV. BaP023 Select compounds of interest from the metabolic profile of participant in fg/ml plasma following oral dosing with 100 ng [14 C]-benzo[a]pyrene using UPLC/AMS

Time(h)	**0.25**	**0.5**	**1**	**1.5**	**2**	**3**	**4**	**8**	**24**	**48**
Compounds										
Tetrols		12.58	16.67	14.93	10.47	8.34	6.41	18.49	8.04	3.79
9,10-dihydrodiol		8.80	10.09	9.20	6.69	4.84	3.58	7.63	2.99	1.61
7,8-dihydrodiol		8.04	9.01	7.79	4.51	4.27	3.86	4.79	1.63	0.86
3-OH		9.91	8.69	8.29	12.15	6.08	4.02	1.52		
BaP	1.06	5.21	2.80	1.85	1.82	1.48	1.63	2.75	1.77	0.98
DBC	3.13	2.26	2.26	2.19	2.37	2.95	4.15	5.63	4.03	3.09

Table V. BaP028 Select compounds of interest from the metabolic profile of participant in fg/ml plasma following oral dosing with 100 ng [14 C]-benzo[a]pyrene using UPLC/AMS

Time(h)	**0.25**	**0.5**	**1**	**1.5**	**2**	**3**	**4**	**8**	**24**	**48**
Compounds										
Tetrols		15.67	29.09	15.00	21.67	11.16	8.04	7.12	6.60	13.07
9,10-dihydrodiol		11.95	23.57	14.24	17.97	9.56	6.00	6.95	6.56	15.42
7,8-dihydrodiol		11.56	10.23	5.77	7.16	3.23	2.71	1.46	1.31	3.28
3-OH		19.86	9.94	3.45	2.17	0.64	0.94			
BaP		13.25	2.96	4.87	2.87	2.78	3.38	2.78	1.52	2.57
DBC		3.39	1.18	2.86	3.64	5.11	3.48	5.25	6.43	14.86

The metabolic profiles from UPLC/AMS were derived from the plasma of the participants. Table III is the metabolic profile of participant BaP022 (*CYP1B1**3/*3), Table IV belong to BaP023 (*CYP1B1**1/*3), and Table V is from BaP028 (*CYP1B1**1/*1). There were no significant trend indicating that participants with *CYP1B1*1/*1* wildtype allele has increased CYP1B1 expression. The data did not demonstrate that participants with *CYP1B1**1/*1 allele were able to detoxify B[a]P more efficiently. Some *CYP1B1**3/*3 participants had fewer toxic compounds in their plasma than the wildtype.

Previous studies suggest that the CYP1B1*3 polymorphism is associated with increased cancer incidences (Rylander-Rudqvist et al., 2003). There are many other genetic variables that may impact the metabolic abilities of these individuals. The presence or absence of certain genes may have an independent or compounding effect on the detoxification of xenobiotics. In the future, it may be of interest to genotype for various other SNPs that are associated with elevating cancer risks. CYP1A1 is vital in metabolizing xenobiotics. CYP1A1 SNPs are reported to increase incidences of breast cancer (Androutsopoulos et al., 2009). The nucleotide excision repair (NER) gene is a safeguard against DNA damage that may cause cancer. Polymorphisms in the NER gene may result in an inability to repair DNA (Zhu et al., 2018). In the future, the urine of participants will be analyzed to determine the carcinogenic risk associated with the presence or absence of the GSTM1 gene.

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