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# CYP1A2 and NAT2 phenotyping and 3-aminobiphenyl and 4-aminobiphenyl hemoglobin adduct levels in smokers and non-smokers ☆

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**Abstract**

Some aromatic amines are considered to be putative bladder carcinogens. Hemoglobin (Hb) adducts of 3-aminobiphenyl (3-ABP) and 4-aminobiphenyl (4-ABP) have been used as biomarkers of exposure to aromatic amines from cigarette smoke. One of the goals of this study was to determine intra- and inter-individual variability in 3-ABP and 4-ABP Hb adducts and to explore the predictability of ABP Hb adduct levels based on caffeine phenotyping. The study was conducted in adult smokers (S, $n = 65$) and non-smokers (NS, $n = 65$). The subjects were phenotyped for CYP1A2 and NAT2 using urinary caffeine metabolites. Blood samples were collected twice within 6 weeks and adducts measured by GC/MS. The levels of 4-ABP Hb adducts were significantly ($p < 0.0001$) greater in S (34.5 ± 21.06 pg/g Hb) compared to NS (6.3 ± 3.02 pg/g Hb). The levels of 3-ABP Hb adducts were below the limit of quantification (BLOQ) in most (82%) of the NS and about 10-fold lower in S (3.6 ± 3.29 pg/g Hb) compared to 4-ABP Hb adducts. No differences were observed in the adduct levels between weeks 1 and 6 in the smokers, suggesting that a single sample would be adequate to monitor cigarette smoke exposure. The regression model developed with CYP1A2, NAT2 phenotype and number of cigarettes smoked (NCIG) accounted for 47% of the variability in 3-ABP adducts, whereas 32% variability in 4-ABP adducts was accounted by CYP1A2 and NCIG. The ratio of 4-ABP Hb adducts in adult S:NS was ~5:1, whereas 3-ABP Hb adducts levels were BLOQ in some S, exhibited large interindividual variability (~91% compared to 57% for 4-ABP Hb) and poor dose response relationship. Therefore, 4-ABP Hb adduct levels may be a more useful biomarker of aminobiphenyl exposure from cigarette smoke.


*Keywords:* Aromatic amines; 3-Aminobiphenyl; 4-Aminobiphenyl; Biomarkers; Bladder cancer; Cigarette smoke; Cytochrome *P*4501A2; Exposure study; Hemoglobin adducts; Phenotype; *N*-acetyltransferase; Polymorphism; Tobacco smoke carcinogens; Risk

## Introduction

Human exposure to aromatic amines can occur from various sources including dietary, environmental exposure and tobacco smoke inhalation (Sabbioni and Neumann, 1990; Chiang et al., 1999; Platzek et al., 1999; Myers et al., 1996; Dallinga et al., 1998). Aminobiphenyls (ABP) are one of the groups of aromatic amines that arise from cigarette smoke. Of the various ABP, 4-ABP is classified by IARC (2004) as a Group 1 carcinogen (sufficient evidence of carcinogenicity in humans) and human exposure to 4-ABP occurs from cigarette smoke (Bryant et al., 1987) as well as other sources (Sugimura, 1982); 3-ABP on the other hand is not classified as a carcinogen by IARC, and exposure to 3-ABP appears to be primarily from cigarette smoke (Bryant et al., 1988a).

Hepatic *N*-hydroxylation by cytochrome *P*4501A2 (CYP1A2) is considered to be one of the critical step in the metabolic activation of 4-ABP (Vineis et al., 1990; Sabbioni, 1992). However, CYP1A2 might not be the sole CYP450 involved in 4-ABP *N*-hydroxylation (Kimura et al., 1999). Extra-hepatic *N*-hydroxylation by flavine adenine dinucleotide-containing monooxygenases (FMO) and prostaglandin H synthase plays

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only a minor role in humans (Sabbioni, 1992). The reaction of the parent amine with the *N*-acetyltransferase (NAT) and the glucuronidation of the *N*-hydroxylarylamine are considered to be detoxification reactions that compete with the *N*-hydroxylation (Lilienblum et al., 1987; Poupko et al., 1979). Some of the glucuronide conjugates can be hydrolyzed under the acid conditions prevailing in the urine (pH 5) to reliberate the hydroxylamine (Kadlubar et al., 1977; Poupko et al., 1979; Sarkar et al., 2002). The *N*-hydroxylamine in blood is heme-mediated oxidized to the nitrosoarene, which reacts with the sulfhydryl group of cysteine in hemoglobin (Hb), resulting in a stable Hb adduct. It has been suggested that transport of the *N*-hydroxylamine to the bladder (Poupko et al., 1979; Bartsch et al., 1990) followed by hydrolysis in the acid environment of the human bladder results in the formation of electrophilic nitrenium ions involved in critical DNA-binding reactions (Kadlubar et al., 1977). Studies have shown a correlation between Hb adduct and DNA adduct levels (Dallinga et al., 1998; Vineis et al., 1996; Kadlubar et al., 1991), suggesting that ABP Hb adducts could provide a measure of the biologically effective dose. Although 3-ABP is activated by CYP1A2 forming the *N*-hydroxylamine as well as the Hb adducts, its *N*-hydroxylamine is not a mutagen (Sabbioni, 1993).

The biologically effective dose of the ultimate carcinogen depends on the rate of formation of the *N*-hydroxylamine, which is mainly influenced by the balance between *N*-acetylation and *N*-hydroxylation of the parent amine (Vineis et al., 1990). *N*-Acetylation of arylamines is catalyzed by cytosolic arylamine *N*-acetyltransferase (NAT) coded for by two gene loci, NAT1 and NAT2 (Probst-Hensch et al., 2000). The latter is responsible for acetylator polymorphism in humans resulting in rapid, intermediate and slow acetylator phenotypes (Grant et al., 1990). In addition, the CYP1A2 activity is inducible, particularly showing a significant increase in smokers as well as in individuals consuming charbroiled meat (Fontana et al., 1999). Due to the presence of allelic variants of NAT1 and NAT2 and the induction of CYP1A2 in smokers, this balance can be perturbed in some individuals. Since both enzyme activities can be readily estimated from urinary metabolite levels of a single substrate like caffeine (Butler et al., 1992), it may be possible to predict the levels of ABP Hb adducts based on a simple non-invasive test. Furthermore, there is little knowledge regarding the variability in the levels of the two ABP Hb adducts in the same individual over different time periods. Therefore, the purpose of this study was to determine the intra- and inter-individual variability of 3-ABP and 4-ABP Hb adduct levels and to investigate whether these biomarkers can be used to differentiate between adult smokers of 3.0 to 6.9 mg tar (FTC) cigarettes and adult non-smokers. An additional purpose was to develop a model to predict 3- and 4-ABP Hb adducts levels based on CYP1A2 and NAT2 activity.

## Materials and methods

### Study population

The study was approved by the Covance Clinical Research Unit Institutional Review Board, and informed consent was obtained from all volunteers. The study was performed by and conducted at Covance Laboratories Inc., Madison, Wisconsin, USA. Of the 140 subjects enrolled, 10 subjects (3 non-smokers and 7 smokers) were excluded from the data listings and 14 subjects (3 non-smokers and 11 smokers) excluded from ABP data analysis due to violations of specific inclusion or exclusion criteria (e.g., pregnancy, medication or urine cotinine levels for non-smokers of greater than 50 μg/l). The study population consisted of 65 healthy adult smokers (37 females and 28 males) and 65 healthy non-smokers (35 females and 30 males). All the smokers enrolled in the study had smoked at least 1 cigarette (tar range of 3.0–6.9 mg per cigarette tested under FTC (Federal Trade Commission testing conditions) every day for over the last 12 months at the time of the sampling. Exclusion criteria included clinically significant renal, liver, metabolic, cardiac or pulmonary disease; evidence of hepatitis B or C or HIV infection; or illicit drug use. Women of childbearing potential were excluded if they were pregnant, lactating or intending to get pregnant during the study. Questionnaires were used to obtain information on smoking, diet, medication, possible occupational exposure and coffee consumption. Additional data from this study have been reported elsewhere (Zedler et al., in press), however, the number of subjects included in the current analysis is different than previously reported. In this analysis, we have reported only blood biomarkers with a long half-life, whereas the subjects excluded in the previous report were due to issues (e.g., urine sampling for nicotine and metabolites and other biomarkers of exposure) which were not relevant for this study.

### Study conduct

*Blood collection for ABP Hb adduct analysis.* The subjects were enrolled after successfully completing the screening visit and were asked to visit the clinic site on two occasions on week 1 and week 6 to be scheduled with minimal interruption of their daily routines. Alterations to regular smoking behaviors were minimized by allowing them to smoke in designated areas in the clinic.

Peripheral blood samples (10 ml) were obtained by venipuncture and placed in ethylene diamine tetra-acetic acid (EDTA) tubes. Erythrocytes were isolated according to the method described by Bryant et al. (1987a), with minor modifications. After centrifugation, the packed red blood cells were washed 3 times with 0.85% saline and stored at −20 °C until further use.

*Urine collection for CYP1A2 and NAT2 phenotype analysis.* Caffeine phenotyping was carried out during the enrolment period; subjects refrained from consuming alcoholic drinks and food or caffeine containing beverages for a minimum of 8 h before and during the day of urine collection. After emptying their bladder, subjects ingested 200 mg of caffeine (No-Doz, 2 tablets at 100 mg each) with 250 ml of water. Consumption of liquids was not restricted during the study. All urine formed in 4 to 5 h was collected, and urine volumes were recorded. A 10-ml aliquot of the collected urine was adjusted to pH 3.5 by addition of 200 mg of ascorbic acid, immediately frozen, and stored at −20 °C until analysis.

### Chemicals

3-Aminobiphenyl (99.5%) was purchased from Tokyo Chemical Industry Co. Ltd. (TCI, Tokyo, Japan), 4-aminobiphenyl (98.7%) from Sigma-Aldrich Chemie (Seelze, Germany), 3-aminobiphenyl-$d_9$ (>98%) from Toronto Research Chemicals Inc. (TRC, Toronto, Canada) and 4-aminobiphenyl-$d_9$ (99.4%) from CDN Isotopes Inc. (QMX Laboratories Limited, Thazted, Essex, UK) Caffeine (100%), 1,7-dimethylxanthine (100%), 1,7-dimethyluric acid (99.6%) and vanillic acid (99%) were purchased from Sigma Company (St. Louis, MO). 1-Methylxanthine (99.5%) was supplied by ICN Biomedicals and AFMU (95%) by TRC. Solvents used were of HPLC or ACS grade, and the chemicals were of analytical or HPLC grade with the purity of the reagents was stated to be at least 99%, unless stated otherwise.

### Determination of 3-ABP and 4-ABP hemoglobin adducts

*Isolation of hemoglobin.* After thawing, the erythrocytes were lysed by adding 3 volumes of ice-cold deionized water. After 20 min, the solution was buffered with one-third the volume of a 0.67 M phosphate buffer (pH 6.6) prior to ultra-centrifugation (38,000 *g*, 30 min at 4 °C) to remove the cell debris. The remaining hemoglobin solution was dialyzed (Visking dialysis tubing 27/32, Medicell International Limited, London) against 6 changes of deionized water