

# Nonsmoking-related Arylamine Exposure and Bladder Cancer Risk<sup>1</sup>

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## Abstract

**Roughly one-half of bladder cancer incidence in the United States can be attributed to known causes, mainly cigarette smoking, and it has been hypothesized that the aromatic amines in tobacco smoke are important etiological agents. Nonsmokers are also exposed, through unknown sources, to many of the same carcinogenic aromatic amines that are present in cigarette smoke. Previous epidemiological studies have not tested whether either of these aromatic amine exposures are associated with cancer risk. We conducted a population-based case-control study in Los Angeles County, California, involving 761 case patients with bladder cancer and 770 individually matched control subjects. In-person interviews provided information on tobacco smoking and other potential risk factors. Quantitative analysis of hemoglobin adducts of 4- and 3-aminobiphenyl (ABP) was used to assess aromatic amine exposure. Adducts of both aminobiphenyls were significantly higher in cases than in controls, independent of cigarette smoking at the time of blood collection and lifetime smoking history. Adjustment for other risk factors as well as for metabolic differences did not materially alter the associations. Our findings strengthen the connection between exposure to aromatic amines in tobacco smoke and cigarette smoking-related bladder cancer and suggest that environmental exposure to arylamines may account for a significant proportion of nonsmoking-related bladder cancer in the general population.**

## Introduction

Epidemiological investigations have found associations between exposure to aromatic amines and bladder cancer, with compelling evidence for human carcinogenicity coming from

studies of occupational exposure to 4-ABP,<sup>3</sup> 2-naphthylamine, and benzidine (1). These three compounds are now classified and regulated as human bladder carcinogens. There is also suggestive epidemiological evidence for the carcinogenicity of other aromatic amines such as *o*-toluidine (2).

As a result of the great reduction of occupational exposure to aromatic amines that has occurred since these compounds became known as human carcinogens, the most important cause of human bladder cancer that epidemiological studies now detect is cigarette smoking. After the discovery that aromatic amines are constituents of cigarette smoke, the hypothesis was advanced that exposure to the aromatic amines in tobacco smoke is a principal mechanism by which cigarette smoking induces bladder cancer (3).

Aromatic amine exposure well below the levels resulting from cigarette smoking can now be detected with sensitive and specific biomarker measurements (4). Aromatic amines are oxidized *in vivo* to *N*-hydroxylamines that, upon reaching the circulation, react with hemoglobin to form adducts that may be sufficiently stable to persist for as long as the adducted hemoglobin remains in circulation. As a result, quantitative analysis of the adducts in a blood specimen can be used to assess exposure during the several-month period before blood collection (5). Oxidation of aromatic amines to *N*-hydroxylamines is generally thought to be required for their carcinogenic activity (6, 7). The aromatic amine-hemoglobin adduct biomarker may, therefore, be more informative about cancer risk than questionnaire-based exposure assessment.

Cross-sectional population studies have revealed that exposure to 4-ABP, as indicated by the presence of its hemoglobin adduct in individuals of all ages, even neonates, is nearly universal (8–10). Adduct levels are higher in smokers than in nonsmokers (11–13). Some, but not all, studies have demonstrated an association between passive smoke exposure and the presence of adducts in the hemoglobin of nonsmokers (14–17). These and other studies also suggest that the nature of most sources of arylamines in nonsmokers is unclear.

We initiated a population-based case-control study of bladder cancer in Los Angeles County in 1992 to test several hypotheses concerning the etiology of this disease. Blood specimens were collected from consenting participants and subjected to quantitative analysis for hemoglobin adducts of 4-ABP as well as the isomeric 3-ABP. Here, we examine the relationship between bladder cancer risk and levels of hemoglobin adducts of these two amines as a function of smoking habits, with adjustment for known and suspected risk factors for bladder cancer that include metabolic phenotype or genotype.

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<sup>3</sup> The abbreviations used are: 4-ABP, 4-aminobiphenyl; NAT2, *N*-acetyltransferase 2; GSTM1, glutathione *S*-transferase M1; OR, odds ratio; CI, confidence interval; 4-NB, 4-nitrobiphenyl.

## Materials and Methods

**Study Design.** Recruitment of subjects has previously been described in detail (13). Briefly, cases were identified through the Los Angeles County Surveillance Program, the population-based Surveillance, Epidemiology, and End Results (SEER) cancer registry of Los Angeles County. Between January 1, 1987 and April 30, 1996, 2098 histologically confirmed bladder cancers were diagnosed among non-Asians between the ages of 25 and 64 years. Among these, 175 patients died before we could contact them or were too ill to be interviewed. Permission to contact 74 patients was denied by their physicians. Two hundred sixty-seven patients refused to be interviewed. Thus, we interviewed 75% (1582/2098) of all eligible patients.

For each interviewed case patient, we sought to recruit a control subject who was matched by gender, date of birth (within 5 years), race (non-Hispanic white, Hispanic, or African-American), and neighborhood of residence at the time of cancer diagnosis. To search for these neighborhood control subjects, we followed an invariant procedure that defines a sequence of houses on specified neighborhood blocks. We attempted to identify the sex, age and race of all of the inhabitants of each housing unit; not at home units were systematically revisited to complete the census. When we failed to find a match after canvassing 150 housing units, we excluded race from the matching criteria. If a match based on this relaxed criterion could not be found within a maximum of 300 housing units, the case was dropped from the study. Sixty-eight cases were excluded from the study because of the lack of a matched control, and 20 controls were not matched to the index case by race. All of the study subjects signed informed consent forms approved by the University of Southern California Human Subjects Committee.

In-person structured interviews were conducted in each subject's home. The questionnaire requested information up to 2 years before the diagnosis of bladder cancer for case patients or 2 years before the diagnosis of cancer of the index case patient for the matched control subject. Information was requested on demographic characteristics, lifetime use of tobacco products and alcohol, usual adult dietary habits, lifetime occupational history, prior medical conditions, and prior use of medications.

Beginning in January 1992, all of the case patients ( $n = 1044$ ) and their matched control subjects ( $n = 979$ ) were asked for a blood sample donation at the end of the in-person interview. We obtained a blood sample from 761 (73%) case patients (55% of total eligible cases) and from 770 (79%) control subjects. Subjects also were asked to collect an overnight urine sample (ending with the first morning void) after consumption of two cups of instant coffee (about 70 mg of caffeine) in the afternoon (between 3:00 and 6:00 p.m.).

**Laboratory Measurements.** Blood specimens were collected in heparinized tubes and were subsequently fractionated into plasma, buffy coat, and erythrocytes, which were stored at  $-80^{\circ}\text{C}$  before analysis. Erythrocyte samples were sent on dry ice to the Massachusetts Institute of Technology, identifiable only by their code numbers, where they underwent quantitative analysis for hemoglobin adducts of 4- and 3-ABP. Details of the analytical procedure used in this study have been reported previously (18). Cases and their matched controls were always tested together. For singletons (*i.e.*, cases without matched controls or controls with missing index cases), number of cases and number of controls were always similar in a given batch. All of the subjects who donated blood were asked detailed questions about their use of tobacco products during the immediately preceding 2 months. NAT2 and CYP1A2 phenotypes were determined by analysis of the urinary caffeine metabolites

Table 1 Demographic and other selected characteristics of bladder cancer patients and control subjects

	Cases		Controls	
	<i>n</i>	%	<i>n</i>	%
Total subjects	761	100.0	770	100.0
Sex				
Male	603	79.2	605	78.6
Female	158	20.8	165	21.4
Race				
Non-Latino white	669	87.9	692	89.9
Latino white	55	7.2	51	6.6
African-American/Other	37	4.9	27	3.5
Age (yr) at cancer diagnosis <sup>a</sup>				
$\leq 49$	143	18.8	154	20.0
50–59	329	43.2	320	41.6
$\geq 60$	289	38.0	296	38.4
Mean	55.7		55.8	
Cigarette smoking status <sup>b</sup>				
Never smoker	142	18.7	280	36.4
Ex-smoker	289	38.0	347	45.0
Current smoker	330	43.3	143	18.6
Use of permanent hair dyes, women only				
No	88	55.7	112	67.9
Yes	70	44.3	53	32.1
Regular use of NSAIDs <sup>c</sup>				
No	486	63.9	462	60.0
Yes	275	36.1	308	40.0

<sup>a</sup> For controls, age at date of cancer diagnosis of the index case.

<sup>b</sup> At 2 years before cancer diagnosis of the index case (see "Materials and Methods" for details).

<sup>c</sup> NSAIDs nonsteroidal anti-inflammatory drugs. Regular use = two or more times a week for 1 month or longer.

as described previously (9). *GSTM1*, *GSTP1*, *GSTT1*, and *NAT1* genotypes were determined as described previously (19, 20).

**Statistical Analysis.** The distributions of 4- and 3-ABP hemoglobin adduct levels in our study population were markedly skewed; therefore, formal statistical testing was performed on logarithmically transformed values of adduct levels, and geometric (as opposed to arithmetic) mean values are presented. The analysis of covariance method was used to compare adduct levels between cases and controls while adjusting for the effects of age, sex, laboratory batch, and cigarette smoking on adduct levels (21). Additional adjustments of other risk factors for bladder cancer identified in the Los Angeles Study also were performed. All of the *P*s are two-sided.

In addition, we used conditional logistic regression methods (22) to examine associations between hemoglobin adduct levels and bladder cancer risk. A matched set consisted of all of the cases and controls tested in a single laboratory batch. Age, sex, and level of education were included as covariates in all of the models. The associations were measured by ORs and their corresponding 95% CIs and 2-sided *P*s. Study subjects were grouped into quartiles based on the distribution of values in control subjects, separately for those who smoked and those who did not smoke at the time of blood draw (see Appendix A). The linear trend tests for adduct-cancer associations were based on ordinal values (0–3) for the quartiles.

## Results

Relevant demographic and other characteristics of the study subjects are detailed in Table 1. Roughly 80% of the cases were men and close to 90% of them were non-Latino whites. Adduct

Table 2 Geometric mean (95% CI) levels of 4- and 3-ABP-hemoglobin (Hb) adducts (pg/g Hb) among bladder cancer patients and control subjects<sup>a</sup>

	Cases	Controls	2-sided <i>P</i> (case-control difference)
<b>4-ABP Hb adducts</b>			
Total subjects <sup>b</sup>	40.5 (38.1–43.0)	29.9 (28.1–31.7)	<0.0001
Nonsmokers at blood draw	33.5 (31.0–36.2)	25.3 (23.5–27.3)	<0.0001
Lifetime nonsmokers	26.1 (23.4–29.0)	21.4 (19.8–23.2)	0.002
Smokers at blood draw <sup>b</sup>	98.0 (89.2–107.7)	75.2 (67.0–84.5)	<0.0001
Further adjusted for other risk factors <sup>c</sup>			
Total subjects <sup>b</sup>	39.2 (36.6–42.0)	30.2 (28.1–32.5)	<0.0001
Nonsmokers at blood draw	29.9 (27.7–32.2)	22.7 (21.1–24.4)	<0.0001
Lifetime nonsmokers	26.7 (23.7–30.0)	22.1 (20.1–24.3)	0.007
Smokers at blood draw <sup>b</sup>	97.7 (87.7–109.0)	75.5 (65.8–86.6)	0.001
<b>3-ABP Hb adducts</b>			
Total subjects <sup>b</sup>	1.30 (1.18–1.44)	0.80 (0.70–0.91)	<0.0001
Nonsmokers at blood draw	0.71 (0.61–0.81)	0.46 (0.38–0.55)	<0.0001
Lifetime nonsmokers	0.42 (0.32–0.53)	0.34 (0.27–0.42)	0.19
Smokers at blood draw <sup>b</sup>	6.06 (5.14–7.12)	3.46 (2.75–4.30)	<0.0001
Further adjusted for other risk factors <sup>c</sup>			
Total subjects <sup>b</sup>	1.22 (1.08–1.36)	0.86 (0.74–0.99)	<0.0001
Nonsmokers at blood draw	0.59 (0.50–0.68)	0.38 (0.31–0.46)	0.0004
Lifetime nonsmokers	0.44 (0.32–0.58)	0.36 (0.27–0.46)	0.25
Smokers at blood draw <sup>b</sup>	5.72 (4.77–6.83)	3.80 (2.96–4.83)	0.002

<sup>a</sup> Laboratory batch, age, sex, and level of education were adjusted for in all runs.

<sup>b</sup> Further adjusted for average number of cigarettes smoked per day during the 2 months before blood draw.

<sup>c</sup> The other risk factors were: average number of cigarettes smoked per day, number of years of smoking, current smoker (yes, no), regular use of nonsteroidal anti-inflammatory drugs (yes, no), total times of use of permanent hair dyes over lifetime, dietary intake of carotenoids (in quintiles), NAT2 phenotype (rapid, slow), and *GSTM1* genotype (null, non-null). Exposures were based on information up to 2 years before cancer diagnosis of the index case (see "Materials and Methods" for details).

levels of 4-ABP in all of the subjects ranged from 5.1 to 7400 pg/g hemoglobin. The highest value is greater by an order of magnitude than any we have observed previously, but because there were very few exceptionally high values (~1%), the geometric mean is not notably different from values observed in other studies. Subjects with the 18 highest values (>400 pg/g) were all cases, but only 4 of them were current smokers. None of the values in control subjects were outside the range reported in previous studies of healthy subjects.

Table 2 presents geometric mean values of 4-ABP hemoglobin adducts as well as adducts of 3-ABP among cases and controls as a function of smoking status. Adducts of 3-ABP were included in this study because 3-ABP, although isomeric with 4-ABP, has markedly different toxicological properties, and it is likely that the environmental origins of the two amines are substantially different. Results obtained from analysis of 3-ABP adducts thus has potential value for interpretation of 4-ABP adduct results.

In agreement with several previous studies (12, 13, 23), adducts of both 4- and 3-ABP were elevated in smokers as compared with nonsmokers. Also, levels of 4-ABP adducts were 15–60 times higher than their 3-ABP counterparts. Whereas 3-ABP adducts were negligibly low among nonsmokers, 4-ABP adducts were relatively substantial in nonsmokers (about seven times the 3-ABP adduct levels in smokers).

The main finding presented in Table 2 is that adducts of both 4- and 3-ABP are significantly higher in cases than in controls, independent of the level of tobacco smoking at the time of blood donation. Risk or protective factors for bladder cancer identified in this study population include cigarette smoking (13), regular use of nonsteroidal anti-inflammatory drugs (24), use of permanent hair dyes (25, 26), dietary intake of carotenoids,<sup>4</sup> NAT2 phenotype,<sup>4</sup> and *GSTM1* genotype.<sup>4</sup>

Additional adjustment for all of these factors did not reduce the case-control differences in 4- and 3-ABP hemoglobin adducts. Among lifelong nonsmokers, the statistically significant case-control difference in 4-ABP hemoglobin adducts persisted. 3-ABP hemoglobin adducts, on the other hand, no longer showed a case-control difference in lifelong nonsmokers. Some (14–16) but not all (17) studies have detected the presence of 4- or 3-ABP hemoglobin adducts in individuals positive for passive smoke exposure. In this study population, there were no associations between indices of passive smoke exposure and 4- or 3-ABP hemoglobin adducts among nonsmokers at the time of blood draw.<sup>4</sup> Thus, passive smoke exposure was not considered as a potential confounding variable. Certain occupational groups have been consistently shown to exhibit an elevated risk of bladder cancer (1). Further adjustment for a history of high-risk occupations (truck/bus/taxi driver, aluminum product worker, hairdresser) yielded results similar to those without this additional adjustment.

Adducts of 4- and 3-ABP were strongly correlated ( $r = 0.57$ ), as we have observed in previous investigations. Adducts of 4-ABP exhibited a stronger association with bladder cancer risk than did 3-ABP adducts. After adjustment for level of 4-ABP adducts along with other risk or protective factors, 3-ABP adducts no longer showed an association with bladder cancer risk ( $P = 0.60$ ). On the other hand, 4-ABP adducts remained statistically related to bladder cancer risk after adjustment for 3-ABP adducts ( $P = 0.0001$ ).

Table 3 shows bladder cancer risk estimates by quartiles of ABP adduct levels, separately for smokers *versus* nonsmokers at the time of blood draw. Consistent with the findings presented in Table 2, there was statistically significant increasing risk with increasing adduct levels, irrespective of smoking status at blood draw. For example, among nonsmokers, those at the highest quartile of 4-ABP adducts were more than three times as likely to develop bladder cancer relative to the subjects in the lowest quartile (OR, 3.2; 95% CI, 2.1–4.9).

<sup>4</sup> Unpublished observations.

Table 3 4- and 3-ABP-hemoglobin (Hb) adduct levels in relation to bladder cancer<sup>a</sup>

ABP adduct	Quartile <sup>b</sup>				2-sided <i>P</i> for linear trend
	1st	2nd	3rd	4th	
4-ABP Hb adducts					
Nonsmokers at blood draw, OR (95% CI)	1.00	1.56 (1.09–2.25)	1.70 (1.17–2.49)	3.50 (2.42–5.05)	<0.0001
Smokers at blood draw, OR (95% CI) <sup>c</sup>	1.00	0.83 (0.40–1.71)	1.60 (0.79–3.26)	3.22 (1.54–6.74)	0.0004
Further adjusted for other risk factors <sup>d</sup>					
Nonsmokers at blood draw, OR (95% CI)	1.00	1.43 (0.94–2.19)	1.57 (1.004–2.44)	3.19 (2.08–4.90)	<0.0001
Smokers at blood draw, OR (95% CI) <sup>c</sup>	1.00	1.35 (0.50–3.59)	2.53 (0.99–6.45)	3.76 (1.46–9.72)	0.003
3-ABP Hb adducts					
Nonsmokers at blood draw, OR (95% CI)	1.00	1.07 (0.64–1.81)	1.36 (0.88–2.11)	2.44 (1.65–3.61)	<0.0001
Smokers at blood draw, OR (95% CI) <sup>c</sup>	1.00	1.58 (0.75–3.30)	1.50 (0.70–3.18)	4.19 (2.03–8.63)	0.0001
Further adjusted for other risk factors <sup>d</sup>					
Nonsmokers at blood draw, OR (95% CI)	1.00	1.02 (0.56–1.86)	1.16 (0.70–1.92)	2.10 (1.34–3.29)	0.003
Smokers at blood draw, OR (95% CI) <sup>c</sup>	1.00	2.66 (0.98–7.21)	1.89 (0.69–5.19)	5.62 (1.99–15.83)	0.004

<sup>a</sup> Based on conditional logistic regression method. A matched set consisted of all cases and controls tested in a single laboratory batch. Age, sex, and level of education were included as covariates in all of the runs.

<sup>b</sup> See appendix A for quartile cut-points.

<sup>c</sup> Further adjusted for average number of cigarettes smoked per day during the 2 months prior to blood draw.

<sup>d</sup> The other risk factors were average number of cigarettes smoked per day, number of years of smoking, current smoker (yes, no), regular use of nonsteroidal anti-inflammatory drugs (yes, no), total times of use of permanent hair dyes over lifetime, dietary intake of carotenoids (in quartiles), NAT2 phenotype (rapid, slow), and *GSTM1* genotype (null, non-null). Exposures were based on information up to 2 years before cancer diagnosis of the index case (see "Materials and Methods" for details).

## Discussion

Results of this population-based investigation of aromatic amine exposure and bladder cancer risk clearly indicate that case patients were exposed to higher levels of metabolically activated 4- and 3-ABP than their matched controls. The association between risk and 3-ABP disappeared after adjustment for 4-ABP. Neither smoking at the time of blood draw nor lifetime smoking history accounted for the case-control differences. Independent of smoking status at the time of blood draw, cases exhibited elevated adducts compared with controls, and the magnitude of the relative difference (30–40% higher in cases) was comparable between smokers and nonsmokers. Elevated adduct levels may reflect exposure to higher doses of aromatic amines, greater metabolic conversion of amine to *N*-hydroxylamine, or a combination of both factors. As adjustment for metabolic differences did not substantially change the observed case-control differences, much of the difference in adduct levels of 4- and 3-ABP is likely to represent differences in exposures to these two chemicals.

Cigarette smoke is an important source of 4- and 3-ABP exposure. Active smokers appear to be exposed to ~3- to 10-fold higher amounts of 4- and 3-ABP than nonsmokers. Whether 4- and 3-ABP and other aromatic amines are the principal agents responsible for the apparent carcinogenicity of cigarette smoke for the bladder is difficult to test epidemiologically, because of the complex mixture of toxic and carcinogenic substances in tobacco smoke. Our finding that nonsmokers exhibit higher 4- and 3-ABP hemoglobin adducts if they are bladder cancer cases compared with control subjects strengthens the hypothesis that arylamines in cigarette smoke are responsible, at least in part, for bladder cancer development in smokers.

More significantly, our results suggest that 4- and 3-ABP exposure at levels lower than those experienced by smokers are linked to bladder cancer risk. 4-ABP is one of the most potent bladder carcinogens in animals. Previous studies have indicated widespread exposure to 4-ABP in humans other than through exposure to cigarette smoking; the sources of such nonsmoking related exposure remain unclear (9–11, 27).

Several exposure scenarios that could result in formation of human hemoglobin adducts of 4-ABP can be described. 4-nitrobiphenyl (4-NB) is a product of incomplete combustion

that has been identified as a component of kerosene heater emissions (28) and diesel engine exhaust (29), and exposure to 4-NB can result in the production 4-ABP hemoglobin adducts (30); therefore, exposure to airborne 4-NB is one possibility. Human intestinal microbiota are capable of generating 4-ABP from at least one azo dye (31). Therefore, it is possible that exposure to one or more colorants is responsible for some human 4-ABP exposure. Finally, there is a recent report that fumes from heated cooking oils contain 4-ABP (32), which raises the possibility that food preparation can lead to 4-ABP exposure. The limited information contained in these studies does not provide a sufficient basis for meaningful exposure assessment at this time; we, therefore, do not speculate on the relative importance of each of these possible scenarios. Pinpointing the additional sources of 4-ABP exposure in the environment should be of the highest scientific importance.

The present study has one major limitation inherent in most case-control studies. Assessment of ABP hemoglobin adducts was performed on blood samples taken after cancer diagnosis in cases. It is possible that the postdiagnostic profile in ABP adducts among the cases does not accurately reflect the group's prediagnostic profile. Furthermore, the carcinogenic process is believed to take decades to complete, and, thus, the relevant exposure periods are far removed from the time of blood draw. It is unknown whether recent exposures in study subjects, as captured by the ABP hemoglobin adducts, generally reflect exposure levels in decades past.

Cigarette smoking accounts for no more than 50% of bladder cancer cases in the United States (13), but research over the past several decades has had little success in identifying other major causes. This investigation provides the first substantial evidence that environmental exposure to aromatic amines unrelated to smoking may account for a significant portion of bladder cancer in the general population.

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## Appendices

Appendix A Quartile cut-points of ABP hemoglobin (Hb) adducts (pg/g hemoglobin)

ABP adduct	Quartiles			
	1st	2nd	3rd	4th
4-ABP Hb adducts				
Nonsmoker at blood draw	<15.3 (89/164) <sup>a</sup>	15.3–20.3 (114/159)	20.4–28.4 (117/162)	≥28.5 (228/161)
Smoker at blood draw	<55.3 (35/31)	55.3–77.3 (30/31)	77.4–108.0 (57/31)	≥108.1 (91/31)
3-ABP Hb adducts				
Nonsmoker at blood draw	ND <sup>b</sup> (358/471)	0.2–0.4 (37/52)	0.5–1.0 (53/62)	≥1.1 (100/61)
Smoker at blood draw	<2.0 (31/31)	2.0–3.8 (42/31)	3.9–7.3 (43/32)	≥7.4 (97/30)

<sup>a</sup> Number of cases/Number of controls in parentheses.

<sup>b</sup> ND, Nondetectable. Quartiles 2–4 represent tertiles among controls with positive 3-ABP Hb adducts.

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