

## Short Communication

# Bulky DNA Adduct Formation and Risk of Bladder Cancer

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## Abstract

Exposure to polycyclic aromatic hydrocarbons (PAHs) has been associated with risk of bladder cancer and with increased bulky DNA adduct levels in several studies, mainly in smokers. We investigated the relation between bulky PAH-DNA adducts in peripheral blood mononuclear cells and bladder cancer in nonsmoking subjects from a large hospital-based case-control study in Spain. Additionally, we examined the association between DNA adduct formation and several air pollution proxies. The study comprised 76 nonsmoking cases and 76 individually matched controls by sex, region of residence, age, and smoking status (never, former). To maximize the relevance of the DNA adduct measurement as a proxy of PAH exposure, subjects selected had not changed residence, occupation, and major lifestyle factors during the last 10 years. Bulky DNA adducts were measured using the

<sup>32</sup>P-postlabeling technique, nuclease P1 treatment. The percentage of detectable adducts was higher in controls (41%) than in cases (32%) with an odds ratio of 0.75 (95% confidence interval, 0.36-1.58). In an analysis limited to controls, a higher percentage of DNA adducts was found among those whose last residence was in a big city (50%) compared with those living in villages (19%;  $P = 0.04$ ). No consistent associations were found for other markers of air pollution. In this study, among nonsmokers with stable environmental and lifestyle factors, bulky DNA adducts were not associated with bladder cancer risk. Results do not support an association of bladder cancer risk with low-level exposure to PAHs as measured through the formation of bulky DNA adducts in peripheral mononuclear cells. (Cancer Epidemiol Biomarkers Prev 2007;16(10):2155-9)

## Introduction

Polycyclic aromatic hydrocarbons (PAH), formed during incomplete combustion, are ubiquitous in the environment and account for an important proportion of atmospheric pollution due to traffic, heating, and industry emissions. Exposure to PAHs occurs mainly through smoking (active and passive), diet, inhalation of polluted air, and dermal contact in certain occupations and activities (1, 2).

There is evidence suggesting an association between PAHs and bladder cancer in humans (3). Excess risk of bladder cancer has been observed frequently among drivers of trucks, buses, or taxi cabs (4, 5) and among aluminum workers (6, 7). Although these occupational settings have exposure to PAHs, they also contain exposure to nitro-PAHs and in the case of aluminum workers also aromatic amines, making it difficult to attribute the elevated risk specifically to PAHs. PAH exposure has been related with bulky DNA adduct levels (8) and as a logical extension should therefore be related to bladder cancer risk if PAH exposure is associated with the disease. In a recent study, however, such a relation was not found, except among current smokers (9).

To further investigate the potential association between PAH exposure and bladder cancer, we investigated the relation of bulky DNA adduct levels in mononuclear cells, measured by <sup>32</sup>P-postlabeling, and bladder cancer risk among nonsmoking participants of a hospital-based case-control study. We selected only nonsmokers so as to avoid possible confounding by smoking, as smoking is

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related to bulky DNA adducts and to bladder cancer by mechanisms that are not specific to PAH exposure (10, 11).

## Materials and Methods

**Selection of Subjects.** The Spanish Bladder Cancer Study is a hospital-based case-control study conducted from 1998 to 2001 that enrolled 1,219 cases and 1,271 controls from 18 hospitals in five regions of Spain (Barcelona, Valles/Bages, Alicante, Tenerife, and Asturias; ref. 12). Cases were patients newly diagnosed with and histologically confirmed bladder cancer. Controls were patients admitted to the participating hospitals with diagnoses thought to be unrelated to the exposure of interest, mainly trauma and minor surgery. Controls were individually matched with cases by 5-year categories, sex, and region.

Information on lifestyle factors was obtained using computer-aided personal interview administered at the hospital. Information was collected on tobacco use, lifetime occupational and residential history, environmental exposures, and other potential bladder carcinogens. Information on dietary habits was obtained through a food frequency questionnaire. The participation rate for the computer-aided personal interview was 85%, and 96% of subjects who completed the interview gave a blood sample.

A subgroup of the total study subjects was selected for DNA adduct analysis. After limiting the population to subjects with at least 40 million lymphocytes available, were nonsmokers or former smokers for at least 10 years before interview, and had not changed their residence or occupation (for those employed) during the 10 years before interview/diagnosis, we ended up with 158 cases (29 women) and 228 controls (20 women). Out of the eligible subjects, 76 cases (65 males and 11 females) were randomly selected and individually matched to 76 controls by sex, age, smoking status [former (for >10 years) or never smoker], and region (when possible). Each region comprises wide geographic areas with both rural and urban environments so as to avoid having cases and controls coming from very similar environments.

**DNA Extraction and <sup>32</sup>P-Postlabeling.** Peripheral blood mononuclear cells were isolated from the whole blood using a Ficoll gradient and cryopreserved in DMSO. DNA was extracted from the peripheral blood mononuclear cells using the chloroform/isoamyl alcohol method (13).

DNA samples were analyzed in duplicate using the <sup>32</sup>P-postlabeling technique with nuclease P1 enrichment (14). In addition, blind duplicates were randomly inserted for quality control purposes. DNA (4 μg) was hydrolyzed by incubation for 3 h at 37°C using 2.5 units of micrococcal endonuclease (Sigma) and 0.25 μg of calf spleen phosphodiesterase (Sigma). Nuclease P1 was added (0.75 unit) to the digested DNA and samples were again incubated for 40 min at 37°C to enhance the sensitivity of the assay. Labeling of adducted nucleotides was done by adding 50 μCi per sample of [<sup>32</sup>P]ATP and incubating with T4 polynucleotide kinase (5 units/μL) for 40 min at 37°C. Potato apyrase (2 μL) was added to

the mixture and incubated for 40 min at 37°C to hydrolyze any unreacted ATP. Eighty percent of the sample volume was spotted on a 10 × 10 cm polyethyleneimine cellulose plate, and adducts were mapped with a three-directional TLC using (a) 0.65 mol/L sodium phosphate (pH 6.0); (b) 3.6 mol/L lithium formate and 8.5 mol/L urea; and (c) 0.8 mol/L lithium chloride, 0.5 mol/L Tris-HCl, and 8.5 mol/L urea (pH 8.0). Plates were developed in 1.5 mol/L sodium phosphate (pH 6).

DNA adducts were visualized by autoradiography and quantified using Cerenkov counting. The first spot detected was assigned number 1, and each spot detected in the same position was equally labeled. Other spots detected in different positions in the chromatogram were numbered consecutively according to each new appearance in the plates. Plates were placed in autoradiography cassettes containing intensifying screens and exposed at -80°C for 48 h. Spots were excised from the plates and counted in plastic scintillation vials containing 5 mL 70% ethanol. Appropriate blank areas of the chromatogram were also excised to provide a background adjustment.

Calculation for relative adduct labeling (RAL) was done using the following formula (14):

$$\text{RAL} = \frac{\text{adduct count per minute (cpm)} \times 10^6}{1.25 \times 10^6 \text{ cpm/pmol (ATP)} \times 3,240 \text{ pmol (dNP)} / \mu\text{g DNA}}$$

A benzo(a)pyrene diol epoxide-DNA adduct standard was run with the samples as a positive control. Benzo(a)pyrene-dG-3'-monophosphate was supplied by the National Cancer Institute, Division of Cancer Biology, Chemical and Physical Carcinogenesis Branch (64 FR 72090).

**Statistical Analyses.** Samples were assayed blind to case-control status. Adduct cpm results were corrected for background radioactivity by taking the average background cpm level of TLC plates in the same run ( $n = 16$ ). Limit of detection of the <sup>32</sup>P-postlabeling was calculated based on the observed variability in the background cpm levels (limit of detection = thrice the SD). Limit of detection was 25 cpm (corresponding to ~1 RAL), and thus, adduct cpm levels above 1 RAL, after background correction, were considered detectable. The median coefficient of variation between blinded duplicates (14 pairs) for adduct 1, adduct 3, and total adducts was 31%, 42%, and 35%, respectively. The intraclass correlation coefficient of adduct 1, adduct 3, and total adducts was 0.96, 0.90, and 0.89, respectively.

Conditional logistic regression was used to estimate the association of bulky DNA adducts with risk of bladder cancer. Analyses were based on the presence ( $\geq 1$  RAL) or absence ( $< 1$  RAL) of adducts (yes/no) and as continuous measure after log transformation. In these analyses, nondetectable adduct levels were substituted with the limit of detection (1 RAL) divided by the square root of two. Odds ratios were adjusted for a priori high-risk occupations (metal workers, hairdressers and barbers, painters, chemical, leather, transport, and rubber industries; ref. 5). Differences in adduct levels due to lifestyle and environmental exposures were analyzed only among the controls by the Wilcoxon two-sample test

**Table 1. Main characteristics of the study population**

	Cases ( <i>n</i> = 76)		Controls ( <i>n</i> = 76)	
	<i>n</i>	%	<i>n</i>	%
Gender, male	65	86	65	86
Age, mean (SD)	71 (9)		70 (10)	
Male	71 (6)		71 (6)	
Female	68 (10)		65 (11)	
Region				
Barcelona	10	13	9	12
Valles/Bages	12	16	14	18
Alicante	5	7	3	4
Tenerife	13	17	10	13
Asturias	36	47	40	53
Education				
Less than primary school	39	52	44	58
Primary school	28	37	27	36
High school or higher	8	11	4	5
Other	0	0	1	1
Missing	1		0	
Smoking				
Never smokers	31	41	31	41
Former smokers	45	59	45	59

NOTE: Cases and controls were matched by sex, age, and smoking status (former or never smoker). Controls were also matched to cases by region of residence whenever possible.

and the Student's *t* test. Statistical analyses were carried out using Stata software, version 8.2. Diet was also evaluated in relation with adduct levels. As we did not find any association of adduct levels with fruit and vegetable intake, estimated benzo(*a*)pyrene, or heterocyclic amines (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline, and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) intake results are not presented.

## Results

Table 1 presents the main characteristics of the study population. The distribution of the study population by gender was similar to that of the Spanish Bladder Cancer Study, with 86% males and a mean age of 70 years.

Adduct 1 and adduct 3 (numbering based on first occurrence in the experiments) were the most prevalent adducts, with 32% and 9% of detectable adducts, respectively. These two spots coincided with the two main spots of the benzo(*a*)pyrene diol epoxide standard. The remaining spots (*n* = 5) represented <5% of

detectable adducts in the samples and were not analyzed individually. Table 2 shows the percentage of individuals with detectable adducts stratified by case status, median RAL level, and the corresponding 10th and 90th percentiles of adducts among the subjects in whom adduct levels were detectable. For adduct 1, adduct 3, and total adducts, percentage of individuals with detectable levels was lower in cases than in controls, showing odds ratios below 1, albeit none of the differences was statistically significant. When using the logarithm of the levels of adducts instead of the dichotomous variable of detectable versus nondetectable adducts, the results were essentially similar (data not shown).

DNA adducts were also analyzed among controls as possible markers of exposure to air pollution among the general population. Table 3 shows the percentage of individuals with detectable adduct levels according to three of the variables of air pollution studied: proximity to industries, windows facing traffic, and size of the city of residence. We show both lifetime indices as well as air pollution indices for the last residence (average duration in last residence is 40 years). Only the size of the city showed a small increase in the percentage of detectables for subjects living more than 40 years in a big city or metropolitan area compared with those subjects who spend more than 25 years in a small village (from 50% to 19%, respectively, considering only the last residence and from 57% to 33%, respectively, taking into account the lifetime index).

## Discussion

We examined bulky DNA adducts in peripheral mononuclear cells of nonsmoking bladder cancer cases and controls, who were selected among subjects with a stable lifestyle in relation to place of residence and occupation in the past 10 years.

This selection strategy was chosen to increase the historical relevance of the measured adduct levels and thus reduce the inherent exposure misclassification. All subjects included in the study were never or long-term former smokers. No difference in adduct levels was observed between these two groups and risk analyses stratified by smoking status resulted in similar observations. Controls tended to have a higher percentage of detectable adduct levels than cases, but differences were not statistically significant. These results are consistent with a previous study among nonsmokers (9) and seem

**Table 2. Percentage of detectable adducts and median adduct levels (only for the detectables) for adducts 1 and 3 and total adducts and conditional logistic regressions**

	Cases ( <i>n</i> = 76)		Controls ( <i>n</i> = 76)		OR (95% CI)
	% Detectable adducts	Median RAL (10-90th percentile)	% Detectable adducts	Median RAL (10-90th percentile)	
Adduct 1	29	1.5 (1.1-64.9)	36	1.8 (1.2-5.5)	0.86 (0.43-1.74)
Adduct 3	4	5.7 (1.1-6.9)	13	1.7 (1.2-9.3)	0.15 (0.02-1.18)
Total adducts	32	1.5 (1.1-83.9)	41	2.5 (1.3-9.7)	0.75 (0.36-1.58)

NOTE: Matching variables: age, gender, region, and smoking status (never/former); adjusted for high-risk occupations. The other adducts (2 and 4 to 7) were not considered because of their low frequencies of detectable adducts. RAL expressed as adducted nucleotides per 10<sup>9</sup> normal nucleotides. Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.

**Table 3. Comparison of air pollution variables with percentage of detectable adducts, both lifetime and last residence variables**

	<i>n</i> total	Level of exposure to air pollution variables			<i>P</i> ( $\chi^2$ )	<i>P</i> (trend)
		Low	Intermediate	High		
		<i>n</i> (% detectables)	<i>n</i> (% detectables)	<i>n</i> (% detectables)		
<b>Lifetime exposure</b>						
Having an industry close to the residence	76	46 (39)	11 (27)	19 (52)	0.370	0.413
Having windows facing traffic street at home	76	44 (43)	15 (47)	17 (29)	0.541	0.400
Size of the city of residence	75	30 (33)	24 (38)	21 (57)	0.212	0.102
<b>Last residence</b>						
Having an industry close to the residence	62	51 (43)	2 (100)	9 (11)	0.042	0.143
Having windows facing traffic street at home	62	37 (43)	12 (58)	13 (15)	0.076	0.171
Size of the city of residence	74	21 (19)	43 (51)	10 (50)	0.040	0.037

NOTE: Only controls are included in the analysis. Low exposure: (a) no industries in the proximity of the residence, (b) not having windows facing traffic street, and (c) >25 y living in a small village. High exposure: (a) >30 y living close to an industry, (b) >30 y with windows facing a traffic street, and (c) >40 y living in a big city. Intermediate exposure: subjects that did not fit in any of the two categories (e.g., low and high) were classified as "intermediate exposed."

to indicate that DNA adducts measured in mononuclear cells are not associated with bladder cancer risk. Our finding might be also in keeping with those for lung cancer. Tang et al. (11) found a relation between carcinogen-DNA adducts and lung cancer only among current smokers but not among former and nonsmokers. They concluded that DNA adduct levels could serve as a measure of biological susceptibility, which would manifest most clearly while exposure is ongoing.

In addition, we evaluated bulky DNA adducts as a biomarker of exposure, with the nuclease P1 method of <sup>32</sup>P-postlabeling, which is sensitive to many PAH carcinogen-DNA adducts (15). A meta-analysis of grouped data had previously shown a correlation between low-level benzo(a)pyrene exposure and level of DNA adducts (16) but not at the individual level. In our study, one of the criteria used for the selection of subjects was not having changed the residence in the past 10 years. Living in the proximity of an industrial plant or having windows facing a traffic street did not seem to be associated with the level of DNA adducts. Size of the city of residence as a proxy for living in an urban or rural setting, was positively correlated with the percentage of detectable adducts, especially when taking into account the last residence rather than lifetime residences. PAHs in air originate mainly from traffic exhaust (17). Unfortunately, no quantitative data were available on traffic intensity to further explore this potential association. Besides differences in air quality, the size of the city could also reflect differences in lifestyles between urban and more rural environments, although we have explored and/or adjusted for the main factors that can influence the results, such as smoking, diet, and occupation.

The upper confidence intervals of odds ratios for adduct associations decrease the probability of a substantial effect (upper 95% confidence interval ~1.6) of PAH on risk of bladder cancer in this population, which was presumably exposed to relatively low levels of PAHs. We cannot exclude that the absence of a relation between DNA adducts and bladder cancer risk is partly due to exposure misclassification resulting most likely in a bias toward the null. However, reliability of our measurements was high with intraclass correlation

coefficients between 0.89 and 0.96. The coefficient of variation, on the other hand, was ~35% but was similar in cases and controls, and therefore, odds ratios and other comparisons between these two groups should not have been biased. Due to the hospital-based case-control design of the study, subjects might have changed their usual lifestyle habits due to illness. However, subjects were enrolled quickly after hospital admission, and as such, the potential changes in dietary habits will probably not have influenced the results to a large extent, especially as we measured bulky DNA adducts in mononuclear cells that have an estimated half-life of at least several weeks to a few months (18).

In conclusion, despite the small numbers, our study is one of the few studies of DNA adducts and bladder cancer risk among nonsmokers. We reported that bulky DNA adducts were not associated with bladder cancer risk in this carefully selected population of nonsmokers with stable lifestyles. Results from this study do not support an association of bladder cancer risk with low-level exposure to PAHs measured through the formation of bulky DNA adducts, at least among nonsmokers.

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