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Biomarkers of genotoxicity of air pollution (the AULIS project): bulky DNA adducts in subjects with moderate to low exposures to airborne polycyclic aromatic hydrocarbons and their relationship to environmental tobacco smoke and other parameters

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The levels of bulky DNA adducts were measured by ^{32}P-post-labelling in lymphocytes of 194 non-smoking students living in the city of Athens and the region of Halkida, Greece, once in the winter and again in the following summer. Personal exposures to particulate-bound polycyclic aromatic hydrocarbons (PAH) were significantly higher in Athens subjects during both seasons. There was hardly any diagonal radioactive zone in the pattern of DNA adducts observed. Highest adduct levels were observed in a sub-group of subjects living in or near the Halkida Institute campus, which was located in rural surroundings with a minimal burden of urban air pollution. The remaining Halkida subjects had intermediate levels, while Athens subjects showed the lowest levels. This trend, which was observed over both monitoring seasons, consistently paralleled the variation in three markers of exposure to environmental tobacco smoke (ETS), namely (i) declared times of exposure to ETS during the 24 h prior to blood donation, (ii) plasma cotinine levels and (iii) chrysene/benzo[*g*,*h*,*i*]perylene ratios in the profile of personal PAH exposure. Furthermore, among the Halkida campus area subjects (but not the remaining subjects) positive correlations were observed between DNA adducts and (i) measured personal exposures to chrysene or benzo[*a*]pyrene, (ii) time of declared ETS exposure and (iii) chrysene/benzo[*g*,*h*,*i*]perylene ratios. These correlations suggest that, for a group suffering minimal exposure to urban air pollution, exposure to ETS was a significant determinant of the observed DNA damage. Gender had a consistent and significant effect on adduct levels (males having higher levels), which remained significant even after multiple regression analysis. Habitual consumption of roasted meat was significantly associated with an enhancement of adduct levels and the effect was strengthened when only individuals unexposed to ETS were taken into consideration. No significant effects were observed for other dietary parameters or factors reflecting exposure to air pollution.

Abbreviations: B[*a*]A, benzo[*a*]anthracene; B[*b*]F, benzo[*b*]fluoranthene; B[*k*]F, benzo[*k*]fluoranthene; B[*a*]P, benzo[*a*]pyrene; BPer, benzo[*g*,*h*,*i*,]perylene; CHRYS, chrysene; ETS, environmental tobacco smoke; IND, indeno[1,2,3,*d*]pyrene; PAH, polycyclic aromatic hydrocarbons; $PM_{2.5}$, particulate matter <2.5 μm; TEI, Technical Educational Institute; TLAD, time–location–activity diary.

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Introduction

Epidemiological studies have indicated that the relative risk of developing lung cancer is ~1.5-fold higher in urban areas than in rural areas, even after adjusting for tobacco smoking (1). Atmospheric pollution has been generally recognized as a health hazard and substantial evidence exists for increased rates of morbidity and mortality from respiratory and pulmonary diseases following severe air pollution episodes (2). However, the long-term effects of urban air pollution on human health, including any possible contribution to lung cancer, are still inadequately understood (1).

Urban air quality is heavily influenced by a variety of anthropogenic activities. Power plants and other industrial activities, motor vehicles and residential heating sources all release a complex mixture of gaseous and particulate-bound compounds into the urban atmosphere. Polycyclic aromatic hydrocarbons (PAH) constitute a large and, from the human cancer aetiology point of view, a particularly important category of emitted compounds because: (i) they can be metabolically activated to reactive intermediates that can bind covalently to DNA to form adducts which can lead to mutations and tumor initiation; (ii) many PAH are mutagenic and carcinogenic in animals; (iii) they may react with other primary or secondary air pollutants (NO_x and O_3) and generate other potentially carcinogenic chemicals. In addition to ambient air, other sources (especially mainstream or sidestream tobacco smoke as well as dietary sources) can contribute to human exposure to PAH (3,4).

Increased risks of lung and bladder cancer have been demonstrated, by means of classical epidemiological studies, in populations occupationally exposed to levels of PAH orders of magnitude higher than those commonly found in urban air (5). In contrast, epidemiological studies have failed to provide conclusive evidence of increased cancer risk in populations who, for occupational reasons, suffer extended exposure to urban air pollution, such as bus and truck drivers and railroad workers (reviewed in ref. 6). Given that some studies have suggested short-term health effects even at levels of urban air pollution below the current guidelines and standards, it is of prime interest to investigate the chronic effects of urban air

pollution (especially urban air PAH) at relatively low levels, such as those commonly found in most metropolitan areas of the Western world.

Over the past decade, molecular epidemiology studies using biomarkers of exposure and early biological effects have provided additional evidence of genotoxic effects of atmospheric PAH under conditions of heavy air pollution. For example, studies on the levels of PAH–DNA adducts and chromosomal damage have demonstrated in many cases an increased risk of genetic damage in populations occupationally exposed to high levels of PAH mixtures generated from either industrial or urban sources, as well as from tobacco smoking (7–11, reviewed in ref. 12). Attempts to extend the search for analogous effects to cohorts derived from the general population exposed to urban air pollution have yielded supporting evidence in cases of populations living in regions with high levels of pollution. For example, a study on individuals living in an area of heavy industrial pollution in Silesia, Poland, with atmospheric concentrations of pollutants well above the limits of ambient air pollution accepted in most Western countries, showed quantitative associations between PAH exposure, DNA adducts and a number of biomarkers of effect related to genotoxicity (13). An analogous association between exposure to urban air PAH and DNA adducts was also observed in Teplice, a district of Northern Bohemia heavily polluted by intensive brown coal combustion (14). On the other hand, a recent study in the Italian city of Genoa on individuals suffering exposure to lower levels of air pollution failed to demonstrate a relationship between personal exposure to airborne PAH and DNA adducts in blood lymphocytes (15).

The AULIS project (within which the currently reported work was carried out) is a large scale molecular epidemiological study conducted to investigate the quantitative relationships between exposure of the general population to urban air pollution in a Western European region and various biomarkers of exposure, susceptibility and early biological effects. The study involved two cohorts of Technical Educational Institute (TEI) students, one living in Athens, a densely populated city of nearly 4 000 000 inhabitants with moderate to high levels of atmospheric pollution, and the other living in or near Halkida, a nearby town of ~25 000 inhabitants anticipated to have lower levels of ambient air pollution. These cohorts were investigated in terms of their personal exposure to airborne particulate matter <2.5 μm ($PM_{2.5}$) and associated PAH (eight carcinogenic PAH were measured) and the levels of various biomarkers of genotoxicity. The conclusions of the personal exposure monitoring study, already reported (16), can be summarized as follows.

i. $PM_{2.5}$ exposures were higher for Halkida subjects during winter but not during summer (median winter values, 40.5 μg/m^3 for Athens, 58.9 μg/m^3 for Halkida; median summer values, 32.5 μg/m^3 for Athens, 31.6 μg/m^3 for Halkida), while PAH exposures were higher for Athens subjects, particularly during the summer (median winter values, 7.81 ng/m^3 for Athens, 6.24 ng/m^3 for Halkida; median summer values, 4.47 ng/m^3 for Athens, 1.62 ng/m^3 for Halkida).

ii. While the PAH exposure profiles at the two locations were broadly similar, the relative abundances of the lighter PAH [chrysene (CHRYS), benzo[*a*]anthracene (B[*a*]A), benzo[*b*]fluoranthene (B[*b*]F) and benzo[*k*]fluoranthene (B[*k*]F)] were consistently and significantly higher and those of heavier PAH [benzo[*g*,*h*,*i*,]perylene (BPer) and indeno[1,2,3,*c*,*d*]pyrene (IND)] lower among Halkida subjects.

iii. Higher relative abundances of lighter PAH (as indicated by the CHRYS/BPer ratio) in the personal PAH exposure profile correlated well with markers of personal exposure to environmental tobacco smoke (ETS) and appear to constitute a fingerprint of ETS exposure. Recent ETS exposure, as reflected in declared time spent in the presence of smokers, plasma cotinine levels and PAH profiles (CHRYS/BPer ratio), was higher in Halkida subjects, particularly among a subgroup of subjects living in or near the students' residence on the TEI campus ('Halkida campus area' sub-group).

Here we present the results of measurements of bulky DNA adducts in blood lymphocytes and their association with personal exposures to $PM_{2.5}$ and associated PAH as well as other exposure-related variables.

Materials and methods

Field study

Details of the design and conduct of the study are described elsewhere (17). Briefly, 194 students (18–28 years old) attending the TEI of Athens (117 subjects) and Halkida (77 subjects) participated in the study, which took place over two winter periods (November 1996–February 1997, November 1997–February 1998) and two summer periods (May–September 1997 and 1998). All subjects declared that they had not smoked for at least 6 months prior to their participation in the study. Athens subjects lived in the greater urban region of Athens (including the city and its suburbs) and attended Athens TEI, which is centrally located in the city. Halkida subjects lived in the town of Halkida or the surrounding area and attended Halkida TEI, which is located in a rural area, 12 km from the town. All participants filled in a personal history questionnaire giving information on dietary habits, residence and other activities that might be expected to influence genotoxic exposure and biomarker levels and, for a period of 4 days, kept a time–location–activity dairy (TLAD), in which they noted their location and activities every 15 min. During the last 24 h of this 4 day period they carried a personal monitor for $PM_{2.5}$. On the morning of day 4 they provided a sample of blood and answered a 24 h recall questionnaire giving further information on their activities and movements during this period.

DNA isolation

Blood samples were collected in CPT vacutainer tubes (Beckton Dickinson) and lymphocytes were isolated according to the manufacturer's instructions within 6 h of collection. Lymphocyte pellets were stored at –80°C. DNA isolation was performed as previously described by Gupta *et al*. (18). Lymphocytes were resuspended in a solution of 10 mM Tris–HCl, 100 mM EDTA, 0.5% SDS, pH 8, and treated with proteinase K, followed by phenol extraction and ethanol precipitation of the DNA. Contaminant RNA was then hydrolyzed with RNase A and RNase T1, followed by a second proteinase K treatment. DNA was finally phenol extracted and ethanol precipitated. DNA concentration and purity were assessed spectrophotometrically by measuring UV absorbance at 260 and 280 nm. All DNA samples had a 260/280 ratio within the range 1.76–1.84 and were kept at –80°C until analysis.

^{32}P-post-labelling

The nuclease P1 enrichment procedure described by Reddy and Randerath (19) was employed with minor modifications. The butanol enrichment procedure was performed as previously described (20). A BPDE–DNA standard was included in both analyses to correct for assay variability. Some samples were analysed in two independent experiments and the variability was found to be $<20\%$. The limit of detection was 0.1 adducts/10^8 nt.

PAH analysis

PAH extraction from the $PM_{2.5}$ and HPLC analysis using fluorescence detection were performed as previously described (16).

Plasma cotinine measurements

Cotinine levels were analysed by radioimmunoassay using the RIA set provided by Brandeis University (Waltham, MA) (16,21). Plasma was isolated from heparinized blood by 10 min centrifugation at 1000 *g*. Seven cotinine standards (Sigma) in the range 0.05–5 ng/ml were used in duplicate to construct the standard curve on a log–logit scale. The variability between experiments was checked by using three plasma samples with known levels of cotinine: low (1 ng/ml), average (10 ng /ml) and high (100 ng/ml). All samples were analysed in duplicate. If the difference between duplicates was $>15\%$, the analysis was repeated. All samples had plasma cotinine levels

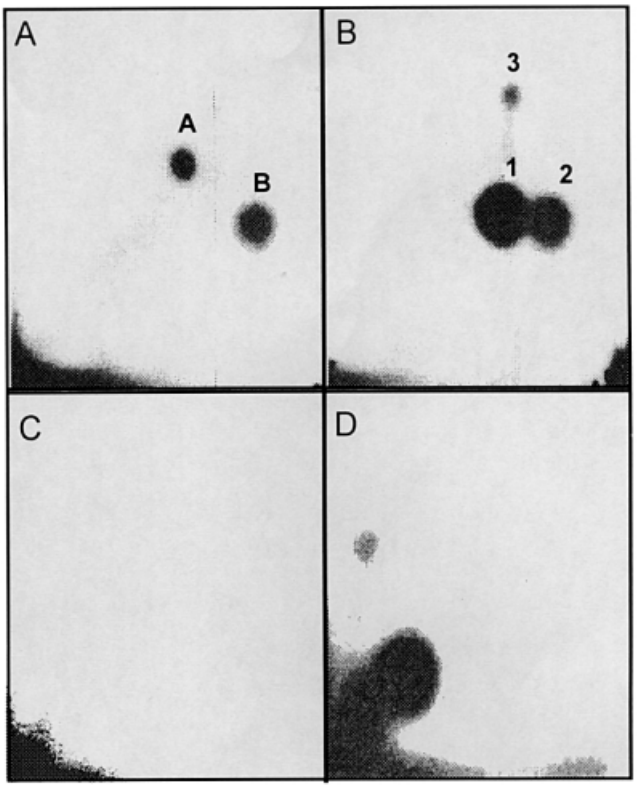

Fig. 1. Typical autoradiograms of ^{32}P-post-labelled human DNA isolated from lymphocytes of environmentally exposed individuals. (**A**) Nuclease P1 method (two discrete spots, designated A and B, are shown); (**B**) the same DNA analysed by the butanol extraction enrichment method (three discrete spots, designated 1–3, are shown); (**C**) water blank (nuclease P1); (**D**) B[*a*]P rat liver standard from a rat treated orally with a single dose of 100 mg/kg body wt B[*a*]P (nuclease P1). After digestion of the DNA, covalently modified nucleotides were enriched and labelled with [γ-^{32}P]ATP and separated by PEI–cellulose TLC. Screen enhanced autoradiography was performed at –80°C for 3–5 days.

between the limit of detection (0.05 ng/ml) and 20 ng/ml and this range was considered to be the active range for passive smokers.

Statistical analysis

All values were natural log transformed in order to obtain a normal distribution. Student's *t*-test for independent or paired samples, one way ANOVA or univariate or multivariate linear regression analysis was used.

Results

Two hundred and three subjects were initially enrolled in the study. Nine individuals that were found to have cotinine levels >20 ng/ml in either collection period were considered as possible active smokers and for this reason all data from them were excluded from the study (16).

^{32}P-post-labelling using nuclease P1 enrichment was employed for the analysis of bulky adducts in all DNA samples. The adduct pattern observed was consistently characterized by the presence of only two distinct spots (designated adducts A and B) and almost complete absence of a diagonal radioactive zone (Figure 1A). During the second winter period a third spot (adduct C) was observed in a number of samples (n = 45) with a high total DNA adduct level (23 from Athens and 22 from Halkida). Similar patterns with distinct spots and no diagonal radioactive zone were observed in a number of samples, which were also analysed using the butanol extraction, ^{32}P-post-labelling method (Figure 1B). None of the adducts co-migrated with the main benzo[*a*]pyrene (B[*a*]P) diol epoxide-derived DNA adduct or with other well-characterized PAH–DNA adducts (standards derived from incubations of eight carcinogenic PAH with calf thymus DNA were used; 22). Calf thymus DNA repurified using the same method of DNA purification as employed for the unknown samples and analysed in parallel with them did not give the two characteristic spots. A diagonal radioactive zone was observed when, as a positive control, lymphocyte DNA from a heavy smoker living in Athens was analysed for DNA adducts.

The levels of the two adducts commonly observed (A and B) were highly correlated with each other (r = 0.77, $P < 0.001$). Adduct C was also correlated with the other two adducts (r = 0.66, $P < 0.001$ and r = 0.39, P = 0.008 for the correlations with adducts A and B, respectively). The adduct levels were low, with a mean adduct A level per 10^8 nt of 0.54 (median 0.39, geometric mean 0.43, range 0.07–3.84), a mean adduct B level per 10^8 nt of 0.60 (median 0.50, geometric mean 0.48, range 0.03–3.88) and a mean adduct C level per 10^8 nt of 0.07 (median 0.0, geometric mean 0.00, range 0.00–3.35). Halkida subjects exhibited higher mean levels of all adducts than Athens subjects regardless of the year (1996, 1997 or 1998) or season (winter or summer) of sample collection (Table I). A multiple linear regression model showed that the differences in adduct levels between the two locations were highly significant ($P < 0.001$) after controlling for both season and year (results not shown).

Effect of personal exposure to $PM_{2.5}$ and PAH

No statistically significant correlation was observed between individual or total DNA adduct levels and personal exposure to $PM_{2.5}$ or total PAH when all the samples, regardless of location, were taken into consideration (Figure 2). Similarly, no association was observed when the samples were classified separately according to location or season (data not shown). However, when personal exposure to individual PAH was stratified by quartiles, a linear trend for adducts A and B versus exposure to CHRYS was observed only among Halkida subjects (all individuals), which tended to be more significant during the winter season (Table II). A similar trend was observed for B[*a*]A (another light PAH) and B[*a*]P, but not for BPer, a PAH considered to constitute a fingerprint for traffic exhaust emissions (data not shown).

Seasonal variation

As already noted, a seasonal variation in personal exposure to $PM_{2.5}$ and PAH was observed, exposures being significantly higher during the winter season (16). A similar, but non-significant, trend towards higher adduct levels during the winter was seen at both locations (Table I) and during both years of the study (data not shown).

Intra-individual correlation

During the study all subjects were monitored for PAH analysis and sampled for biomarker analysis twice, once during the winter and once during the following summer. Thus, by having two measurements for every individual it was possible to assess the correlation of the ranking of each subject for his/her adduct levels, as well as exposure parameters, between the two monitoring seasons. As shown in Table III, a strong correlation was observed for all adducts only among subjects belonging to the Halkida campus area cohort. This correlation indicates that subjects ranking high for their winter adduct levels also tended to rank high for their summer adduct levels. The same cohort showed the strongest intra-individual

Table I. DNA adducts detected by the nuclease P1 ^{32}P-post-labelling method (adducts A, B and C) by location only, location and season or location and year of sample collection

		Adduct A/10^8	Adduct B/10 nt^8 nt	Total (A+B)/10^8 nt	Total (A+B+C)/10^8 nt
Athens					
Total	*n*	225	225	225	225
	Mean (SD)	0.47 (0.37)	0.54 (0.38)	1.01 (0.68)	1.25 (1.19)
	Median	0.36	0.45	0.83	0.94
Halkida					
Total	*n*	150	150	150	150
	Mean (SD)	0.64 (0.54)	0.71 (0.57)	1.35 (1.00)	1.54 (1.19)
	Median	0.48	0.58	1.11	1.25
Athens					
Winter	*n*	111	111	111	111
	Mean (SD)	0.52 (0.44)	0.58 (0.44)	1.10 (0.78)	1.25 (1.05)
	Median	0.36	0.48	0.90	0.94
Halkida					
Winter	*n*	77	77	77	77
	Mean (SD)	0.72 (0.64)	0.68 (0.46)	1.39 (1.02)	1.54 (1.19)
	Median	0.45	0.61	1.24	1.25
Athens					
Summer	*n*	114	114	114	
	Mean (SD)	0.42 (0.29)	0.49 (0.30)	0.92 (0.56)	
	Median	0.36	0.44	0.81	
Halkida					
Summer	*n*	73	73	73	
	Mean (SD)	0.56 (0.39)	0.74 (0.67)	1.30 (0.99)	
	Median	0.48	0.54	0.98	
Athens					
First year	*n*	111	111	111	
	Mean (SD)	0.48 (0.32)	0.61 (0.35)	1.09 (0.62)	
	Median	0.42	0.53	0.97	
Halkida					
First year	*n*	75	75	75	
	Mean (SD)	0.56 (0.29)	0.77 (0.54)	1.33 (0.74)	
	Median	0.48	0.65	1.20	
Athens					
Second year	*n*	114	114	114	114
	Mean (SD)	0.47 (0.42)	0.46 (0.39)	0.93 (0.73)	1.07 (1.04)
	Median	0.33	0.33	0.66	0.73
Halkida					
Second year	*n*	75	75	75	75
	Mean (SD)	0.72 (0.70)	0.64 (0.60)	1.36 (1.22)	1.51 (1.37)
	Median	0.46	0.48	0.99	1.05

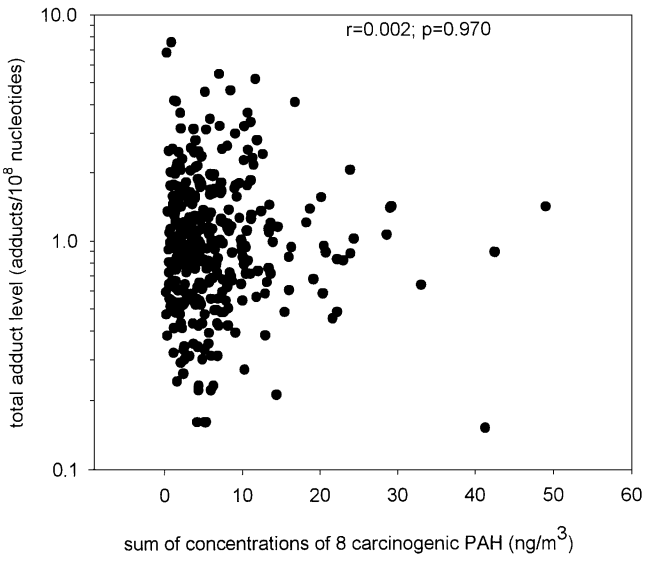

Fig. 2. Relationship between DNA adduct levels and personal exposure to the sum of eight carcinogenic PAH: B[*a*]A, CHRYS, B[*b*]F, B[*k*]F, B[a]P, benzo[*a*,*h*]anthracene, BPer and IND.

correlations for declared ETS exposure, $PM_{2.5}$ and total PAH exposure, as well as for most individual PAH (e.g. B[*a*]P and BPer) exposures and the BPer/B[*a*]P ratio (a characteristic fingerprint of exposure to traffic; 16) (Table III). This indicates that for this cohort only each individual tended to maintain a consistent behaviour pattern determining exposure and DNA adduct levels over the period covered between the two monitoring seasons (~6 months). Plasma cotinine levels showed significant correlations between the two monitoring seasons for all three sub-groups of subjects (Athens, Halkida minus Halkida campus area and Halkida campus area) (Table III), while no correlations were observed for CHRYS exposure or the CHRYS/BPer ratio (a marker of ETS exposure; 16).

Effects of location and ETS exposure

Comparisons of DNA adduct levels in subjects in Athens and Halkida (excluding the campus area) and subjects living in the Halkida campus area show a highly significant trend in the order Athens < Halkida < Halkida campus area ($P < 0.001$). For adducts A and B individually (data not shown) as well as the total adduct level (A+B+C) the trend was consistently observed during all four sampling seasons (Figure 3). The variation parallels the highly significant trend ($P < 0.001$) observed for three markers of exposure to ETS, i.e. declared

Table II. Linear trend of DNA adduct levels detected by the nuclease P1 method across quartiles of personal exposure to CHRYS

CHRYS exposure quartile	*n*	Geometric mean (A)	Geometric mean (B)	Total geometric mean (A+B+C)
Athens, both seasons				
1[a]	49	0.37	0.40	0.82
2	52	0.49	0.49	1.07
3	51	0.36	0.44	0.84
4	53	0.33	0.40	0.77
P[b]		0.091	0.638	0.245
Halkida, both seasons				
1	34	0.42	0.46	0.94
2	36	0.50	0.51	1.10
3	36	0.47	0.65	1.16
4	33	0.65	0.65	1.42
P		0.033	0.018	0.019
Athens, winter				
<0.33 ng/m^3	24	0.34	0.38	0.81
0.33–0.62	25	0.56	0.53	1.30
0.620–1.09	26	0.35	0.43	0.85
>1.09	27	0.35	0.41	0.88
P		0.660	0.986	0.712
Halkida, winter				
<0.48 ng/m^3	18	0.45	0.40	0.95
0.48–0.90	19	0.46	0.45	1.05
0.90–1.30	19	0.51	0.73	1.30
>1.30	18	0.68	0.63	1.51
P		0.127	0.025	0.055
Athens, summer				
<0.14 ng/m^3	25	0.40	0.42	0.83
0.14–0.21	27	0.43	0.46	0.89
0.210–0.35	25	0.37	0.46	0.84
>0.35	26	0.29	0.37	0.67
P		0.029	0.467	0.129
Halkida, summer				
<0.06 ng/m^3	16	0.38	0.53	0.93
0.06–0.13	17	0.56	0.59	1.17
0.13–0.21	17	0.42	0.57	1.03
>0.21	15	0.60	0.69	1.32
P		0.130	0.326	0.193

Levels expressed as adducts per 10^8 nt.
[a]Classification from 1 to 4 in the pooled summer and winter samples was based on the values in the winter quartile values for winter samples and in the summer quartile values for summer samples.
[b]*P*, significance by linear regression analysis.

time of exposure during the 24 h prior to blood donation, relative abundance of CHRYS in the PAH exposure profile (expressed as the CHRYS/BPer ratio) and plasma cotinine concentrations (Figure 3A–C), discussed in detail in Georgiadis *et al.* (16). The parallel variation in ETS exposure and DNA adducts between the above three locations suggests that ETS exposure may have been a significant determinant of DNA adduct levels. In order to examine this possibility further, the relationship between DNA adduct levels and parameters of ETS exposure at the level of individual subjects was examined. When subjects from all three locations (Athens, Halkida and Halkida campus area) were pooled, no significant associations between DNA adduct levels and declared ETS exposure during the last 24 h prior to blood donation or with plasma cotinine levels were obtained (Table IV). However, when subjects were divided according to location and season, positive associations were revealed for the winter samples of the sub-group of subjects residing in the Halkida campus area. For this sub-group and season only, a nearly significant linear trend emerged between adduct levels and declared ETS exposure (Figure 4A). A similar trend was also observed in the same sub-group

Table III. Winter versus summer correlations of subject ranking according to adduct levels and exposure parameters in Athens, Halkida (minus campus area) and Halkida campus area residents

Variable	Athens			Halkida (minus campus)			Halkida campus area		
	n	*r*	*P*	*n*	*r*	*P*	*n*	*r*	*P*
Adduct A	108	–0.043	0.659	34	0.060	0.735	39	0.305	0.059
Adduct B	108	0.058	0.549	34	0.245	0.162	39	0.451	0.004
(A+B)[a]	108	0.015	0.876	34	0.212	0.229	39	0.431	0.006
Cotinine	113	0.322	0.001	34	0.324	0.062	39	0.298	0.065
ETS	114	0.133	0.159	36	0.290	0.086	39	0.483	0.002
$PM_{2.5}$	106	0.010	0.916	28	0.207	0.291	33	0.302	0.088
PAH	90	–0.051	0.633	31	–0.094	0.623	32	0.351	0.053
CHRYS	95	–0.134	0.196	31	–0.193	0.308	34	0.179	0.318
B[*a*]P	103	–0.038	0.705	33	–0.117	0.522	37	0.304	0.072
BPer	103	–0.025	0.802	33	–0.008	0.967	37	0.433	0.008
BPer/B[*a*]P	102	0.170	0.087	33	–0.211	0.239	37	0.310	0.062
CHRYS/BPer	95	–0.074	0.481	31	–0.080	0.669	34	0.012	0.945

[a]Adduct C was not included in the total adduct level because this adduct was undetectable in all summer samples.

and season between adduct levels and CHRYS/BPer ratio (a marker for ETS exposure; 16) (Figure 4B). For the same sub-group, significant associations were observed between adduct levels and exposure to CHRYS (Figure 4C) and B[*a*]P (data not shown), which seemed to be better than those observed for the other cohorts. In contrast, no association with plasma cotinine levels was observed even for this sub-group and season (results not shown).

Effects of gender, lifestyle, dietary habits and urban air pollution

An excess of all DNA adducts was observed in males as compared with females, which was significant for the Halkida cohort (Table V). The gender effect remained significant even after controlling for body mass index and declared ETS exposure (data not shown).

The adduct data were further analysed using information obtained from the personal history questionnaire. Univariate analysis did not reveal any statistically significant effects of location of residence (rural, suburban or urban), its distance from streets with heavy traffic or type of indoor heating. Similarly, no effect was observed for most of the parameters obtained from the TLAD or the 24 h recall questionnaire, including time spent indoors or outdoors, walking or biking (data not shown). Likewise, cooking during the last 24 h did not have any effect on adduct levels. However, a significant increase in the levels of DNA adducts was observed in individuals who had been engaged in physical exercise indoors or outdoors during the previous 24 h. Furthermore, recent (previous 24 h) or habitual consumption of barbecued or grilled meat (meat, fish or poultry) seems to almost significantly enhance the levels of adduct A (Table VI). No correlation was observed between adduct levels and recent (last 24 h) or habitual consumption of fruits and vegetables.

Because ETS exposure seemed to be an important determinant of adduct levels, which might have confounded the effects of ambient air pollution, the above-mentioned analyses were also performed only for the group of students that fulfilled the following criteria: (i) no ETS exposure by declaration and (ii) plasma cotinine levels <1.5 μg/ml. For these samples (93 out

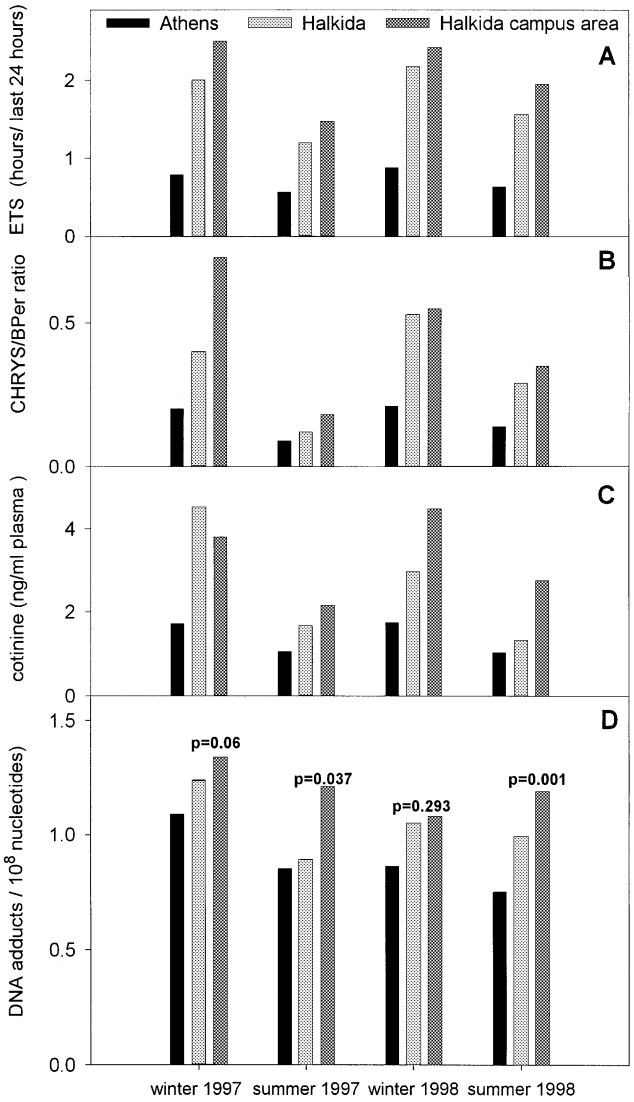

Fig. 3. Comparison of (**A**) ETS exposure during the last 24 h, (**B**) CHRYS/BPer ratio, (**C**) cotinine in plasma and (**D**) total adduct levels (A+B+C) at the three locations (Athens, Halkida and Halkida campus area) and during the four phases of the study (winter 1997, summer 1997, winter 1998 and summer 1998). *P*, significance for linear trend.

Table IV. Linear regression analysis (for all samples) of DNA adduct levels with cotinine levels in plasma or declared hours of ETS exposure during the last 24 h before blood donation

	Cotinine		ETS	
	r	*P*	*r*	*P*
Adduct A	–0.010	0.394	0.026	0.622
Adduct B	–0.005	0.645	–0.025	0.627
Total (A+B+C)	–0.010	0.428	–0.007	0.900

of the total of 388 samples) the correlation between adduct levels and habitual consumption of grilled meat was strengthened (Table VII) relative to that obtained for the pooled samples (Table VI). No other significant differences in the correlations between adduct levels and lifestyle parameters were apparent between the two groups (total and ETS unexposed) (data not shown).

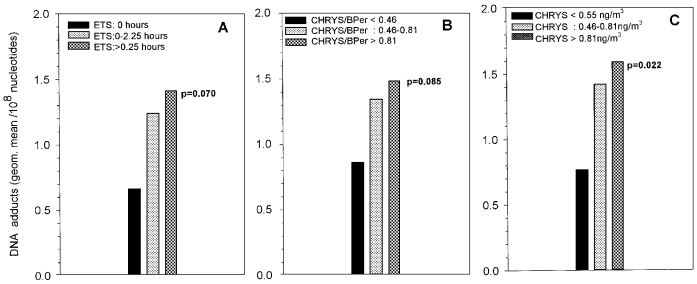

Fig. 4. Correlation of the winter total DNA adduct levels (A+B+C) observed in individuals living in the Halkida campus area with (**A**) tertiles of declared ETS exposure during the last 24 h, (**B**) tertiles of CHRYS/BPer ratios and (**C**) tertiles of CHRYS concentration.

Table V. Effect of gender on adduct levels

	Gender	*n*	Geometric mean (A)	Geometric mean (B)	Total geometric mean (A+B+C)
Total	Male	112	0.52	0.57	1.08
	Female	263	0.39	0.44	0.89
	P		<0.001	0.001	<0.001
Athens	Male	38	0.41	0.47	0.94
	Female	187	0.38	0.43	0.86
	P		0.408	0.384	0.431
Halkida	Male	74	0.59	0.63	1.30
	Female	76	0.41	0.48	0.96
	P		0.003	0.022	0.007

Levels expressed as adducts per 10^8 nt. *P*, significance from Student's *t*-test on natural log transformed values.

In a multiple linear regression model, where location, gender and recent exercise were included, the former two variables were highly statistical significant determinants of adduct levels, while the effect of exercise remained significant only for adduct A (Table VII).

Discussion

Many molecular epidemiology studies have examined the genotoxic effects of human exposure to airborne PAH, bulky DNA adducts being the most widely used biomarker of exposure. High levels of such adducts have been detected in white blood cells of individuals occupationally exposed to high levels of PAH (reviewed in ref. 12). The AULIS project has focused on the examination of genetic damage that relatively low levels of PAH, such as those found in the urban air of Western European countries, may cause. The 24 h median personal exposures to particulate-bound PAH of the two cohorts examined within this study were 7.81 ng/m^3 for Athens and 6.24 ng/m^3 for Halkida during the winter and 4.47 ng/m^3 for Athens and 1.62 ng/m^3 for Halkida during the summer (16), levels which are among the lowest encountered in comparable published biomarker field studies.

^{32}P-post-labelling of lymphocyte DNA using either the nuclease P1 enrichment or the butanol extraction method consistently resulted in a picture characterized by the presence of only two spots (with the exception of a few samples which showed a third spot) and by the absence of a diagonal radioactive zone (Figure 1). Attempts were made to identify the nature of adducts A and B observed by the nuclease P1 method. However, neither of these adducts co-migrated with the main B[*a*]P diol epoxide-derived DNA adduct or with the

Table VI. Effects of activities and diet during the last 24 h and habitual diet on adduct levels

Parameter	Level	*n*	Geometric mean (A)	*P*	Geometric mean (B)	*P*	Total geometric mean	*P*
Activity during the last 24 h								
Exercise	No	307	0.40	0.004	0.47	0.117	0.92	0.016
	Yes	68	0.52		0.54		1.18	
Diet during the last 24 h								
Grilled meat	No	272	0.40	0.010	0.48	0.775	0.93	0.139
	Once	97	0.47		0.43		1.04	
	Twice	6	0.68		0.62		1.37	
Fruits or vegetables	No	91	0.45	0.973	0.47	0.007	1.02	0.173
	Once	147	0.39		0.42		0.86	
	More than once	137	0.44		0.57		1.05	
Habitual diet								
Grilled meat	Almost never	126	0.38	0.074	0.46	0.207	0.88	0.126
	Rarely	142	0.44		0.48	0.99		
	Often	67	0.44	0.49		0.98		
	Almost every day	39	0.46	0.53		1.11		
Fruits or vegetables	Almost never	29	0.46	0.228	0.47	0.504	1.05	0.322
	Rarely	107	0.46	0.47	1.05			
	Often	70	0.39		0.44	0.86		
	Almost every day	169	0.41	0.47	0.95			

Levels expressed as adducts per 10^8 nt. *P*, significance by Student's *t*-test on natural log transformed values (exercise) or by linear regression analysis.

Table VII. Effect of habitual eating of grilled meat on adduct levels in samples from individuals not exposed to ETS (plasma cotinine levels <1.5 μg/ml and ETS by declaration = 0).

Parameter	Level	*n* (93)	Geometric mean (A)	*P*	Geometric mean (B)	*P*	Total geometric mean	*P*
Grilled meat	Almost never	35	0.35	0.053	0.48	0.139	0.88	0.088
	Rarely	33	0.41		0.47		0.94	
	Often	16	0.50		0.57		1.15	
	Almost every day	9	0.50		0.68		1.21	

Levels expressed as adducts per 10^8 nt. *P*, significance by linear regression analysis.

DNA adducts generated by other carcinogenic PAH (22). ^{32}P-post-labelling analysis of DNA from individuals exposed to high levels of airborne genotoxins (smokers and individuals living in highly polluted regions) usually gives a more complex picture, consisting of multiple spots and a characteristic diagonal radioactive zone (23). The much simpler picture, and the low adduct frequency (<1 adduct/10^8 nt), observed in the current study are compatible with a population suffering a relatively low genotoxic burden, in agreement with the low levels of personal PAH exposure observed. A somewhat similar autoradiographic pattern was reported for subjects in Genoa with personal PAH exposures and DNA adduct levels comparable with those observed in the current study (15). Randerath *et al*. (24) have also reported a reproducible pattern of three ^{32}P-labelled spots in DNA samples obtained from human brain, two of which showed a positive correlation with age (I-spots).

While the qualitative picture of DNA adducts was highly conserved among most of the samples examined regardless of location and season, there was substantial (>100-fold) inter-individual variation in adduct levels. No statistically significant correlation was observed between adduct levels and personal exposures to $PM_{2.5}$ or total PAH, either for all subjects together (Figure 2) or for subjects divided according to location or season. Furthermore, significantly higher mean levels were consistently observed for all adducts in Halkida subjects (Table I), even though mean personal exposures to PAH were significantly lower than those of Athens subjects, suggesting that the observed bulky adducts were not PAH–DNA adducts. Positive associations between PAH exposure and PAH-specific or bulky DNA adducts have been reported in individuals exposed to high levels of PAH resulting from ambient air pollution (13,14,25). Those studies involved populations in Silesia, Poland and Teplice, and the Czech Republic, who were exposed to PAH concentrations significantly higher than those of the present study. On the other hand, studies conducted on populations with lower exposure levels have given contradictory results. For example, while higher DNA adduct levels were observed in bus drivers working in the central part of Copenhagen than in a similar group driving in suburban areas (20,26), DNA adducts were not elevated in bus drivers of central Stockholm as compared with a less exposed control group (27) or in street vendors in Milan, Italy, working in areas of high versus low traffic density (28). In the previously mentioned study in Genoa (15) higher adduct levels were observed in police officers than in less exposed office controls, but this difference was seen only during the summer and there was no significant correlation with personal PAH exposures. These results, in agreement with ours, suggest that in non-smoking populations exposed to relatively low levels of ambient air pollution the contribution of airborne PAH to genotoxic damage may not be readily detectable (by ^{32}P-post-labelling methods) above the background damage caused by other genotoxic exposures of exogenous or endogenous origin.

While no correlations between DNA adduct levels and total

PAH exposures were found in the present study, evidence of associations with exposure to individual PAH compounds was found specifically among Halkida subjects. For example, when subjects were stratified according to their exposure to CHRYS, a PAH which is abundant in sidestream tobacco smoke (29), a dose–response relationship with adduct levels emerged only in Halkida, and only during the winter (Table II). A similar correlation was observed with the levels of other PAH such as B[*a*]A and B[*a*]P. Such a correlation was not, however, observed with BPer, a PAH which is considered as a marker of automobile exhaust emissions (30). Interestingly, these mentioned correlations were mainly localized in a sub-group of students living in the Halkida campus area, located in a relatively isolated and non-industrialized region (see below).

Halkida was initially selected as a location anticipated to have atmospheric PAH pollution qualitatively similar to, but quantitatively lower than, Athens, in order to broaden the range of exposures to be investigated. However, although total PAH exposures in Halkida did tend to be significantly lower, the PAH exposure profile of Halkida subjects showed a consistent deviation in detail from that of Athens subjects. Characteristically, Halkida subjects, while having lower mean exposures to total as well as most individual PAH than Athens subjects during the winter, had higher relative and absolute exposures to lighter PAH such as CHRYS (16). This observation, which indicates that different PAH sources made different contributions to personal exposure at the two locations, along with the observation of higher adduct levels in Halkida, might suggest a possible causal link between particular PAH sources and DNA adduct levels. Furthermore, the observation of correlations between adduct levels and CHRYS only in Halkida would suggest that in Athens the contribution of the responsible source(s) was too small to allow its characteristic markers (e.g. CHRYS content) to be discernible from the background of PAH emissions arising from other sources. It also suggests that if the observed association is a causal one, the DNA damage does not arise directly from the specific PAH with which it correlates but rather that the latter act as a marker for other genotoxins emitted by the responsible source.

In a previous paper (16) the PAH profile differences between Athens and Halkida were shown to be related to the higher exposure to ETS among Halkida subjects. In view of the observed associations of the Halkida-type PAH profile with DNA damage, reported in this paper, it seems possible that this damage could be related to ETS exposure. This possibility is strengthened by a series of observations made in a sub-cohort of subjects living in the Halkida Institute campus area. This site is located 12 km from the town, in a rural environment with a low traffic burden and no industrial activity. For subjects belonging to this sub-cohort the following observations were made.

i. While having relatively low $PM_{2.5}$ and total PAH exposures, they exhibited the highest exposure to ETS (according to the trend campus area > rest of Halkida > Athens), as indicated by their declared time in the presence of smokers, PAH profiles and plasma cotinine concentrations (Figure 3A–C).

ii. In contrast to the other sub-cohorts, they exhibited strong inter-individual (winter versus summer) correlations for all parameters related to short-term exposure to $PM_{2.5}$, PAH and ETS (Table III). This means that, for reasons related to living environment and lifestyle, their relative individual exposures to these agents were less varied than for the other sub-cohorts. It also suggests that the available short-term exposure measurements from this sub-cohort reflected long-term exposure (over the period of ~6 months between the winter and summer samplings) better than for the remaining cohorts. In view of the above, it may seem surprising that winter and summer values for both CHRYS and CHRYS/BPer were not correlated in any of the three cohorts, including the campus area cohort. However, this can be explained by the altered distribution of CHRYS between the gaseous and particulate phases during the summer (31), which greatly reduces particulate-bound CHRYS during that season to levels often just above the limit of detection (range 0.02–0.42 ng/m^3).

iii. The same trends that were observed regarding exposure (especially ETS exposure) were also seen for DNA adducts. DNA damage levels varied according to the trend campus area > rest of Halkida > Athens consistently over all four observation periods (Figure 3D) and a strong inter-individual (winter versus summer) correlation of DNA adduct levels was observed for the campus area sub-cohort (Table III).

iv. For the campus area sub-cohort the winter DNA adduct levels showed nearly significant associations with the declared time of ETS exposure (Figure 4A) and with the CHRYS/BPer ratio (a marker of ETS exposure) (Figure 4B). In addition, the correlation of absolute CHRYS or B[*a*]P exposure levels was stronger within the Halkida campus area sub-group than that observed for the rest of the Halkida subjects (Figure 4C).

While not constituting conclusive evidence for a role of ETS in the induction of the observed DNA adducts, these observations are fully consistent with the hypothesis that chronic exposure to ETS was a major determinant of such damage. The absence of a correlation between ETS exposure and DNA adduct levels among the other cohorts would be attributed to the inadequacy of the available exposure measurements to reflect long-term exposure to ETS.

The absence of a correlation of DNA adduct levels with ETS exposure during the summer period may be explained by a number of factors.

i. During the winter season more time is spent indoors, allowing the declared time spent in the presence of smokers to reflect more accurately actual exposure to ETS. This is supported by the observation of higher correlations between declared ETS exposure and plasma cotinine concentrations and CHRYS/BPer ratios during the winter (16).

ii. High temperatures and solar radiation during the summer can result in selective photodegradation and chemical modification of the more reactive PAH, including B[*a*]P. In addition, high temperatures may alter the particulate/gaseous phase distribution of the more volatile PAH such as CHRYS (31), thus resulting in a reduction in their particulate-bound concentration without a true reduction in their total concentrations.

The possibility that other lifestyle parameters common to subjects living in the campus area (e.g. food obtained from the local refectory) might have contributed to the effects specifically observed among them was examined, but no supporting evidence was found using data obtained from their dietary questionnaire or the TLAD.

Despite the evidence for an ETS–DNA damage link, no correlation of DNA adduct levels with plasma cotinine concentrations was found, even among subjects in the Halkida campus area cohort. However, the degree to which cotinine is a quantitative or semi-quantitative indicator of ETS exposure, particularly at low levels of exposure, has not yet been substantiated (32–36) and the lack of correlation of plasma

cotinine with adduct levels may reflect inter-individual differences in nicotine metabolism.

Most studies on human populations have shown a positive correlation between active smoking and DNA adduct levels (detected by ^{32}P-post-labelling methods) in organs like lungs, placenta and lymphocytes (37–43), while the association was not always consistent for white blood cell DNA (44–46). On the other hand, few studies dealing with the effects of ETS on biomarkers of genotoxicity of PAH have been reported. In a study of mothers and newborns the levels of maternal white blood cell PAH–DNA adducts were reported to be higher in subjects declaring exposure to ETS as compared with those reporting no exposure (47). However, no quantitative association was seen between adduct levels and the self-reported number of cigarettes/day of passive smoking, while no association was observed between white blood cell PAH–DNA adducts of newborns and maternal ETS exposure. ETS exposure was reported to increase the B[*a*]P–albumin adducts in one study (48) but not in others (49–51). Finally, in a recent study in young children it was found that ETS was associated with increased 4-aminobiphenyl– and PAH–albumin adducts as well as sister chromatid exchange frequency (52).

Adduct levels tended to be higher in the winter than in the summer, a trend parallel to the strong seasonal variation in $PM_{2.5}$ and PAH exposures (Table I). However, this trend was neither consistent nor statistically significant. The absence of a significant seasonal variation in DNA adduct levels observed in this study is in contrast to the substantial variation (higher levels observed in the winter) previously reported for residents of Silesia, Poland, who were exposed to very high levels of ambient air pollution. Our observation also differs from those of the Genoa study mentioned above (15), in which higher adduct levels were observed in police officers during the summer, even though PAH exposures were lower during this season, an effect that was attributed to possible seasonal variation in aryl hydrocarbon hydroxylase inducibility or to photochemical reactions of PAH in the atmosphere during the summer.

Lifestyle, dietary habits and urban pollution

Females had lower adduct levels than males, the difference being more significant among Halkida subjects (Table V). This gender effect was not related to height, weight or body mass index differences. It remained significant even after controlling for declared ETS exposure and therefore seems likely to reflect other behavioural or intrinsic differences between the two sexes. An opposite gender effect (females having higher adducts than males) has been observed in lung tissue of smokers (53–54). It has to be noted that the gender-related I-compounds are believed to represent DNA damage of endogenous origin (55).

Examination of the data in the 24 h recall questionnaire and the TLAD shows that residence location (urban, suburban or rural), traffic density and habitual or previous 4 days walking or biking had no effect on adduct levels, even for the subgroup of individuals that declared no ETS exposure during the last 24 h before blood donation. However, the time spent exercising indoors or outdoors during the last 24 h before sample collection seems to have an enhancing effect on adduct levels (Table VI). This association remained statistically significant even after controlling for area and gender. Although there is no previous report of an association of physical exercise with the induction of bulky DNA adducts, increased amounts of DNA strand breaks following physical exercise, possibly related to oxidative stress, have been previously demonstrated (56). Poulsen *et al*. (57) have also reported increased 8-hydroxyguanine levels after intense exercise. It is interesting to note that the levels of type II I-compounds have also been shown to be associated with oxidative stress (56).

In addition to inhalation of polluted air, diet is an important route of genotoxins, and PAH intake in particular, which, under some circumstances, can make a substantial contribution to overall PAH exposure. While no effects on DNA adduct levels were found for habitual or recent consumption of fruits and vegetables or fried meat (meat, fish or poultry), habitual or recent consumption of barbecued or grilled meat was associated with an increase in DNA adduct levels to an almost significant degree (Table VI). Among individuals not exposed to ETS, habitual consumption of grilled meat seemed to have an even greater effect on the adduct levels (Table VII) than that observed for the pooled samples. In a previous study DNA adducts in fire fighters were related to consumption of charbroiled food (58). In another investigation volunteers consuming 280 g charbroiled beef for 7 days showed elevated DNA adduct levels (59), although studies dealing with intra-individual variations in PAH–DNA adducts and consumption of grilled meat are limited and negative results also exist (51). It has to be recognized that diet might be an important confounder of PAH exposure, particularly in non-occupationally exposed populations suffering moderate to low urban pollution.

The effects of gender and exercise imply the possibility that DNA adducts observed in the current study are related to I-like compounds of endogenous origin. On the other hand, the evidence obtained strongly suggests a role for ETS exposure. In this context it is notable that Gupta *et al*. (60) reported that mainstream and sidestream tobacco smoke can enhance pre-existing DNA adducts (I-compounds) in the lungs of rodents, possibly as a response to oxidative stress, thus suggesting a way whereby tobacco smoke exposure may modulate endogenous DNA damage levels.

In conclusion, we have found that the levels of bulky DNA damage detected by ^{32}P-post-labelling in lymphocytes of non-smoking subjects living in urban areas and suffering moderate to low levels of personal exposure to particulate-bound PAH (i) consisted of a discreet number (2–3) of adducts present at levels below 1 per 10^8 nt, (ii) were higher in males than in females, (iii) did not show a significant group- or individual-specific correlation with exposure to ambient air PAH pollution and (iv) showed a correlation with the extent of recent exposure to ETS only within a specific cohort living in an area with the lowest ambient pollution burden.

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