Dose-Response Modeling of Occupational Exposure to Polycyclic Aromatic Hydrocarbons with Biomarkers of Exposure and Effect

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Abstract

In regulatory toxicology, the dose-response relationship between occupational exposure and biomarkers is of importance in setting threshold values. We analyzed the relationships between occupational exposure to polycyclic aromatic hydrocarbons (PAH) and various biomarkers of internal exposure and DNA damage with data from 284 highly exposed male workers. Personal exposure to phenanthrene and other PAHs was measured during shift and correlated with the sum of 1−, 2+9−, 3−, and 4-hydroxyphenanthrenes in post-shift urine. PAHs and hydroxyphenanthrenes were associated with DNA damage assessed in WBC as 8-oxo-7,8-dihydro-2′-deoxyguanosine/10^6 dGuo and strand breaks by Comet assay as Olive tail moment. Hydroxyphenanthrenes correlated with phenanthrene (Spearman $r_s = 0.70$; $P < 0.0001$). No correlations could be found between strand breaks and exposure (r_s = 0.01, $P < 0.0001$ for PAHs; $r_s = -0.03$, $P = 0.68$ for hydroxyphenanthrenes). Correlations with 8-oxo-7,8-dihydro-2′-deoxyguanosine/10^6 dGuo were weakly negative ($r_s = -0.22$, $P = 0.004$ for PAHs) or flat ($r_s = -0.08$, $P = 0.31$ for hydroxyphenanthrenes). Linear splines were applied to assess the relationships between the log-transformed variables. All regression models were adjusted for smoking and type of industry. For hydroxyphenanthrenes, 51.7% of the variance could be explained by phenanthrene and other predictors. Up to 0.77 μg/m^3 phenanthrene, no association could be found with hydroxyphenanthrenes. Above that point, hydroxyphenanthrenes increased by a factor of 1.47 under a doubling of phenanthrene exposure (slope, 0.56; 95% confidence interval, 0.47-0.64). Hydroxyphenanthrenes may be recommended as biomarker of occupational PAH exposure, whereas biomarkers of DNA damage in blood did not show a dose-response relation to PAH exposure. (Cancer Epidemiol Biomarkers Prev 2007;16(9):1863–73)

Introduction

Polycyclic aromatic hydrocarbons (PAH) are formed during incomplete combustion and constitute a mixture of various compounds of different carcinogenic potency. Their chemistry and formation have been reviewed by the IARC (1). Several marker compounds have been selected for air monitoring in environmental and occupational settings and risk assessment (2). Among these, phenanthrene is one of the most abundant compounds, and benzo(*a*)pyrene [B(a)P] is a historical marker compound for carcinogenic PAHs. Human biomonitoring of internal levels of PAH exposure has become integral part of large population surveys and of studies in occupational epidemiology. Only a few biomarkers of exposure have been established. Monohydroxylated PAHs, mostly 1-hydroxypyrene (1-OHP) but also various hydroxyphenanthrenes, have been studied as biomarkers of detoxification pathways in urine (3). Both parent compounds of these metabolites, pyrene and phenanthrene, are not considered as carcinogenic PAH compounds. In human biomonitoring, urinary metabolites of carcinogenic PAHs have not yet been established as common markers of exposure. PAH compounds require metabolic activation to exert their carcinogenicity (4). Phenanthrene but not pyrene contains a bay region and might thus serve as surrogate for

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the bay-region diol epoxide pathway (5). More recently, urinary biomarkers of that metabolic activation pathway of phenanthrene have been proposed for human biomonitoring (5, 6).

The carcinogenic effects of activated PAHs have been attributed to DNA adduct formation (7, 8). Occupational exposure to PAHs has been associated with an increased risk of lung cancer (9, 10). Human biomonitoring determines biomarkers of DNA damage usually in WBC as proxy to characterize high-risk populations because the lungs are hardly accessible. Some noninvasive methods like inducing sputum allow the collection of epithelial cells from the airways but protocols to use these cells in the Comet assay or other methods are still to be established. In a review of biomonitoring studies, associations between exposure to PAH and various adducts could be shown but usually only at group level with a wide variation between methods and laboratories (11).

Analyses at group level do not provide information about the strength and shape of the functional relation between exposure and biomarker. Regulatory toxicology needs suitable statistical modeling of the dose-response function at the individual level. The fraction of variance explained by exposure and the slope or deviations from linearity are important features of these associations. Both dose and response variables should be determined with sufficient precision. Measurements below or near the limit of quantitation or visual data that a prone to observer bias can increase the variance and may thus mask an underlying dose-response function.

To characterize the associations between airborne occupational exposure to PAHs and biomarkers of internal exposure and DNA exposure, a cross-sectional study was conducted in occupational settings with high PAH exposure (12, 13). We analyzed the dose-response relationships between external and internal PAH exposure and between internal exposure and DNA damage.

Materials and Methods

Study Design. A cross-sectional study was conducted in Germany between March 1999 and December 2003 in different occupational settings. The study population comprised 284 men occupationally exposed to PAHs during manufacture of refractory products (n = 99) and graphite electrodes (n = 92), coke oven works (n = 63), tar distillation (n = 18), and feeding converters during steel production (n = 12). Each worker was equipped with a personal air sampler during the shift. After the shift, the workers provided a spot urine sample to measure the internal dose and two blood samples to assess oxidative DNA damage and DNA single- and double-strand breaks. Data of biomarkers of DNA damage were available for 177 [for 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo)] and 174 workers [for Olive tail moment (OTM)], respectively. A structured questionnaire was applied in a face-to-face interview to assess demographic characteristics and smoking habits. All study subjects provided a written informed consent before investigation. The study was approved by the ethics commission of the Ruhr-Universität Bochum and conducted in accordance with the definitions of the declaration of Helsinki.

Assessment of Occupational Exposure to Airborne PAHs. Personal air sampling was conducted in the worker's breathing zone on average for 2 h to assess airborne exposure to PAHs for a working shift. Samples were collected with battery-operated personal air sampling pumps at a sampling rate of 2 L/min and using a filter and a sorbent tube to trap particle-bound and volatile PAHs. Sixteen U.S. EPA PAHs were analyzed according to method 5506 published by the U.S. National Institute for Occupational Safety and Health (14). The limits of quantification (LOQ) ranged between 0.007 to 0.51 $\mu g/m^3$ for the different PAHs.

Analytic Methods for Urinary Metabolites. Spot urine samples were collected at the end of the work shift in polypropylene tubes, coded, and frozen at −20°C until analysis. All analyses were carried out in a blinded fashion and by using standardized and quality-controlled analytic procedures. The determination of 1-OHP and the sum of 1-, 2+9-, 3-, and 4-hydroxyphenanthrenes was carried out by two-dimensional high-performance liquid chromatography and fluorescence detection, described in detail elsewhere (12, 15). Briefly, after enzymatic hydrolysis, the metabolites were enriched on a precolumn consisting of copper phthalocyanine-modified silica gel, separated on a RP-C18 column, and quantified by fluorescence detection. The LOQs were in the range between 24 and 96 ng/L depending on the metabolites. Urinary o-cotinine was determined by gas chromatography with nitrogen-specific detection after a liquid/liquid extraction of the urine samples, according to a procedure described previously (16). Urinary creatinine (crn) was determined photometrically according to the Jaffé method (17). The concentration of hydroxyphenanthrenes was corrected for crn. For quality control, hydroxyphenanthrenes and 1-OHP were determined in a subset of samples in two laboratories.

Blood Collection and Determination of Genotoxic Biomarkers. 8-oxodGuo was analyzed in WBC to determine oxidative DNA exposure. Whole blood samples (9 mL) were collected by venipuncture in tubes containing EDTA as an anticoagulant. The tubes were immediately frozen at −80°C. DNA from WBC was isolated within 2 days and frozen at −80°C. DNA extraction and 8-oxodGuo determination were carried out using a previously published procedure (12, 18) with slight modifications (19). DNA and nucleosides were prepared in darkness using argon to minimize oxygen exposure of the buffer solutions. In brief, WBC were collected by centrifugation of blood diluted with 45 mL of a solution with 0.9% NH_4Cl at 215 × g for 20 min at 4°C. The washing was repeated, and the pellets were stored at −80°C until processing. DNA was extracted with chloroform from a mixture of pellets and extraction buffer [1 mol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.5% SDS (pH 7.4)]. After two centrifugation steps at 500 × g for 30 min, DNA was precipitated with cooled ethanol. The extracted DNA was stored at −80°C until processing. The DNA was stepwise digested with 20 μg nuclease P1 (Sigma) for 30 min at 37°C, followed by 20 μL Tris-HCl (pH 7.5), and by 1.2 units alkaline phosphatase (Sigma) for 60 min at 37°C. Nucleosides were separated by centrifugation at 12,000 rpm for 90 min using a Microcon YM-3 filter (Millipore). Nucleosides were detected with a Shimadzu high-performance

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liquid chromatography/UV equipment connected to a Coulochem II (model 5200) electrochemical detector (ESA). Quality was controlled by a standard compound, with calibration curves, and analyses in duplicate.

Alkaline single-cell gel electrophoresis (Comet assay) was carried out to determine DNA single- and double-strand breaks as well as alkaline labile sites. Whole blood samples (5 mL) were collected in a tube containing heparin as an anticoagulant. Lymphocytes were isolated by a standard method of centrifugation on a Ficoll density gradient. A modified protocol of the method by Östling and Johanson (20) and Singh et al. (21) was used as described previously (22). The migration of DNA during electrophoresis was quantified as OTM with arbitrary units. OTM was determined with Komet software version 4.0 (Kinetic Imaging) as the product of the amount of DNA in the tail and the mean distance of migration in the tail (23). Median OTM was calculated from 51 cells per slide, using two different slides prepared from each subject.

Data Analysis. The distributions of the variables were presented with geometric mean, median, and the 5th, 10th, 25th, 75th, 90th, and 95th percentiles. Crude correlations between the variables selected for analyses were estimated with Spearman correlation coefficients (r_s). Statistical modeling of the associations between external and internal dose as well as the dose-response relationships between exposure and effect were carried out for phenanthrene, hydroxyphenanthrenes, 8-oxodGuo, and OTM. Measurements below LOQ were set to half of LOQ. We controlled for potential confounders in the regression models and in the graphical presentation of the individual outcomes. Current smoking was assessed as a binary variable (yes/no) with a urinary *o*-cotinine concentration of 100 μg/L as cutoff. The smoking status of 65 workers could only be obtained from the questionnaire. However, *o*-cotinine levels were in good agreement with the self-assessed smoking status from the questionnaire (concordance, 94.9%) for those persons where both *o*-cotinine levels in urine and questionnaire information were available. Because occupational settings may differ by PAH composition and other exposure circumstances, the type of setting was implemented as categorical variable in all models. Age and ethnicity were not associated with the outcome variables in this study population and were not included in the models. Tests were conducted two sided using a significance level of $\alpha = 5\%$. All calculations were done with SAS/STAT and SAS/IML software, version 9.1 (SAS Institute, Inc.).

For the modeling of dose-response relationships, the study variables were log transformed to achieve a normal distribution. The following model was chosen:

$$y = \mu + x'\beta + X_c\beta_c + \varepsilon \qquad \text{(A)}$$

with y the vector of the log-transformed outcome, x' the vector of the log-transformed exposure variable, X_c the design matrix of the potential confounders, μ the overall mean, β and β_c the regression variables for the exposure variable and the potential confounders, and ε an error term following a normal distribution.

To identify a deviation from linearity in the dose-response relationship between the log-transformed outcome and the log-transformed exposure in Eq. A, linear regression splines were applied by using the truncated power basis of the linear splines (24):

$$y = \mu + x'\beta + \sum_{i=1}^{K}[x' - k_i]_+\beta_i + X_c\beta_c + \varepsilon \qquad \text{(B)}$$

with K the number of knots, k_i the position of the ith knot, $[z]_+$ the truncated power (i.e., $[z]_+ = z$, if $z \geq 0$; else 0), and β_i the regression variable of the ith truncated power. The slope of the regression line is equal to β in the first segment and $\beta + \Sigma_{i=1}^{j-1}\beta_i$ in the jth ($j \geq 2$) segment.

One problem of using splines is the location of the knots. Here, a nonlinear model was fitted with the knots as regression variables. Model (3) turns out to be separable (i.e., for any given knot estimates of the other regression variables can be obtained by usual linear regression; ref. 25). In this approach, the knots are the only nonlinear variables in the model. Hence, the residual sum of squares was calculated as function of the knots only and minimized using the Levenberg-Marquardt algorithm as implemented in SAS/IML. To reduce the possibility of ending in a local minimum, the

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Table 1. Distribution of the study variables and of selected exposure variables in male workers with occupational exposure to PAHs

	n	n < LOQ*	GM	GSD	Median	Percentiles					
						5th	10th	25th	75th	90th	95th
Sum of 16 EPA PAHs (μg/m^3)	284	—	34.7	4.45	30.2	3.23	5.30	13.9	90.6	240	531
Dibenz(*a*,*h*)anthracene (μg/m^3)	284	151	0.07	7.06	0.08	0.01	0.01	0.01	0.28	0.78	1.69
B(a)P (μg/m^3)	284	68	0.38	7.04	0.42	0.02	0.03	0.09	1.44	5.31	11.9
Phenanthrene (μg/m^3)	284	5	4.81	4.88	5.07	0.34	0.61	1.79	13.2	37.2	65.4
1-OHP (μg/g crn)	284	0	5.33	3.68	5.61	0.51	1.07	2.16	12.7	26.8	38.5
Sum of OH-phenanthrenes (μg/g crn)	284	0	9.49	3.19	10.0	1.51	2.11	4.20	21.2	44.4	64.3
	177†	0	12.3	3.07	12.3	2.02	2.53	5.38	30.4	55.2	73.3
8-oxodGuo/10^6 dGuo	177†	0	5.67	1.59	5.70	2.69	3.11	4.34	7.20	10.4	11.7
OTM	174†	0	2.28	1.53	2.27	1.21	1.35	1.70	3.01	3.78	4.77

Abbreviations: GM, geometric mean; GSD, geometric SD; OH-phenanthrenes, hydroxyphenanthrenes, CRN, creatinine; OTM, olive tail moment.
*Observations below LOQ were set to half of LOQ.
†Restricted to observations with available blood samples.

Table 2. Spearman rank correlations of 284 personal measurements of phenanthrene in air during a working shift with other U.S. EPA PAH

	r_s*	$n_{>LOQ}$	$r_{s>LOQ}$†		r_s*	$n_{>LOQ}$	$r_{s>LOQ}$†
Sum of 15 PAHs	0.80	—	—	Benz(*a*)anthracene	0.42	228	0.71
Anthracene	0.95	268	0.88	Benzo(*k*)fluoranthene	0.35	197	0.57
Fluoranthene	0.81	251	0.73	Benzo(*b*)fluoranthene	0.34	210	0.52
Fluorene	0.79	219	0.64	B(a)P	0.34	216	0.65
Pyrene	0.70	236	0.74	Indeno(1,2,3-cd)pyrene	0.28	184	0.51
Acenaphthene	0.54	124	0.51	Dibenz(*a,h*)anthracene	0.24	133	0.30
Naphthalene	0.49	208	0.41	Benzo(*g,h,i*)perylene	0.24	147	0.49
Chrysene	0.46	229	0.61	Acenaphthylene	—‡	44	—‡

*r_s, Spearman rank correlation coefficients; all correlations with $P < 0.0001$.
†$r_{s>LOQ}$, Spearman rank correlation coefficients for values above the LOQ; all correlations with $P < 0.0001$.
‡Not calculated because the majority of measurements were below LOQ.

optimization procedure was done on a grid of initial points. To avoid overfitting, models were considered only if each segment contained at least 10% of the observations between two consecutive knots. As consequence, knot positions could only be determined between the 10th and 90th percentile of the exposure variable. To test whether the inclusion of an additional knot resulted in a better model fit, an F test was applied that compared the residual sum of squares with and without the examined knot and including the other variables. If the consecutive inclusion of two knots did not improve the model fit, the model selection procedure was stopped. The separation of the model and the nonlinear optimization was done with PROC IML and the CALL NLPLM routine.

Results

The analysis of the relationship between external (phenanthrene) and internal exposure (hydroxyphenanthrenes) was carried out with data from 284 workers. The corresponding analysis between PAHs or hydroxyphenanthrenes and genotoxic effects was based on 177 (for 8-oxodGuo) and 174 workers (for OTM), respectively. The distribution of the study variables and selected other exposure variables is depicted in Table 1. Several measurements of carcinogenic compounds, such as B(a)P, were below LOQ, whereas phenanthrene was a prominent PAH compound with only five measurements below LOQ. For all variables, geometric means corresponded well to the median values supporting the log transformation chosen for regression analyses.

Overall, phenanthrene correlated strongly with the other PAH compounds and their sum (r_s = 0.80; $P <$ 0.0001; Table 2). For acenaphthylene, the majority of measurements were below the LOQ; therefore, no correlation coefficient was calculated. The weak correlation of phenanthrene with several individual PAHs, such as B(a)P and dibenz(*a,h*)anthracene, may partly be due to the large number of measurements below the LOQ for those compounds. These correlations became stronger when restricted to data with measurements above LOQ.

Table 3 shows the correlations between various measures of exposure and biomarkers of DNA damage

Table 3. Spearman rank correlations between exposure to PAH compounds and biomarkers of internal exposure and DNA damage in highly PAH-exposed workers

Measure of exposure	Urinary biomarkers of internal exposure					
	1-OHP			OH-phenanthrenes		
	$n_{>LOQ}$	$r_{s>LOQ}$*	P	$n_{>LOQ}$	$r_{s>LOQ}$*	P
Phenanthrene	279	0.49	<0.0001	279	0.70	<0.0001
Pyrene	236	0.45	<0.0001	236	0.32	<0.0001
B(a)P	216	0.24	0.0003	216	0.13	0.06
Sum of 16 PAHs	251	0.34	<0.0001	251	0.60	<0.0001
Measure of exposure	**Biomarkers of DNA damage in WBC**					
	OTM			8-oxodGuo/10^6 dGuo		
	$n_{>LOQ}$	$r_{s>LOQ}$*	P	$n_{>LOQ}$	$r_{s>LOQ}$*	P
Phenanthrene	173	0.00	0.99	176	−0.15	0.049
Pyrene	149	−0.09	0.28	152	−0.06	0.47
B(a)P	127	−0.02	0.86	129	−0.09	0.31
Sum of 16 PAHs	174	0.00	0.97	177	−0.20	0.008
OH-phenanthrenes	174	−0.03	0.68	177	−0.08	0.31
1-OHP	174	−0.12	0.11	177	0.06	0.42

*$r_{s>LOQ}$, Spearman rank correlation coefficients for values above the LOQ.

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Table 4. Model selection procedure with linear splines of the association between log-transformed variables of airborne phenanthrene and the sum of 1-, 2+9-, 3- and 4-hydroxyphenanthrenes in spot urine samples of 284 workers exposed to PAH

Model*	Estimate	95% CI	$r^{2\dagger}$ (%)	RSS	Test vs previous model		
					F	df	P
No knots			49.1	196.13	—	277	—
$\beta^{\ddagger}$	0.47§	0.40-0.54					
One knot			51.7	184.30	7.33	275	0.0008
Knot	−0.26	−1.04 to 0.52					
$\beta^{\ddagger}$	−0.06	−0.45 to 0.33					
$\beta + \beta_1^{\parallel}$	0.56§	0.47-0.64					
Two knots			52.0	183.33	0.73	273	0.48
Three knots			52.6	180.98	1.76	271	0.17

Abbreviations: RSS, residual sum of square; df, degrees of freedom of the denominator of the F test.
*Adjusted for type of industry and current smoking.
†Coefficient of determination.
‡Slope of regression line in the first segment.
§$P < 0.0001$.
‖Slope of regression line in the second segment.

in blood. Urinary hydroxyphenanthrenes correlated strongly with airborne phenanthrene ($r_s = 0.70$; $P < 0.0001$) and the sum of PAHs ($r_s = 0.60$; $P < 0.0001$), whereas 1-OHP correlated less pronounced with PAHs ($r_s = 0.34$; $P < 0.0001$). The correlation of both metabolites with B(a)P was only marginal respectively weak ($r_s = 0.13$, $P = 0.06$ for hydroxyphenanthrenes; $r_s = 0.24$, $P = 0.0003$ for 1-OHP). Various measures of PAH exposure did not correlate with strand breaks assessed with OTM in WBC (e.g., $r_s = 0.00$ for phenanthrene as well as for hydroxyphenanthrenes). Significant negative correlations were observed between exposure to PAH and DNA adducts assessed with 8-oxodGuo ($r_s = -0.15$, $P = 0.049$ for phenanthrene; $r_s = -0.20$, $P = 0.008$ for PAHs).

To model the shape of the association between the log-transformed variables phenanthrene and hydroxyphenanthrenes, we investigated deviations from overall linearity with linear splines and a successively increasing number of knots. The results of the model selection procedure are given in Table 4. By modeling an overall linear relationship between phenanthrene and hydroxyphenanthrenes with adjustment for type of industry and current smoking, 49.1% of the variance of hydroxyphenanthrenes could be explained (without confounders, 46.6%; data not shown). Phenanthrene showed a highly significant effect on hydroxyphenanthrenes [regression coefficient $\hat{\beta} = 0.47$; 95% confidence interval (95% CI), 0.40-0.54], which represents an increase of hydroxyphenanthrenes by a factor of 1.38 after doubling of the external exposure to phenanthrene. The inclusion of one knot increased the explained variance to 51.7% and resulted in a significant improve of the model fit ($P = 0.0008$). The best-fitted knot was located at −0.26 ln ($\mu g/m^3$ phenanthrene; 95% CI, −1.04 to 0.52) representing 0.77 $\mu g/m^3$ phenanthrene (95% CI, 0.35-1.68 $\mu g/m^3$). The knot corresponded to an internal exposure of ~3 μg/g crn hydroxyphenanthrenes. Thirteen percent of the workers had internal exposure levels below that value. The knot indicates that the dose-response function can be divided into two parts. Up to that knot, no association between phenanthrene and hydroxyphenanthrenes could be found ($\hat{\beta} = -0.06$; 95% CI, −0.45 to 0.33), whereas a significant linear increase between the log-transformed external and internal exposure variables was found above 0.77 $\mu g/m^3$ ($\hat{\beta} + \hat{\beta}_1 = 0.56$; 95% CI, 0.47-0.64; Fig. 1A). Hence, hydroxyphenanthrenes increased by a factor of $2^{0.56} = 1.47$ in that segment after doubling of the external exposure to phenanthrene. The inclusion of two further knots did not improve the model fit significantly ($P = 0.48$ for two knots; $P = 0.17$ for three knots). As a consequence, the model selection procedure was stopped, and the one-knot model turned out as best fit for the given data.

Similarly, Table 5 shows the model selection procedure for the association between the log-transformed variables for internal exposure (hydroxyphenanthrenes) and oxidative DNA exposure (8-oxodGuo) as well as hydroxyphenanthrenes and DNA single- and double-strand breaks (OTM). By analyzing the overall linear relationship of hydroxyphenanthrenes on 8-oxodGuo without confounders, hydroxyphenanthrenes explained only 0.1% of the variance of 8-oxodGuo (data not shown). The inclusion of type of industry and current smoking explained 42.1% of the variance of 8-oxodGuo. An association of hydroxyphenanthrenes with 8-oxodGuo could not be found ($\hat{\beta} = -0.04$; 95% CI, −0.09 to 0.01; Fig. 1B). The inclusion of knots did not improve the model fit significantly ($P = 0.07$ for one versus no knot; $P = 0.38$ for two versus one knot; $P = 0.13$ for two versus no knot). With one and two additional knots, 43.9% and 44.5% of the variance of 8-oxodGuo could be explained, respectively. Hence, the model selection procedure was stopped. Because the inclusion of one knot resulted in a marginally better fit, the estimates were presented in Table 5. No associations were found modeling phenanthrene or PAH instead of hydroxyphenanthrenes (data not shown).

By modeling an overall linear relationship of the log-transformed variables hydroxyphenanthrenes on OTM without adjustment for potential confounders, hydroxyphenanthrenes could not explain the variance of OTM ($r^2 = 0.0\%$). After inclusion of current smoking and type of industry, the model explained 11.5% of the variance of OTM, but no association between hydroxyphenanthrenes and OTM was observed ($\hat{\beta} = 0.01$; 95% CI, −0.05 to 0.07;

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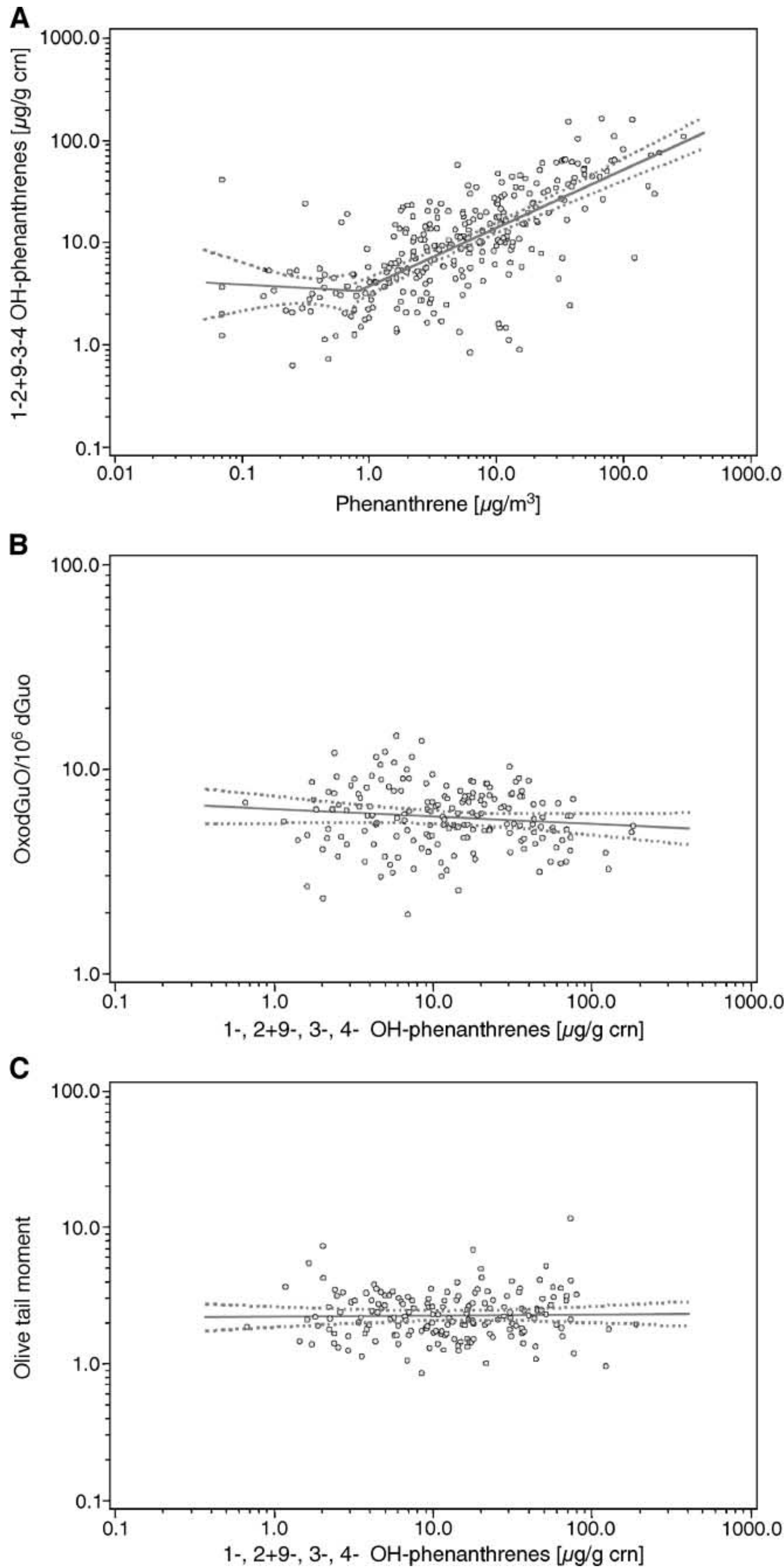

Figure 1. Dose-response curves with 95% confidence bands for exposure and effect variables modeled with linear splines and adjusted for type of industry and current smoking. **A.** Airborne phenanthrene and urinary hydroxyphenanthrenes (*OH-phenanthrenes*). **B.** Urinary hydroxyphenanthrenes and 8-oxodGuo in WBC. **C.** Urinary hydroxyphenanthrenes and strand breaks assessed with OTM in lymphocytes.

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Table 5. Model selection procedure with linear splines of the association between log-transformed variables of internal exposure to PAHs (assessed with the sum of 1-, 2+9-, 3- and 4-hydroxyphenanthrenes in spot urine samples) and DNA exposure (8-oxodGuo/10^6 dGuo in WBC and DNA strand breaks in blood lymphocytes) in 177 and 174 German workers, respectively

Model*	Estimate	95% CI	r^2† (%)	Test vs previous model			
				RSS	F	df	P
8-oxodGuo/10^6 dGuo							
No knots			42.1	21.66	—	171	—
β‡	−0.04	−0.09 to 0.01					
One knot			43.9	21.00	2.65	169	0.07
Knot	3.44	2.60-4.27					
β‡	0.01	−0.06 to 0.08					
$\beta + \beta_1$§	−0.25	−0.50 to 0.002					
Two knots			44.5	20.76	0.99	167	0.38
OTM							
No knots			11.5	27.27	—	168	—
β‡	0.01	−0.05 to 0.07					
One knot			13.1	26.78	1.51	166	0.22
Two knots			14.7	26.28	1.57	164	0.21

Abbreviation: RSS, residual sum of squares.
*Adjusted for type of industry and current smoking.
†Coefficient of determination.
‡Slope of regression line in the first segment.
§Slope of regression line in the second segment.

Fig. 1C). As for 8-oxodGuo, the inclusion of knots did not improve the model fit significantly. The variance of OTM (13.1% and 14.7%) could be explained with one and two additional knots (P = 0.22 for one knot versus none; P = 0.21 for two versus one knot; P = 0.19 for two knots versus none). Again, similar results were observed modeling phenanthrene or PAH instead of hydroxyphenanthrenes (data not shown).

Discussion

The statistical analysis presented here was carried out to characterize the dose-response relationships between measures of exposure and various biomarkers with data from a large group of German workers occupationally exposed to PAH. Overall, there was a strong association between external and internal exposure where the parent agent phenanthrene explained ~50% of the variance of its metabolites hydroxyphenanthrenes. Doubling of external exposure to phenanthrene resulted in an increase of hydroxyphenanthrenes excretion by a factor of 1.47 indicating that other metabolic pathways have to be taken into account (26, 27). On the other hand, we could not detect genotoxic effects of airborne PAHs in the blood assessed with DNA strand breaks and DNA adducts.

This biomonitoring study was conducted in occupational settings with high exposure to PAHs such as coke production and manufacture of refractory products and graphite electrodes. A detailed description of PAH exposure in these different occupational settings will be given elsewhere. Exposure to 16 PAH compounds were determined with personal measurements during a working shift. The distributions of all measurements were highly skewed. The lower quartile of the B(a)P concentrations was 85 ng/m^3 and exceeded typical environmental settings where levels of <10 ng/m^3 have been measured (10). The upper quartile of 1,435 ng/m^3 B(a)P was higher than in many occupational settings. However, exposure can be as high as 100 μg/m^3 on the top side of coke ovens or in pot rooms of aluminum smelters (28).

In the present study, we determined 1-OHP and hydroxyphenanthrenes as metabolites of pyrene and phenanthrene, respectively, to serve as biomarkers of internal PAH exposure in the post-shift urines. We also determined 1,6-+1,8-dihydroxypyrene and isomeric phenanthrene-dihydrodiols among a subgroup of workers, but statistical modeling was limited due to the smaller sample size. As observed for airborne PAH compounds, the metabolite levels were also highly skewed. The 5th percentile of 1-OHP concentrations in these German workers corresponded to the 95th percentile in U.S. men investigated in the 1999 to 2000 National Health and Nutrition Examination Survey (29). Compared with smokers from the general German population, these workers showed on average 30-fold higher levels of urinary 1-OHP (30). For hydroxyphenanthrenes, the levels were ~10-fold higher than in German smokers (31).

PAHs comprise a mixture of more than 100 compounds, where several marker compounds have been suggested for air monitoring and a few metabolites as biomarkers of exposure in human biomonitoring. Therefore, the dose-response modeling of PAH effects relies on marker variables. Their analytic quantitation should be possible with sufficient precision. The large fraction of measurements below the LOQ rendered B(a)P and other carcinogenic PAHs less suitable for regression analyses. Phenanthrene was one of the most prominent PAH compounds and correlated well with the sum of PAH compounds in our study. Regarding marker compounds for internal exposure, hydroxyphenanthrenes were measured with a higher precision than 1-OHP according to an interlaboratory comparison conducted for that study. hydroxyphenanthrenes correlated more closely with its parent compound as well as with the remaining PAH compounds than 1-OHP. Further, hydroxyphenanthrenes levels were less affected by smoking and type

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of industry than 1-OHP as shown in a previous analysis of covariance (13). In addition, in other study, occupational PAH exposure was also found less clearly associated with 1-OHP levels than with other metabolites of phenanthrene (32). Thus, we selected phenanthrene as marker for external and hydroxyphenanthrenes as surrogate for internal exposure to PAH in dose-response modeling.

Here, we modeled dose-response functions with individual data. When using data at the individual level, regression models can be applied to test the slope against unity across the dose axis without the need of a reference group. Studies that only report grouped data might suffer from inappropriate reference groups or batch problems when the samples of exposed workers and referents are not analyzed in parallel. Comparisons of exposed and nonexposed subjects at group level can lead to various misinterpretations when the groups are in average different by physical workload or other potential confounders.

The strength and shape of a functional relationship of a biomarker with exposure data are important variables in regulatory toxicology especially when evaluating exposure limits. To better characterize the shape of the dose-response function, we applied linear regression splines. Splines are piecewise polynomials that join in a smooth way at the so-called knots and are a flexible way to fit an unknown functional form (24). For most applications of this method, cubic splines are chosen (33). However, this method does not provide interpretable variables (34). A more promising approach can be linear splines that are piecewise linear functions, which are connected at the knots (35, 36). That leads to a simple parametric model as recommended by Steenland and Deddens (34) because a knot could be interpreted as point on the dose axis where a function alters its characteristic. A methodologic problem is the location of the knots. Rosenberg et al. (36) used quantiles as knot locations, whereas Molinari et al. (35) entered the knots as variables in the model and determined their distribution by bootstrapping. Here, a nonlinear model was fitted with the knots as regression variables. For the relationship between phenanthrene and hydroxyphenanthrenes, one knot was identified at 0.77 $\mu g/m^3$ phenanthrene that corresponded to an internal exposure of ~ 3 μg/g crn hydroxyphenanthrenes in these workers. Although only 13% of workers had lower levels of internal exposure, this concentration was above the 95th percentile of hydroxyphenanthrenes in the general German population (31). In that lower dose segment, there was no association between external and internal exposure. In the upper dose segment, there was a clear linear increase of internal exposure by external exposure at the log-transformed scales.

The functional relation between phenanthrene and hydroxyphenanthrenes was thus characterized by two segments, a low-dose segment with no association and a high-dose segment with linearity at the log-transformed scales. The location of a knot that separates these segments is data driven, and interpretation should be done with caution (37). One possibility of an interpretation of a knot is mechanistically as a toxicokinetic threshold (35). Such thresholds are discussed for several agents (38, 39). In addition, methodologic issues should be considered about a higher uncertainty of data in the low-dose range. We compared measurements of hydroxyphenanthrenes from two laboratories and found a lower reliability of measurements at lower doses (data not shown). PAHs from nonoccupational sources may confound the association especially at lower doses, whereas occupational exposure at high doses may override confounding from smoking or diet. Further, the margins of the dose-response curve are a specific problem when using splines. Therefore, besides mechanistic causes for a deviation from linearity, also artifacts have to be taken into account when a knot is located near the lower margin of the dose range. Here, the lower precision of hydroxyphenanthrenes measurements at lower concentrations is a plausible explanation for that lacking association. This lower precision in a dose range that is typical for most occupational and environmental settings should be taken into account when using hydroxyphenanthrenes but especially 1-OHP as quantitative measure of internal PAH exposure.

There was a clear linear trend at the log-transformed scales at high exposure levels but the internal exposure levels increased only by a factor of ~ 1.5 after doubling of the external exposure. This raises the question whether metabolic pathways other than the detoxification of phenanthrene to hydroxyphenanthrenes could be preferred at higher exposure levels and if so, which other urinary metabolites of phenanthrene should be determined. Of particular interest is the metabolic activation pathway of phenanthrene where phenanthrene 1,2-dihydrodiol (6) and *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (5) can be determined as urinary biomarkers. Exposure to phenanthrene seems to induce the diol epoxide pathway of phenanthrene (40). When starting our study in 1999, metabolites of that pathway were not yet common markers in human biomonitoring but should be analyzed together with hydroxyphenanthrenes in the future.

The final model with phenanthrene, current smoking, and type of industry explained $\sim 50\%$ of the variance of hydroxyphenanthrenes. The unexplained variance of that association between external and internal exposure may have several causes besides the generation of metabolites other than hydroxyphenanthrenes and the precision of measurements as already discussed. General reasons for exposure variability and its effect on exposure assessment have been reviewed (41, 42). Sufficient information on the measurement strategy should be provided to estimate the uncertainties of the exposure variable. In this study, external exposure was assessed with personal measurements in the worker's breathing zone during a working shift. However, measurements were only taken for 2 h on average. Exposure variability during the working shift may have led to exposure misclassification. A few workers used protective equipment. Measurements not taken behind a mask also contributed to a lower correlation between external and internal exposure. Dermal exposure can be another important route of PAH exposure in certain occupational settings (43, 44). In our study, dermal exposure was not assessed, but protective clothing was used in occupational settings with relevant dermal exposure. Another limitation is the assessment of internal exposure with spot urine samples because the collection of 24-h urine samples was not feasible in the occupational setting. In addition, errors of hydroxyphenanthrenes measurements can contribute to the unexplained

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variance (45). Further, insufficiently controlled confounders may add to the residual confounding. Smoking was implemented only as a categorical variable (current smokers and nonsmokers) because *o*-cotinine measurements were not available for all workers. However, models with *o*-cotinine concentrations did not significantly improve the regression model. For the majority of workers, the occupational exposure levels were much higher than smoking-related PAH exposure. hydroxyphenanthrenes showed a stronger correlation with phenanthrene exposure than 1-OHP with pyrene and was less affected than 1-OHP by smoking and type of industry (13). Other potential confounders, such as age and Caucasian ethnicity, had no relevant effect on hydroxyphenanthrenes in this study (13). In addition, genetic polymorphisms may modulate the individual levels of PAH metabolites (46-52). We found no clear effect of various sequence variations on the metabolite levels in our study as published elsewhere (13).

PAHs have been shown to form adducts with DNA (53). The associations of markers of adduct formation with PAH exposure have been reviewed in epidemiologic studies (54), and the authors concluded that 8-oxodGuo may serve as biomarker of oxidative DNA damage in settings with PAH exposures. But a few conflicting results were reported (55) and critical comments were added from the methodologic point of view (56). Concentrations of 8-oxodGuo in this study were found to be higher than in construction and bitumen-exposed workers, when measured with the same method and in the same laboratory (57). However, we could not find an association between 8-oxodGuo in WBC and any measure of exposure at the level of individual data in highly PAH-exposed workers. Modeling of B(a)P instead of hydroxyphenanthrenes did not improve the correlation with 8-oxodGuo (data not shown). In addition, the large multicenter study EXPAH could not show a clear effect of occupational PAH exposure or smoking on 8-oxodGuo (58). A recent study among policemen and bus drivers showed only a weak correlation between individual PAH exposure levels with 8-oxodGuo in WBC (59). Further, our findings are consistent with a previous study on 8-oxodGuo in blood and 1-OHP in urine (60). However, they are in contrast to a significant association between urinary 8-oxodGuo and 1-OHP reported by Wu et al. (61).

Various possible causes for the lacking or even negative association of PAH exposure with 8-oxodGuo in WBC have to be taken into account. The type of industry was a relevant predictor of 8-oxodGuo in our study where high levels of oxidative damage occurred in the manufacture of graphite electrodes, although PAH concentrations were lower than in the other settings. Detailed results about the levels of exposure and DNA damage in the different occupational settings will be published elsewhere. A cohort study in an Italian graphite electrode plant revealed a pronounced excess mortality for silicosis (62), indicating the potential effect of other carcinogens, such as crystalline silica. 8-oxodGuo is probably best considered as a biomarker of general oxidative stress and may be influenced by factors such as coexposure to other agents at the workplace, physical activities, or diet (63) rather than a marker of occupational exposure to PAHs. Besides the potential confounding and the general problem of detecting DNA damage of lung carcinogens in WBC, also methodologic problems of measuring 8-oxodGuo have to be considered. The European Standards Committee on Oxidative Damage reported on an interlaboratory comparison of base oxidation in human lymphocyte DNA (56). Accurate measurement of 8-oxodGuo is hampered by the ease with which guanine is oxidized during preparation of DNA for analyses. In addition to adventitious oxidations, DNA repair is another concern when transporting and processing the biological samples (56).

The Comet assay has been used with increasingly popularity to assess DNA damage in various occupational settings but mostly of small study size and at group level only. Moller et al. (64) concluded that it is difficult to provide a firm conclusion from the available studies. In our study, DNA single- and double-strand breaks in lymphocytes were not positively associated with hydroxyphenanthrenes or other measures of PAH exposure. Several reasons may have contributed to the failure to observe positive associations. Although the Comet assay is considered a suitable assay for the DNA-damaging potential of an agent, the assay also shares the methodologic problems of a suitable cell type for airborne carcinogens and the control of DNA repair and apoptosis in human biomonitoring (65). Lymphocytes may not be sufficiently specific to assess occupational airborne exposure to PAH. A meta-analysis of the genotoxic effects of smoking, the most important risk factor for lung cancer, revealed only minor or lacking genotoxic effects when assessed with the Comet assay (66). The procurement of a suitable surrogate tissue is therefore still a major challenge (67). Apart from the target tissue, the definition of a suitable outcome variable for DNA strand breaks with Comet assay is another uncertainty because the segmentation of DNA fragments in head and tail regions can vary (68, 69). Further, it is common practice to prepare two slides per subject and to analyze only ~ 50 single cells per slide. A comparison of the results from two slides revealed only a reasonable intraclass correlation coefficient and indicated that this number of cells might be not sufficient for precise measurements in human biomonitoring (data not shown). On the side of exposure hydroxyphenanthrenes is only a proxy for internal exposure to a complex mixture of PAHs from various sources. Although hydroxyphenanthrenes are not a genotoxic compound, hydroxyphenanthrenes are overall linked with PAH exposure via strong correlations with phenanthrene and the sum of PAHs. Again, the associations with DNA exposure and damage did not improve after direct implementation of B(a)P as exposure variable (data not shown). In addition, the controlling for confounders, such as smoking or type of industry, did not strengthen the relationship. Moreover, occupational exposure was much higher than smoking-related exposure to PAHs and should therefore far outweigh any confounding effects of smoking on the dose-response associations.

In conclusion, there was a strong relationship between external and internal exposure to PAHs, with linearity in the medium- to high-dose range at the log-transformed scales where a doubling of exposure to phenanthrene resulted in a 1.5-fold increase of hydroxyphenanthrenes. hydroxyphenanthrenes are an appropriate candidate to assess internal exposure to phenanthrene in biomonitoring studies, together with biomarkers for the metabolic

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activation pathway. Uncertainties in the low-dose range remain to be elucidated where the lower imprecision of measurements has to be taken into account. The lacking of a dose-dependent association of PAH exposure with DNA adducts and DNA strand breaks in WBC, however, needs a critical debate about blood as medium for lung carcinogens and the validity of these biomarkers of genotoxic effects in human biomonitoring. There is an urgent need not only for standardization of the methods but also for the use of cells that are better proxies for the target organ than WBC.

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