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O^6-methylguanine DNA adducts associated with occupational nitrosamine exposure

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Occupational nitrosamine exposures from a rubber vehicle seal (VS) curing operation were compared with the peripheral blood lymphocyte concentrations of two nitrosamine-related DNA adducts, N^7-methylguanine (N^7mdG) and O^6-methylguanine (O^6mdG), and with the activity of the enzyme that repairs O^6mdG adducts, O^6-alkylguanine-DNA alkyltransferase (AGT). The occupational personal breathing zone (PBZ) nitrosamine exposures ranged from 0.4 to 9.3 μg/m^3 in the VS area, from 0.1–2 μg/m^3 in an area remote from the VS and were not detected at a nearby rubber plant. Workers from all three of these locations had detectable concentrations of N^7mdG adducts, ranging from 0.1 to 133.2 adducts/10^7 deoxyguanosine nucleosides. Although N^7mdG concentrations were elevated for those who worked in the VS area (median 3.60 compared with 1.44), the difference was not statistically significant after controlling for confounding factors. The O^6mdG adduct concentrations were much lower than those of N^7mdG, ranging from non-detectable to 12.7 O^6mdG adducts/10^7 deoxyguanosine nucleosides and many of the participants (40/78 successfully analyzed) did not have detectable amounts of these adducts (limit of detection 0.03 O^6mdG adducts/10^7 deoxyguanosine nucleosides). Analysis of the ordinal exposure categories (high, medium/high, medium/low, low and no exposure) yielded a statistically significant association with having detectable O^6mdG adducts (Kendall's $\tau b = -0.253$, asymptotic SE = 0.096). There was no significant association between AGT activity and nitrosamine exposure or exposure category ($P > 0.30$). Although no association was found between PBZ exposure and either the N^7mdG adduct concentrations or AGT activity, the significant positive association between working in and near the VS department and the presence of O^6mdG adducts, which have mutagenic potential, provides evidence to link nitrosamine exposure one step closer to human cancer by demonstrating an association between external nitrosamine exposures and cancer-related biological effects.

Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; BMI, body mass index; dG, deoxyguanosine 3′-monophosphate; NDBA, nitrosodibutylamine; NDEA, nitrosodiethylamine; NDMA, nitrosodimethylamine; NDPA, nitrosodipropylamine; NIOSH, National Institute for Occupational Safety and Health; N^7mdG, N^7-methylguanine; NMOR, nitrosomorpholine; NPIP, nitrosopiperidine; NPYR, nitrosopyrrolidine; O^6mdG, O^6-methylguanine; PBZ, personal breathing zone; RFLP, restriction fragment length polymorphism; SIM, selected ion monitoring; VS, vehicle seal.

Introduction

Rubber industry workers have a significantly increased incidence of several types of cancer (1,2) and nitrosamines are one suspected cause because they have been documented in the rubber industry and many are suspected human carcinogens (3–15). The IARC considers nitrosodimethylamine (NDMA) and nitrosodiethylamine (NDEA) to be probable human carcinogens (Group 2A) and nitrosodipropylamine (NDPA), nitrosodibutylamine (NDBA), nitrosopiperidine (NPIP), nitrosopyrrolidine (NPYR) and nitrosomorpholine (NMOR) to be possible human carcinogens (Group 2B) (3). However, the variety of external and internal exposures, from food, household products, industrial products, tobacco products and industrial processes, creates a complicated matrix of total nitrosamine exposure making it extremely difficult to perform a conclusive epidemiological study. Nevertheless, there is a plethora of circumstantial evidence suggesting that nitrosamines cause cancer in humans. Since 1956, when Magee and Barnes demonstrated the carcinogenic potential of NDMA in rats (7), nitrosamines have been studied extensively, but mostly in laboratory animals. Species of mammals, birds, fish and amphibia have been tested and none has been resistant; carcinogenic effects have been demonstrated in 29 organs (8); the tumor sites depend on the specific nitrosamine, the species tested and the route of administration. Studies have revealed that various human tissues can metabolize nitrosamines into DNA-binding compounds (9), that human and rodent liver tissue metabolize nitrosamines in a similar manner (9) and that experimental animals form DNA adducts similar to those detected in human studies (10–12). Also, the few human DNA adduct studies have revealed higher levels of nitrosamine-related DNA adducts in cancer cases than in controls (13,14). This study is the first to report the presence of nitrosamine-related DNA adducts associated with occupational nitrosamine exposures.

Both the Occupational Safety and Health Administration and National Institute for Occupational Safety and Health (NIOSH) consider one nitrosamine, NDMA, to be an occupational carcinogen (15), but neither have established a numerical exposure limit. Der Ausschuβ für Gefahrstoffe in Germany has a limit for total occupational nitrosamine exposure of 1 μg/m^3 for general industry and of 2.5 μg/m^3 for certain processes, such as rubber vulcanization (16). However, American plants are not required to adhere to any nitrosamine exposure limits. For that reason, it was important to demonstrate the potential health risk when occupational nitrosamine exposures were detected during a NIOSH health hazard evaluation in a rubber vehicle seal plant (17). With review and approval from the NIOSH Human Subjects Review Board, a follow-up evaluation was conducted to measure the concentration of two

nitrosamine-related DNA adducts [N^7-methyldeoxyguanosine (N^7mdG) and O^6-methyldeoxyguanosine (O^6mdG)] in peripheral lymphocytes from blood samples collected at the same time as personal breathing zone (PBZ) air samples. Quantitating these DNA adducts was a way to measure the biologically effective dose. The suspected mechanism of carcinogenesis is that nitrosamines, from exogenous or endogenous sources, are metabolized into reactive intermediates which can then covalently bind to macromolecules, including DNA, potentially resulting in genetic mutations, which can be a first step in carcinogenesis. The majority of DNA adducts (70–90%) from methylating nitrosamines are N^7mdG, which has a half-life of ~150 h (18). The O^6mdG adduct is more actively removed from DNA and is therefore not as stable. This adduct, however, may have more of a mutagenic potential than the N^7mdG adduct and may play more of a role in carcinogenesis (12,19,20).

Several important factors to consider when assessing the DNA adducts associated with occupational nitrosamine exposures are that: (i) there are many non-occupational sources of nitrosamines; (ii) nitrosamines are not the only cause of methylated DNA; (iii) there is individual variability in the activity of nitrosamine metabolizing enzymes; (iv) there is individual variability in the activity of DNA repair enzymes. The first two factors were partially addressed by including unexposed workers in the study and assessing non-occupational nitrosamine exposures. To help to address the third factor, the genotype of the cytochrome P450IIE1 gene (*CYP2E1*), which is responsible for encoding an enzyme that metabolizes nitrosamines and (21) has been associated with an increased amount of nitrosamine-related DNA adducts and an increased risk of lung cancer (22–24), was determined for all participants. To address the fourth factor, the activity of O^6-alkylguanine-DNA alkyltransferase (AGT) was measured. This is a repair enzyme that removes O^6mdG adducts in a 1:1 stoichiometric irreversible reaction (25–27). A large interindividual variation in AGT activity is suspected to be due to genetic differences and a decreased activity could increase the risk of cancer from exposure to nitrosamines. Since the reaction is irreversible, it is possible for high exposures to nitrosamines to reduce the repair activity by exhausting the supply of the repair enzyme (28,29).

Materials and methods

Study population

The study population was selected from a rubber plant that manufactured automotive vehicle seals (Plant A) and from a nearby plant of the same company that manufactured other rubber automotive parts (Plant B). Employees were considered eligible to participate in this study if they were current first shift employees and had been working in the same department for at least 6 months. Eighty-five volunteers participated in the study; 64 of the 164 eligible employees from Plant A where the nitrosamine exposures were documented and 21 of the 35 eligible employees from Department T of Plant B, where nitrosamines were not detected. The nitrosamine exposures in Plant A were from a salt bath curing operation in the vehicle seal (VS) department and participants from this plant were divided into exposure categories based on where they worked in relation to the salt baths: Category I, VS employees who worked along the salt bath lines (17 workers); Category II, VS employees who did not work along the salt bath lines (15 workers); Category III, non-VS employees who worked frequently in and near the VS area (20 workers); Category IV, non-VS employees who worked remote from the VS area (12 workers). Category V was employees from Plant B (21 workers).

Data collection

Prior to the study, each participant completed a consent form and also a self-administered questionnaire that assessed non-occupational nitrosamine exposures and other factors that might influence nitrosamine metabolism: age, height and weight [converted to body mass index (BMI)], tobacco use, alcohol consumption, diet, medical history and fertilizer and pesticide exposures. These factors were evaluated for any confounding effect. Any factors determined to be confounders were controlled for in the analyses.

For the collection of a full shift PBZ air sample, participants wore a Gillian high flow personal air sampling pump that pulled air through a Thermosorb/N medium tube at a rate of 1 l/min. At the end of the work shift, each participant provided a 40 ml venous blood sample, which was packaged and immediately driven back to the laboratory.

Nitrosamine air sample analysis

The air samples were analyzed in a NIOSH laboratory for NDMA, NDEA, NDPA, NDBA, NPIP, NPYR and NMOR. Samples were prepared by back-flushing the Thermosorb/N sorbent tubes with 1.9 ml of a solution of 80% dichloromethane and 20% methanol. A mass spectrometer was operated in the selected ion monitoring (SIM) mode at a resolution of 3500 and specific *N*-nitrosamines were detected by monitoring the characteristic NO^+ ion at *m/z* 29.998 during the expected chromatographic elution time of the analyte. If the mass spectrometer response occurred at the elution time of the analyte, an additional confirmation step was performed. For this, the mass spectrometer was set to monitor the molecular ion of the analyte during analysis; a postive response confirmed its presence. The gas chromatograph was equipped with a 30 m×0.25 mm HP-INNOWAX capillary column and the oven temperature was programed from 55 to 185°C at 15°C/min. GC/SIM analysis conditions were optimized with 1 µl injections of a liquid standard (Thermedics Inc.). The area response obtained for a sample peak was compared with the area of the standard to estimate the concentration. Quality assurance samples were prepared by injecting 5 µl of the standard directly onto a fresh Thermosorb/N tube and allowing them to stand at room temperature overnight before desorption.

N^7-methylguanine and O^6-methylguanine DNA adduct analyses

Lymphocytes were isolated from whole blood using Histopaque and were stored in liquid nitrogen until DNA isolation. DNA was isolated from lymphocytes using a Gentra DNA isolation kit. The quantification of DNA was performed by UV spectrophotometry. Hydrolysis of DNA was conducted by the method of Kato *et al.* (30). DNA bases were separated using the method of Shields *et al.* (31). Briefly, DNA bases were separated using a Waters HPLC with a Waters Model 484 UV detector fitted with a Beckman Altex Ultrasphere ion pair column and an Ultrasphere guard column. The mobile phase was 0.1 M triethylamine, pH 7.0, and 1% acetonitrile mixed isocratically for 20 min. The acetonitrile concentration was increased to 5% over 10 min, then allowed to mix isocratically for 10 min. Afterwards, the acetonitrile concentration was increased to 10% over 10 min. The flow rate was 1 ml/min and UV detection was at 254 nm.

Three milliliter fractions of N^7mdG and deoxyguanosine 3′-monophosphate (dG) and 6 ml fractions of O^6mdG were collected and concentrated using vacuum centrifugation. The fraction containing N^7mdG was further purified on a Hewlett Packard HPLC with a Nova Pak C-18 reverse phase column equipped with a Hewlett Packard Diode Array UV detector at 254 nm. The mobile phase was 0.05 M ammonium formate:methanol and was run at 1 ml/min. Three milliliter fractions of N^7mdG were again collected and concentrated using vacuum centrifugation. The dried fractions were dissolved in 1 ml of water to which 1 µl of the corresponding dG fraction diluted 1:100. N^7mdG fractions were post-labeled using the method of Kato *et al.* (30). Deoxyguanosine and N^7mdG were localized using a radioisotope image analyzer and the radioactivity measured. For N^7mdG, standards were synthesized and used at two different steps. N^7mdG was synthesized by the method of Shields *et al.* (31), post-labeled and developed chromatographically as above for construction of a standard curve. Standard values ranged from 1×10^3 to 1×10^8 N^7mdG/10^7 dG residues. Authentic N^7mdG 3′,5′-bisphosphate was enzymatically synthesized from N^7mdG and used to prespot the TLC plates prior to sample application so as to be able to visualize the location of the N^7mdG adduct by UV.

Dried fractions of both O^6mdG and dG were hydrolyzed to O^6-methylguanine (O^6mdG) and guanine, respectively, by solubilization in 1 ml 0.1 N HCl for 1 h at 60°C. The O^6mdG and guanine fractions were concentrated by vacuum filtration, solubilized in water and filtered. O^6mdG levels were determined using a Dionex HPLC equipped with an ESA Coulochem Model 5100A electrochemical detector and fitted with a Supelco C-18 reverse phase column. The mobile phase (1 ml/min) was 100 mM sodium acetate, pH 7, and 5% methanol. Osmosis-purified water for the preparation of the sodium acetate was further purified by filtering through a Waters C-18 Sep Pak cartridge to remove additional organic contaminants. The detector was set at 500–650 mV. The chromatographic conditions were the same for guanine except that the detector was set at 500–800 mV. Guanine could not be used as an internal standard as above due to an interfering peak present in the O^6mdG fractions. Standard curves for O^6mdG and guanine were prepared

Table I. Average concentration of PBZ exposures to nitrosamines

Exposure category (*n*)	Nitrosamine concentration (μg/m^3, mean ± SD)			
	NDMA	NPIP	NMOR	Total
I (17)	2.54 ± 1.37	1.59 ± 0.73	0.30 ± 0.15	4.44 ± 2.15
II (15)	1.22 ± 0.60	1.05 ± 0.27	0.70 ± 0.70	2.97 ± 1.16
III (20)	1.27 ± 0.85	0.88 ± 0.51	0.17 ± 0.13	2.32 ± 1.37
IV (12)	0.42 ± 0.52	0.21 ± 0.24	0.05 ± 0.05	0.67 ± 0.69
V (21)	ND	ND	ND	ND

ND denotes that no nitrosamine was detected; the minimum detectable concentration was 0.01 μg/m^3. Category I, high; Category II, medium/high; Category III, medium/low; Category IV, low; Category V, no exposure.

using concentrations that ranged from 60 to 6000 fmol for O^{6}mdG and from 28 to 2800 fmol for guanine.

AGT activity analysis

AGT activity was determined by the transfer of radioactivity from ^{3}H-labeled O^{6}mdG (32) from DNA substrate to cell extract protein isolated from peripheral lymphocytes using a slight modification of the method described by Preuss *et al.* (33). Lymphocytes were isolated from whole blood using Histopaque. Cell extracts were prepared by sonication of the cell suspensions. Cellular protein was concentrated by Centricon 10 or Microcon 10 concentrators then incubated with ^{3}H-labeled DNA. The protein was precipitated and washed. Radioactivity associated with the precipitated protein was determined using a liquid scintillation counter and the AGT activity was expressed as fmol [^{3}H]methyl transferred to precipitated protein per mg total cell extract protein.

Genotype analysis

Small amounts (0.5–1.0 μg) of the isolated DNA was used to perform a restriction fragment length polymorphism (RFLP) analysis. DNA was amplified by PCR using primers appropriate to the regions of interest within the *CYP2E1* gene. The region of the gene encompassing intron 6 contains a *Dra*I polymorphism. For analysis of RFLPs, aliquots of the amplified DNA were digested with the restriction enzyme *Dra*I and analyzed by agarose gel electrophoresis. DNA was visualized by ethidium bromide fluorescence and the size of the DNA ascertained by comparison with authentic size standards. The presence of a *Dra*I restriction site yields a 572 bp fragment; in the absence of the site an 874 bp fragment resulted. DNA from individuals that are heterozygous for the restriction site display a mixture of each of the bands.

Statistical analysis

Linear regression was used to evaluate the associations between N^{7}mdG concentration and nitrosamine exposure and AGT activity and nitrosamine exposure. The N^{7}mdG data was log transformed for the regression analyses because of the right skewed distribution of the data and the scatter plots involving N^{7}mdG and exposure. The AGT data were not transformed for the regression analyses because doing so did not improve the distribution or scatter plots. Logistic regression was used to evaluate the relationship between O^{6}mdG and nitrosamine exposures. A dichotomized version of the O^{6}mdG data was used because more than half of the participants did not have detectable values (34). The nitrosamine exposures used in the regression analyses were PBZ measurements or the exposure categories. Potential confounders (questionnaire data, genotype and AGT activity for O^{6}mdG only) were considered in multivariable models only if they demonstrated a relationship with both the response and the exposure variable at the $P \leqslant 0.30$ level of significance.

Results

Air sampling

The air sampling results are summarized in Table I. Only NDMA, NMOR and NPIP were detected; the other four analytes were not detected in any samples. Every worker sampled in Plant A had detectable nitrosamine exposure, even those in areas remote from the VS area (Category IV). There was an expected reduction in exposure for those workers further away from the salt bath lines (Figure 1). In the VS area (Categories I–III), the PBZ total nitrosamine concentrations ranged from 0.4 to 9.3 μg/m^3 and 54% of the results were higher than the German occupational standard of 2.5 μg/m^3 for rubber vulcanizing and processing industries (11). Nitrosamines were not detected in any of the samples collected at Plant B.

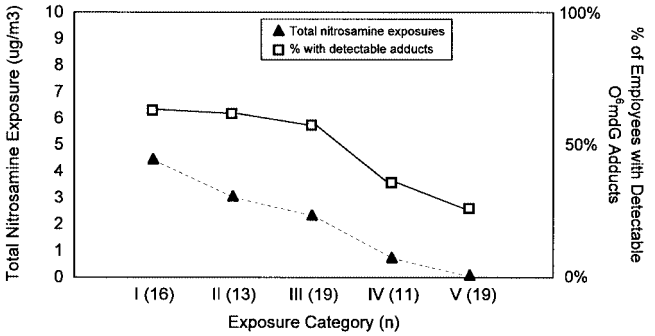

Fig. 1. Average PBZ total nitrosamine exposures and percent of employees with detectable O^{6}mdG adducts by exposure category.

Table II. Average concentration of N^{7}mdG by exposure category

Exposure category (*n*)	N^{7}mdG (adducts/10^7 dG nucleosides)	
	GM ± GSD	Range
I (17)	3.6 ± 10.7	0.1–72.4
II (15)	5.0 ± 8.2	0.3–133.2
III (20)	2.8 ± 6.1	0.1–79.7
IV (12)	2.0 ± 13.3	0.1–69.7
V (21)	1.2 ± 11.3	0.1–128.2

Category I, high; Category II, medium/high; Category III, medium/low; Category IV, low; Category V, no exposure. GM, geometric mean.

N^7-methylguanine adduct concentrations

The N^{7}mdG adduct concentrations measured in this study ranged from 0.1–133.2 adducts/10^7 dG nucleosides (Table II); every participant had detectable concentrations of these adducts. Although N^{7}mdG concentrations were elevated for those who worked in and near the VS area (Categories I–III, median 3.6; Categories IV and V, median 1.4), the difference was not statistically significant after controlling for confounding factors. The average N^{7}mdG concentrations are in the same range as those published in a study by Mustosen and Hemminki (35) that documented significantly higher concentrations of these adducts in peripheral lymphocytes of smokers (23.6 adducts/10^7 dG nucleosides) than in non-smokers (13.5 adducts/10^7 dG nucleosides). The individual results varied markedly within each exposure group. For example, the range of adduct concentrations for those who worked in and near the VS area (Categories I–III) was

Table III. Average concentration of AGT activity by exposure category

Exposure category (*n*)	AGT (fmol/mg protein)	
	Mean ± SD	Range
I (13)	203.82 ± 138.01	0.00–422.18
II (10)	179.88 ± 167.97	0.00–507.87
III (15)	154.08 ± 121.12	0.00–456.63
IV (6)	168.71 ± 165.20	20.04–485.03
V (21)	209.26 ± 158.66	24.59–809.43

Not every participant's blood sample was successfully analyzed for AGT activity, so the number of analyzed samples (*n*) is included for each category. Category I, high; Category II, medium/high; Category III, medium/low; Category IV, low; Category V, no exposure.

0.1–133.2 adducts/10^7 dG nucleosides and the range for those who did not work anywhere near the VS area (Categories IV and V) was similarly 0.1–128.2 adducts/10^7 dG nucleosides.

O^6-methylguanine adduct concentrations

The O^6mdG adduct concentrations were much lower than the N^7mdG results, ranging from non-detectable to 12.7 O^6mdG adducts/10^7 dG nucleosides, and many of the participants (40/78 successfully analyzed) did not have detectable amounts of O^6mdG adducts (limit of detection 0.03 O^6mdG adducts/10^7 dG nucleosides). Therefore, the response was analyzed as a dichotomous variable (presence of detectable O^6mdG adducts) using logistic regression. When the continuous exposures (PBZ measurements of NDMA, NMOR, NPIP and total nitrosamines) were analyzed, there was no significant association with the presence of O^6mdG adducts ($P > 0.10$ controlling for age and BMI). However, analysis of the ordinal exposure categories (Category I, high; Category II, medium/high; Category III, medium/low; Category IV, low; Category V, no exposure) yielded a statistically significant association with having detectable O^6mdG adducts (Kendall's $\tau b = -0.253$, asymptotic SE = 0.096) (Figure 1). A limitation of this test is that it does not control for confounding factors. Categories I–III had similar percentages of employees with detectable O^6mdG adducts (63, 62 and 58%, respectively) and these three categories all represented employees who worked routinely in the VS area of the plant. If employees are categorized dichotomously by VS area (Categories I–III, 60% had detectable O^6mdG adducts) and non-VS area (Categories IV and V, 30% had detectable O^6mdG adducts), there is a significant association ($P = 0.02$), controlling for age and BMI.

AGT activity

The AGT activity results ranged from 0 to 809.43 fmol/mg protein (Table III). There was no significant association between AGT activity and PBZ nitrosamine exposure nor was there a significant difference in average AGT activity between exposure categories ($P > 0.30$). However, there was large variability within each exposure group, as illustrated by the averages and standard deviations (Table III). The AGT activity results yielded no evidence of a confounding effect on the DNA adduct and nitrosamine exposure relationships and therefore was not controlled for in the other analyses.

Genotype

A genotype of DD means that there are two normal copies of the *CYP2E1* gene; a genotype of CD means that one copy of the gene is normal and one is a variant; a genotype of CC means that both copies of the gene are variant. Fifteen participants had the CD genotype and 70 had the DD genotype. The 15 participants with the CD genotype might be expected to have higher DNA adduct concentrations from the same exposures than the DD genotype participants (21), but because genotype yielded no evidence of a confounding effect, it was not controlled for in these analyses.

Discussion

The fact that no significant association was found between N^7mdG adduct concentration and PBZ exposure could be due to the many exogenous and endogenous nitrosamine exposures. Relative to all the other nitrosamine exposures, the occupational exposure may not be significant for the formation of N^7mdG or occupational exposure may be significant for the formation of N^7mdG, but this study may not have been able to assess the other exposures well enough to document this significant association. Also, because of the 150 h half-life of this adduct, 1–2 weeks of exposure data might be necessary to document any significant association between N^7mdG concentration and exposure, but such data collection was not feasible during this study. The former possibility is more plausible than the latter since the category IV and V results were not significantly different to the category I–III results, however both possibilities need further exploration. Another possibility is that the wide range and non-significance of N^7mdG adduct concentrations could be the result of radiolabeled compounds that co-chromatographed with N^7mdG, even though several steps were taken to purify the N^7mdG.

Similarly, the occupational nitrosamine exposures in this study may not have been high enough to elicit a decrease in AGT activity or to elicit one that was distinguishable from what might be caused by non-occupational nitrosamine exposures. In other studies that demonstrated a lowered AGT activity with nitrosamine exposure, the exposure concentrations were not documented, but it is probable that they were higher since the exposed populations were tire storage employees (29), clinical workers handling chemotherapeutic agents (29) and cancer patients treated with methylating chemotherapeutic agents (28). Also, the variability in AGT activity was not high in these groups, as it was among the exposure groups in this study.

In conclusion, when conducting this study there were many variables to consider, such as multiple external nitrosamine exposures, as well as endogenous formation of nitrosamines, metabolism of nitrosamines into compounds that will bind to DNA and DNA repair mechanisms, all of which vary by individual. Spot samples of external occupational nitrosamine exposures were collected and other external exposures were qualitatively assessed using a questionnaire. However, genotype of one enzyme and activity of one repair enzyme were the only assessments of internal variability; other tests were not feasible during this study. Another weakness was not having a large enough population to perform random sampling. Even with these limitations, there was a significant positive association between occupational nitrosamine exposure and having detectable O^6mdG adducts in peripheral lymphocytes. A significant association does not prove a cause–effect relationship, but since this DNA adduct has mutagenic potential and may play a role in carcinogenesis (19), the findings support the biological plausibility of an association between occupational nitrosamine exposure and cancer. These results are the first documentation of a cancer-related biomarker associated with occupational nitrosamine exposure and more studies are

necessary so that nitrosamines will be recognized by the broader community as occupational carcinogens and occupational exposure limits will be set by countries other than Germany.

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