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# Crotonaldehyde exposure in U.S. tobacco smokers and nonsmokers: NHANES 2005–2006 and 2011–2012

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## Abstract

**Introduction—**Crotonaldehyde is an α,β-unsaturated carbonyl compound that is a potent eye, respiratory, and skin irritant. Crotonaldehyde is a major constituent of tobacco smoke and its exposure can be quantified using its urinary metabolite N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HPMM). A large-scale biomonitoring study is needed to determine HPMM levels, as a measure of crotonaldehyde exposure, in the general U.S. population.

**Materials and methods—**Urine samples were obtained as part of the National Health and Nutrition Examination Survey 2005–2006 and 2011–2012 from participants who were at least six-years-old (N = 4,692). Samples were analyzed for HPMM using ultra performance liquid chromatography - tandem mass spectrometry. Exclusive tobacco smokers were distinguished from non-tobacco users through a combination of self-reporting and serum cotinine data.

**Results—**Detection rate of HPMM among eligible samples was 99.9%. Sample-weighted, median urinary HPMM levels for smokers and non-users were 1.61 and 0.313 mg/g creatinine, respectively. Multivariable regression analysis among smokers showed that HPMM was positively associated with serum cotinine, after controlling for survey year, urinary creatinine, age, sex, race, poverty level, body mass index, pre-exam fasting time, and food intake. Other significant predictors of urinary HPMM include sex (female > male), age (children > non-user adults), race (non-Hispanic Blacks < non-Hispanic Whites).

**Conclusions—**This study characterizes U.S. population exposure to crotonaldehyde and confirms that tobacco smoke is a major exposure source. Urinary HPMM levels were significantly

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**Institutional Review Board Approval**

The National Health and Nutrition Examination Survey (NHANES) is a program of studies designed to assess the health and nutritional status of adults and children in the United States. The survey is unique in that it combines interviews and physical examinations. NHANES is a major program of the National Center for Health Statistics (NCHS). NCHS is part of the Centers for Disease Control and Prevention (CDC) and has the responsibility for producing vital and health statistics for the nation. NCHS has obtained approval to conduct the survey from its Research Ethics Review Board. All approvals can be found at the following link: https://www.cdc.gov/nchs/nhanes/irba98.htm.

higher among exclusive combusted tobacco users compared to non-users, and serum cotinine and cigarettes per day were significant predictors of increased urinary HPMM. This study also found that sex, age, ethnicity, pre-exam fasting time, and fruit consumption are related to urinary HPMM levels.

**Keywords**

crotonaldehyde; HPMM; tobacco smoke exposure; NHANES; biomonitoring

## INTRODUCTION

Crotonaldehyde (2-butenal), an α,β-unsaturated carbonyl compound, is a colorless liquid with a pungent odor. It exists as the *cis* and the *trans* isomers; commercial crotonaldehyde consists of >95% *trans* isomer (IARC, 1995). It is mainly used in the manufacturing of sorbic acid and n-butanol. It is a potent eye, respiratory, and skin irritant (Coenraads et al., 1975). The occupational short term exposure limit (STEL) for crotonaldehyde is 0.3 ppm according to the American Conference of Governmental Industrial Hygienists (ACGIH, 2015).

Crotonaldehyde reacts with deoxyguanosine in DNA to generate 1,$N^2$-propanodeoxyguanosine adducts that may lead to genetic mutations (Chung et al., 1984). These adducts have been found in human lung tissues (Zhang et al., 2006). In rats, crotonaldehyde forms non-neoplastic and neoplastic liver lesions including hepatocellular carcinomas (Chung and Hecht, 1986). However, no human data associates carcinogenicity with crotonaldehyde exposure; thus the International Agency for Research on Cancer classifies the compound as group 3, not classifiable as to its carcinogenicity in human (IARC, 1995). In contrast, the U.S. Environmental Protection Agency (EPA) lists crotonaldehyde as a possible human carcinogen (group C) based on limited animal data and supporting genotoxicity data (EPA, 1991).

A major source of crotonaldehyde exposure is cigarette smoke (Counts et al., 2004). The amount of the compound in cigarette smoke varies from 1–53 μg per cigarette, depending on the machine smoking protocol used for measurement and the cigarette brand filter ventilation (Pazo et al., 2016). Crotonaldehyde is also found in smokeless tobacco, engine exhaust, and wood combustion (Destaillats et al., 2002; IARC, 1995; Masiol and Harrison, 2014; Stepanov et al., 2008). Crotonaldehyde occurs naturally in many foods (Feron et al., 1991; Kensler et al., 2012), such as fruits (e.g., apples, guavas, grapes, strawberries and tomatoes), vegetables (e.g., cabbage, cauliflower, Brussels sprouts, carrots and celery leaves), dairy products (e.g., bread, cheese and milk), animal proteins (e.g., meat and fish), alcoholic beverages (e.g., beer and wine), heated cooking oils, and chips. Additionally, endogenous lipid peroxidation could result in crotonaldehyde exposures in humans (Nair et al., 2007; Niki, 2009; Voulgaridou et al., 2011). Crotonaldehyde can also form *in vivo* as a metabolite of N-nitrosopyrrolidine and 1,3-butadiene (Elfarra et al., 1991; Wang et al., 1988).

Crotonaldehyde is metabolized primarily to N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HPMM) and to a lesser extent, 2-carboxy-1-methylethylmercapturic acid, both of

which are excreted via the urine in rats (Gray and Barnsley, 1971). The identification of HPMM as a major crotonaldehyde metabolite is supported by the HPMM structural homologue, N-acetyl-S-(3-hydroxypropyl)-L-cysteine (HPMA), being identified as a primary metabolite of crotonaldehyde's three carbon structural homologue acrolein (Parent et al., 1998). Urinary HPMM levels are proportional to crotonaldehyde exposure (Carmella et al., 2013), and it is a useful biomarker for smoking-related exposure (Scherer et al., 2007). Cigarette smokers have higher urinary HPMM compared to non-smokers (Pluym et al., 2015; Scherer et al., 2007). Carmella et al. also demonstrated that urinary HPMM decreases significantly in the first three days after a smoker ceases smoking (Carmella et al., 2009).

Although there are studies on crotonaldehyde exposure among smokers, there are no large-scale biomonitoring studies assessing exposure in the general population. Moreover, the effect of diet on crotonaldehyde exposure has not been assessed systematically. These gaps prompted us to examine crotonaldehyde exposure in a representative sample of the U.S. population. In this study, we measured HPMM concentrations in urine samples provided by participants in the 2005–06 and 2011–12 cycles of the National Health and Nutrition Examination Survey (NHANES). Multivariable regression models were used to determine the influence of demographic variables (e.g., age, sex, and race) on HPMM concentrations, as well as the effects of certain lifestyle factors, such as obesity, tobacco use, and diet. Thus, this biomonitoring study characterizes crotonaldehyde exposure in the U.S. population and explores different exposure sources and modifiers.

## MATERIAL AND METHODS

### 1.1. Study design

NHANES is a population-based survey designed to assess the health and nutritional status of adults and children in the United States (https://www.cdc.gov/nchs/nhanes/index.htm). The survey is based on cross-sectional observation of a complex, multistage probability sample representative of the civilian, non-institutionalized U.S. population. The survey collects questionnaire data, physical examination data, and biological samples. NHANES is conducted by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC). The study protocol was reviewed and approved by a CDC institutional review board, and informed written consent is obtained from all study participants before they participate in the study.

Spot urine samples were collected from participants in two NHANES survey cycles—a one-half subsample of participants ≥ 12 years old from NHANES 2005–2006 and a one-third subsample of participants ≥ 6 years old from NHANES 2011–2012—and were measured for HPMM to determine crotonaldehyde exposure.

### 1.2. Chemical analysis

The collected urine samples were stored at −70 °C until analysis. Urinary HPMM concentrations were measured using ultra high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) according to a published procedure (Alwis et al., 2012). Briefly, urine samples were analyzed at 1:10

dilution (a mixture of 50 μL urine, 25 μL $^{2}H_{3}$-HPMM internal standard, and 425 μL 15 mM ammonium acetate, pH 6.8). Liquid chromatography was performed using an ACQUITY UPLC HSS T3 Column, 1.8 μm, 2.1 mm × 150 mm, with mobile phases containing 15 mM ammonium acetate, pH 6.8 (solvent A) and acetonitrile (solvent B). The eluate was ionized using ESI technique. The mass spectrometer was operated in scheduled multiple reaction monitoring (SMRM) mode for negative ions; mass-to-charge (m/z) transitions were monitored at 234→105 for HPMM and 237→105 for the internal standard, $^{2}H_{3}$-HPMM. Urinary HPMM concentrations were calculated from a linear calibration curve obtained by plotting the relative response factor (ratio of the peak area of native analyte to the peak area of the corresponding internal standard) as a function of the native standard concentration. The limit of detection (LOD) in urine was 2.0 ng/mL for HPMM (Alwis et al., 2012).

### 1.3. Statistical analysis

The crotonaldehyde metabolite HPMM was measured in spot urine samples collected from 5,815 participants in the one-third environmental subsample of NHANES 2005–2006 and 2011–2012. Many of these study participants were likely exposed to crotonaldehyde as a component of tobacco smoke; therefore we categorized tobacco smoke exposure based on a combination of questionnaire and serum cotinine data (Pirkle et al. 1996). Study participants were identified as exclusive users of combusted tobacco products (named “exclusive combusted tobacco users” or “exclusive tobacco smokers”) if they had serum cotinine >10 ng/mL and responded “yes” to question SMQ680 (tobacco or nicotine use within 5 days prior to NHANES physical examination), “yes” to at least one of SMQ690A–SMQ690C (cigarettes, pipes, cigars), and “no” to all of SMQ690D–SMQ690F (smokeless tobacco and nicotine delivery products). Participants were identified as non-users of tobacco products if they answered “no” to either SMQ680 or SMD020 (smoked 100 cigarettes in life), or answered “never smoked cigarettes regularly” to SMD030 (age started smoking regularly). Non-users were confirmed by a serum cotinine measurement ≤10 ng/ml. Alternatively, participants missing responses for SMQ680, SMD020, and SMD030 were classified as non-users if they had serum cotinine ≤10 ng/mL. Participants were excluded from analysis because of missing serum cotinine data (N = 284), for not having answered SMQ680 (230 participants), or missing data for other variables used in the regression models (N = 609), leaving 4,692 study participants eligible for statistical analysis.

Reported results met the accuracy and precision specifications of the quality control/quality assurance program of the CDC National Center for Environmental Health, Division of Laboratory Sciences (Caudill et al., 2008). Measurements below the limit-of-detection (LOD) were imputed with the quotient of the LOD divided by the square root of two (Hornung and Reed, 1990).

Because NHANES participants are recruited through a multistage sampling design, it is necessary to account for this complex design to estimate variances properly and to produce unbiased, nationally representative statistics. Robust estimation may be accomplished by applying survey sample weights to each participant’s data and using Taylor series linearization to produce variance estimates. We used this estimation approach as it was implemented in the DESCRIPT subroutine of the statistical software package SUDAAN®,

Version 11.0.0 (Research Triangle Institute 2012), called from the SAS statistical software application, Version 9.3, as well as the SURVEYREG subroutine of SAS 9.3 (SAS Institute 2010). Sample-weighted linear regression models stratified by tobacco use status (exclusive combusted tobacco users vs. non-users) were fit to NHANES data from the 2005–2006 and 2011–2012 survey cycles (NHANES), where the dependent variable was urinary HPMM concentration (ng/mL). Because the distribution of urinary measurements was highly right-skewed and would have adversely affected hypothesis testing, urinary HPMM concentration data were reported as geometric means and transformed with the natural log for evaluating the statistical significance of regression slopes. The *p*-values for slopes from the natural log of the urinary HPMM concentration regression models are reported. To facilitate interpretability, however, we report slopes and their 95% confidence intervals estimated from identical regression models of untransformed urinary concentration data. Statistical significance was set to $\alpha \leq 0.05$.

Potential confounders were included in the regression models: age, sex, race/ethnicity, body mass index (BMI), poverty level (the ratio of family income to poverty), food intake, and hours of pre-exam fasting. Information for these potential confounders was self-reported. Age (year) was categorized into the following ranges: 6–11, 12–19, 20–39, 40–59, and ≥ 60. While standard definitions for underweight (BMI < 18.5), healthy weight (18.5 ≤ BMI < 25), and overweight/obese (BMI ≥ 25) applied to adults 20 years of age and older, participants younger than 20 were classified as underweight, healthy weight, and overweight/obese if they were below the 5$^{th}$ percentile, between the 5$^{th}$ and 85$^{th}$ percentile, and above the 85$^{th}$ percentile, respectively, for their sex and age (https://www.cdc.gov/healthyweight/assessing/bmi). Poverty level was determined by whether the ratio of a family's income to poverty (INDFMPIR) was greater or less than the poverty threshold, which is represented by the ratio of 1, according to NHANES (https://www.cdc.gov/nchs/nhanes/index.htm).

Food intake was reported with a 24-hour dietary recall on the same day blood and urine samples were taken (DR1IFF_G). Each food recalled was reported by NHANES with a quantity, nutritional information, and an 8-digit code, which uniquely identifies the type of food in the USDA Food and Nutrient Database for Dietary Studies (FNDDS) database. Regression variables were produced by summing the mass of the individual food group consumed by each participant, with any participant reporting no consumption given a zero. Food categories consist of nine food groups identified by the USDA corresponding to the first digit of the FNDDS food code as well as the following independently derived categories. The smoked meat category was constructed based on the USDA's "What's in the Foods You Eat" search tool and by using the search term "smoked," "barbecue" (which is synonymous with smoking), and "pastrami" (which is by definition smoke-cured) and including all dishes. The brewed coffee category was constructed by using the search term "coffee" and including drinks that are mostly coffee (e.g., regular coffee and espresso), but excluding things such as lattes that are mostly milk. The cruciferous vegetables category was constructed by using every vegetable listed on the Wikipedia page for cruciferous vegetables as a search term. Self-reported hours of pre-exam fasting was included in the model as a continuous predictor and potential confounder of the association between diet and crotonaldehyde exposure, ranging as high as several days.

In addition, urinary biomarker concentrations can be influenced by urine dilution, which can vary markedly from void to void and may confound statistical inference (Barr et al., 2005). Urine dilution can be accounted for by scaling urinary analyte concentration to the urinary concentration of creatinine, a compound formed endogenously by lean body mass and excreted at a fairly constant rate. Summary statistics of urinary concentrations are reported as the ratio of HPMM to creatinine (mg/g creatinine). For the regression models, however, we accounted for urinary dilution by including urinary creatinine (mg/dL) as a model predictor.

Serum cotinine was used as a continuous variable to evaluate the association between urinary HPMM concentration and tobacco smoke exposure in the regression model for both exclusive combusted tobacco users and non-users. Among non-users, tobacco smoke exposure is primarily attributed to second-hand smoke (SHS), which is associated with serum cotinine levels in the range of 0.05–10 ng/mL (Homa et al., 2015). To directly associate urinary biomarker concentrations with the frequency of cigarette smoking, we ran the same regression model but replaced serum cotinine with self-reported average number of cigarettes smoked per day (CPD) over the five days preceding the exam. We kept the dual users, who reported CPD as well as use of other combusted tobacco products, in this model to maintain consistency throughout the study. This variable was classified in ranges of 1–10 CPD (0.5 pack), 11–20 (1 pack), and > 20 (> 1 packs), where the reference category was comprised of participants with serum cotinine ≤ 0.05 ng/mL. CPD was only assigned in subjects with no missing cotinine values. In the CPD model, participants were excluded if they were neither exclusive combusted tobacco users nor non-users (N = 230), could not be assigned a CPD value (N = 499), or had missing data for other variables used in the regression model (N = 584 participants), leaving 4,502 participants eligible for statistical analysis.

## RESULTS

HPMM was detected in 99.9% of the urine samples measured in NHANES 2005–2006 and 2011–2012 cycles. Shown in Table 1 are sample-weighted demographic distributions for this study for exclusive combusted tobacco users (~20% of the population) and non-users.

Sample-weighted summary statistics for urinary HPMM concentrations among participants are presented in Table 2. A detailed analysis is available in the online supplementary material (Table A.2 for non-users and Table A.3 for exclusive combusted tobacco users). The median urinary HPMM concentration for exclusive combusted tobacco users (1.63 mg/g creatinine) was higher than for non-users (0.313 mg/g creatinine). We observed the similar shift in median HPMM level (the green bar) in Figure 1, which shows the percentage distribution of HPMM among combusted tobacco smokers and non-users. In this figure, the distribution among tobacco smokers shows a bimodality, which is only present for the creatinine-adjusted HPMM data.

The median value of urinary HPMM concentrations typically increased with age except among non-users aged 6–11, who had the highest concentration of HPMM among the non-users. Interestingly, median concentration of HPMM was higher among females compared

with males for both exclusive combusted tobacco smokers and non-users. Among different racial groups, Mexican Americans and non-Hispanic Blacks had the lowest concentration of urinary HPMM among exclusive combusted tobacco users and non-users respectively, whereas non-Hispanic Whites had the highest levels in both groups. In an unstratified multivariable regression model, urinary HPMM was significantly higher by 2214 ng/mL among exclusive smokers compared to non-users, controlling for survey year, urinary creatinine, age, sex, race, poverty level, body mass index, pre-exam fasting time, and food intake.

Results of the multivariable regression analysis for non-users are shown in Table 3. In this model, serum cotinine was not a strong predictor ($p = 0.0823$) of urinary HPMM concentrations after controlling for survey year, urinary creatinine, age, sex, race/ethnicity, BMI, poverty level, food intake, and pre-exam fasting time. The model also showed no sex differences between female and male subjects ($p = 0.0616$). Using the age group 20–39 as a reference, we determined that HPMM levels were significantly higher among all age groups, except 12–19, which was not significantly different. When non-users were grouped according to race, only non-Hispanic blacks ($p < 0.0001$) had statistically lower HPMM values compared with non-Hispanic whites. Subjects' BMI and poverty status had no effect on urinary HPMM excretion. Among different food categories, only fruits ($p = 0.0014$) showed significant positive correlations with HPMM levels. Pre-exam fasting time showed strong negative correlation ($p < 0.0001$) with urinary HPMM excretion.

Results of multivariable regression analysis for the exclusive combusted tobacco smokers are presented in Table 4. In contrast to non-users, serum cotinine in exclusive combusted tobacco users, was a strong predictor ($p = 0.0014$) of urinary HPMM concentrations after controlling for other regression variables. The model also showed sex differences: female subjects ($p = 0.0135$) had significantly higher HPMM levels compared with males. Using the age group 20–39 as a reference, we determined that HPMM levels were significantly lower for the group 12–19 ($p = 0.0048$), whereas they were higher for both 40–59 ($p = 0.0001$) and ≥ 60 ($p = 0.0003$) groups. When these smokers were grouped according to race, Mexican Americans ($p = 0.0104$) and non-Hispanic Blacks ($p < 0.0001$) had significantly lower HPMM values compared with non-Hispanic whites. Subjects below the poverty level had significantly higher levels of HPMM in their urine samples ($p = 0.0007$) compared with those above poverty level. When compared with healthy weight individuals, overweight ($p = 0.0365$) populations had significantly lower urinary HPMM concentrations. Unlike non-users, fruits were not a strong predictor of HPMM concentrations among exclusive combusted tobacco users. Pre-exam fasting time showed strong negative correlation ($p = 0.0003$) with urinary excretion of HPMM among tobacco smokers as well.

Since serum cotinine showed a significant positive correlation with urinary HPMM concentrations among exclusive combusted tobacco users, we also examined the relationship between the metabolite and CPD. Figure 2 shows that HPMM level increases with increasing CPD.

We further ran a multivariable regression model combining exclusive combusted tobacco users and non-users, where the variable cotinine was replaced by CPD (Table A.1). When

adjusted for survey year, urinary creatinine, age, sex, race/ethnicity, BMI, poverty level, food intake, and pre-exam fasting time, Table A.1 showed that all exclusive combusted tobacco users had significantly higher urinary HPMM levels compared with subjects with no tobacco smoke exposure (cotinine ≤ 0.05 ng/mL). Similar to Figure 2, a dose-dependent increment of slope was observed with respect to CPD. The variables, sex and BMI, followed similar trends as described in the model in Table 4 for exclusive combusted tobacco users. For example, females had higher HPMM levels than men, and overweight people had lower levels than healthy individuals. Similar to non-users (Table 3), all age groups had significantly higher HPMM levels compared with the group aged 20–39, except for the 12–19 year olds, who were not statistically different. Only non-Hispanic Blacks had significantly lower HPMM values than the non-Hispanic Whites. Fruits ($p = 0.0327$) showed significant positive correlation with HPMM levels, as seen among non-users. Likewise, pre-exam fasting time was negatively correlated with HPMM concentrations.

Additionally, crotonaldehyde is a homologue of acrolein (α,β-unsaturated aldehydes), and both are major components of cigarette smoke; thus exposure to those two aldehydes will likely be positively correlated. Similar to crotonaldehyde, the majority of absorbed acrolein is metabolized and excreted in the urine as mercapturic acid conjugates, HPMA as well as CEMA (Parent et al., 1998). Therefore, we investigated the correlations between their respective metabolites, HPMM from crotonaldehyde and HPMA and CEMA from acrolein (Figure 3). Both HPMA (coefficient = 0.81) and CEMA (coefficient = 0.63) showed strong correlations with HPMM.

## DISCUSSION

In this report, the detection rate of HPMM was 99.9% of urine samples collected from a representative sampling of the U.S. population. This finding likely reflects widespread population exposure to crotonaldehyde from endogenous sources, such as lipid peroxidation (Nair et al., 2007; Niki, 2009; Voulgaridou et al., 2011), and exogenous sources, including vehicle exhaust (Destaillats et al., 2002), diet (Feron et al., 1991), and tobacco smoke (Pazo et al., 2016).

In this first biomonitoring evaluation of crotonaldehyde exposure in the U.S. population, we find that tobacco smoke is a major source of crotonaldehyde exposure: the median value of HPMM in exclusive combusted tobacco users was five times higher than in non-users (Table 2). The percentage distribution of the population depicted a similar median shift between tobacco smokers and non-users (Figure 1). Furthermore, among exclusive combusted tobacco users, data analysis revealed a significant correlation between HPMM and serum cotinine (Table 4), and HPMM and CPD (Figure 2 and Table A.1).

As shown in Table 2, children (6–11 YO) had the highest urinary HPMM levels of non-users. This trend persisted even after adjusting for other important predictors, such as creatinine and cotinine, in the model in Table 3. This could be due to their relatively larger surface area to body weight ratio, which can lead to higher toxicant exposure dose in children compared with adults (Bearer, 1995). Another explanation is that young children have higher levels of crotonaldehyde exposure because they have higher levels of

secondhand smoke exposure compared with older age groups (CDC, 2010). It is also of note that in all three regression models (Tables 3, 4, and A.1), non-Hispanic Blacks had significantly lower HPMM concentrations compared with non-Hispanic Whites. Such differences among racial groups were also observed by Park et al. (Park et al. 2015). Additionally, female smokers had significantly higher HPMM levels than their male counterparts (Table 4). This sex-related bias could result from the sex differences in pharmacodynamics and pharmacokinetics often observed in drug metabolism (Soldin and Mattison, 2009). Another source of the differences in urine HPMM levels among different non-user subpopulations could be endogenous formation of crotonaldehyde during oxidation of lipids by reactive oxygen/nitrogen species; for example different race/ethnicities may have different rates of inflammatory conditions such as Crohn's Disease (Nair et al., 2007; Niki, 2009; Voulgaridou et al., 2011). Researchers suggested that ω-3 polyunsaturated fatty acids may be the main precursor of endogenous crotonaldehyde and its subsequently formed DNA adduct (i.e., 1,$N^2$-propano-2′-deoxyguanosine) (Pan and Chung, 2002). However, because 1,$N^2$-propano-2′-deoxyguanosine can also be generated from DNA adduct formation with two acetaldehyde molecules (Wang et al., 2000), its *in vivo* utility as an exposure biomarker for either endogenous or background crotonaldehyde remains uncertain.

BMI and income to poverty ratio variables were significant predictors of HPMM in smokers, but not in non-users (Table 4). The lack of consistency of these associations between the two models indicates that these findings may be spurious. However, obesity has previously been associated with decreased smoke exposure biomarkers in cotinine-adjusted models of smokers (Vesper et al., 2013). Obese smokers had significantly lower HPMM concentrations than healthy weight individuals (Table 4). Another predictor that was only significant in the smoker model is poverty: Tobacco smokers below the poverty level had significantly higher HPMM concentrations compared with smokers above poverty level (Table 4). This difference may be attributable to other lifestyle factors, such as usage of alcohol, medicines, and other smoked products (e.g. hookah or marijuana), which could affect crotonaldehyde exposure and the pharmacokinetic profiles of absorbed crotonaldehyde.

As described above, almost everybody in the population would have detectable levels of HPMM in their urine. In part, this could be due to the natural occurrence of crotonaldehyde in diets (Feron et al., 1991). In order to identify different dietary exposure sources of crotonaldehyde, we included several food groups in our regression model. Among different food groups, fruits showed significant positive correlation with HPMM concentrations in non-users (Table 3). This finding corroborates the existing literature listing many fruits—such as apples, guavas, grapes, strawberries and tomatoes—as natural sources of crotonaldehyde (Feron et al., 1991). In the model for exclusive combusted tobacco users, the effect of fruits was not significant, possibly because the magnitude of crotonaldehyde from fruit intake is less than the magnitude from tobacco smoke. We also evaluated the possibility that consumption of alcohol, toast, or smoked foods could affect urinary HPMM, but found no relation (data not shown). The overall relevance of dietary intake of crotonaldehyde was underscored by the finding that urinary HPMM level decreased with increasing fasting time in all models (Tables 3, 4, and A.1).

Crotonaldehyde is a homologue of acrolein, and thus is similarly formed (e.g. pyrolysis or combustion) and metabolized (e.g., formation of glutathione conjugates and DNA adducts) (Horiyama et al., 2016; Pan and Chung, 2002). Both α,β-unsaturated aldehydes are major components of cigarette smoke (Pazo et al., 2016) and could be formed endogenously as a byproduct of lipid peroxidation (Nair et al., 2007; Niki, 2009; Voulgaridou et al., 2011). As expected based on the common formation and metabolism of these aldehydes, significant correlations were found between the urinary metabolites of crotonaldehyde (HPMM) and acrolein (HPMA and CEMA).

The strengths of this study include the robust characterization of crotonaldehyde exposure (by measuring its urinary metabolite HPMM), as it examined tobacco users and non-users in a large representative sampling of the U.S. population (NHANES participants). The NHANES study is conducted as a series of surveys focusing on different population groups or health topics in a sustainable and reliable manner. Because NHANES is an ongoing program, the information collected contributes to annual estimates in topic areas included in the survey. For small population groups and less prevalent conditions and diseases, data must be accumulated over several years to provide adequate estimates. The continuous design allows increased flexibility in survey content (https://www.cdc.gov/nchs/nhanes/about_nhanes.htm). Our study focuses on the two latest available surveys surveying VOC metabolites in urine (2005–2006–2011–2012).

Our study, however, suffers from some limitations. We report on crotonaldehyde exposure as determined by metabolite quantification after *in vivo* crotonaldehyde epoxidation, followed by glutathione conjugation, rather than measurements of native crotonaldehyde. However, since crotonaldehyde is a reactive species, its native presence in biological fluids may not provide useful information about the extent of exposures. Additionally, the NHANES study is cross-sectional, where participants are selected to be representative of the U.S. population, and may occasionally not be representative. Nevertheless, the sample study size minimizes these occurrences, providing reliable estimates of environmental exposures, dietary and smoking information on the U.S. population.

## CONCLUSIONS

This report characterizes the urinary levels of HPMM in a representative sample of the U.S. population and validates tobacco smoke as a major source of crotonaldehyde exposure. Demographic variables, such as age, sex and race, showed distinct effects on crotonaldehyde exposure. Although crotonaldehyde naturally occurs in many foods, increased urinary HPMM was significantly associated only with fruit consumption among non-users, but not in exclusive combusted tobacco users, suggesting the magnitude of crotonaldehyde from fruit intake is less than the magnitude from tobacco smoke. Future work could possibly elucidate differences in urinary HPMM excretion and hence potential toxicological effects of crotonaldehyde related to different variables (e.g., age, sex, race, and diet). Additionally, analysis of urinary HPMM in future NHANES cycles will allow us to track changes in crotonaldehyde exposure pertaining to potential regulatory/policy changes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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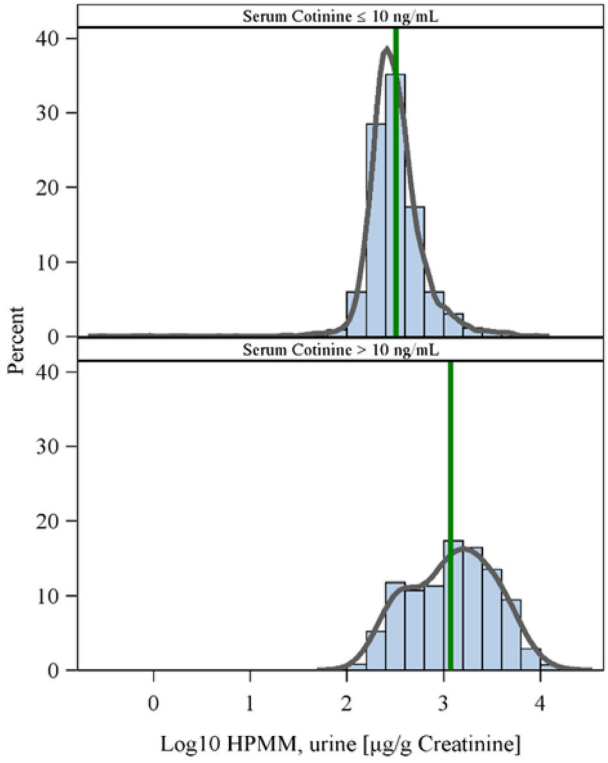



**Fig. 1.**

Percentage distribution (not sample-weighted) of urinary HPMM concentrations (µg/g creatinine) among non-users and exclusive combusted tobacco users. Urinary HPMM concentration data were log (base 10) transformed.

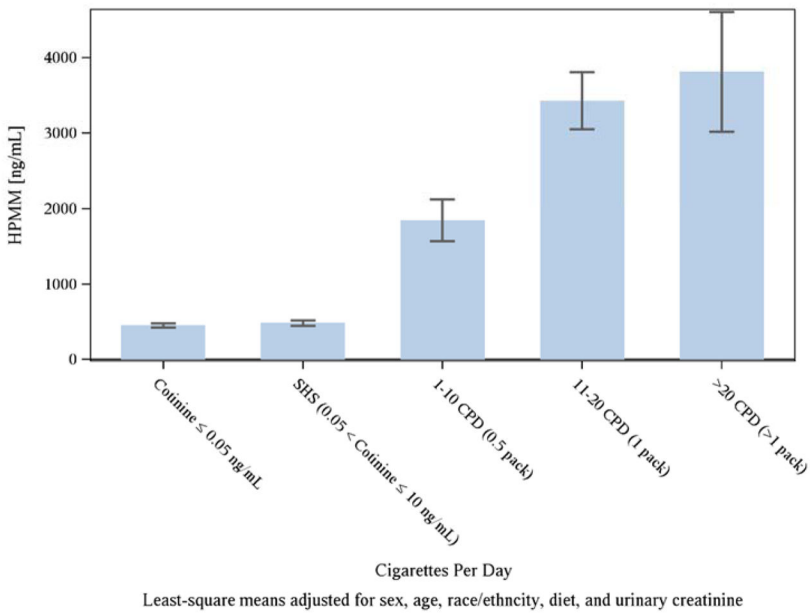
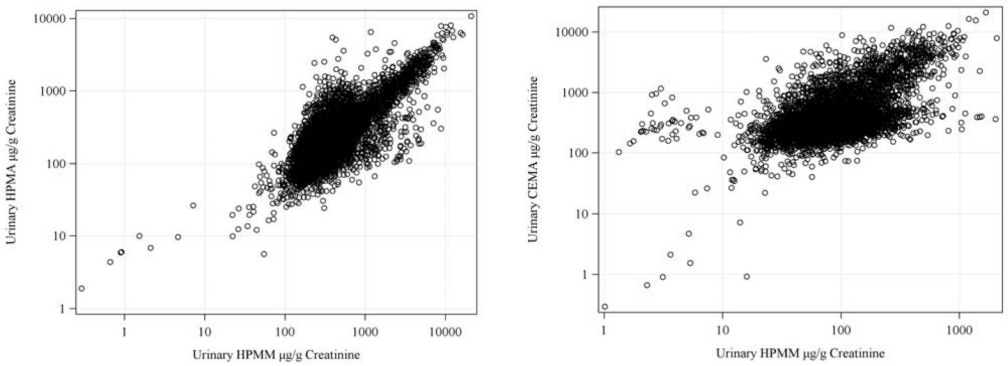



**Fig. 2.**

Least-square means of urinary HPMM concentrations for different numbers of cigarettes smoked per day (CPD) categories, adjusted for all other regression variables (e.g., age, sex, race/ethnicity, etc.).




(a) HPMA vs HPMM,
Pearson correlation coefficient = 0.81

(b) CEMA vs HPMM,
Pearson correlation coefficient = 0.63

**Fig. 3.**

Scatterplot diagrams showing correlations between HPMA and HPMM (a) & CEMA and HPMM (b). Data were adjusted for urinary creatinine and log (base 10) transformed.

**Table 1**

Sample-weighted demographic distributions of the NHANES 2005–2006 and 2011–2012 participants (sample sizes not weighted), N = 4692.

| Predictor | Level | Exclusive Combusted Tobacco Users | | Non-users | |
|---|---|---|---|---|---|
| | | Sample Size | Percent [95% CI] | Sample Size | Percent [95% CI] |
| Survey year | 2005–2006 | 569 | 55.3 [49.7:60.8] | 2,261 | 46.2 [50.8:51.7] |
| | 2011–2012 | 298 | 44.7 [39.2:50.3] | 1,564 | 53.8 [48.3:59.2] |
| Age | 6–11 | 0 | 0.0 [0.0] | 276 | 5.1 [4.2:6.1] |
| | 12–19 | 133 | 6.6 [5.4:8.0] | 1,002 | 14.0 [12.3:15.8] |
| | 20–39 | 306 | 38.5 [33.4:43.9] | 969 | 29.5 [26.7:32.3] |
| | 40–59 | 279 | 41.5 [36.7:46.4] | 757 | 30.7 [28.2:33.3] |
| | ≥ 60 | 149 | 13.4 [10.4:17.2] | 821 | 20.8 [18.0:24.0] |
| Sex | Male | 513 | 55.8 [51.3:60.3] | 1,760 | 46.6 [45.0:48.3] |
| | Female | 354 | 44.2 [39.7:48.7] | 2,065 | 53.4 [51.7:55.0] |
| Race/Ethnicity | Non-Hispanic White | 403 | 71.2 [65.1:76.7] | 1,448 | 67.8 [62.7:72.5] |
| | Mexican American | 97 | 5.4 [3.6:8.0] | 829 | 9.5 [7.2:12.3] |
| | Non-Hispanic Black | 275 | 13.6 [10.0:18.4] | 957 | 10.9 [8.1:14.5] |
| | Other Race – Including Multi-Racial | 92 | 9.7 [7.1:13.1] | 591 | 11.8 [9.8:14.1] |
| BMI | Underweight | 25 | 2.9 [1.6:5.1] | 67 | 1.4 [1.0:2.1] |
| | Healthy weight | 293 | 33.5 [28.1:39.3] | 1,468 | 35.4 [32.1:38.9] |
| | Overweight/Obese | 549 | 63.6 [58.3:68.6] | 2,290 | 63.1 [59.5:66.7] |
| Below Poverty Threshold | No | 621 | 80.1 [75.9:83.8] | 2,972 | 87.0 [83.9:89.6] |
| | Yes | 246 | 19.9 [16.2:24.1] | 853 | 13.0 [10.4:16.1] |

CI: Confidence interval; BMI: Body mass index.

## Table 2

Sample-weighted median ($25^{th}$, $75^{th}$ percentile) urinary HPMM concentrations (mg/g creatinine).

| Variable | Level | Exclusive Combusted Tobacco Users | Non-Users |
|---|---|---|---|
| | All | 1.63 [0.680, 3.29] | 0.313 [0.231, 0.451] |
| Age (yr) | 6–11 | N/A | 0.423 [0.324, 0.511] |
| | 12–19 | 0.607 [0.398, 1.32] | 0.259 [0.204, 0.350] |
| | 20–39 | 1.17 [0.552, 2.16] | 0.275 [0.211, 0.400] |
| | 40–59 | 2.25 [0.936, 4.04] | 0.329 [0.241, 0.479] |
| | ≥ 60 | 2.24 [1.12, 4.09] | 0.375 [0.277, 0.542] |
| Sex | Male | 1.28 [0.580, 2.62] | 0.290 [0.218, 0.414] |
| | Female | 2.03 [0.910, 3.92] | 0.332 [0.245, 0.484] |
| Race/Ethnicity | Non-Hispanic White | 1.88 [0.836, 3.62] | 0.330 [0.239, 0.476] |
| | Mexican American | 0.694 [0.369, 1.82] | 0.306 [0.227, 0.423] |
| | Non-Hispanic Black | 1.07 [0.489, 1.87] | 0.253 [0.195, 0.356] |
| | Other Race - Including Multi-Racial | 1.44 [0.394, 2.61] | 0.303 [0.236, 0.459] |
| BMI | Underweight | 1.73 [1.09, 4.28] | 0.385 [0.249, 0.444] |
| | Healthy weight | 1.97 [0.863, 3.45] | 0.323 [0.235, 0.486] |
| | Overweight/Obese | 1.44 [0.626, 3.02] | 0.306 [0.227, 0.426] |
| Poverty Status | No | 1.57 [0.655, 3.31] | 0.316 [0.232, 0.453] |
| | Yes | 1.67 [0.906, 3.20] | 0.297 [0.227, 0.434] |

BMI: Body mass index

## Table 3

Sample-weighted multiple regression slopes for urinary HPMM concentrations among non-users (N = 3825). The *p*-value was estimated from identical models, where the dependent variable was natural log-transformed.

| Predictor | Level | Slope [95% CI] | *p*-Value |
|---|---|---|---|
| Intercept | | 84.18 [−35.44:203.80] | < 0.0001 |
| NHANES Cycle | 2005–2006 | −96.50 [−143.71: −49.29] | < 0.0001 |
| | 2011–2012 | Ref. | |
| Creatinine, urine [mg/dL] | Slope | 3.47 [3.08:3.87] | < 0.0001 |
| Cotinine, serum [ng/mL] | Slope | 18.52 [−3.53:40.58] | 0.0823 |
| Age (yr) | 6–11 | 109.22 [22.28:196.16] | 0.0007 |
| | 12–19 | 0.37 [−66.98:67.72] | 0.5339 |
| | 20–39 | Ref. | |
| | 40–59 | 120.89 [44.08:197.71] | 0.0002 |
| | ≥ 60 | 211.67 [147.82:275.52] | < 0.0001 |
| Sex | Male | Ref. | |
| | Female | 62.49 [13.42:111.55] | 0.0616 |
| Race/Ethnicity | Non-Hispanic White | Ref. | |
| | Mexican American | 72.52 [5.07:139.97] | 0.1313 |
| | Non-Hispanic Black | −115.88 [−167.27: −64.49] | < 0.0001 |
| | Other Race - Including Multi-Racial | 2.07 [−56.25:60.39] | 0.6281 |
| BMI | Underweight | 125.80 [−195.61:447.21] | 0.5463 |
| | Healthy weight | Ref. | |
| | Overweight/Obese | −23.22 [−59.77:13.33] | 0.1618 |
| Poverty Status | No | Ref. | |
| | Yes | 57.85 [−20.89:136.59] | 0.6539 |
| Milk Products [kg] | Slope | −19.18 [−141.52:103.15] | 0.9556 |
| Meat, Poultry [kg] | Slope | −27.80 [−114.76:59.17] | 0.8175 |
| Eggs [kg] | Slope | −87.83 [−490.49:314.83] | 0.8060 |
| Legumes, Nuts, Seeds [kg] | Slope | 70.68 [−75.28:216.64] | 0.2989 |
| Grain Products [kg] | Slope | 57.50 [−6.55:121.55] | 0.0952 |
| Fruits [kg] | Slope | 136.79 [71.09:202.49] | 0.0014 |
| Vegetables [kg] | Slope | −12.26 [−109.85:85.33] | 0.7297 |
| Fats, Oils, Salad Dressings [kg] | Slope | −107.74 [−929.65:714.17] | 0.6119 |
| Sugars, Sweets, Beverages [kg] | Slope | 3.66 [−8.17:15.49] | 0.3400 |
| Pre-exam Fasting Time [hr] | Slope | −18.91 [−22.49: −15.33] | < 0.0001 |

CI: Confidence interval; BMI: Body mass index

## Table 4

Sample-weighted multiple regression slopes for urinary HPMM concentrations among exclusive combusted tobacco users (N=867). The *p*-value was estimated from identical models, where the dependent variable was natural log-transformed.

| Predictor | Level | Slope [95% CI] | *p*-Value |
|---|---|---|---|
| Intercept | | −377.44 [−1378.76:623.88] | < 0.0001 |
| NHANES Cycle | 2005–2006 | −42.51 [−359.69:274.67] | 0.4540 |
| | 2011–2012 | Ref. | |
| Creatinine, urine [mg/dL] | Slope | 15.47 [12.37:18.56] | < 0.0001 |
| Cotinine, serum [ng/mL] | Slope | 4.49 [1.80:7.17] | 0.0014 |
| Age (yr) | 6–11 | N/A | |
| | 12–19 | −508.62 [−1109.69:92.45] | 0.0048 |
| | 20–39 | Ref. | |
| | 40–59 | 965.23 [672.74:1257.71] | 0.0001 |
| | ≥ 60 | 1248.30 [442.67:2053.93] | 0.0003 |
| Sex | Male | Ref. | |
| | Female | 553.52 [119.90:987.14] | 0.0135 |
| Race/Ethnicity | Non-Hispanic White | Ref. | |
| | Mexican American | −674.53 [−1206.22: −142.84] | 0.0104 |
| | Non-Hispanic Black | −843.50 [−1227.95: −459.05] | < 0.0001 |
| | Other Race - Including Multi-Racial | −111.66 [−1174.11:950.80] | 0.2247 |
| BMI | Underweight | 1034.52 [−352.87:2421.92] | 0.1140 |
| | Healthy weight | Ref. | |
| | Overweight/Obese | −317.85 [−623.53: −12.16] | 0.0365 |
| Poverty Status | No | Ref. | |
| | Yes | 359.93 [−32.77:752.64] | 0.0007 |
| Milk Products [kg] | Slope | −43.87 [−482.02:394.29] | 0.9142 |
| Meat, Poultry [kg] | Slope | −391.24 [−1214.25:431.77] | 0.3726 |
| Eggs [kg] | Slope | −419.65 [−4193.11:3353.82] | 0.1078 |
| Legumes, Nuts, Seeds [kg] | Slope | −523.58 [−3164.64:2117.48] | 0.4399 |
| Grain Products [kg] | Slope | −62.57 [−783.57:658.43] | 0.1558 |
| Fruits [kg] | Slope | −403.66 [−903.36:96.04] | 0.0582 |
| Vegetables [kg] | Slope | −783.27 [−1769.90:203.35] | 0.1181 |
| Fats, Oils, Salad Dressings [kg] | Slope | −4712.55 [−10261.80:836.69] | 0.3549 |
| Sugars, Sweets, Beverages [kg] | Slope | 60.88 [−38.93:160.68] | 0.8801 |
| Pre-exam Fasting Time [hr] | Slope | −61.88 [−89.67: −34.09] | 0.0003 |

CI: Confidence interval; BMI: Body mass index

# Carcinogenicity of acrolein, crotonaldehyde, and arecoline

In October–November, 2020, a Working Group of 20 scientists from ten countries met remotely at the invitation of the International Agency for Research on Cancer (IARC) to finalise their evaluations of the carcinogenicity of acrolein, crotonaldehyde, and arecoline. Acrolein was classified as "probably carcinogenic to humans" (Group 2A) on the basis of "sufficient" evidence of carcinogenicity in experimental animals and "strong" mechanistic evidence. Crotonaldehyde and arecoline were classified as "possibly carcinogenic to humans" (Group 2B) on the basis of "strong" mechanistic evidence. For all three agents, the evidence regarding cancer in humans was "inadequate"; no data were available for arecoline, and the few available studies of cancer in humans for acrolein and crotonaldehyde were generally small or uninformative. These assessments will be published in volume 128 of the IARC Monographs.[1]

Acrolein is a high-production-volume chemical used in the manufacture of numerous chemical products, and as a herbicide in recirculating water systems. Tobacco smoke is a major source of acrolein exposure in the general population. Other sources of exposure include emissions from combustion of fuels, wood, and plastics, and from ambient air pollution and electronic cigarette vapour. Acrolein is generated in kitchens during high-temperature roasting and deep-fat frying. Acrolein is also formed endogenously. Firefighters are exposed occupationally. Acrolein reacts with glutathione and is primarily excreted in urine as 3-hydroxypropylmercapturic acid and 2-carboxyethylmercapturic acid. The carcinogenicity of inhaled acrolein was shown in two rodent species. It induced malignant lymphoma in female B6D2F$_1$/Crlj mice,[2] and increased the incidence of rare nasal cavity rhabdomyoma and squamous cell carcinoma combined in female F344/DuCrlCrlj rats.[3] A strongly electrophilic α,β-unsaturated aldehyde (enal), acrolein readily forms DNA adducts including the cyclic adducts α- and γ-hydroxy-1,$N^2$-propanodeoxyguanosine. γ-Hydroxy-1,$N^2$-propanodeoxyguanosine has been detected in DNA from various human biospecimens, including lung, liver, brain, urothelial mucosa, and saliva.[4,5] Elevated levels of these DNA adducts are seen in people who smoke tobacco[4] and under chronic inflammatory conditions. In acrolein-treated human lung cells, acrolein–DNA adducts are preferentially formed at *TP53* mutational hotspots for lung cancer.[6] In human primary cells, acrolein induces DNA strand breaks and DNA–protein cross-links. Across many in-vitro experimental systems, acrolein is genotoxic, inducing DNA strand breaks, DNA–protein cross-links, mutations, and chromosomal damage. The mutagenicity of acrolein has also been shown in experiments with plasmid DNA. In addition, acrolein directly inhibits proteins in three major DNA-repair pathways, inducing concentration-dependent inhibition of nucleotide excision repair,[6] base excision repair, and mismatch repair in human primary cells. Furthermore, acrolein induces oxidative stress markers in vitro and in vivo, and increases 8-hydroxy-2′-deoxyguanosine in rodent lung DNA. It also induces chronic inflammation in rodents.[2,3] Acrolein is immunosuppressive, altering bacterial-induced mortality, bactericidal activity, and innate immune function in exposed rodents. Acrolein alters cell proliferation, cell death, or nutrient supply, inhibiting tumour suppressor genes and activating proto-oncogenes in cultured human and rodent cells, and inducing hyperplasia and metaplasia in the rodent respiratory system.[2,3] Overall, there is "strong" evidence that acrolein exhibits multiple key characteristics of carcinogens, primarily from studies with human primary cells and studies in experimental systems, supported by studies in humans on DNA adducts.

Crotonaldehyde is a high-production-volume chemical that is widely used for synthesizing chemical agents used in the pharmaceutical, rubber, chemical, and leather industries, as well as in food production and agriculture. Tobacco smoke is a major source of crotonaldehyde exposure in the general population. Crotonaldehyde is also formed during combustion of vehicle fuels and wood, and in thermal treatment of foodstuffs. It is found in cooking fires, ambient air pollution, electronic cigarette vapour, some foods and heated cooking oils, and it is also formed endogenously. Occupational exposures to crotonaldehyde occur among firefighters, coke-oven workers, and workers in aldehyde manufacture, garages, and toll booths. Crotonaldehyde is efficiently conjugated with glutathione and is primarily excreted in urine as 3-hydroxy-1-methylpropylmercapturic acid and 2-carboxy-1-methylethylmercapturic acid. There is "limited" evidence in experimental animals for the carcinogenicity of crotonaldehyde. Crotonaldehyde administered in drinking water increased the incidence of hepatocellular adenoma and carcinoma combined in one sex of one species, the male F344 rat.[7] It also induced benign tumours of the nasal cavity in male F344/DuCrj rats exposed by inhalation. An electrophilic α,β-unsaturated aldehyde (enal), crotonaldehyde forms cyclic adducts in DNA as well as DNA interstrand and DNA–protein cross-links. α-Methyl-γ-hydroxy-1,$N^2$-propanodeoxyguanosine has been detected in human saliva, urine, blood, mammary tissue, oral (gingival) tissue, liver, and placenta.[4,5] Adduct levels were significantly elevated in tobacco smokers.[4] Crotonaldehyde-derived DNA adducts have also been detected in human cells in vitro and in rodents. Crotonaldehyde is genotoxic,

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exhibiting clastogenicity in human primary cells and human cell lines,[8] dominant lethality and chromosomal aberrations in rodents, and gene mutations in cultured rodent cells, *Drosophila melanogaster*, *Salmonella typhimurium*, and plasmid systems. In addition, crotonaldehyde induces oxidative stress in human endothelial and bronchial epithelial cells and in the lungs of rats. Crotonaldehyde also induces chronic inflammation in the nasal respiratory epithelium in rats and mice. Overall, there is "strong" evidence that crotonaldehyde exhibits multiple key characteristics of carcinogens, from studies in human primary cells and in various experimental systems, supported by studies in humans on DNA adducts.

Arecoline is the primary active ingredient of the areca nut, which is "carcinogenic to humans" (Group 1).[9] At least 10% of the global population, primarily in south-eastern Asia, chew areca nut for its mild psychoactive effects.[9] Arecoline has been used medicinally as an anthelmintic and is still applied in the form of areca nut preparation in traditional Chinese and Ayurveda medicines. Arecoline is readily absorbed and can be detected in the saliva, blood, urine, hair, and breast milk of people who chew areca nut. It is rapidly metabolised by human flavin-containing monooxygenases and is excreted as mercapturic acids. *N*-Nitrosamines, a class of carcinogenic agents that are known to be metabolically activated to alkylating agents, are formed by the reaction of arecoline with sodium nitrite and have been identified in the saliva of people who chew areca nut. There is "limited" evidence for the carcinogenicity of arecoline in experimental animals. In two gavage studies in mice, arecoline increased the incidence of total tumours.[9] In co-carcinogenicity studies, arecoline induced malignant oesophageal tumours in mice and benign oesophageal and tongue tumours in rats. There is "strong" evidence from studies in human primary cells and various experimental systems that arecoline exhibits key characteristics of carcinogens. Arecoline is an electrophilic α,β-unsaturated ester. It is genotoxic, inducing DNA strand breaks, micronucleus formation, chromosomal aberrations, and sister-chromatid exchanges in human primary and cultured cells.[10,11] Arecoline induces chromosomal damage in other experimental systems both in vitro and in vivo, and induces gene mutations in vitro in mammalian cells and in bacteria. Arecoline alters the mutation spectrum in a transgenic mouse mutation assay. The arecoline metabolites arecaidine and arecoline-*N*-oxide are also genotoxic. In human cell lines in vitro, arecoline alters DNA repair.[11] Arecoline also induces oxidative stress in human primary cells, and in various experimental systems.

We declare no competing interests.

*IARC Monographs Vol 128 group*

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# WHO launches strategy to accelerate elimination of cervical cancer

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On Nov 17, 2020, WHO launched a global initiative to accelerate the elimination of cervical cancer, and set up an historical milestone—through adoption of a resolution by 194 countries at the World Health Assembly—that pledged, for the first time, to eliminate a malignant disease by pursuing three important steps: vaccination, screening, and treatment.

By 2050, 40% of new cervical cancer cases and 5 million related deaths could be prevented with successful implementation of vaccination, screening, and treatment of the disease, according to WHO at the launch of the Global Strategy to Accelerate the Elimination of Cervical Cancer initiative. Cervical cancer is the fourth most common malignancy in women worldwide, with an incidence that is approximately two-times higher in low-income

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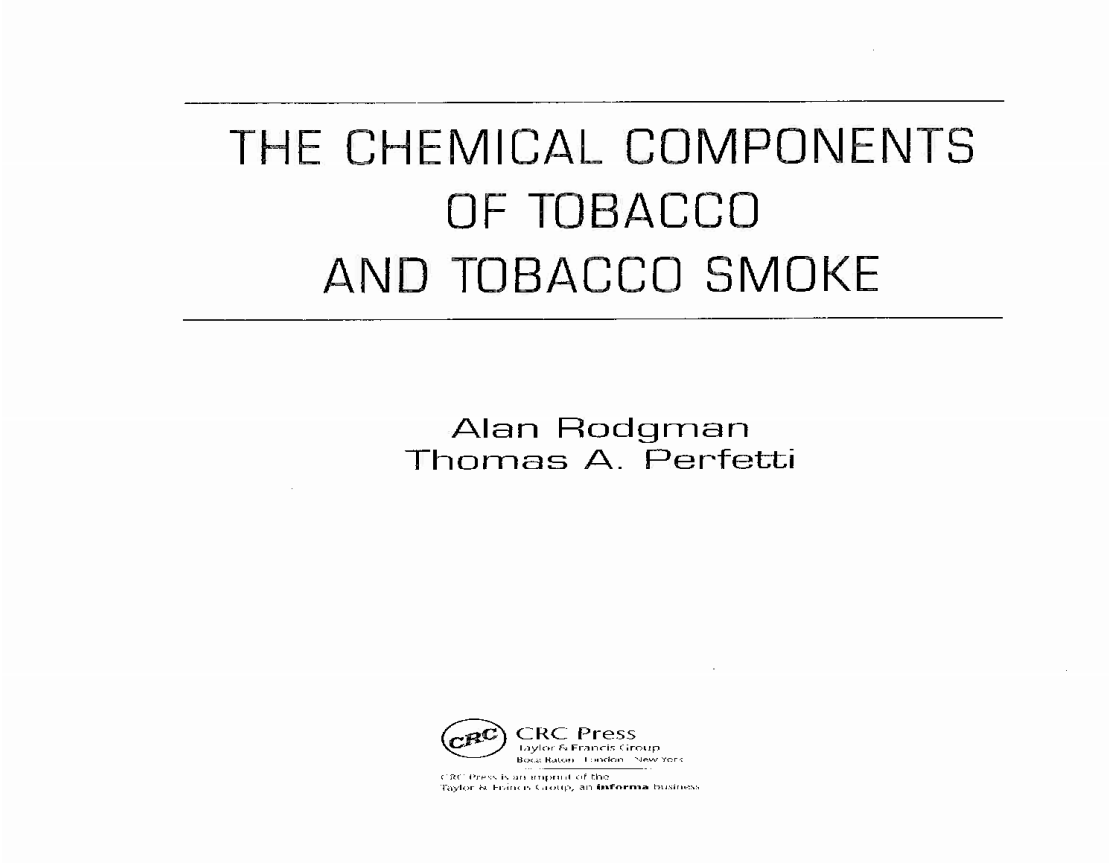

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OF TOBACCO
AND TOBACCO SMOKE

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