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Research Article

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Variation in Levels of the Lung Carcinogen NNAL and Its Glucuronides in the Urine of Cigarette Smokers from Five Ethnic Groups with Differing Risks for Lung Cancer

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Abstract

Background: Results of the Multiethnic Cohort (MEC) study demonstrated that, for the same quantity of cigarettes smoked, African Americans and Native Hawaiians have a higher risk of lung cancer compared with whites, whereas Latinos and Japanese Americans have a lower risk. We hypothesize that the uptake and/or metabolism of the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) could explain the differences in lung cancer risk.

Methods: We measured urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides and their sum (total NNAL), biomarkers of NNK uptake, in 2,252 smokers from the MEC. Ethnic-specific geometric means were compared adjusting for age at urine collection, sex, creatinine and total nicotine equivalents, a marker of total nicotine uptake.

Results: African Americans had the highest median total NNAL levels (1.80 pmol/mL urine) and Japanese Americans had the lowest (0.914 pmol/mL urine), with intermediate values in the other three groups. Geometric mean of total NNAL in African Americans was also highest, and in Japanese Americans it was lowest; Japanese American geometric mean was statistically different from whites ($P = 0.004$).

Conclusions: African Americans had higher levels of total NNAL per mL urine than whites, while Japanese Americans had lower levels, consistent with lung cancer risk among smokers in these groups. However, our data were not consistent with the high and low lung cancer risks of Native Hawaiian and Latino smokers, respectively.

Impact: The higher lung cancer susceptibility of African-American smokers and the lower susceptibility of Japanese-American smokers compared with whites can be explained in part by exposure to the potent lung carcinogen NNK. *Cancer Epidemiol Biomarkers Prev; 24(3); 561–9. ©2014 AACR.*

Introduction

Ethnic and racial differences in the risk of lung cancer due to smoking have been consistently observed in epidemiologic studies. In one major prospective study, differences in the risk of lung cancer due to cigarette smoking were investigated among self-identified African American, Native Hawaiian, white, Latino, and Japanese-American men and women in the Multiethnic Cohort (MEC; ref. 1). For the same quantity of cigarettes smoked, African Americans and Native Hawaiians were more susceptible to lung cancer than whites, while Japanese Americans and Latinos were less susceptible. These differences were observed for all histologic types of lung cancer and in both men and women, and were most evident at lower numbers of cigarettes smoked per day. Such relationships were not evident in nonsmokers. These results are consistent with the SEER data and with a number of earlier studies which examined ethnic and racial differences in lung cancer incidence (2–12).

Genetic factors that could influence both extents of smoking and lung cancer risk have been extensively investigated. Meta-analyses of the association between smoking quantity and multiple genetic variants on chromosome 15q25 have been conducted. Distinct genes in the *CHRNA5-CHRNA3-CHRNB4* region were associated with quantity smoked in people of European ancestry, Asians, and African Americans (13). Other studies have investigated ethnic differences in polymorphisms in genes involved in carcinogen metabolism such as *CYP1A1* (14), *CYP1B1* (15), and *GSTM1* (16), and in DNA repair (17) and DNA damage signaling (18), with respect to lung cancer incidence. Socioeconomic aspects of the observed ethnic differences have also been investigated (19–21). A clear explanation for the observations cited above in the MEC has not yet emerged from these studies although it is recognized that some of these factors likely contribute.

Our working hypothesis is that there are ethnic and racial differences in the uptake and/or metabolism of tobacco smoke carcinogens that could explain the differences in lung cancer risk. In the study reported here, we examined this hypothesis with respect to metabolites of the tobacco-specific lung carcinogen

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4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK is a powerful systemic pulmonary carcinogen in laboratory animals and is considered, together with the related compound *N*′-nitrosonornicotine (NNN), "carcinogenic to humans" by the International Agency for Research on Cancer (22). NNK is metabolized to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in laboratory animals and in humans. NNAL and its glucuronides (NNAL-Glucs, the sum of NNAL-*O*-Gluc and NNAL-*N*-Gluc) are excreted in urine and have been widely used as biomarkers of NNK uptake in smokers (23–28). All smokers have NNAL and NNAL-Glucs (the sum of which is termed total NNAL) in their urine. NNAL has carcinogenic activity similar to that of NNK, whereas NNAL-Glucs are considered to be noncarcinogenic detoxification products (29, 30). The ratio NNAL-Glucs:NNAL has been proposed as a marker of NNK detoxification potential (31).

The relationship of tobacco smoke NNK to urinary total NNAL has been studied. One report demonstrated a significant relationship between mouth level exposure to NNK, assessed by analysis of cigarette butts, and urinary total NNAL in smokers from Australia, Canada, the United Kingdom, and the United States, where popular cigarette brands have different levels of NNK in their smoke (32). Population-based studies have demonstrated lower levels of total NNAL in the urine of smokers in Canada than in the United States, consistent with the lower levels of NNK in Canadian cigarettes than in popular U.S. cigarettes (26, 33). Chinese cigarette smokers also have lower total NNAL levels in their urine than smokers in the United States, because levels of NNK in the smoke of Chinese cigarettes are lower than in popular American blended cigarettes (34–36). These large studies all demonstrate that NNK levels in cigarette smoke are consistent with NNAL levels in urine, although one small study found the opposite (37). The relationship of total NNAL to lung cancer risk in smokers has been demonstrated in three nested case–control studies within prospective cohorts in which urine samples were collected years before lung cancer occurred and frozen for later analysis (34, 38, 39). Collectively, these results demonstrate that urinary total NNAL is a reliable measure of NNK exposure and most likely of lung cancer risk in smokers.

Several published studies have examined possible racial differences in uptake and metabolism of NNK in smokers, as reflected by urinary levels of NNAL and NNAL-Glucs. Richie and colleagues and Muscat and colleagues quantified free NNAL and NNAL-Glucs in the urine of 162 African American and white smokers who lived in a suburban New York community (31, 40). Overall, there were no significant differences in free NNAL, NNAL-Glucs, or total NNAL between the racial groups. The ratio NNAL-Glucs:NNAL was significantly higher in white than in African-American women. A significantly higher NNAL-Glucs:NNAL ratio in whites than in African Americans was also reported by Sarkar and colleagues in a study of 3,203 smokers in the tobacco industry-sponsored "Total Exposure Study" (41). The latter study also observed significantly higher levels of total NNAL per day in whites than African Americans (2.22 vs. 1.64 nmol/24 hours), but levels of total NNAL per cigarette were significantly higher in African Americans than in whites (27). Xia and colleagues examined total NNAL levels in the NHANES study of 1373 tobacco users, mainly smokers (26). Total NNAL, expressed per mL or per mg creatinine, was significantly higher in whites than African Americans, and total NNAL per cigarette was significantly higher in African Americans than in whites. Benowitz and colleagues (42), in a study of 128 smokers, found significantly higher total NNAL per mg creatinine in whites than African Americans (1.2 vs. 0.9 pmol/mg creatinine), but no differences in total NNAL per cigarette. An earlier study by our group compared total NNAL in whites, Native Hawaiians, and Japanese Americans (43). Significantly higher levels were found in whites than in either of the other groups, whereas the ratio NNAL-Gluc:NNAL was significantly lower in Japanese Americans and Native Hawaiians than in European Americans.

In this study, we used a validated liquid chromatography–tandem mass spectrometry (LC/MS-MS) method (28) to analyze NNAL and NNAL-Glucs in urine samples from 357 African Americans, 325 Native Hawaiians, 434 whites, 442 Latinos, and 694 Japanese Americans.

Materials and Methods

Subjects

This study was approved by the Institutional Review Boards of the University of Southern California (Los Angeles, CA), the University of Hawaii (Honolulu, HI), and the University of Minnesota (Minneapolis, MN). Subjects were participants in the MEC, a prospective cohort study established to investigate the association of lifestyle and genetic factors with chronic diseases in a multiethnic population (44). Briefly, the cohort is comprised of 215,251 men and women between the ages of 45 to 75 at baseline, primarily belonging to five ethnic/racial groups: African Americans, Japanese Americans, Latinos, Native Hawaiians, and whites. Between 1993 and 1996, potential participants were identified in Hawaii and California (primarily Los Angeles County) through drivers' license files, voter registration lists, and Health Care Financing Administration files. Each participant completed a mailed, self-administered questionnaire regarding demographic, dietary, lifestyle, smoking history, and other exposure factors.

This specific study comprises a subgroup of the MEC participants who were cancer-free current smokers at the time of urine collection. Approximately 10 years after cohort entry, 2,393 current smokers with no cancer diagnosis participated in the MEC biospecimen subcohort by providing a blood sample and overnight (subjects recruited in Hawaii) or first morning urine (subjects recruited in California) and completing an epidemiologic questionnaire that included a history of daily cigarette smoking during the past 2 weeks, smoking duration, and a record of current medications. The overnight urine collection started between 5 and 9 pm (depending on the subject) and included all urine passed during the night as well as the first morning urine. All urine was kept on ice until processing. Aliquots were subsequently stored in a −80°C freezer until analysis. The overnight or first morning urine was used to measure nicotine and NNK metabolites. In a pilot study of 10 smokers who collected their urine overnight as in this study, we compared urinary total NNAL levels measured from first morning urine versus overnight urine. We found that total NNAL per creatinine was comparable between the two types of urine samples.

The subjects in this study were almost certainly daily smokers because they reported the average number of cigarettes smoked per day during the past 2 weeks; also, they were excluded if their total nicotine equivalent levels were below 1.27 nmol/mL (see below).

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Analysis of total NNAL and free NNAL

The LC/MS-MS method and its validation parameters have been described in detail (28). Briefly, 80 μL of urine was used for the "free NNAL" assay and 40 μL for the "total NNAL" assay. To each aliquot was added 0.1 pmol [$^{13}C_6$]NNAL as internal standard.

The "total NNAL" aliquots were incubated with β-glucuronidase after which the remaining steps were identical for both aliquots. Partial purification was accomplished by solid-phase extractions on 96-well plates. These steps were followed by LC/MS-MS analysis with selected reaction monitoring for m/z 210.12 → m/z 93.14 for NNAL quantitation and m/z 216.14 → m/z 98.14 for [$^{13}C_6$]NNAL quantitation. Amounts of NNAL-Glucs (the sum of NNAL-*O*-Gluc and NNAL-*N*-Gluc) were determined by subtraction of free NNAL from total NNAL.

The analysts were blinded to the origin of all urine samples. Among the 2,252 study samples were 220 undiluted urine samples (10 aliquots each from 22 volunteers outside of the MEC). The coefficients of variation (CV) across all duplicates calculated from these samples were 16.2% for total NNAL and 16.6% for free NNAL. The CVs for duplicates within the same batch were 8.4% and 9.7%, respectively. The interclass correlation across the 220 duplicate samples was 99% with a 95% CI: 98%–99%.

For this study, we included samples with total nicotine equivalents (the sum of nicotine, cotinine, 3′-hydroxycotinine and their glucuronides, and nicotine-*N*-oxide) >1.27 nmol/mL (4 times the limit of quantitation), determined by LC/MS-MS (45), in which total NNAL was detected. Thus, 2,252 samples were retained for analysis. Among these 2,252 samples, 2,174 subjects had free NNAL measured. Three of the 2,174 subjects had free NNAL values greater than their total NNAL levels. Two of these 3 subjects had free NNAL values greater than 20% of their total NNAL levels and therefore their free NNAL was coded as missing. The one subject with free NNAL less than 20% more than total NNAL was considered to be within the margin of error and was recoded as having a free NNAL measure of 0 pmol/mL. Subsequently, for the statistical analyses, free NNAL data were available for 2,172 participants.

Creatinine (Cr) analysis

Cr was analyzed using a colorimetric microplate assay (CRE34-K01) purchased from Eagle Bioscience (http://stores.eaglebio.com/creatinine-microplate-assay-kit).

Statistical analysis

Total NNAL and free NNAL concentrations in urine were calculated in pmol/mL and pmol/Cr. In the latter, total and free NNAL (pmol/mL) were divided by Cr (mg/dL) and multiplied by 100. NNAL glucuronidation ratio was calculated from (total NNAL − free NNAL)/free NNAL.

Among the subjects retained for this analysis, 11 participants were missing BMI and 42 participants had missing values for cigarettes per day at the time of urine collection. The missing values were imputed by the Markov Chain Monte Carlo method using the PROC MI statement from the SAS v9.2 software (SAS institute; ref. 46). Imputed values were based on age of cohort entry, race/ethnicity, time between cohort entry and time of blood draw, and BMI at baseline and at blood draw (for missing BMI), or number of cigarettes per day at baseline and at blood draw and smoking duration (for missing cigarettes per day). Ten datasets were created for the imputed missing values. The mean value across all 10 datasets was used to replace the missing value.

In initial analyses, results were stratified by sex; however, patterns of NNAL distributions by race/ethnicity did not differ and therefore the results have been combined for ease of presentation; the stratified results are presented in the Supplementary Tables. Pearson partial correlation coefficients, adjusting for age, sex and race/ethnicity, and Cr levels (natural log), were computed to examine the correlation of total and free NNAL with measures of smoking (cigarettes per day and total nicotine equivalents). To compare the distributions of total NNAL and free NNAL levels across race/ethnicity, the Wilcoxon–Mann–Whitney test was employed. To examine ethnic/racial differences for measures of NNAL and NNAL glucuronidation ratio, the covariate-adjusted geometric means were computed for each ethnic/racial group at the mean covariate vector. We used two multivariable linear models: (i) adjusted for the following predictors: age at time of urine collection (continuous), sex, race, and creatinine levels (natural log) and (ii) additionally adjusted for total nicotine equivalents. Values of all metabolites were transformed by taking the natural log to better meet model assumptions. In instances where the metabolite value was transformed, values presented in the tables are back-transformed as geometric means to their natural scale for ease of interpretation.

Results

Characteristics of the study population are summarized in Table 1. Cr levels, age and BMI at time of urine collection differed by race/ethnicity ($P < 0.05$), with the exception of Japanese Americans in whom age at urine collection and Cr were not statistically different from those of whites ($P \geq 0.32$). Median number of cigarettes smoked per day ranged from 8 (Latinos) to 20 (whites) while total nicotine equivalents ranged from 27.3 (Japanese Americans) to 44.7 (African Americans) (45). The distribution of these measures of smoking differed across race/ethnicity ($P < 0.05$), with the exception of Latinos who had a similar level of total nicotine equivalents to that of whites ($P = 0.12$). The distribution of smoking by race/ethnicity was similar in males and females; whites reported smoking the highest number of cigarettes per day and Latinos the least (Supplementary Table S1). When quantity of smoking was measured by total nicotine equivalents, African Americans had the highest and Japanese Americans had the lowest levels (Table 1). Other characteristics of the study population are summarized in Table 1 with stratification by sex in Supplementary Table S1.

Total NNAL ($r = 0.70$, $P < 0.0001$) and free NNAL ($r = 0.60$, $P < 0.0001$) were highly correlated with total nicotine equivalents measured in the same samples (45). The ethnic-specific correlations between total NNAL and total nicotine equivalents ranged from $r = 0.64$ (African Americans) to $r = 0.77$ (Latinos), while those for free NNAL ranged from $r = 0.49$ (African Americans) to $r = 0.69$ (whites).

Median values and ranges of total NNAL (pmol/mL urine), free NNAL (pmol/mL urine), and NNAL-glucuronidation ratio are presented in Table 2 and Fig. 1A (for total NNAL in natural log scale) for the five ethnic groups. The highest level of total NNAL was found in African Americans (1.80 pmol/mL urine) and the lowest in Japanese Americans (0.914 pmol/mL urine), with intermediate values in the other three groups. Levels of total NNAL in African Americans were significantly greater than in whites ($P < 0.001$) while in Japanese Americans levels of total NNAL were significantly less than in whites ($P < 0.001$). Levels of

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Table 1. Main characteristics for study participants stratified by race/ethnicity[a]

	African Americans ($n = 357$) N (%)	Native Hawaiians ($n = 325$) N (%)	Whites ($n = 434$) N (%)	Latinos ($n = 442$) N (%)	Japanese Americans ($n = 694$) N (%)
Sex					
Males	109 (30.5)	118 (36.3)	191 (44.0)	231 (52.1)	396 (57.1)
Females	248 (69.5)	207 (63.7)	243 (56.0)	211 (47.9)	298 (42.9)
Median (interquartile)					
Age (years)	64.0 (59.0–69.0)	60.0 (56.0–65.0)	62.0 (58.0–68.0)	65.0 (61.0–70.0)	62.0 (58.0–69.0)
BMI (kg/m^2)	27.0 (23.4–30.9)	26.8 (24.1–30.8)	24.8 (22.0–28.2)	26.5 (24.0–29.9)	24.4 (21.9–27.0)
Cr (mg/dL)	90.0 (55.0–141)	60.0 (38.0–91.0)	54.0 (33.0–86.0)	77.0 (50.0–117)	56.0 (35.0–89.0)
Age at smoking initiation	24.5 (19.5–30.5)	21.5 (17.5–27.5)	21.5 (18.5–26.5)	23.5 (19.5–30.0)	22.0 (18.5–26.5)
Duration of smoking (years)	37.5 (34.5–46.5)	37.5 (33.5–46.5)	43.5 (35.5–46.5)	43.5 (34.5–48.0)	42.5 (35.5–46.5)
Cigarettes per day	10.0 (6.00–15.0)	15.0 (9.00–20.0)	20.0 (10.0–20.0)	8.00 (4.00–12.0)	12.0 (10.0–20.0)
Total nicotine equivalents (nmol/mL)	44.7 (28.3–74.1)	31.3 (20.2–48.0)	36.9 (22.2–61.7)	33.1 (21.6–54.0)	27.3 (16.1–43.5)
Ethanol (g/day)	1.87 (0.00–15.5)	1.47 (0.00–10.3)	6.55 (0.00–25.9)	1.28 (0.00–13.1)	0.392 (0.00–12.3)
Total vegetable (g/kcal/day)	124 (88.8–172)	133 (93.4–167)	136 (100–180)	150 (112–206)	122 (91.4–165)
Total fruit (g/kcal/day)	107 (50.7–186)	74.7 (38.1–135)	86.9 (41.1–172)	90.6 (43.4–171)	75.5 (33.2–134)

[a]Includes all study participants with total NNAL measured.

total NNAL (pmol/mL urine) in African Americans, whites, and Japanese Americans are also shown in Fig. 1B, which illustrates the higher proportion of African Americans with higher levels of total NNAL versus the higher proportion of Japanese Americans with lower levels, while whites are intermediate.

Median values of free NNAL (pmol/mL urine) were also greatest in African Americans and lowest in Japanese Americans while the other groups had intermediate values (Table 2). The differences between African Americans and whites ($P < 0.001$) and between Japanese Americans and whites ($P = 0.003$) were significant.

All five ethnic groups had significantly different NNAL glucuronidation ratios ($P < 0.05$), ranging from the highest in African Americans (median = 2.82) to the lowest in Japanese Americans (median = 2.02; Table 2). The ratios in all groups were significantly different from that in whites (median = 2.58). The distributions of the ratios are also illustrated in Fig. 2. African Americans had a higher proportion of high ratios whereas Japanese Americans and Native Hawaiians had a greater proportion of lower ratios.

Table 2. Median and interquartile range of total NNAL, free NNAL, and NNAL glucuronidation ratio, stratified by race/ethnicity

	N	Median	Interquartile range	P when compared with whites
Total NNAL (pmol/mL)				
African Americans	357	1.80	1.01–2.88	<0.001
Native Hawaiians	325	1.08	0.664–1.68	0.016
Whites	434	1.19	0.725–2.17	
Latinos	442	1.22	0.701–2.12	0.99
Japanese Americans	694	0.914	0.532–1.46	<0.001
P			<0.001	
Free NNAL (pmol/mL)				
African Americans	345	0.441	0.268–0.698	<0.001
Native Hawaiians	315	0.323	0.208–0.509	0.53
Whites	419	0.347	0.208–0.551	
Latinos	421	0.371	0.216–0.596	0.28
Japanese Americans	672	0.304	0.188–0.453	0.0031
P			<0.001	
NNAL glucuronidation ratio ((total NNAL − free NNAL)/free NNAL)				
African Americans	345	2.82	2.03–4.06	0.014
Native Hawaiians	315	2.17	1.65–2.84	<0.001
Whites	419	2.58	1.99–3.50	
Latinos	421	2.45	1.78–3.36	0.048
Japanese Americans	672	2.02	1.51–2.77	<0.001
P			<0.001	

NOTE: *P* value using the Wilcoxon–Mann–Whitney test.

This pattern of higher values of total and free NNAL in African Americans, lower values in Japanese Americans, and intermediate values in the other groups was consistent across sex (Supplementary Table S2). In all racial/ethnic groups, women had slightly lower total and free NNAL measures. Significant differences across sex were only detected in total NNAL and free NNAL in whites and Japanese Americans ($P < 0.001$). There was no difference across sex for NNAL glucuronidation ratios.

When median total NNAL was expressed per total nicotine equivalents, the differences among the ethnic groups were considerably weakened and were only significant for Latinos, when compared with whites (Supplementary Table S3). This is a consequence of the strong correlation between total nicotine equivalents and total NNAL. In addition, these values did not differ by sex ($P \geq 0.14$).

When median total NNAL was expressed per cigarettes per day (Supplementary Table S4), the highest levels were found in African Americans, with the lowest levels in whites and Japanese Americans. The difference between African Americans and whites was significant ($P < 0.001$). Levels of total NNAL in Latinos were also significantly higher than in whites when expressed this way. The highest levels of free NNAL per cigarettes per day were observed in African Americans and Latinos, and were significant when compared with whites ($P < 0.001$).

Expressing total NNAL and free NNAL per mg Cr had a strong effect on the values (Table 3). When viewed in this way, levels of total NNAL were highest in whites followed by African Americans, Native Hawaiians, Latinos, and Japanese Americans. Levels in all groups were significantly different from those in whites ($P < 0.001$). Free NNAL values were similarly affected (Table 3). These results are a consequence of the significant differences in Cr among the groups; African Americans had the highest levels (median = 90 mg/dL) and whites the lowest (median = 54 mg/dL; Table 1).

Geometric means of total NNAL, expressed per mL urine, in the five ethnic groups are presented in Table 4. In Model 1, these values are adjusted for age, sex, and Cr (natural log) while in Model 2 they are additionally adjusted for total nicotine equivalents. In Model 1, levels of total NNAL were similar in African Americans and whites (geometric means = 1.35 pmol/mL), with the lowest levels

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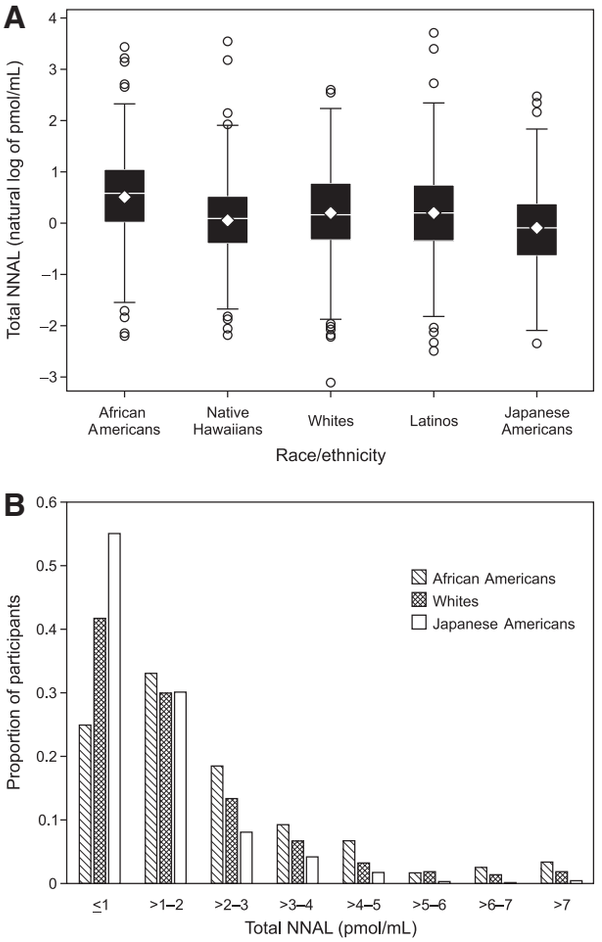

Figure 1.
A, median levels of total NNAL (natural log of pmol/mL) in the urine of 357 African Americans, 325 Native Hawaiians, 434 whites, 442 Latinos, and 694 Japanese-American subjects from the MEC study. The box represents the interquartile range. The line across the box represents the median. The diamond in the box represents the mean. The bottom and top whiskers represent the first and 99th percentile. The circles outside the whiskers represent outliers. B, proportion of subjects among African Americans (▨), whites (▩), and Japanese Americans (□) with varying median levels of total NNAL in urine.

in Japanese Americans (geometric mean = 0.984 pmol/mL; P comparing across all race/ethnicity <0.0001). In Model 2, the highest levels of total NNAL were found in African Americans (geometric mean = 1.23 pmol/mL) and the lowest in Japanese Americans (geometric mean = 1.07 pmol/mL), with whites having intermediate values (geometric mean = 1.18 pmol/mL; P value comparing across all race/ethnicity = 0.0038). Levels in African Americans and whites were not significantly different, whereas Japanese Americans had significantly lower amounts of total NNAL than whites in both models ($P \leq 0.004$). For free NNAL, in Model 1, the lowest values were found for Latinos and Japanese Americans (geometric means = 0.325 and 0.315 pmol/mL, respectively; $P \leq 0.02$ compared with whites) while in Model 2 there were no significant differences ($P \geq 0.42$). NNAL glucuronidation ratio was significantly different when compared across all racial/ethnic groups, with the highest ratios in African Americans and the lowest in Japanese Americans in both models (Model 2: geometric means for African Americans = 2.84 and for Japanese Americans = 2.12 pmol/mL). When compared with whites, significant statistical differences were found in Native Hawaiians and Japanese Americans ($P \leq 0.02$).

Discussion

The subjects in this study smoked from 8–20 cigarettes per day and it was at these relatively low levels of smoking that clearly significant differences in relative risks for smoking-related lung cancer were previously observed, with African Americans and Native Hawaiians having significantly greater risk for lung cancer than whites, while risks for Latinos and Japanese Americans were significantly less than those of whites (1). In a concurrent study with the one reported here, we have analyzed total nicotine equivalents in the urine of the same subjects (45). The median level of total nicotine equivalents was highest in African Americans and lowest in Japanese Americans with intermediate levels in whites, Native Hawaiians, and Latinos. In the study reported here, we found a strong correlation between levels of total NNAL ($R = 0.70$; $P < 0.0001$) and total nicotine equivalents and between levels of free NNAL ($R = 0.60$; $P < 0.0001$) and total nicotine equivalents. The correlation between total NNAL and various nicotine metabolites including total nicotine equivalents has been seen in previous studies (24, 27, 42, 47) and supports the validity of our analytical methods, which were performed by analysts blinded to sample identity and in different laboratories. The correlation between total nicotine equivalents and total NNAL originates from the facts that nicotine and NNK are structurally related tobacco-specific compounds and that NNAL is a metabolite of NNK, easily produced in all systems studied to date including humans (22, 48). Thus, as a consequence of the relationship between total nicotine equivalents and total NNAL, we see the highest median levels of total NNAL, expressed per mL urine, in African Americans, intermediate levels in whites, and the lowest in Japanese Americans (Table 2, Fig. 1A and B). The median levels of total NNAL were nearly twice as great in African

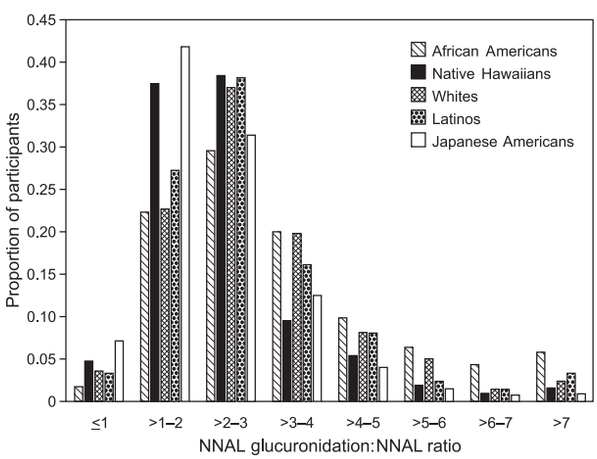

Figure 2.
Proportion of subjects with varying urinary NNAL-glucuronides: free NNAL ratio in 345 African Americans (▨), 315 Native Hawaiians (■), 419 whites (▩), 421 Latinos (▩), and 672 Japanese Americans (□).

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Table 3. Median and interquartile range of total and free NNAL per mg Cr, stratified by race/ethnicity

	N	Median	Interquartile range	*P* when compared with whites
Total NNAL/creatinine				
African Americans	357	1.97	1.28–2.91	<0.001
Native Hawaiians	325	1.87	1.26–2.74	<0.001
Whites	434	2.45	1.57–3.62	
Latinos	442	1.77	1.02–2.78	<0.001
Japanese Americans	694	1.65	1.06–2.49	<0.001
P			<0.001	
Free NNAL/creatinine				
African Americans	345	0.485	0.310–0.739	<0.001
Native Hawaiians	315	0.593	0.359–0.862	0.0067
Whites	419	0.676	0.427–1.00	
Latinos	421	0.515	0.304–0.804	<0.001
Japanese Americans	672	0.545	0.348–0.802	<0.001
P			<0.001	

NOTE: *P* value using the Wilcoxon–Mann–Whitney test.

Americans as in Japanese Americans. Levels of free NNAL were also highest in African Americans, intermediate in whites, and lowest in Japanese Americans. These results in African Americans, whites, and Japanese Americans are consistent with their lung cancer risk, and are plausible because NNK, the tobacco smoke precursor to metabolically formed NNAL, as well as NNAL itself, are powerful lung carcinogens in laboratory animals (22, 48). In one recent study in F-344 rats, administration of 5 ppm of NNK or enantiomers of NNAL in the drinking water daily for 90 weeks produced a nearly 100% incidence of lung tumors, confirming earlier data (29, 49). Multiple laboratory animal tests consistently demonstrate robust induction of lung tumors by NNK, which is the only tobacco smoke constituent with organoselectivity for induction of lung cancer in multiple species (22, 48). Thus, the uptake of NNK shown here by analysis of total NNAL in urine is fully consistent with the results of the MEC showing highest risk of lung cancer in African American smokers, intermediate risk in whites, and lowest in Japanese-American smokers. We also observed that African Americans had significantly higher levels of total and free NNAL than whites or Japanese Americans per cigarette smoked. However, our total and free NNAL data for Native Hawaiians and Latinos are not consistent with the epidemiologic results in the MEC, as the levels in Native Hawaiians were lower than those in whites while the amounts in Latinos were not different from those in whites (Table 2). This indicates the involvement of other factors, so far unidentified.

Cr is widely used to adjust urinary metabolite concentrations thus circumventing the potential problems inherent in use of volume, which may be affected by hydration levels and other variables (50). But urinary Cr levels are also affected by multiple factors including gender, muscle mass, age, and red meat intake. Notably, one study of more than 20,000 participants in the Third National Health and Nutrition Examination Survey (NHANES) found that race/ethnicity affected urinary Cr levels (50). Non-Hispanic blacks had significantly greater concentrations of urinary Cr than did non-Hispanic whites and Mexican Americans. We also found significant differences in urinary Cr levels among the ethnic groups in this study, except between whites and Japanese Americans. Mean Cr levels in African Americans were 1.6–1.7 times greater than in whites and Japanese Americans and 1.5 times greater than in Native Hawaiians. Thus, when expressed per mg Cr, the highest levels of total NNAL were in whites with successively decreasing levels in African Americans, Native Hawaiians, Latinos, and Japanese Americans, respectively (Table 3). The switch in order between African Americans and whites depending on the mode of expression (per mL urine vs. per mg Cr) appears to be due mainly to the higher Cr levels in African Americans. The use of Cr as one denominator for expressing the results of this study was necessitated by the different methods of urine collection in the MEC. Most of the samples from African Americans were first morning voids whereas those from Native Hawaiians and Japanese Americans were overnight collections. We have compared these two methods of collection in a small study and they produced statistically identical results when total NNAL was expressed per Cr, but not when it was expressed per mL urine. Overall, when the MEC results presented here were adjusted for age, sex, Cr, and total nicotine equivalents, African Americans had the highest, whites intermediate, and Japanese Americans the lowest levels of total NNAL (Table 4) and the differences across ethnicity were significant although the African American versus white difference was not.

Our results for total NNAL levels in African Americans versus whites are not fully consistent with two other large studies. In data from the 2007–2008 NHANES study, non-Hispanic whites had significantly higher levels of total NNAL than non-Hispanic blacks, when the data were expressed per mL urine after adjusting for other factors including Cr (26). In the "Total Exposure Study," whites had significantly higher levels of total NNAL than blacks per 24 hours (51). However, both studies found significantly higher levels of total NNAL per cigarette smoked per day in blacks than in whites, similar to our results (Table 4). It is not clear why some results of these studies differ, but it could be related to differing subject recruiting strategies. The sampling in the NHANES study was based on a complex, multistage probability strategy which includes counties in the United States, household segments within the counties, and patients from selected households (26). It included smokers ranging from age 12 to >65 years, from 4 ethnic groups: non-Hispanic white, non-Hispanic black, Mexican American, and "other." The Total Exposure Study focused on subjects 21 years or older and in generally good health from 31 states in the United States (51). Our study specifically recruited a subset of cancer-free MEC participants with an average age in their 60s. Thus, our results are relevant to those that are at greater risk of lung cancer in the general U. S. population.

Since our initial report of lower NNAL glucuronidation ratio in African Americans versus whites (31), this parameter has been investigated with divergent results. An extension of the original study found a lower NNAL glucuronidation ratio only in African-American women (40). The results of the "Total Exposure Study" of more than 3000 smokers also showed a lower NNAL glucuronidation ratio in blacks than in whites (41). Our results in this study are different. We found a significantly higher NNAL glucuronidation ratio in African Americans than in whites, 2.82 vs. 2.58 ($P = 0.014$; Table 2). The reasons for these diverse results are not clear, but seem to indicate that differences in glucuronidation of NNAL may not be important as an explanation for differing lung cancer risks between African Americans and whites.

Our data provide plausible explanations for the differences in lung cancer risk among African Americans, whites, and Japanese Americans in the MEC. African Americans among our subjects

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Table 4. Geometric means (95% CIs) of total NNAL, free NNAL, and NNAL glucuronidation ratio, stratified by race/ethnicity

		Model 1		Model 2	
	N	**Geometric means**	**95% CI**	**Geometric means**	**95% CI**
Total NNAL (pmol/mL)					
African Americans	357	1.35	1.25–1.45	1.23	1.15–1.30
Native Hawaiians	325	1.10	1.02–1.19[b]	1.14	1.07–1.21
Whites	434	1.35	1.26–1.45	1.18	1.12–1.25
Latinos	442	1.09	1.02–1.17[b]	1.16	1.10–1.22
Japanese Americans	694	0.984	0.933–1.04[b]	1.07	1.02–1.12[b]
P[a]			<0.001		0.0038
Free NNAL (pmol/mL)					
African Americans	345	0.342	0.316–0.370	0.317	0.295–0.339
Native Hawaiians	315	0.332	0.306–0.359	0.343	0.320–0.368
Whites	419	0.366	0.341–0.392	0.327	0.308–0.348
Latinos	421	0.325	0.303–0.348[b]	0.339	0.319–0.360
Japanese Americans	672	0.315	0.298–0.333[b]	0.338	0.322–0.355
P[a]			0.017		0.45
NNAL glucuronidation ratio					
African Americans	345	2.90	2.63–3.18	2.84	2.59–3.13
Native Hawaiians	315	2.24	2.04–2.47[b]	2.26	2.05–2.49[b]
Whites	419	2.71	2.49–2.95	2.64	2.43–2.87
Latinos	421	2.43	2.23–2.64	2.45	2.25–2.66
Japanese Americans	672	2.09	1.96–2.23[b]	2.12	1.99–2.27[b]
P[a]			<0.001		<0.001

NOTE: Model 1: adjusted for age, sex, and Cr (natural log). Model 2: model 1 additionally adjusted for total nicotine equivalents.
[a]*P* comparing the difference across race/ethnicity.
[b]When compared with whites $P < 0.05$.

seem to smoke differently from whites, extracting more nicotine and NNK per cigarette than do whites. This is supported by the data in Supplementary Table S4. Benowitz and colleagues also observed that people who smoke fewer cigarettes per day smoke each cigarette more intensively and that this effect appears stronger in blacks than in whites (42). Japanese Americans on the other hand have a generally lower ability to metabolize nicotine, as clearly shown by both phenotyping and genotyping methods (3). Therefore, they need to extract less nicotine per cigarette with a consequent reduction in exposure to NNK. This is supported by the data in Tables 1 and 4. Currently, however, we have no cogent explanation for the relationship of nicotine metabolite and NNAL levels to lung cancer risk in Native Hawaiians and Latinos.

There are some limitations to this study. First, only one urine sample was collected and the collection methods were not fully consistent as noted above. We do not know if a given individual in this study would have consistent biomarker data over a period of time, although we have carried out longitudinal studies of total NNAL in other subjects and the coefficient of variation over a one year period was 30% (52). In addition, in our current study, we have serial overnight urine samples from 270 participants (median time between samples = 0.7 years) with total NNAL measured (43). The Pearson partial correlation, adjusted for age, sex, race, creatinine at both time points and time between urine collection, for these serial measures of NNAL was $r = 0.37$. The CV for these serial samples was 42.5%. Second, the lower proportions of male smokers among African Americans and Native Hawaiians in our study probably reflect the difficulty in recruiting African-American and Native Hawaiian smokers for research studies. These proportions were consistent with those in the greater MEC study population, as were the average number of cigarettes per day smoked by sex and race/ethnicity (44). Third, we do not have biomarker data on subjects who eventually presented with lung cancer. Rather, our subjects were cancer-free MEC participants of the five major race/ethnic groups. We are planning studies to relate prospective levels of total NNAL and total nicotine equivalents to lung cancer in the MEC. We note, however, that we have found significant relationships between levels of total NNAL and total cotinine and risk of lung cancer in the Shanghai Cohort Study and the Singapore Chinese Health Study (53). A notable strength of this study, on the other hand, is the MEC itself, which has the potential to provide significant insights on lung cancer etiology in diverse ethnic groups.

In summary, we present unique data on total NNAL levels in the urine of smokers from five ethnic groups with differing susceptibility to lung cancer. African Americans had higher levels (per mL of urine) of NNAL, a potent lung carcinogen, compared with whites, who in turn had higher levels than Japanese Americans. Our data provide a plausible explanation for the greater lung cancer susceptibility of African Americans, compared with whites, and of whites compared with Japanese Americans in the MEC. However, our data could not provide an explanation for the intermediate lung cancer risks of Native Hawaiians and Latinos. The reasons for these differences in NNAL levels in these racial/ethnic groups have yet to be elucidated, but are likely a result of a combination of biologic and environmental effects on smoking behavior and metabolism in these populations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D.O. Stram, L. Le Marchand, S.S. Hecht
Development of methodology: S.L. Park, X. Ming, E. Vielguth, L. Le Marchand, S.G. Carmella
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.L. Park, X. Ming, E. Vielguth, L. Le Marchand, S.G. Carmella
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.L. Park, X. Ming, D.O. Stram, L. Le Marchand, S.S. Hecht

Writing, review, and/or revision of the manuscript: S.L. Park, D.O. Stram, L. Le Marchand, S.S. Hecht
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Le Marchand
Study supervision: L. Le Marchand

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