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Association between NNAL and Mortality in U.S. Adults, NHANES 2007-2014 with 2015 Mortality Follow-Up

Nathan M. Holt[1*], Ryan A. Black[1], Qiwei Liang[1], Saul Shiffman[2]

[1]Regulatory Science, Juul Labs, Inc., Washington, DC, USA
[2]Pinney Associates, Inc., Pittsburgh, USA
Email: *nate.holt@juul.com

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Abstract

Cigarette smoking is a leading cause of premature mortality, attributable to chronic exposure to toxic compounds in cigarette smoke, including tobacco-specific nitrosamines, which are known carcinogens. This research aims to assess the association between NNAL, a metabolite of the tobacco-specific nitrosamine NNK, and mortality. Data from 14,766 U.S. adults aged 21 - 79 in the National Health and Nutrition Examination Survey (2007-2014) included smoking status and urinary NNAL concentration at the time of examination. These data were linked to participants' subsequent mortality status as recorded in the public-use Linked Mortality File (through 2015). Cox proportional hazards regression models assessed the relative risk of all-cause, cancer, cardiovascular disease (CVD), and other-causes mortality for increasing levels of natural log (creatinine-adjusted NNAL). In the whole sample, a unit increase in log (NNAL) is associated with a 20% higher risk of all-cause (HR = 1.20; 95% CI: 1.16 - 1.24), cancer (HR = 1.20; 95% CI: 1.14 - 1.26), CVD (HR = 1.21; 95% CI: 1.12 - 1.31) and other-causes (HR = 1.20; 95% CI: 1.15 - 1.25) mortality. Among current smokers, a unit increase in log (NNAL) is associated with 44% higher cancer mortality risk (HR = 1.44; 95% CI: 1.08 - 1.92) and a 96% higher CVD mortality risk (HR = 1.96; 95% CI: 1.20 - 3.20). Risks of all-cause and other-causes mortality, but neither cancer nor CVD mortality, were positively associated with NNAL among never and former smokers. Inferences are limited by the observational nature of the data, and by the focus on a single biomarker of tobacco-related exposure. The findings suggest that urinary NNAL concentration is acting as a proxy for exposure to the toxicants in cigarette smoke rather than as a biomarker of disease-specific mortality risk.

Keywords

NHANES, NNAL, Mortality, Smoking, Cigarettes

1. Introduction

Cigarette smoking is a major cause of preventable disease and mortality. Although smokers are motivated to smoke to obtain nicotine, the adverse effects of cigarette smoking are caused by exposure to other constituents of tobacco smoke, most of which are generated by the process of combustion [1]. Research has demonstrated that cigarette smokers are at an increased risk of cancer, cardiovascular disease, chronic obstructive pulmonary disease (COPD), and other diseases through their exposure to these harmful constituents [2]. In addition to nicotine, which is not a primary driver of smoking-related morbidity or mortality [2], cigarette smoke contains thousands of other chemicals including more than sixty carcinogens, including polycyclic aromatic hydrocarbons (PAHs), which are not specific to tobacco, but are produced in combustion of any plant matter, as well as tobacco-specific nitrosamines (TSNAs) ([2] and [3]). Besides helping to explain the process by which smoking causes disease and increased mortality risk, these substances or their metabolites provide objective biomarkers of exposure to cigarette smoke.

TSNAs are a particularly important class of toxicants and biomarkers because, unlike many other toxicants in cigarette smoke (e.g., PAHs), which would be produced in the combustion of any plant matter, TSNAs are specific to tobacco [2]. Furthermore, TSNAs such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are potent carcinogens ([2], p. 227). In animal models, NNK has been shown to cause tumors of the lung, pancreas, liver, and nasal cavity [2]. In particular, NNK has been shown to cause tumors of the lung, nasal cavity and liver in rats; tumors of the lung, nasal cavity, and trachea of hamsters; and lung tumors in mice [4], demonstrating its causal role in cancer in animal models, where causality can be unambiguously established. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), the main metabolite of NNK, is typically used to measure NNK exposure in humans [2]. Consistent with the carcinogenicity of NNK, and its metabolic substrate, NNAL, in animal models, observational studies have shown that NNAL concentrations in humans are associated with lung cancer risk ([2] [5] [6] [7] [8] and [9]).

In contrast to animal model experiments designed to assess the causal relationship between NNK exposure and carcinogenesis, however, human studies are epidemiological and observational in nature [10] and generally assess the association between NNK exposure measured by its metabolite, NNAL, and subsequent morbidity and mortality outcomes. Several such epidemiological studies have reported evidence of a positive association between levels of biomarkers of tobacco exposure and subsequent morbidity and mortality risk in the popula-

tions of Norway [5], China and Singapore ([6] and [7]), Scotland [8], and South Korea [9]. Few large-sample studies report on the association between baseline measurements of biomarkers of tobacco exposure and subsequent morbidity or mortality outcomes in the United States of America (U.S.) population. In a case-control study of 200 subjects, Church *et al.* report a positive association between baseline levels of NNAL and the odds of subsequent lung cancer morbidity [11]. Another study found an association among nonsmokers between baseline cotinine measurements—as noted, a biomarker of tobacco exposure strongly correlated with many harmful constituents of tobacco smoke—and subsequent mortality outcomes [12]. The present study is a more recent and nationally representative assessment of the association between NNAL and subsequent mortality outcomes in the U.S. population that accounts for follow-up time using Cox proportional hazards regression analysis.

In this study, we examine the relationship between urinary NNAL concentration and mortality risks in U.S. adults using the National Health and Nutrition Examination Survey (NHANES; [13]) 2007-2014 linked to the public-use Linked Mortality File (LMF; [14]) with follow-up through 2015. Mortality risks were descriptively summarized across ordinal categories of creatinine-adjusted urinary NNAL (nanograms per gram creatinine; ng/g-cr), for the whole sample, and also stratifying by self-reported smoking status. Cox proportional hazards regression analyses were used to estimate the relative mortality risk associated with increasing levels of urinary NNAL after controlling for sex (Unadjusted Analysis), and additional models were used to control for other sociodemographic characteristics (Adjusted Analysis). This analytic method takes into account more information than a logistic regression analysis by accounting for follow-up time—attained age at death or censoring—in addition to a dichotomous vital status outcome, and has been shown to be more efficient than logistic regression analysis [15].

While TSNAs have demonstrated specific roles in carcinogenesis, their presence may also serve more generally as indicators of exposure to tobacco. Because TSNAs, PAHs and other toxicants are all present in tobacco smoke, the various specific toxicants are highly correlated, and may serve as indicators of smoke exposure. In cigarette smokers, NNAL is strongly correlated ($r > 0.85$) with nicotine biomarkers, which are in turn strongly correlated ($r > 0.50$) with biomarkers of other components of tobacco smoke, including NNN, 2-CYMA, and 3-HPMA [16]. Thus, any single biomarker may act as a disease-specific, causal predictor of the effect of cigarette smoking (e.g., NNAL causing cancer) but also as a surrogate measure of overall tobacco exposure. Accordingly, even though NNAL is known to be a carcinogen, and not reported as a contributor to other diseases [17], we assess the relationship of NNAL to non-cancer risks, such as cardiovascular risks, to determine whether the association is disease-specific, or is disease non-specific, and thus likely reflecting overall tobacco smoke exposure including other toxicants.

2. Methods

2.1. Overview of Data Sources

The cross-sectional NHANES has been administered by the U.S. Centers for Disease Control and Prevention (CDC) National Center for Health Statistics (NCHS) since 1999, and includes interview and examination components [13]. Respondent interviews provide demographics and tobacco use history. Biological specimens are collected at Mobile Examination Center (MEC) examinations. NCHS also provides public-use and restricted-use LMFs that contain all-cause and cause-specific mortality status as well as follow-up time for respondents to NHANES and other survey programs [14]. Public-use LMFs are updated periodically. The present analysis uses the public-use LMF with mortality follow-up through December 31, 2015. This LMF contains interval-censored follow-up time, vital status, and leading cause of death coarsened into three categories for NHANES 2007-2014 respondents: mortality due to malignant neoplasms, diseases of the heart, and all-other-causes. Respondents ineligible for public-use mortality follow-up include those less than 18 years old at interview, those with insufficient identifying information, and those for whom mortality linkage could present an identification risk.

2.2. Self-Reported Smoking Status

At interview, respondents reporting having smoked < 100 cigarettes in their lifetime were classified as never smokers. All others were classified as ever-smokers: those reporting smoking "every day" or "some days" as current cigarette smokers, those smoking "not at all" as former cigarette smokers. NNAL levels have been shown to vary by tobacco product and frequency of product use [18]. In NHANES 2007-2014, other tobacco product use data were only collected at the MEC examination, which was conducted an average of two weeks after the interview and at which urine samples were collected [19]. To mitigate variation arising from transitory tobacco use behavior between the interview and urine collection, respondents reporting tobacco use at MEC examination inconsistent with their interview responses were excluded, such that never and former smokers reported no MEC past-5-day use of cigarettes and current smokers reported cigarette smoking on at least one of the five preceding days. Respondents reporting any past 5-day use at examination of other tobacco or nicotine products (pipe tobacco, cigars, chew, snuff, nicotine replacement therapy [NRT] and electronic nicotine delivery systems [ENDS]) were excluded to eliminate confounding exposures. Analyses were performed stratified by smoking status and among all adult never, current and former smokers ("full sample"). As NNAL concentration is expected to more directly measure actual exposures, including among self-reported nonsmokers, analyses do not control for measures of smoking intensity (cigarettes per day, pack-years).

2.3. Urinary NNAL and Creatinine

NNAL and creatinine were measured in the urine samples collected at the

NHANES MEC examination, with urinary NNAL concentration first measured in NHANES 2007-2008. Urinary creatinine was used to adjust NNAL measurements for urinary dilution, and analyses were performed using creatinine-adjusted NNAL (NNAL, ng/g-cr). For respondents with NNAL measurements below the lower limit of detection (LLOD), NNAL was imputed using the LLOD for NNAL divided by the square root of two [20]. The LLOD varied within and between NHANES 2007-2012 survey cycles but was a constant 0.0006 ng/mL in NHANES 2013-2014. Henceforth we use BLLOD to denote NNAL measurements below the LLOD. Descriptive and graphical analysis of creatinine-adjusted NNAL measurements showed the data are skewed right and thus values were natural log-transformed for analysis [log (NNAL)]. Research has shown that urinary NNAL concentration is strongly correlated with concentrations of total nicotine equivalents and other biomarkers of tobacco exposure [16]. To avoid multicollinearity in the Cox proportional hazards regression design matrix, mortality risks associated with NNAL are estimated without controlling for other biomarkers of tobacco exposure.

2.4. Vital Status, Follow-Up Time, and Underlying Leading Cause of Death

The public-use LMF provided vital status, follow-up time, and leading cause of death, classified as: malignant neoplasms ("cancer", ICD-10 code range: C00-C97); diseases of the heart ("cardiovascular disease" [CVD], ICD-10 code range: I00-I09, I11, I13, and I20-I51); or other causes (all other ICD-10 codes, including pulmonary causes, and non-disease causes such as suicide and car accidents) [14].

2.5. Analysis Subpopulation

The analysis subpopulation is limited to NHANES 2007-2014 respondents aged 21 to 79 years old at interview, with non-missing sociodemographic characteristics, urinary NNAL and creatinine measurements; eligible for mortality follow-up; and with non-missing follow-up time and known underlying cause of death. Respondents aged 80 years and older at interview, for whom age is top-coded at 80, were excluded from the analysis subpopulation because attained age at last follow-up, a crucial variable in Cox proportional hazards regression analysis, is censored. Also, after age 80, overall mortality increases, and the differential impact of tobacco exposures on premature mortality declines.

Mortality analyses were performed to assess the dose-response relationship between NNAL and mortality outcomes. Crude mortality rates were estimated marginally over and stratifying by smoking status, and marginally over and stratified by ordinal NNAL category. To assess the relative mortality risk associated with increasing levels of log (NNAL), Cox proportional hazards regression analyses were performed marginally over and stratified by smoking status. Sensitivity analyses were performed using ordinal NNAL categories. Analyses marginal over smoking status permit analysis over the gradient of NNAL in the analysis

subpopulation, while analyses stratified by smoking status permit analysis over the NNAL gradient among those with generally higher (current smokers) and lower (former and never smokers) levels of NNAL at examination.

Ordinal NNAL categories were constructed as follows. Respondents with NNAL BLLOD were coded as the lowest of five ordinal categories. The second lowest ordinal category includes respondents with NNAL above the LLOD and less than 2.0 ng/g-cr, the upper cut point approximating the median of the distribution of NNAL among former smokers and never smokers with NNAL above the LLOD (unweighted median = 2.1 ng/g-cr; weighted median = 2.0 ng/g-cr). The third, middle category includes respondents with NNAL at least 2.0 ng/g-cr and less than 200 ng/g-cr, the upper cut point approximating the first tertile of the distribution of adjusted NNAL among self-reported current smokers (unweighted first tertile = 179.1 ng/g-cr; weighted first tertile = 197.1 ng/g-cr). The fourth ordinal category contains respondents with NNAL from 200 ng/g-cr to less than 400 ng/g-cr, approximating the second tertile of the distribution of adjusted NNAL among self-reported current smokers (unweighted second tertile = 398.6 ng/g-cr; weighted second tertile = 435.3 ng/g-cr). The fifth and highest ordinal NNAL category includes respondents with NNAL measurements of 400 ng/g-cr or more, approximating the third tertile of the distribution of NNAL among current smokers.

Analyses were performed using SAS© software (version 9.4, copyright 2016 by SAS Institute, Cary, NC, USA) to account for the multistage probability sampling design and post-stratification weights. Summary statistics and crude mortality rates were estimated using the SURVEYFREQ and SURVEYMEANS procedures. Cox proportional hazards regression analysis was performed using the SURVEYPHREG procedure.

3. Results

3.1. Respondent Characteristics

The analysis population included 14,766 respondents. See Table 1. Respondent demographics are summarized in Table 2. This sample was 52.8% female, and a majority were non-Hispanic white (68.4%), married or living with a partner (65.5%), and had attained more than a high school education (62.5%). Most identified as never smokers (56.4%), with 22.9% identifying as former smokers, and 20.7% as current smokers.

NNAL data are summarized in Table 3. Among self-reported current smokers NNAL measurements were largely consistent with current tobacco use, but measurements for about one in four self-reported never and former smokers were inconsistent with no recent tobacco exposure. Less than 1% of self-reported current smokers had NNAL < 2 ng/g-cr. However, nearly 20% of self-reported never smokers and nearly 30% of self-reported former smokers had NNAL levels ranging from 2 to 200 ng/g-cr, with about 1% of self-reported former smokers and about 0.5% of self-reported never smokers having NNAL levels in the highest

Table 1. Analysis subpopulation flow chart.

Step	Description	Unweighted N Excluded by Step	Unweighted N Remaining after Step
1	NHANES 2007-2014 respondents		40617
2	& Participated in interview and MEC examination	1451	39166
3	& Eligible for mortality follow-up, with non-missing vital status and follow-up time	15315	23851
4	& Aged 21 - 79 at interview	3070	20781
5	& Non-missing sex, race/ethnicity, educational attainment, BMI, marital status, and IPR	2026	18755
6	& Non-missing urinary NNAL and urinary creatinine > 0	1227	17528
7	& Never, current or former cigarette smoker reporting no other past 5-day tobacco or nicotine use at MEC examination	2762	14766

NHANES: National Health and Nutrition Examination Survey; MEC: Mobile Examination Center; BMI: Body Mass Index; IPR: Family Income to Poverty Ratio.

Table 2. Weighted distribution of sociodemographic characteristics by NNAL concentration.

Characteristic	Full Sample N = 14766	BLLOD N = 6124	[LLOD, 2.0) N = 2622	[2.0, 200) N = 3925	[200, 400) N = 978	400+ N = 1117
Smoking Status (%)						
Current Smoker N = 3232	20.7	0.1	0.3	28.6	97.7	96.4
Former Smoker N = 3337	22.9	26.2	26.0	26.9	1.4	1.7
Never Smoker N = 8197	56.4	73.6	73.7	44.5	1.0	1.9
NNAL, ng/g-cr Geometric Mean (SE)	3.26 (0.181)	0.53 (0.009)	0.93 (0.011)	12.39 (0.435)	283.53 (2.237)	699.85 (15.948)
Age at Interview, Years Mean (SE)	46.93 (0.262)	49.52 (0.390)	45.37 (0.476)	43.56 (0.435)	44.96 (0.550)	47.87 (0.508)
Follow-up Time, Years Mean (SE)	4.85 (0.096)	4.96 (0.141)	4.28 (0.089)	4.84 (0.093)	5.04 (0.140)	5.37 (0.508)
Attained Age at Last Follow-up, Years Mean (SE)	51.78 (0.277)	54.49 (0.436)	49.65 (0.470)	48.39 (0.439)	50.00 (0.505)	53.24 (0.500)
Sex (%)						
Male	47.2	42.9	53.0	50.2	53.5	44.9
Female	52.8	57.1	47.0	49.8	46.5	55.1
Race/ethnicity (%)						
Non-Hispanic White	68.4	70.8	63.0	62.8	68.9	84.2
Non-Hispanic Black	10.7	7.4	12.0	15.8	16.1	6.4

Continued

Hispanic	13.8	13.8	18.0	15.2	8.3	4.7
Other	7.0	8.0	6.9	6.2	6.7	4.7
Educational Attainment (%)						
Less than High School	16.1	9.9	15.1	20.4	27.6	32.6
High School Diploma or GED	21.5	16.4	19.9	26.2	32.4	30.1
Some College or Above	62.5	73.7	66.1	53.4	40.0	37.3
Marital Status (%)						
Never Married	17.1	12.0	19.0	25.2	21.0	13.5
Married or Living with Partner	65.5	74.1	66.4	55.0	56.4	54.7
Separated, Divorced, or Widowed	17.4	13.9	14.6	19.8	22.6	31.9
BMI (%)						
<25	29.6	30.9	22.8	27.3	33.1	41.5
[25, 30)	33.1	34.8	33.3	31.5	29.4	30.5
30+	37.3	34.3	43.9	41.1	37.5	28.0
IPR (%)						
<1	14.2	7.0	12.8	20.9	23.6	29.4
[1, 1.5)	11.1	7.3	12.4	14.0	16.6	16.3
[1.5, 2)	8.7	7.3	8.8	11.0	9.0	8.5
2+	66.1	78.5	66.0	54.1	50.7	45.8

Due to rounding, weighted percentages may not sum to 100%. N is the unweighted frequency; SE: Standard Error; BMI: Body Mass Index; IPR: Family Income to Poverty Ratio.

Table 3. Weighted distribution of NNAL concentration by smoking status.

Weighted Row Percentage (N)	Creatinine-adjusted Urinary NNAL Concentration (ng/g-cr)					Row Total
	BLLOD	[LLOD, 2.0)	[2.0, 200)	[200, 400)	400+	
Current Smoker	0.3 (9)	0.2 (9)	33.7 (1191)	29.1 (952)	36.7 (1071)	100% (3232)
Former Smoker	50.5 (1628)	19.9 (685)	28.7 (990)	0.4 (16)	0.6 (18)	100% (3337)
Never Smoker	57.5 (4487)	22.9 (1928)	19.2 (1744)	0.1 (10)	0.3 (28)	100% (8197)
Total N	6124	2622	3925	978	1117	14766

N is the unweighted frequency.

two ordinal categories.

3.2. Crude Mortality Rates

In Table 4, crude mortality rates provide evidence of a dose-response relationship between baseline NNAL and all-cause, cancer, and CVD mortality. For all

Table 4. Weighted mortality rates per 100,000 person-years by NNAL concentration.

Weighted Mortality Rate (95% CI) [Deaths/N]		Creatinine-adjusted Urinary NNAL Concentration (ng/g-cr)					
Cause of Death	**Group**	**Overall**	**BLLOD**	**[LLOD, 2.0)**	**[2.0, 200)**	**[200, 400)**	**400+**
All-cause	Full Sample	609.7 (533.3, 686.0) [629/14766]	527.6 (421.0, 634.2) [242/6124]	417.2 (272.2, 562.3) [63/2622]	626.9 (494.5, 759.4) [175/3925]	962.8 (684.4, 1241.3) [60/978]	1068.3 (824.9, 1311.7) [89/1117]
	Current Smokers	830.8 (682.6, 979.0) [187/3232]	# [0/9]	# [0/9]	500.8 (305.0, 696.7) [46/1191]	964.6 (673.2, 1256.0) [58/952]	1018.8 (772.9, 1264.8) [83/1071]
	Former Smokers	921.0 (748.2, 1093.9) [220/3337]	830.8 (590.5, 1071.1) [104/1628]	665.9 (359.0, 972.7) [29/685]	1182.8 (907.5, 1458.0) [82/990]	# [1/16]	# [4/18]
	Never Smokers	396.8 (313.3, 480.3) [222/8197]	419.9 (316.3, 523.5) [138/4487]	332.3 (168.5, 496.2) [34/1928]	379.4 (221.5, 537.3) [47/1744]	# [1/10]	# [2/28]
Cancer	Full Sample	190.3 (159.7, 220.8) [194/14766]	179.6 (131.2, 228.0) [75/6124]	126.9 (68.3, 185.6) [22/2622]	147.4 (91.9, 202.9) [48/3925]	266.9 (121.2, 412.5) [17/978]	420.9 (270.8, 571.0) [32/1117]
	Current Smokers	278.4 (226.0, 330.8) [61/3232]	# [0/9]	# [0/9]	123.8 (47.2, 200.3) [14/1191]	263.1 (114.0, 412.2) [16/952]	422.1 (271.0, 573.3) [31/1071]
	Former Smokers	261.2 (187.4, 335.0) [68/3337]	238.9 (148.5, 329.2) [31/1628]	210.5 (63.5, 357.5) [10/685]	333.9 (145.2, 522.6) [26/990]	# [1/16]	# [0/18]
	Never Smokers	127.0 (85.9, 168.2) [65/8197]	158.8 (96.4, 221.2) [44/4487]	98.3 (40.4, 156.3) [12/1928]	52.6 (9.5, 95.6) [8/1744]	# [0/10]	# [1/28]
CVD	Full Sample	88.8 (68.2, 109.4) [98/14766]	60.7 (30.8, 90.6) [31/6124]	101.1 (34.9, 167.3) [14/2622]	85.3 (49.7, 121.0) [30/3925]	176.1 (44.7, 307.6) [8/978]	158.6 (53.3, 264.0) [15/1117]
	Current Smokers	130.7 (78.6, 182.8) [30/3232]	# [0/9]	# [0/9]	47.5 (8.6, 86.5) [7/1191]	181.1 (45.9, 316.4) [8/952]	164.3 (56.3, 272.3) [15/1071]
	Former Smokers	150.2 (89.9, 210.5) [39/3337]	142.4 (54.4, 230.5) [18/1628]	196 (24.1, 367.8) [8/685]	142.5 (57.1, 227.9) [13/990]	# [0/16]	# [0/18]
	Never Smokers	47.6 (28.1, 67.1) [29/8197]	31.4 (8.8, 54.1) [13/4487]	68.4 (0.0, 139.0) [6/1928]	75.6 (13.7, 137.5) [10/1744]	# [0/10]	# [0/28]
Other-causes	Full Sample	330.6 (274.1, 387.0) [337/14766]	287.3 (200.3, 374.3) [136/6124]	189.2 (71.4, 306.9) [27/2622]	394.2 (282.7, 505.6) [97/3925]	519.9 (345.4, 694.3) [35/978]	488.7 (302.5, 675.0) [42/1117]

Continued

Current Smokers	421.7 (317.1, 526.3) [96/3232]	# [0/9]	# [0/9]	329.5 (168.7, 490.4) [25/1191]	520.4 (341.6, 699.1) [34/952]	432.4 (257.3, 607.4) [37/1071]
Former Smokers	509.6 (374.0, 645.2) [113/3337]	449.5 (267.1, 631.9) [55/1628]	259.4 (29.5, 489.2) [11/685]	706.4 (430.5, 982.3) [43/990]	# [0/16]	# [4/18]
Never Smokers	222.2 (161.4, 283.1) [128/8197]	229.7 (145.8, 313.6) [81/4487]	165.6 (29.1, 302.1) [16/1928]	251.2 (135.5, 366.9) [29/1744]	# [1/10]	# [1/28]

Cell format: Line 1: Weighted mortality rate per 100,000 person-years; Line 2: (95% confidence interval); Line 3: [unweighted number of deaths/unweighted frequency]; Mortality rates per 100,000 person-years are computed as the 100,000 times the weighted total deaths divided by the weighted total person-years of follow-up; #: Mortality rates based upon < 50 respondents are suppressed; BLLOD: Below the lower limit of detection; LLOD: Lower limit of detection.

mortality outcomes, rates among those with NNAL ≥ 400 ng/g-cr are at least double the rate of those in the BLLOD category. In current smokers, all-cause, cancer and CVD mortality rates among those with NNAL ≥ 400 ng/g-cr are at least twice as large as those with NNAL in [2, 200). There is no consistent mortality risk gradient in never smokers, nor is there an apparent gradient in other-causes mortality risk among current smokers. In former smokers the cancer and other-causes risk is 50% - 100% higher for those with NNAL in [2, 200) in comparison to those with NNAL BLLOD.

3.3. Cox Proportional Hazards Regression Analysis in the Full Sample

In analyses of the full sample, Cox proportional hazards regression analyses provide evidence of a positive association between NNAL and all mortality outcomes (Table 5). In an unadjusted model the all-cause mortality risk is estimated to increase by 20% for a unit increase in log (NNAL) (HR = 1.20; $p < 0.0001$), with the effect slightly attenuated in a fully-adjusted model (HR = 1.16; $p < 0.0001$). The cancer mortality risk is estimated to increase by 20% for a unit increase in log (NNAL) (HR = 1.199; $p < 0.0001$), with the effect slightly attenuated in a fully-adjusted model (HR = 1.166; $p < 0.0001$). There is also a very similar positive association between NNAL and the risks of CVD and other-causes mortality; a unit increase in log (NNAL) is associated with a 21% increase in CVD risk in unadjusted models (HR = 1.214; $p < 0.0001$) and a 16% increase in CVD risk in fully adjusted models (HR = 1.161; $p < 0.0001$). Sensitivity analyses using ordinal NNAL categories were consistent with this; for those with NNAL in the highest two ordinal categories (200+ ng/g-cr) the respective mortality risks are estimated to be 2.5 - 5 times larger than the referent BLLOD NNAL category. See Table 6. Estimated risks were similar, albeit with a more gradually-increasing gradient, in fully-adjusted models.

Table 5. Weighted Cox proportional hazards regression analysis estimated hazard ratios and 95% confidence intervals using log (NNAL).

Cause of Death	Group	log (NNAL) Unit Change Estimated Hazard Ratio	
		Unadjusted Analysis (95% HR CI)	Adjusted Analysis (95% HR CI)
All-cause	Full Sample	**1.202*** (1.162, 1.244)	**1.160*** (1.120, 1.201)
	Current Smokers	1.193 (0.987, 1.442)	-
	Former Smokers	**1.197**** (1.084, 1.322)	-
	Never Smokers	**1.141*** (1.017, 1.280)	-
Cancer	Full Sample	**1.199*** (1.140, 1.260)	**1.166*** (1.106, 1.229)
	Current Smokers	**1.442*** (1.083, 1.921)	-
	Former Smokers	1.797 (0.868, 3.720)	-
	Never Smokers	1.004 (0.747, 1.350)	-
CVD	Full Sample	**1.214*** (1.124, 1.311)	**1.161*** (1.061, 1.270)
	Current Smokers	**1.956**** (1.198, 3.193)	-
	Former Smokers	1.024 (0.874, 1.199)	-
	Never Smokers	1.201 (0.999, 1.443)	-
Other-causes	Full Sample	**1.201*** (1.152, 1.253)	**1.156*** (1.108, 1.205)
	Current Smokers	0.991 (0.805, 1.219)	-
	Former Smokers	**1.282**** (1.135, 1.448)	-
	Never Smokers	**1.196**** (1.057, 1.354)	-

log (NNAL): natural logarithm of creatinine-adjusted NNAL, ng/g-cr. 95% HR CI: 95% confidence interval for the hazard ratio associated with a unit increase in log (NNAL). Unadjusted Analysis controls for sex and log (NNAL). *p-value < 0.05; **p-value < 0.01; ***p-value < 0.0001. Hazard ratios that differ significantly from 1.00 at a 0.05-level are identified in boldface font. Adjusted Analysis controls for log (NNAL), sex, race/ethnicity, educational attainment, body mass index, marital status, and income to poverty ratio.

Table 6. Weighted Cox proportional hazards regression analysis estimated hazard ratios and 95% confidence intervals using ordinal NNAL categories.

Cause of Death	Analysis	Group	Urinary creatinine-adjusted NNAL, ng/g-cr Estimated Hazard Ratio (95% Confidence Interval)				
			BLLOD	[LLOD, 2.0)	[2.0, 200)	[200, 400)	400+
All-cause	Adjusted	Full Sample	1.000 (ref)	0.867 (0.580, 1.297)	1.320 (0.969, 1.798)	**2.426*** ** (1.711, 3.440)	**2.799*** ** (2.045, 3.830)
	Unadjusted	Full Sample	1.000 (ref)	0.959 (0.640, 1.437)	**1.692** ** (1.251, 2.289)	**3.190*** ** (2.251, 4.519)	**3.604*** ** (2.683, 4.841)
		Current Smokers		1.000 (ref)		1.210 (0.743, 1.971)	1.336 (0.832, 2.147)
		Former Smokers	1.000 (ref)	0.909 (0.534, 1.547)		**1.898** ** (1.323, 2.723)	
		Never Smokers	1.000 (ref)	1.036 (0.602, 1.783)		1.220 (0.760, 1.958)	
Cancer	Adjusted	Full Sample	1.000 (ref)	0.795 (0.468, 1.349)	0.994 (0.615, 1.605)	**2.046*** (1.147, 3.648)	**3.387*** ** (2.018, 5.683)
	Unadjusted	Full Sample	1.000 (ref)	0.847 (0.498, 1.441)	1.182 (0.744, 1.879)	**2.585** ** (1.427, 4.685)	**4.083*** ** (2.591, 6.437)
		Current Smokers		1.000 (ref)		1.212 (0.487, 3.019)	2.002 (0.929, 4.317)
		Former Smokers	1.000 (ref)	0.971 (0.439, 2.149)		1.781 (0.857, 3.700)	
		Never Smokers	1.000 (ref)	0.769 (0.363, 1.629)		0.513 (0.236, 1.116)	
CVD	Adjusted	Full Sample	1.000 (ref)	1.639 (0.624, 4.304)	1.425 (0.656, 3.098)	**3.381** ** (1.361, 8.398)	**3.438** ** (1.466, 8.062)
	Unadjusted	Full Sample	1.000 (ref)	1.936 (0.760, 4.932)	**2.081*** (1.042, 4.156)	**4.954** ** (1.968, 12.470)	**4.768*** ** (2.351, 9.671)
		Current Smokers		1.000 (ref)		2.210 (0.822, 5.945)	2.163 (0.780, 5.998)
		Former Smokers	1.000 (ref)	1.491 (0.471, 4.722)		1.291 (0.558, 2.985)	
		Never Smokers	1.000 (ref)	2.816 (0.727, 10.907)		3.519 (0.966, 12.817)	
Other-causes	Adjusted	Full Sample	1.000 (ref)	0.732 (0.371, 1.445)	1.479 (0.928, 2.357)	**2.405** ** (1.488, 3.888)	**2.331** ** (1.493, 3.641)
	Unadjusted	Full Sample	1.000 (ref)	0.814 (0.412, 1.609)	**1.925** ** (1.226, 3.021)	**3.186*** ** (1.959, 5.181)	**3.043*** ** (1.993, 4.646)
		Current Smokers		1.000 (ref)		1.069 (0.617, 1.851)	0.930 (0.461, 1.873)

Continued

Former Smokers	1.000 (ref)	0.675 (0.253, 1.805)	**2.145**** (1.281, 3.590)
Never Smokers	1.000 (ref)	0.976 (0.406, 2.345)	1.422 (0.799, 2.531)

Unadjusted Analysis controls for NNAL category and sex. Adjusted Analysis controls for NNAL category, sex, race/ethnicity, educational attainment, body mass index, marital status, and income to poverty ratio. BLLOD: Below the lower limit of detection; LLOD: Lower limit of detection; 95% HR CI: 95% hazard ratio confidence interval; *p-value < 0.05; **p-value < 0.01; ***p-value < 0.0001. Hazard ratios that differ significantly from 1.00 at a 0.05-level are identified in boldface font.

3.4. Cox Proportional Hazards Regression Analysis Stratified by Smoking Status

Among current smokers, Cox proportional hazards regression analyses provide evidence of a positive association between NNAL and subsequent cancer and CVD mortality risk; a one-unit increase in log (NNAL) is associated with a 44% (HR = 1.442; p = 0.0131) increase in cancer mortality risk and a 96% increase in heart disease mortality risk (HR = 1.956; p = 0.008). See Table 5. Among never and former smokers, NNAL is positively associated with an increased risk of all-cause mortality; in former smokers the all-cause risk increased by 20% (HR = 1.197; p = 0.0006) and in never smokers the all-cause risk increased by 14% (HR = 1.141; p = 0.0256) for a unit increase in log (NNAL). There is no evidence of a cancer mortality risk gradient with respect to log (NNAL) in former and never smokers. There is also no evidence of an association between log (NNAL) and other-causes mortality risk among current smokers (HR = 0.991; p = 0.9272), even in the presence of evidence suggesting a positive association between other-causes mortality risk and log (NNAL) among never smokers (HR = 1.196; p = 0.0052) and former smokers (HR = 1.282; p = 0.0001). Sensitivity analyses using ordinal NNAL categories provide limited evidence of an association between NNAL and mortality risk.

4. Discussion

In this analysis we estimated the association between NNAL, a tobacco-specific, carcinogenic biomarker of tobacco smoke, measured at one point in time, and subsequent mortality outcomes an average of 4.9 years later. We find evidence of a dose-response relationship in U.S. adults between urinary NNAL concentration and all mortality outcomes. This finding suggests that NNAL concentration is acting as a biomarker of tobacco exposure in general, rather than as a biomarker of disease-specific mortality risk. In that respect, it confirms the important role of cigarette smoke exposure in premature mortality, across a range of diseases, and also reinforces the value of biomarkers as quantitative indicators of exposure and therefore of risk.

Among current cigarette smokers we saw a dose-response relationship between urinary NNAL concentration and cancer mortality risk, which was expected based on NNK's status as a known carcinogen. However, we also saw an

equally strong association with CVD mortality, in which NNK is not considered to have a causal role. Among never and former smokers there is evidence of a dose-response relationship between NNAL measurements and all-cause and other-causes mortality risk. In both never smokers and former smokers the estimated other-causes mortality risks associated with NNAL concentration were greater than the respective all-cause mortality risks. This suggests that the observed dose-response relationship in never and former smokers between urinary NNAL concentration and all-cause mortality is driven in part by the observed dose-response relationship with other-causes mortality, one of three outcomes that comprise all-cause mortality in the public-use LMF. We are not aware of a plausible biological basis for this result, particularly as other-causes mortality includes accidents, suicide, cirrhosis, nephritis, and other causes not plausibly related to smoke exposure. It is also possible that the association is confounded by other behavioral or psychosocial factors that, for example, affect both environmental exposures and other mortality risks such as diabetes or drug dependence.

Furthermore, the source of tobacco exposure in these nonsmokers is unknown; never and former smokers twice reported no recent tobacco or nicotine use and yet about one in four had a urinary NNAL concentration of at least 2 ng/g-cr. This finding is consistent with [21], who reported that 25% of non-tobacco users are expected to have NNAL > 2.2 ng/g-cr, and could be the result of less recent (e.g. >5 days at urine collection) use of other tobacco products, misreporting tobacco use or environmental exposure to tobacco or tobacco smoke.

Overall, the data show that the amount of exposure to tobacco, and particularly tobacco smoke, is strongly related to the risk of premature death from cancer, cardiovascular disease, and other causes. The data also emphasize the importance of taking into account that tobacco smoke contains many toxicants, so that the relationship between a particular compound or biomarker and a particular disease may not be specific, but rather could reflect the aggregate exposure to the many toxicants in tobacco smoke. For this reason, it will be useful to consider multiple biomarkers in relation to multiple disease outcomes.

5. Limitations

This is an observational study and, as such, does not provide a basis for making causal inferences. The analyses described herein are based upon limited follow-up time, maximally nine years for a respondent interviewed in January 2007 and surviving through last follow-up on December 31, 2015. Individuals could have changed smoking status during the follow-up interval. Respondents provided data about past 5-day, but neither lifetime nor less recent, use of other tobacco and nicotine products. Respondents were generally young (mean age at interview = 47 years) and the oldest NHANES respondents aged 80+ at interview were excluded because age at interview is top-coded in public-use data files. Almost all (95.7%) respondents survived to last follow-up. Analyses were thus sub-

ject to a high rate of censoring, which provides limited means to control for potential confounders and may affect model performance, in particular in analyses stratified by smoking status. In many cause-specific analyses stratified by smoking status the estimated parameters and standard errors are large, possibly a result of high rates of censoring and interval-censored follow-up time.

6. Conclusion

We report evidence of a dose-response association between NNAL, a metabolite of the tobacco-specific, carcinogenic nitrosamine NNK, and subsequent mortality risk. There is a large variation in NNAL measurements among current smokers—that could reflect variation in smoking topography, metabolism, or other factors—and levels of NNAL at one point in time are positively associated with mortality risk. The observed association between NNAL and mortality risk is not specific to cancer, suggesting that NNAL concentration may be acting as a biomarker of tobacco exposure in general and not as a disease- and toxicant-specific biomarker of mortality risk. The data again demonstrate the association between tobacco smoke exposure and premature death from a variety of causes, and reinforce the utility of biomarkers as indicators of the degree of tobacco exposure.

Data Availability Statement

The data analyzed in this study are publicly available and were downloaded from the CDC's website.

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Conflicts of Interest

Authors NMH, RAB, and QL are full-time employees of JLI. Through Pinney Associates, author SS provides consulting services on tobacco harm reduction on an exclusive basis to JLI. SS also owns an interest in intellectual property for a novel nicotine medication that has not been developed nor commercialized.

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