

One-Week Kava Dietary Supplementation Increases Both Urinary *N*- and *O*-Glucuronides of NNAL, a Lung Carcinogen Major Metabolite, among Smokers

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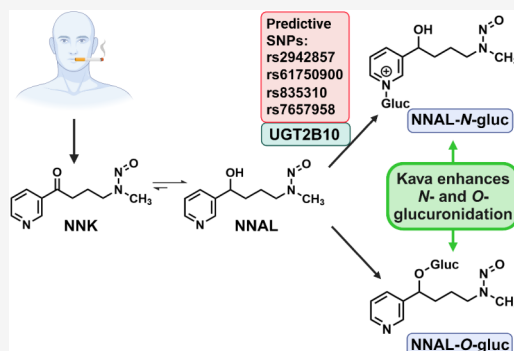


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ABSTRACT: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (commonly known as NNK) is one of the most prevalent and potent pulmonary carcinogens in tobacco products that increases the human lung cancer risk. Kava has the potential to reduce NNK and tobacco smoke-induced lung cancer risk by enhancing urinary excretion of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL, the major metabolite of NNK) and thus reducing NNK-induced DNA damage. In this study, we quantified *N*-glucuronidated NNAL (NNAL-*N*-gluc), *O*-glucuronidated NNAL (NNAL-*O*-gluc), and free NNAL in the urine samples collected before and after 1-week kava dietary supplementation. The results showed that kava increased both NNAL-*N*-glucuronidation and *O*-glucuronidation. Since NNAL-*N*-glucuronidation is dominantly catalyzed by UGT2B10, its representative single-nucleotide polymorphisms (SNPs) were analyzed among the clinical trial participants. Individuals with any of the four analyzed SNPs appear to have a reduced basal capacity in NNAL-*N*-glucuronidation. Among these individuals, kava also resulted in a smaller extent of increases in NNAL-*N*-glucuronidation, suggesting that participants with those UGT2B10 SNPs may not benefit as much from kava with respect to enhancing NNAL-*N*-glucuronidation. In summary, our results provide further evidence that kava enhances NNAL urinary detoxification via an increase in both *N*-glucuronidation and *O*-glucuronidation. UGT2B10 genetic status has not only the potential to predict the basal capacity of the participants in NNAL-*N*-glucuronidation but also potentially the extent of kava benefits.



INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths, with about 1.8 million annual deaths worldwide¹ and over 120,000 deaths in the United States in 2023.² Although improvements in screening and therapy have achieved some success, particularly with the development of immunotherapies, the five-year survival rate among patients with lung cancer in the US barely reached 23% in 2023,³ urging for more effective lung cancer management to reduce lung cancer incidence. Tobacco smoking is the main etiological risk factor for lung cancer, contributing to approximately 80–90% lung cancer cases.⁴ Although tobacco cessation and other efforts aiming to reduce tobacco use are considered the most effective strategy in reducing lung cancer risk, the percentage of adults who smoke in the US has persisted at 13–15% since 2015, translating to ~30 million US adults currently smoking cigarettes.⁵ There is also little indication that this number will decrease significantly in the near future.⁶ Thus, the development of acceptable and cost-effective preventive agents is imperative for these high-risk individuals.^{7,8}

Cigarette smoke contains over 60 known carcinogens, among which 4-(methylnitrosamino)-1-(3-pyridyl)-1-buta-

none (commonly known as NNK) is one of the most prevalent and potent pulmonary carcinogens.^{9,10} NNK induces lung adenoma and adenocarcinoma in several lab animal species.¹¹ As shown in Figure 1, NNK and potentially its main metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) can be metabolically activated by cytochrome P450 enzymes (CYP450s) to generate reactive intermediates, which covalently modify DNA and proteins. Covalent modification of DNA results in DNA damage or DNA adducts,^{12–14} which can lead to mutation if unrepaired. Mutations in critical tumor suppressor genes or oncogenes, such as *TP53* and *RAS*, may initiate lung cancer development.^{15–17} The generation of DNA adducts by tobacco carcinogens is thus considered the root cause of lung carcinogenesis.^{10,11}

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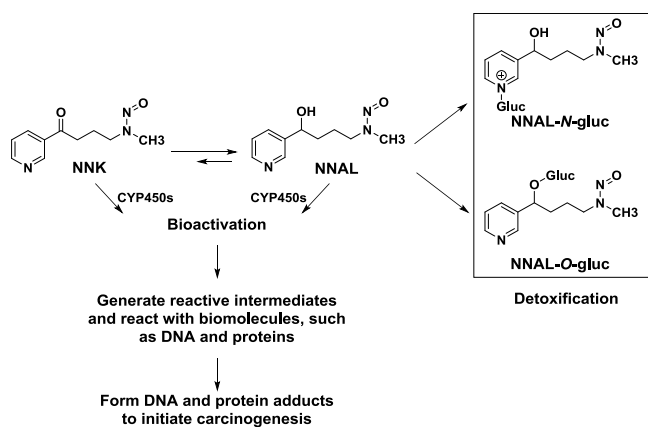


Figure 1. Major bioactivation and detoxification pathways for NNK and NNAL in humans.

NNK and NNAL can also be deactivated and detoxified via metabolism. For instance, NNAL could be glucuronidated at the hydroxy group to form NNAL-O-glucuronide (NNAL-O-gluc) or at the pyridine nitrogen to form NNAL-N-glucuronide (NNAL-N-gluc) (Figure 1), which are catalyzed by various UDP-glucuronosyltransferase (UGT) enzymes.^{18,19} NNAL-N-gluc and NNAL-O-gluc are noncarcinogenic, highly water-soluble, and readily excreted in urine.²⁰ Thus, UGT-mediated glucuronidation of NNAL has been proposed to be an important detoxification pathway for NNK. In humans, both UGT2B10 and UGT1A4 were demonstrated to catalyze NNAL-N-glucuronidation with UGT2B10 likely being the main responsible isoform.^{21,22} UGT2B17, UGT2B7, and UGT1A9 have been reported to be involved in NNAL-O-glucuronidation, although their relative contributions have not been rigorously characterized.^{23–25} Quantitatively, 39–100% of NNK uptake in smokers has been estimated to be converted to NNAL, suggesting that NNAL formation is the major metabolism of NNK.²⁶ Carmella et al. reported that NNAL-N-gluc, NNAL-O-gluc, and free NNAL account for (mean \pm SD) 26.5 \pm 6.2%, 32.1 \pm 17.6%, and 41.4 \pm 16.6% of the mass balance of total NNAL in the urine of smokers,²⁷ while Murphy et al. recently reported that they, respectively, account for 20–23%, 46–54%, and 26–32% of total urinary NNAL mass balance.²⁸ Significant interindividual variations in the relative levels of NNAL-N-gluc and NNAL-O-gluc were also observed in *ex vivo* liver microsomal assays.^{22,29} These variations may in part be related to the genomic differences in the responsible UGTs, such as single-nucleotide polymorphisms (SNPs).²⁹ For instance, Lazarus et al. found that functional SNP variants in UGT2B10, UGT2B17, and UGT2B7 were associated with different levels of NNAL-N- or O-glucuronidation.^{21,23,24} Smokers with different functional SNPs of UGT isoforms may have varying detoxification capacities of NNAL, and thus, different lung cancer risks.

Lung cancer preventive agents that can enhance NNAL glucuronidation have the potential to promote NNAL detoxification in smokers and reduce lung cancer risk. The extent of such benefits may depend on the genetic status of the responsible UGTs. Kava, an herbal product prepared from *Piper methysticum*, has demonstrated promising lung cancer preventive efficacy in preclinical animal models.^{30–33} This is consistent with its intriguing epidemiological observations that regions with higher kava consumption appear to have lower cancer incidence. Additionally, cancer incidence rates in these

regions were lower among males than females, and traditionally, kava is mainly consumed by males.^{34–37} Our mechanistic studies in A/J mice showed that kava reduces NNK/NNAL-induced lung DNA damage^{30,33} and increases the ratio of urinary NNAL-O-gluc to urinary-free NNAL, presumably enhancing NNAL urinary clearance.³⁸ Since mice do not have the ability to catalyze the formation of NNAL-N-gluc, kava's effects on NNAL-N-glucuronidation have never been characterized.³⁸

Kava's potential to increase NNAL urinary clearance and to reduce DNA damage in humans was observed in our recent pilot clinical trial of kava among active smokers.³⁹ Specifically, 1-week kava dietary supplementation among smokers, with no intention to quit, significantly reduced urinary 3-methyladenine (a labile DNA adduct potentially from NNK⁴⁰ and increased urinary excretion of total NNAL (the sum of free NNAL and its N/O-glucuronides).⁴¹ In this follow-up study, we quantified NNAL-N-gluc, NNAL-O-gluc, and free NNAL in the same urine samples and analyzed representative genetic variations for UGT2B10 using collected peripheral blood mononuclear cells (PBMC). Our data show that kava dietary supplementation increased both NNAL-N-gluc and NNAL-O-gluc relative to free NNAL, providing direct evidence about kava's function in enhancing NNK detoxification, which may reduce lung cancer risk. UGT2B10 SNP status appeared to have the potential to predict basal levels of NNAL-N-gluc and even the extent of kava benefits.

EXPERIMENTAL PROCEDURES

Caution. The International Agency for Research on Cancer (IARC) has listed NNK and NNAL as “group 1” carcinogens that should be handled carefully in well-ventilated hoods with appropriate personal protective equipment.

Chemicals and Reagents. NNK, [D₅]-NNAL, [D₃]-NNAL-O-gluc, and [D₃]-NNAL-N-gluc were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Water, formic acid, methanol, and acetonitrile of LC-MS grade were purchased from Fisher Scientific (Fair Lawn, NJ). Strata-XA reverse phase cartridges (33 μ m, 30 mg sorbent) were purchased from Phenomenex (Torrance, CA). Oasis MCX cartridges (30 μ m, 30 mg sorbent) were purchased from Waters (Milford, MA). The SuperScript IV CellsDirect cDNA Synthesis kit was purchased from Fisher Scientific (Fair Lawn, NJ).

Clinical Trial Description. A detailed description of the clinical trial with the information about the participants has been published elsewhere.³⁹ The analyses of the clinical samples were approved by the University of Florida's Institutional Review Board. Briefly, 21 healthy smokers with no intention to quit provided written, informed consent and consumed kava capsules (standardized to 75 mg kavalactones/capsule) three times daily for 1 week. Participants were requested to avoid consuming cruciferous vegetables, which contain phytochemicals that may alter NNK metabolism. 24 h urine samples were collected before and on the last day of the 1-week kava intervention, split into two portions during two time periods: 0–6 h (roughly the first six h since wake-up) and 6–24 h (the rest of the day). Urine samples were aliquoted and stored at -80 °C until analyses. PBMCs were collected before kava use and stored at -80 °C until analysis.

Quantification of Urinary-Free NNAL, NNAL-N-gluc, and NNAL-O-gluc via UPLC–MS/MS. Free NNAL, NNAL-N-gluc, and NNAL-O-gluc in human urine samples were quantified following a published method with minor modifications.²⁸ Briefly, an internal standard solution containing [D₅]-NNAL, [D₃]-NNAL-O-gluc, and [D₃]-NNAL-N-gluc (10 μ L, the concentration of 10 pg/ μ L for each standard) was added into urine (200 μ L). The urine sample was then mixed with 25 mM ammonium acetate buffer (pH 8.5, 500 μ L). Samples were loaded onto a Strata-XA column (30 mg sorbent) and

Table 1. Genetic Variant Status of 3 SNPs for UGT2B10 among the Enrolled 21 Participants^a

ID	UGT2B10				genetic score
	rs2942857(A/C)	rs835310(C/G)	rs7657958(G/A)	rs61750900(G/T)	
1	1	1	1	1	1
2	0	0	1	1	1
3	0	0	0	0	0
6	0	N/A	0	0	0
8	0	0	N/A	1	1
10	0	0	0	0	0
12	0	0	0	0	0
14	0	0	0	0	0
15	0	0	0	0	0
18	0	0	0	0	0
19	0	0	1	1	1
20	0	0	0	0	0
21	0	0	1	1	1
22	0	0	0	0	0
23	0	0	0	0	0
24	0	1	1	1	1
25	0	0	0	0	0
27	0	0	0	0	0
30	1	1	0	0	1
31	0	1	0	0	1
32	1	0	0	0	1
N =	3	4	5	6	9

^aMissing values are indicated as N/A. The last row N indicates the number of participants carrying the alternative allele for SNP data.

separated into NNAL-*N*-gluc, free NNAL, and NNAL-*O*-gluc as described.²⁸ NNAL-*N*-gluc fraction and NNAL-*O*-gluc fraction were incubated with α -glucuronidase (*E. coli* Sigma, 1600 units) at 37 °C overnight. The samples were dried via speed-vac, reconstituted in 2% formic acid in H₂O (1 mL), and then subjected to an MCX column (30 mg sorbent) extraction. The NNAL samples were eluted with 2.5% ammonium hydroxide in MeOH (1 mL) and dried via speed-vac. The residues were dissolved in 10 mM ammonium acetate (30 μ L) with 25 μ L injected for UPLC/MS-MS analysis. NNAL and the internal standard in each fraction accordingly were quantified by targeted UPLC/MS-MS analysis on Dionex Ultimate 3000 RS and a Q Exactive Hybrid Quadrupole Orbitrap Mass Spectrometer using parallel reaction monitoring (PRM) as previously described.⁴¹ In our study, we included three technical replicates for each biological sample in order to assess the inherent variability of the experimental setup and confirm the precision of our measurements.

DNA Isolation and Genotyping. DNA was extracted from blood lymphocytes using a FlexiGene DNA kit (Qiagen, Valencia, CA). The DNA concentration and quality were assessed by a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Genotyping of the UGT2B10 (rs2942857, A/C), (rs835310, C/G), (rs7657958, G/A), and (rs61750900, G/T) polymorphisms was performed by a TaqMan assay on a QuantStudio 12k Flex (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA) Real-Time PCR, genotyping platform. The genetic variants for the participants are summarized in Table 1.

Data Quality Control. To evaluate experimental consistency in the quantification of urinary NNAL metabolites, we assessed the correlations of all technical replicates using Pearson correlation analysis. The correlation coefficients for the technical replicates of the same biological samples are greater than 0.97 (Figure S1), indicating high experimental consistency. Furthermore, we benchmarked the levels of variation among the three technical replicates using the coefficient of variation (CV), which is calculated as the standard deviation of the three technical replicates divided by their mean. This analysis was repeated for all 168 samples (21 biological samples \times 2 gluc types \times 2 time periods \times 2 visits). The range of CVs is from 0.02 to 0.1, corroborating the high experimental consistency among the technical replicates. Therefore, we averaged the measured values from

the three technical replicates for each biological sample, which was used for other data analyses.

Statistical Analysis. Two-group comparisons were conducted by using a paired *t*-test. Correlation analysis was performed using Pearson correlations. To examine whether the effect of kava (calculated as the gluc value at day 7 minus the gluc value at screening) differed by gluc types (NNAL-*N*-gluc or NNAL-*O*-gluc) or by time of day (0–6 or 6–24 h), we fitted linear mixed models using the R package *lme4*, which can capture the repeated measurements from the same participant.

To examine the association between gene variants and NNAL-gluc values, we performed linear regression analysis, where the NNAL-gluc values were the outcome variable and the gene variant was the predictor, adjusting for age, sex, and race as covariates. This analysis was performed for (1) each genetic variant listed in Table 1, (2) both *N*-gluc and *O*-gluc, respectively, and (3) using the basal gluc value at screening and the change in gluc value (day 7–screening), respectively. To investigate the combined effect of multiple SNP variants within the same gene, we developed a genetic score for UGT2B10. The genetic score was defined as 1 if a participant carried at least one alternative allele for any of the three SNPs rs2942857, rs835310, or rs61750900 and as 0 if the participant had the reference alleles for all three SNPs. To further integrate the genetic variant association results (i.e., *p*-values) across different times of day (0–6 or 6–24 h), Fisher's method was adopted, which is a meta-analysis to examine the hypothesis that a genetic variant association exists in at least one time period per day. All statistical analyses were performed using R software (version 4.2).

RESULTS

Demographics. The demographic characteristics of the study participants have been reported previously.³⁹ Briefly, the 21 enrolled participants include seven females and 14 males with an age range of 24–71 years. Thirteen of them were Caucasian Americans, seven were African Americans, and one was Native American.

Basal Levels of Urinary-Free NNAL, NNAL-*N*-gluc, and NNAL-*O*-gluc. Since the participants were expected to have different levels of tobacco smoke exposure and different levels of water intake on different days, we normalized the abundance of NNAL-*N*-gluc and NNAL-*O*-gluc to the corresponding free NNAL in the same urine sample to correct for these variations and defined the ratio as the relative abundance of NNAL-*N*-gluc and NNAL-*O*-gluc, respectively. The relative abundance of NNAL-*N*-gluc and NNAL-*O*-gluc was used for later data analyses, with the level of free NNAL being 1. Among the three metabolites in the 0–6 and 6–24 h urine samples at screening, NNAL-*O*-gluc (2.16 ± 0.64 , 0–6 h; 1.92 ± 0.63 , 6–24 h) was the most abundant followed by NNAL-*N*-gluc (1.40 ± 0.64 , 0–6 h; 1.44 ± 0.58 , 6–24 h) with free NNAL being the least abundant metabolite (Figure 2). These results also suggest that

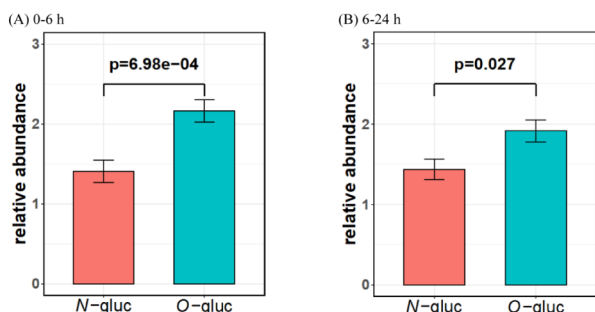


Figure 2. Basal levels of NNAL-*N*-gluc and NNAL-*O*-gluc relative to free NNAL at (A) 0–6 h and (B) 6–24 h, suggesting that NNAL-*O*-gluc is the main form, while free NNAL is the least abundant form.

the relative abundances of NNAL-*O*-gluc, NNAL-*N*-gluc, and free NNAL between these two time periods on the same day were similar (Figure 3). Interestingly, a stronger positive

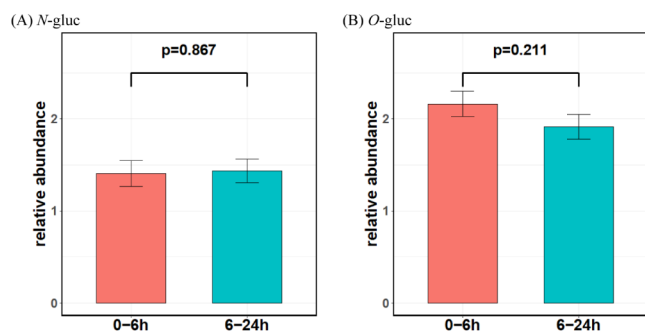


Figure 3. Basal levels of (A) NNAL-*N*-gluc relative to free NNAL between 0 and 6 h and 6–24 h and (B) NNAL-*O*-gluc relative to free NNAL between 0 and 6 h and 6–24 h.

correlation was observed between 0 and 6 h and 6–24 h urine samples among the participants with respect to the basal level of NNAL-*N*-gluc (correlation coefficient $r = 0.67$, $p = 1.03 \times 10^{-4}$), while a weaker correlation was observed for NNAL-*O*-gluc ($r = 0.22$, $p = 0.31$) (Figure 4). Our hypothesis is that NNAL-*N*-glucuronidation is dominantly controlled by a single genetic factor (UGT2B10, with detailed discussion later), while other genetic and environmental factors, such as circadian, may have limited influences, resulting in a stronger correlation between the two time periods. On the other hand, NNAL-*O*-gluc is likely regulated by multiple UGT isoforms,

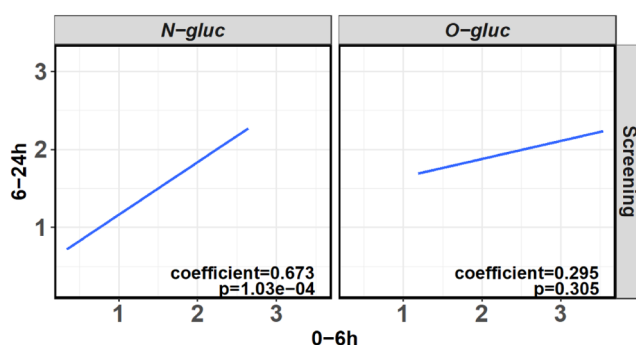


Figure 4. Correlation of NNAL-*N*-gluc relative to free NNAL between 0 and 6 h and 6–24 h samples at screening and that for NNAL-*O*-gluc relative to free NNAL.

and other factors may have more influence on their functions and contributions, leading to weaker correlations.

The Effects of Kava Dietary Supplementation on Urinary NNAL-*N*-gluc and NNAL-*O*-gluc. To evaluate kava's potential in enhancing NNAL detoxification, we compared the relative abundance of NNAL-*N*-gluc and NNAL-*O*-gluc at baseline and after 1 week of kava dietary supplementation. As shown in Figure 5A,B, there were significant increases in the relative abundance of NNAL-*N*-gluc (Figure 5A, ratio of geometric means = 1.58, 95% CI, 1.29–1.97; $p = 0.0003$) and NNAL-*O*-gluc (Figure 5B, ratio of geometric means = 1.27, 95% CI, 1.06–1.51; $p = 0.01$) for the 0–6 h urine samples. Similar results were observed in the 6–24 h urine samples as well, although the magnitudes of the increase were smaller (Figure 5C,D). The extent of the relative increase was not significantly different between NNAL-*N*-gluc and NNAL-*O*-gluc (Figure S2), although a trend of a larger relative increase in NNAL-*N*-gluc was observed in both the 0–6 h (Figure S2A) and 6–24 h samples (Figure S2B). These results suggest that the 1-week dietary supplementation with kava enhanced NNAL detoxification through both *N*-glucuronidation and *O*-glucuronidation. Overall, these data support the potential of kava supplementation in enhancing the detoxification of NNAL via both *N*-glucuronidation and *O*-glucuronidation.

UGT2B10 SNPs. As discussed previously, UGT2B10 has been reported to be mainly responsible for NNAL-*N*-glucuronidation. For this study, we profiled four representative SNPs (rs2942857, rs7657858, rs835310, and rs61750900) for UGT2B10 using DNA samples isolated from PBMCs. Table 1 summarizes the genetic results. Among the four SNPs for UGT2B10, rs7657858 and rs61750900 appear to co-occur or are in linkage disequilibrium except in one participant, where the detection of rs7657858 was not successful. We therefore removed rs7657858 in our later data analyses. Three participants carried the alternative (variant) allele for rs2942857. Four participants had the alternative allele for rs835310. Six participants carried the alternative allele for rs61750900. Given the small number of participants for each SNP allele, we grouped participants with any alternative allele of these SNPs and assigned a genetic score of 1 ($n = 9$), while participants without any alternative allele of these three SNPs were assigned a genetic score of 0 ($n = 12$).

UGT2B10 SNP Status and the Basal Level of NNAL-*N*-gluc. The participants with rs2942857 had statistically significantly lower basal levels of NNAL-*N*-gluc in the 0–6 h urine samples ($\beta = -1.17$, $p = 1.76 \times 10^{-2}$, Figure 6A) and

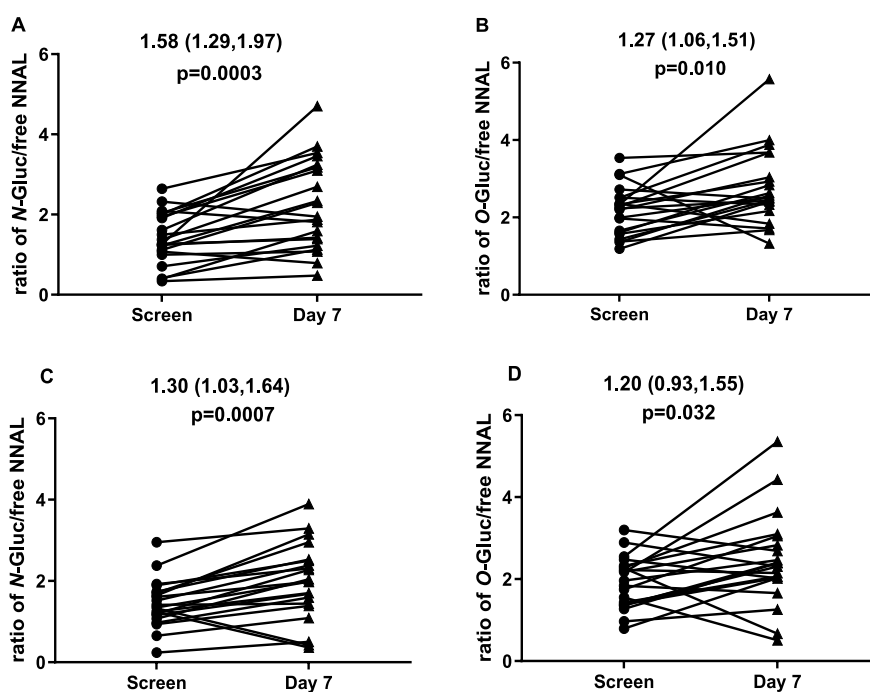


Figure 5. Ratios of NNAL-*N*-gluc and NNAL-*O*-gluc to free NNAL in smokers' urine pre- and post-kava treatment. Urine was collected in the 0–6 h period (A, B) and 6–24 h period (C, D). A two-tailed paired *t*-test was performed for each measure. A ratio of geometric means and 95% CI are presented.

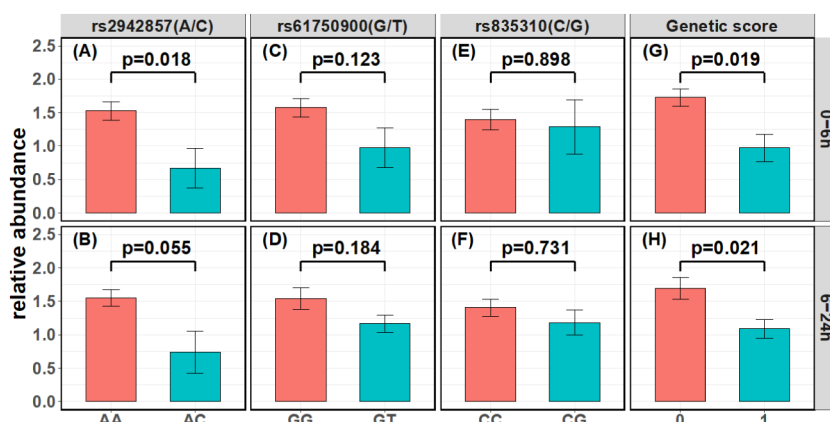


Figure 6. Basal levels of NNAL-*N*-gluc relative to free NNAL among participants with different *UGT2B10* SNP status. (A–H) *p*-values were obtained by a linear regression model, where the NNAL-*N*-gluc value is the outcome variable and *UGT2B10* SNP status is the predictor, adjusting for age, sex, and race as covariates.

nearly significantly lower levels in the 6–24 h urine samples ($\beta = -0.86$, $p = 0.055$, Figure 6B), suggesting that participants with this SNP may have a significantly reduced capacity for NNAL-*N*-glucuronidation. The levels of NNAL-*N*-gluc among participants with the other two SNP alleles do not appear to be significantly different from those without such SNP alleles (Figure 6C–F), which could be due to the small sample size. In terms of the genetic score groups (i.e., carriers of any of the three *UGT2B10* alternative alleles vs noncarriers), there is a significant difference in NNAL-*N*-glucuronidation between these two groups of participants, with $\beta = -0.80$, $p = 0.019$ for 0–6 h and $\beta = -0.696$, $p = 0.021$ for 6–24 h (Figure 6G,H), although the contribution could be dominantly derived from rs2942857. To integrate the results from 0 to 6 and 6–24 h into a 24 h estimation, we further performed Fisher's analysis (Table 2); rs2942857 showed a strong overall association with

the reduced basal level of NNAL-*N*-gluc ($p = 7.7 \times 10^{-3}$). Similarly, the genetic score was also significantly associated with the reduced basal level of NNAL-*N*-gluc ($p = 3.6 \times 10^{-3}$).

UGT2B10 SNP Status and Kava Effects on NNAL-*N*-Glucuronidation. We next analyzed the potential of *UGT2B10* SNPs on kava's effects in enhancing NNAL-*N*-glucuronidation (Figure 7). At 0–6 h, rs617509000 has the largest impact in reducing kava's effect ($\beta = -1.17$, $p = 2.08 \times 10^{-3}$), followed by the genetic score ($\beta = -0.958$, $p = 0.028$). rs835310 ($\beta = -0.56$, $p = 0.43$) and rs2942857 ($\beta = 0.54$, $p = 0.42$) showed the opposite trend; however, it was driven by one participant with both SNPs. This again is the limitation of this study with a small sample size. At 6–24 h, none of these variants were significantly associated with the change in NNAL-*N*-glucuronidation values although the overall trend is that kava's effect appeared to be reduced among individuals

Table 2. Fisher's Meta-Analysis to Integrate 0–6 h and 6–24 h Data for NNAL-N-gluc^a

outcome	variant	<i>p</i> -value at 0–6 h	<i>p</i> -value at 6–24 h	Fisher's <i>p</i> value
N-gluc at screening	rs2942857(A/C)	0.02	0.06	7.7×10^{-3}
	genetic score	0.02	0.02	3.6×10^{-3}
	rs61750900(G/T)	0.12	0.18	0.11
N-gluc changes	rs835310(C/G)	0.90	0.73	0.93
	rs61750900(G/T)	2.07×10^{-3}	0.34	5.9×10^{-3}
	genetic score	0.03	0.46	0.07
	rs835310(C/G)	0.43	0.08	0.14
	rs2942857(A/C)	0.42	0.07	0.14

^aThe null hypothesis of the meta-analysis is that there exists association of the genetic variant and the outcome variable in at least one time of day (0–6 h or 6–24 h).

with any of these SNP alleles. To integrate the results from 0 to 6 and 6–24 h for the 24 h estimation, we further performed meta-analysis using Fisher's analysis (Table 2), which examines the null hypothesis that the genetic association exists in at least one time of day (0–6 or 6–24 h). rs61750900 shows the strongest meta-analysis association ($p = 5.9 \times 10^{-3}$), and the genetic score was marginally significant ($p = 0.07$). The remaining SNPs did not exhibit any significant association in the meta-analysis.

Summary of Genetic Variant Association Results.

Last, we used heatmaps to summarize the associations of individual UGT2B10 genetic variants to the basal level of NNAL-N-gluc and kava's effect (Figure 8). In terms of the association between genetic variants and the NNAL-N-gluc level at baseline, the combined genetic score ($\beta = -0.80$ for 0–6 h) and rs2942857 ($\beta = -1.17$ for 0–6 h) showed negative associations. Additionally, the combined genetic score ($\beta = -0.70$ for 6–24 h) also showed a negative association. For the association between genetic variants and kava's effect on NNAL-N-gluc change, the genetic score ($\beta = -0.96$) and rs61750900 ($\beta = -1.17$) showed a significant negative association only at 0–6 h. In general, these genetic variants show negative associations with the baseline level of NNAL-N-gluc and with a kava-induced increase in NNAL-N-gluc,

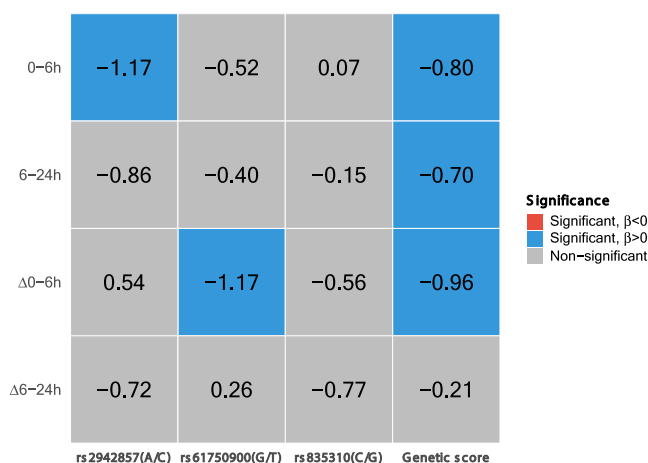


Figure 8. Heatmap for association results for N-gluc. The first 2 rows indicate the association of basal N-gluc with the genetic variant at 0–6 h and 6–24 h. The last 2 rows indicate the association of the change in N-gluc (day 7–screening) with the genetic variant at 0–6 h and 6–24 h. A significant association ($p < 0.05$) is denoted by either a blue color (negative association) or a red color (positive association, none detected). The effect size β (i.e., basal-level difference or kava effect difference explained by the alternative allele) is marked in the heatmap.

indicating that individuals with these genetic variants may have compromised NNAL-N-glucuronidation capacity and reduce the ability of kava to enhance NNAL-N-glucuronidation in such participants.

DISCUSSION

This pilot study systematically analyzed the effects of kava on NNAL-N-gluc and NNAL-O-gluc among smokers and conducted preliminary analysis of the impact of UGT2B10 genetic variants. Results of NNAL-N-gluc, NNAL-O-gluc, and free NNAL in the urine samples from our clinical trial suggested that 1-week kava dietary supplementation increased the relative abundance of both NNAL-N-gluc and NNAL-O-gluc. These data support our earlier finding that kava could increase NNAL glucuronidation and enhance its urinary detoxification among smokers,⁴¹ which may reduce their lung cancer risk. The extents of increase between 0 and 6 and 6–24

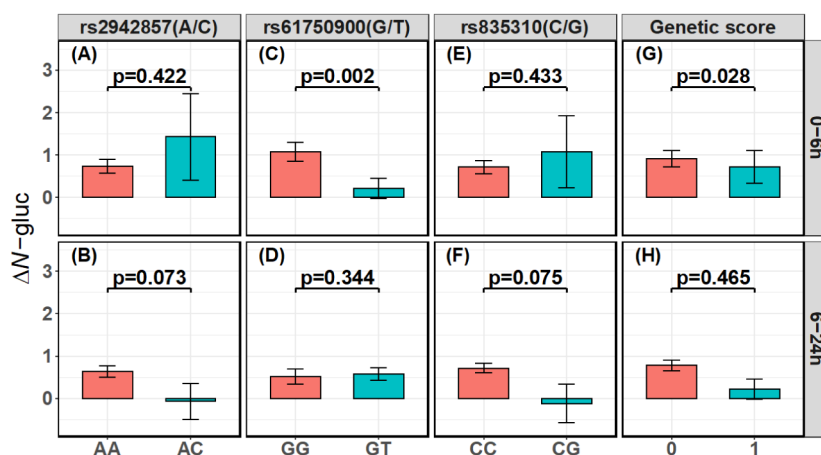


Figure 7. Changes between day 7 and screening of NNAL-N-gluc relative to free NNAL among participants with different UGT2B17 statuses. (A–H) *p*-values were obtained by a linear regression model, where the N-gluc value change is the outcome variable and UGT2B17 variant status is the predictor, adjusting for age, sex, and race as covariates.

h appear to be similar, indicating that future spot urinary analysis may be able to recapitulate the 24 h urinary analysis. Future prospective trials with placebo controls are needed to determine the extent to which kava's increase in NNAL glucuronidation reduces lung cancer incidence.

Kava may also have differential benefits in enhancing NNAL-N-glucuronidation among individuals who smoke depending on their genotype. Our results further support that UGT2B10 is likely the dominant UGT isoform responsible for NNAL-N-glucuronidation, and the UGT2B10 SNP status has the potential to predict basal levels of NNAL-N-gluc and the extent of kava benefits.

This pilot study has several limitations. First, the study was based on a single-arm trial. Therefore, the potential placebo effect cannot be completely ruled out. Second, our small sample size may have led to various confounding factors that influenced the results and conclusions, particularly with respect to UGT genetic variations, where the number of participants carrying alternative alleles was very small (Table 1). There are also other SNPs reported for UGT2B10 and several UGT isoforms that have been reported to contribute to NNAL-O-glucuronidation, which have not been analyzed in these samples. A study with more participants is needed to better understand the effects of these genetic variations on kava's benefits. We are currently performing additional double-blind randomized placebo controlled 4-week kava trials among smokers, targeting to enroll more participants. The clinical samples to be collected will allow a more comprehensive investigation into kava's potential in enhancing the detoxification of NNK and the effects of UGT genetic variations.

In summary, our data show that kava dietary supplementation increased both NNAL-N-gluc and NNAL-O-gluc relative to free NNAL, providing direct evidence about kava's benefits in enhancing NNK detoxification, which may reduce lung cancer risk. UGT2B10 SNP status appeared to have the potential to predict basal levels of NNAL-N-gluc and even the extent of kava benefits.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.chemrestox.4c00109>.

Figure S1, Pearson correlation heatmap for individual technical replicates; Figure S2, the effects of kava on NNAL glucuronidation for 0–6 h and 6–24 h urine samples (PDF)

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Author Contributions

[▽]Q.H. and Z.T. contributed equally. The manuscript was written through contributions of all authors. All authors have read and given approval to the final version of the manuscript for publication. Q.H. was responsible for methodology, investigation, data curation, and writing of the original draft. Z.T. was responsible for data analysis, visualization, and writing of the draft. T.L., Z.H., and C.X. were responsible for conceptualization, funding acquisition, project administration, resource, supervision, and writing, reviewing, and editing of the draft. A.L. and B.F. were responsible for writing, editing, and reviewing of the draft. N.F., R.S., J.M., and F.O. were responsible for funding acquisition, resource, and editing of the draft. CRediT: **Qi Hu** conceptualization, data curation, formal analysis, visualization, writing-original draft; **Zhixin Tang** formal analysis, writing-original draft, writing-review & editing; **Allison Lynch** formal analysis, visualization, writing-review & editing; **Naomi Fujioka** funding acquisition, investigation, resources, writing-review & editing; **Ramzi G. Salloum** funding acquisition, resources, writing-review & editing; **John Malaty** funding acquisition, resources, writing-review & editing; **Frank A. Orlando** funding acquisition, resources, writing-review & editing; **Taimour Langaee** conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, supervision, visualization, writing-review & editing; **Zhiguang Huo** conceptualization, data curation, formal analysis, funding acquisition, investigation, writing-original draft, writing-review & editing; **Chengguo Xing** conceptualization, formal analysis, funding

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNAL-O-gluc	NNAL-O-glucuronide
NNAL-N-gluc	NNAL-N-glucuronide
UGT	UDP-glucuronosyltransferase
UPLC-MS/MS	ultrahigh-performance liquid chromatography-tandem mass spectrometry
PRM	parallel reaction monitoring
SD	standard deviation

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