Protocol Title	STUDY 2: CLINICAL PROTOCOL Metabolism of NNK Among Japanese Americans	
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REVISION HISTORY

Revision #	Version Date	Summary of Changes	Consent Change?

Table of Contents

Study Synopsis

Study Design:	The risk of lung cancer varies by individual and by ethnic/racial group. In this study we will explore how individual differences in the metabolism of a tobacco-specific lung carcinogen may contribute to the variable risk of lung cancer between ethnic/racial groups. In this 10 day clinical trial, Japanese Americans will smoke a cigarette containing deuterium-labeled 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific lung carcinogen. The study cigarette will be smoked for 7 days. This will allow for NNK metabolic profiling and determining the effect of CYP2A6 genotype on the level of NNK α-hydroxylation in Japanese Americans smokers using [pyridine- D4]-NNK containing cigarettes.
Primary Aim:	• To characterize the bioactivation of NNK by α-hydroxylation; • To develop an NNK metabolite profiling method; • To determine the contribution of the enzyme CYP2A6 to NNK bioactivation.
Patient Population:	Japanese American Smokers
Accrual Goal:	20 completed participants
Enrollment Period:	January 1, 2019 through August 30, 2021
ITP #	IU0000492

ABBREVIATIONS/DEFINITIONS

AE: **Adverse Events**: is an undesired harmful effect resulting from a medication or other intervention / study procedure.

α-OHNNK Gluc: α-hydroxymethyl NNK glucuronide

BP: **Blood Pressure:** is the pressure exerted by circulating blood upon the walls of blood vessels and is one of the principal vital signs.

CO: **Carbon Monoxide:** Breath carbon monoxide is the level of carbon monoxide in a person's exhalation.

***CYP2A6*:** This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism. The enzyme (CYP2A6) is known to metabolize nicotine and nitrosamines. Individuals with certain allelic variants are said to have a poor metabolizer phenotype, meaning they do not efficiently metabolize nicotine.

FTND: **Fagerstrom Test for Nicotine Dependence:** is a 6-item standard instrument for assessing the intensity of physical addiction to nicotine and includes an evaluation of cigarette consumption, the compulsion to use, and dependence.

HR: **Heart Rate:** a measure of the number of heart beats per minute (bpm)

MEC **Multiethnic Cohort**

NIAAA: **National Institute on Alcohol Abuse and Alcoholism:** part of the U.S. National Institutes of Health that supports and conducts biomedical and behavioral research on the causes, consequences, treatment, and prevention of alcoholism and alcohol-related problems.

NMR: **Nicotine Metabolite Ratio:** is a urinary measure of the ratio of nicotine metabolites, which indicates speed of nicotine metabolism.

NNAL: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol: Human exposure to NNK can be measured by analyzing the sum of NNAL and its glucuronides (total NNAL) in urine.

NNK 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone: One of the tobacco specific nitrosamines, a potent lung carcinogen in tobacco products, which are formed from nicotine during the curing and processing of tobacco.

[PYRIDINE-D_4]NNK: NNK labeled with deuterium (heavy hydrogen isotope that is non-radioactive and non-hazardous). Adding [pyridine-D_4] to the NNK found cigarette tobacco and spiking study cigarettes allows evaluation of NNK metabolic activation in humans.

SAE: **Serious Adverse Events:** generally, any event which causes death, permanent damage, birth defects, or requires hospitalization is considered an SAE.

TLFB: **Timeline Follow-Back:** is a method that can be used as a clinical and research tool to obtain a variety of quantitative estimates of marijuana, cigarette, and other drug use by asking clients to retrospectively estimate their usage prior to the interview date.

TNE: **Total nicotine equivalents:** a urinary measure of nicotine and its metabolite concentrations (cotinine, trans3'`hydroxycotinine and their glucuronide conjugates plus nicotine N-oxide).

1.0 Objectives

1.1 Purpose:

Smoking is the cause of 90% of all lung cancer but only 11-24% of smokers develop the disease .[1] The risk of lung cancer varies by individual and by ethnic/racial group.[2, 3] In this study we will explore how individual differences in the metabolism of a tobacco-specific lung carcinogen may contribute to the variable risk of lung cancer between ethnic/racial groups. In this protocol we will specifically test the hypothesis that carcinogen bioactivation varies by a smoker's CYP2A6 activity. CYP2A6 activity varies by ethnic group and the activity of this enzyme is associated with lung cancer. Therefore, determining the role of the enzyme in the bioactivation of tobacco carcinogens will contribute to our understanding of the mechanism by which CY2A6 activity influences racial/ethnic differences in lung cancer.

2.0 Background

2.1 Significance of Research Question/Purpose:

The risk of lung cancer for individual smokers and members of different ethnic/racial groups varies significantly. Differences in carcinogen exposure, activation and detoxification contribute to this variable risk. In this study, we will characterize the metabolic pathways of the tobacco-specific lung carcinogen, NNK, in Japanese American (JA) smokers. JA have a high prevalence *CYP2A6* variants that code for low activity and non-functional enzyme. [4, 5] CYP2A6 is the primary enzyme that metabolizes nicotine and a key enzyme involved in the bio-activation of NNK. [6-8]

There are over 20 identified lung carcinogens in tobacco smoke.[1, 9] These include polycyclic aromatic hydrocarbons, NNK, volatiles such as butadiene, metals such as cadmium, and radioactive ^{210}Po.1 Among these the evidence for a role in human lung cancer is strongest for polycyclic aromatic hydrocarbons and NNK, which reproducibly and at modest doses induce lung tumors in laboratory animals.[1, 10, 11] NNK, which is the focus of this project, is unique in that it is tobacco-specific. NNK is considered "carcinogenic to humans" by the International Agency for Research on Cancer and its urinary metabolite NNAL, a biomarker of NNK exposure, has been prospectively associated with lung cancer.[11-13]

The overall hypothesis of the program project grant under which this protocol will be carried out is that the differential lung cancer risk across ethnic/racial groups is due to differences in exposure and response to tobacco smoke carcinogens. In the prior grant period significant differences in carcinogen exposure across ethnic/racial groups were documented. [14, 15] Exposures were highest in African Americans (AA) and lowest in Japanese Americans (JA) compared to Whites. These data are consistent with the relative lung cancer risk in these 3 groups. The lower carcinogen exposure in JA was driven by the influence of CYP2A6 activity on nicotine metabolism.[5] The focus of this study is to determine the influence of CYP2A6 variants on NNK metabolism

2.2 Preliminary Data:

In this project we will characterize the metabolic pathways of the tobacco-specific lung carcinogen, NNK, in JA smoking D_4-pyridyl NNK-containing cigarettes. The rationale for focusing our study on NNK metabolism in JA is: 1) NNK is a tobacco-specific lung carcinogen 2) CYP2A6 is a catalyst of NNK bioactivation (by α-hydroxylation) 3) CYP2A6 activity and genotype is associated with lung cancer, and 4) CYP2A6 copy number variants and other loss of function alleles are present at relatively high frequency in JA compared to other ethnic groups.[5]

D_4-pyridyl NNK-containing cigarettes. Preliminary data from a prior study that was identical in design to the one proposed here, demonstrated a significant level of interindividual variation in NNK α-hydroxylation across smokers. The prior study that used Marlboro Virginia Blend cigarettes (as well as a previously published study with Quest cigarettes[16]), added [pyridine-D_4]NNK to the study cigarettes so that the sum of NNK and [pyridine-D_4]NNK was not higher than the level of NNK in conventional cigarettes. In this study American spirit cigarettes will be used. The beauty of using [pyridine-D_4]NNK is that the products of NNK α-hydroxylation (such as hydroxy acid the primary urinary metabolite, Fig. 2) may be distinguished from common metabolites formed from nicotine. This is critical as nicotine is present in cigarettes at levels that far exceed those of NNK.

In the prior study of African Americans and Whites, the ratio of D_4-hydroxy acid to total D_4-NNAL was used as a measure of the total NNK and NNAL α-hydroxylation (fig 2). This ratio varied about 6 fold among the 114 subjects analyzed, suggesting that significant inter-individual variation occurs. However, since hydroxy acid is a metabolite of both NNAL and NNK this ratio is not ideal to establish a relationship of CYP2A6 activity with NNK bioactivation. In this project, α-hydroxymethyl NNK Gluc will be used a direct measure of the flux of NNK metabolism through the α-hydroxylation pathway. In addition, by carrying out the study in JA smokers with no CYP2A6 activity we will be able to determine the importance of CYPA6 to NNK bioactivation.

Preliminary evidence of an association of P450 2A6 genotype with NNK α-hydroxylation. To test the hypothesis that CYP2A6 activity will influence the extent of NNK metabolism by α-hydroxylation we compared total NNAL levels, corrected for smoking dose (TNE), in smokers homozygous for CYP2A6 loss of function alleles to homozygous "normal" smokers. The geometric mean ratio of total NNAL/TNE excreted by L/L individuals (n=54) was lower, 0.0358 (95% CI, 0.0.33-0.038) than the ratio for N/N individuals, 0.0427 (0.036-0.051). This difference was borderline significant (p=0.09). Our interpretation of these data is that smokers with no CYP2A6 activity metabolize NNK less by α-hydroxylation and more by reduction to NNAL. Given the limitation of these data, (not directly measuring α-hydroxylation, further metabolism of NNAL, and the use of TNE as a proxy for NNK dose) it was encouraging that the effect was in the direction predicted. In this protocol, we will minimize these limitations by quantifying a more direct measure of NNK α-hydroxylation, the α-hydroxymethyl NNK Gluc as well as quantifying the total NNK metabolite profile, and therefore the total dose of NNK more accurately by using [pyridine-D_4] NNK.

2.3 Existing Literature:

Our previous studies support an association of CYP2A6 activity with lung cancer. This association is, at least in part, driven by the influence of CYP2A6 activity on nicotine metabolism and smoking intensity (ref). CYP2A6 also catalyzes the metabolism NNK. [7, 8] Therefore, individuals deficient in CYP2A6 activity may not only smoke less but may also be protected from NNK carcinogenesis

Lung cancer and CYP2A6

Meta-analyses of the association between CYP2A6 genetic polymorphisms and risk of lung cancer reported a statistically significant approximately 50% lower crude odds ratio of lung cancer for poor metabolizers (e.g., carriers of two loss of-function allele or one loss-of-function allele and one decreased-function allele) in Asians smokers.[17, 18] In a recent analysis on the small number of lung cancer cases that have occurred in the multi ethnic cohort (MEC) for whom we have analyzed nicotine metabolism, CYP2A6 activity was associated with an increased risk of lung cancer.[19] Also, in, a genome wide association study by the Transdisciplinary Research in Cancer of the Lung (TRICL) consortium a SNP in CYP2A6, which was associated with lower CYP2A6 activity, was significantly associated with the decreased risk of lung cancer.[20] Importantly, the populations included in the TRICL consortium are predominantly White. Together these studies support an association of CYP2A6 activity with lung cancer. This association is, at least in part, driven by the influence of CYP2A6 activity on nicotine metabolism and smoking intensity. In this project we will begin to test the hypothesis that the relationship of CYP2A6 to lung cancer may also be mediated by its key role in the bioactivation of the tobacco specific lung carcinogen NNK.

Nicotine metabolism

In most smokers the predominant pathway of nicotine metabolism is CYP2A6-catalyzed C-oxidation[15] (Fig. 1). The product of this reaction is oxidized to cotinine, which is then metabolized to *trans* 3'-hydroxycotinine (3-HCOT). Nicotine, cotinine and 3-HCOT are all glucuronidated (Fig. 2). The sum of these metabolites plus nicotine and nicotine *N*-oxide (Fig. 2) is referred to as total nicotine equivalents (TNE) and account for >85% of the nicotine dose. TNE are an objective measure of smoking.[16] CYP2A6 is the primary catalyst of the oxidation of cotinine to 3-HCOT, and the ratio of 3-HCOT to cotinine (in plasma or urine) has been used as a measure of CYP2A6 activity.[15,17] This ratio will be used in the current project to quantify CYP2A6 activity, which will allow us to identify individuals with little or no activity and those with relatively high or normal activity.

In our prior studies of the MEC, nicotine metabolism was comprehensively characterized in five ethnic groups.[18] Nicotine C-oxidation was significantly lower in JA than in Whites or AA. JA excreted the highest concentration of nicotine and the lowest concentration of TNE, adjusted for cigarettes per day (CPD). These data are consistent with the relatively low risk of lung cancer in JA compared to Whites. When TNE are adjusted for CYP2A6 activity (total 3-HCOT/cotinine) there is no longer a statistically significant difference between the TNE excreted by JA and Whites.[19] We analyzed CYP2A6 genotype in these smokers and demonstrated in JA the direct relationship between CYP2A6 genotype and both the 3-hydroxycotinine to cotinine ratio

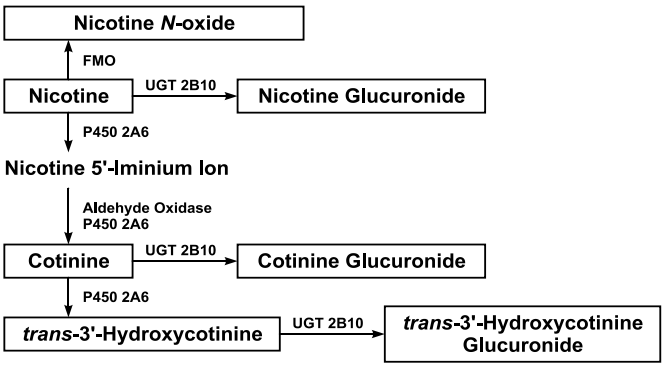


Figure 1. Major pathways of nicotine metabolism.

(CYP2A6 activity) and TNE. These data support the hypothesis that in JA smoking intensity (and therefore carcinogen dose) is significantly influenced by CYP2A6-catalyzed nicotine metabolism.

NNK metabolism

There are three pathways of NNK metabolism; reduction to NNAL, α-hydroxylation and *N*-oxidation[21] (Fig. 2). NNAL, also a lung carcinogen[7] is further metabolized by α-hydroxylation, N-oxidation and two pathways of glucuronidation.[22] NNAL glucuronidation is a detoxification pathway for NNK.[23] Two NNAL glucuronides are formed: NNAL-O-Gluc or NNAL-N-Gluc (Fig. 3). Bioactivation of NNK and NNAL occurs by α-hydroxylation of either the methyl or methylene carbon next to the nitroso group. The α-hydroxy products of these pathways decompose to highly reactive DNA-binding diazohydroxides. The DNA adducts generated, if not repaired may initiate the carcinogenic process. The products of NNK α-hydroxylation excreted in the urine,-hydroxy acid and keto acid - are minor metabolites of nicotine. Therefore, these major urinary NNK metabolites cannot be used as measures of NNK bioactivation (the α-hydroxylation pathway). NNK metabolites that still contain the nitroso group could be used as biomarkers of NNK metabolism. The α-OH-NNK Gluc is the only characterized metabolite of NNK α-hydroxylation, and its level in smokers should be dependent on CYP2A6 activity, and could serve as a measure of NNK bioactivation. The relationship of α-OH-NNK Gluc to total α-hydroxylation will be determined in the two groups (normal and low/no CYP2A6 activity) of JA smokers of [pyridine-D_4]-NNK cigarettes in this protocol.

Cytochrome P450 enzymes (CYP) catalyze the α-hydroxylation of both NNK and NNAL.[21] These enzymes include CYP2A6, CYP2B6 and CYP1A2.[24,25] In human liver microsomes, up to 70% of NNK α-hydroxylation is inhibited by CYP2A6 antibodies.[24] CYP2B6 is similar in catalytic efficiency to CYP2A6, but is typically less abundant in the liver.[24,26] These data support an important (albeit not exclusive) role for CYP2A6 in the catalysis of NNK α-hydroxylation. In this project we will take advantage of the high prevalence of *CYP2A6* copy number variants (CNV) and loss of function alleles in JA smokers to quantify the role of this enzyme in the metabolism of NNK. Our preliminary data (described above) provides some evidence for an association of *CYP2A6* genotype with NNK α-hydroxylation.

We have recently developed a targeted metabolomics approach for urine from rats administered [pyridine-D_4]-NNK (ref). This method will be applied to the analysis of the JA smokers of [pyridine-D_4]-NNK containing cigarettes to determine the relative amounts of all NNK metabolites (fig.2). This will be the first characterization of an NNK metabolic profile in smokers.

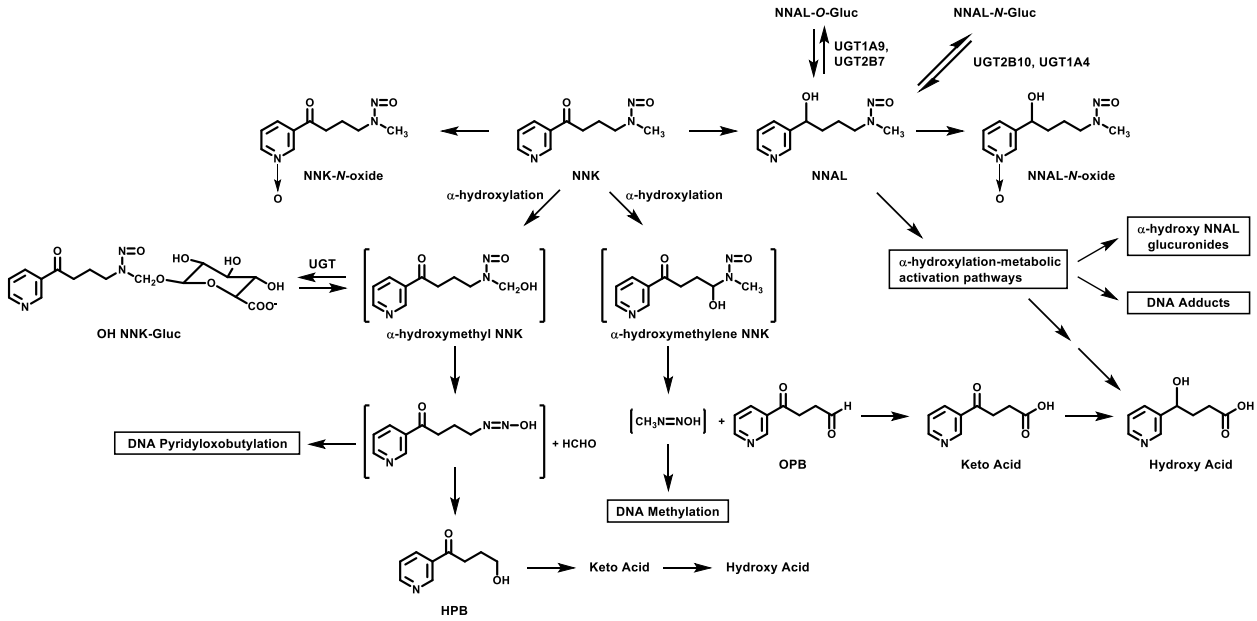


Fig. 2 NNK metabolism

The goals of this study of a small group of JA smoking [pyridine-D_4]-NNK cigarettes is 1) to determine the influence of CYP2A6 activity on NNK a-hydroxylation pathways and 2) to determine if the α-OH-NNK Gluc reflects differences in a-hydroxylation measured in these two groups. This will allow us to determine the contribution of CYP2A6 to NNK bioactivation, and ideally to identify α-OH-NNK Gluc(s) as potential measures of NNK bioactivation. We will validate the NNK metabolic profiling approach in participants with little or no CYP2A6 activity, who we predict will have decreased metabolism through the α-hydroxylation pathway and increased metabolism by NNAL and the NNK-N-oxide pathways.

3.0 Study Endpoints/Events/Outcomes

3.1 Primary Endpoint/Event/Outcome:

To determine the effect of CYP2A6 genotype on the level of NNK α–hydroxylation in JA smokers administered [pyridine-D4]-D4 NNK.

To characterize the NNK metabolic profile in JA smokers receiving [pyridine-D4]-NNK

3.2 Secondary Endpoint(s)/Event(s)/Outcome(s):

NA

4.0 Study Intervention(s)/Investigational Agent(s)

4.1 Description:

The goal of this trial is to determine the effect of CYP2A6 genotype/phenotype on NNK α-hydroxylation in Japanese American (JA) smokers administered [pyridine-D_4]NNK for 7 days. Japanese American smokers (N=20 completers) will be recruited from the 100+ Japanese Americans participating in Study 1: Observation Study part of “Mechanisms of

Ethnic/Racial Differences in Lung Cancer Due to Cigarette Smoking" Clinical and Biomarkers Core.

4.2 Drug/Device Handling:

The study will use a commercial tobacco product that has [pyridine-D_4]NNK added to the tobacco thereby making it an investigational tobacco product. Natural American Spirit-Tan cigarettes (king size, hard pack) are commercially manufactured by the Santa Fe Natural Tobacco Company currently marketed by RJ Reynolds and are available for purchase in retail stores and online. The American Spirits-Tan were chosen because they have approximately 11% of the level of NNK found in other manufactured cigarettes. Natural American Spirit-Tan cigarettes will be modified by injecting [pyridine-D_4]NNK into the tobacco rod of these cigarettes so that the sum of the naturally present NNK and the added [pyridine-D_4]NNK is similar to or below the NNK levels found in typical commercial U.S. cigarettes.

Preparation of study cigarettes
The addition of [pyridine-D_4]NNK to study cigarettes will be carried out by following our previously established methods [16] American Spirit-Tan cigarettes will be purchased by University of Minnesota (king size, hard pack) which contain 0.21 - 0.37 μg NNK/g wet weight tobacco. The cigarettes will be modified by adding 0.300 μg [pyridine-D_4]NNK to each cigarette so that the amount of total (deuterated plus unlabeled) NNK in these cigarettes is below 0.700 μg/g tobacco which is the average NNK level in popular commercial cigarette brands. [Pyridine- D_4]NNK has been synthesized in our laboratory. The addition of [pyridine-D_4]NNK to the cigarettes will be carried out with a specially designed microsyringe applicator system. The prepared cigarettes will be conditioned at 25°C and 60% relative humidity for 2 days, placed back in their original packs, 20 cigarettes per pack, and stored at 4°C until being dispensed to study participants.

The cigarettes will be processed and stored at the Masonic Cancer Center. Once the cigarettes are spiked with the deuterated NNK they will be transferred to the University of Hawai'i study site. The study cigarettes will be kept in a locked refrigerator and only study staff will have access. Detailed records of study cigarettes received, dispensed and returned will be kept. Any study product remaining after the completion of the trial will be destroyed per the University of Hawaii's institutional Chemical Waste procedures.

5.0 Procedures Involved

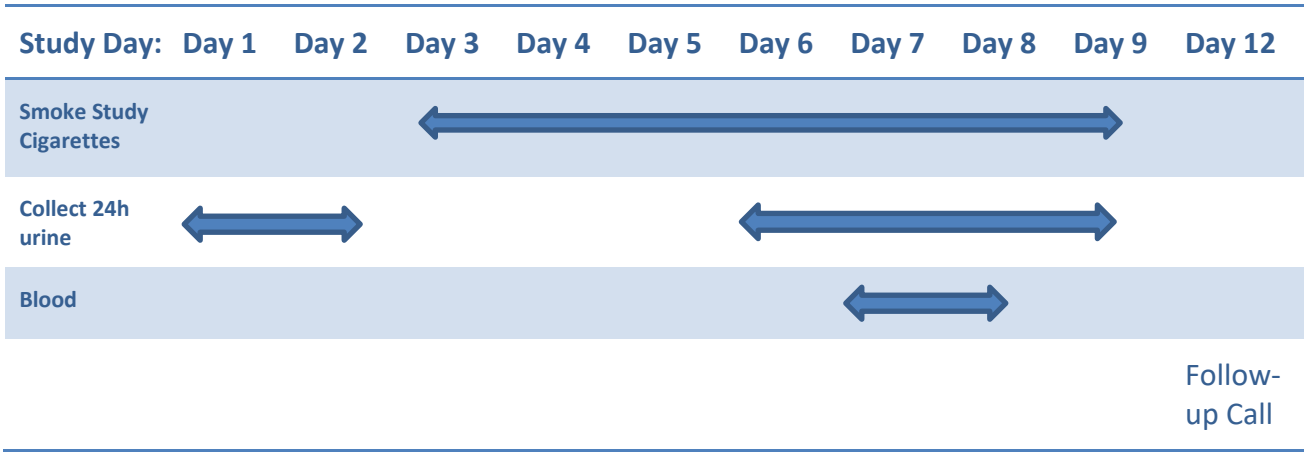
5.1 Study Design:

In this clinical trial, the selection and recruitment will be determined based on the urinary ratios of CYP2A6-genreated nicotine metabolites total 3-HCOT to cotinine. Little or no-activity is defined as a ratio of <0.6 (expected 12% for JA from previous MEC sample and "relatively high" is defined as a ratio of >3.0. (28% had a ratio >3 and 15% <0.6 expected for JA smokers in the previous MEC sample and had a median ratio of 1.8).

Japanese American smokers will be recruited for a study where they will smoke cigarettes that contain [pyridine-D_4] NNK. One group will be smokers with very low or no CYP2A6 activity and the other will have relatively high activity. Participants are selected from Study 1: Observational Study which provided human biological samples and measurements from Native Hawaiian, Japanese Americans and Whites.

In this study, participants will smoke the [D_4-pyridine]-NNK study cigarettes for 7 days and 24 hour urine samples and blood samples will be collected to assess NNK and nicotine metabolism (see Figure 1 for study schema).

Figure 1



5.2 Study Procedures:

Clinical Study visits will be scheduled at the University of Hawai'i Cancer Center clinic, however, if participants are unable to attend the clinic visit, home visits will be completed.

Visit 1 (Baseline)

At the first Clinical Study visit, the following procedures will be administered:

1. Participants will be provided detailed information about the study, and asked to sign the Study 2 Consent Form. Medical History, Concomitant Medications and Tobacco Use Exposure Update will be reviewed for any changes in health since last contact following consent procedures.
2. Timeline Follow-Back will be administered to assess the last two weeks of cigarettes and other tobacco/nicotine products, other combusted products or other drugs.
3. Provide the following supplies and instructions for the collection of the 24 hour baseline urine sample to obtain baseline sample while smoking usual brand cigarettes:

a. Two 24h urine collection 1500 mL containers (no preservative) to collect the urine for **Sample 1** (the second container is provided in case the participant collects over 1500 mL of urine).

b. For women: Specimen collection commode that can be placed over the toilet that will allow easy capture of urine.

c. Instructions to begin the 24 hour urine collection with the second void the day before the next visit and continue to collect ALL urine through the first void of the visit day.

d. Instructions for urine container storage: Keep container in a cool place, such as near the toilet.

e. At Home Urine Collection Questionnaire assessing start and end time of the collection and verifies any missed collections.

4. Obtain vital signs (heart rate, blood pressure).
5. Obtain exhaled alveolar carbon monoxide (CO) levels; obtain time of last cigarette and number of cigarettes smoked the day of the visit.
6. Provide Daily Tobacco Use Diary to record each day's cigarette, other tobacco or nicotine use, and number of alcoholic drinks daily until the end of the study.
    a. Inform participants to refrain from using any other nicotine-containing or combustible product, however ask them to record these products should any be used.
7. Schedule Visit 2 for day of starting study cigarettes. (Provide Visit Calendar Handout).

Procedures to be performed at Visits 2 - 6:

The following procedures will be performed:

1. Administer Health Changes Questionnaire to assess any change in health since last visit.
2. Administer Timeline Follow-Back.
3. Administer Adverse Event checklist to assess for any untoward symptoms.
4. Obtain vital signs (heart rate, blood pressure).
5. Obtain carbon monoxide (CO) levels; obtain time of last cigarette and number of cigarettes smoked the day of the visit.
6. Provide two 24 hour urine sample collection containers for starting the next 24h sample (Visits 3, 4 and 5).
    - Instruct the participant to start the 24h sample starting with the second morning void and ending with the first morning void on the day of the visit. This sample

will be brought into the clinic the day it is completed. The second container will be used to start the next 24h collection with the second void.

7. Collect 24h urine sample and review collection times and volume and verify if any collection points were missed (Visits 2, 4, 5, 6).
8. Collect and review Daily Tobacco Use Diary.

Additional Procedures to be performed at Visit 2, 3, 4, and 5:

1. Dispense deuterated NNK study cigarettes to last until the next visit. Number of cigarettes to be dispensed is based on the maximum number of cigarettes smoked per day as reported in the participant's baseline Daily Tobacco Use Diary.
2. Complete the Study Product Dispensing Log
3. Instruct participant to:
    - Smoke only the study cigarettes and to use no other nicotine-containing products or combustibles over the 7-day intervention period.
    - Smoke approximately the same number of cigarettes per day as when smoking their usual brand of cigarettes and to try to maintain this rate of smoking for the duration of the study.
    - Collect all study cigarette spent filters (butts). Return of all used cigarette butts in dated ziplock collection baggies at next clinic visit. Return all unsmoked cigarettes and empty cigarette packs. This allows for verification of amount of study cigarettes smoked.

Additional Procedures for Visit 4 and 5

1. Blood draw (2 lavender and 1 red top)

Additional Procedures for Visit 6

1. Provide tobacco cessation materials to the participant and advise to set a quit date.
2. Arrange for study compensation.

Phone follow-up – Visit 7

The participant will be called two to four days after going off of the study cigarettes to assess for any health changes or adverse events.

1. Administer Health Changes Questionnaire to assess any change in health since last visit.
2. Administer Adverse Event checklist.

Table of Study Procedures

	CLINICAL STUDY (N=20 Japanese Americans)										
	OBS[1]	BL[2]	Days on Study Cigarettes								
STUDY DAY		**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**11**
Clinic Visits		**1**	**2**			**3**		**4**	**5**	**6**	Phone Follow-up
Telephone screening X											
Consent form	X	X									
Questionnaires[3]	X										
Tobacco Exposure and History Interview	X	X[4]									
Environmental Exposures	X										
48 food recall	X										
First void urine	X										
Oral cell collection	X										
Blood draw	X								X	X	
Body Mass Index (ht & wt)	X										
Medical History		X[4]									
Concomitant Medications		X[4]									
Carbon Monoxide	X	X	X			X		X	X	X	
Vital signs	X	X	X			X		X	X	X	
Adverse effects			X			X		X	X	X	X
Health changes &Timeline Follow-Back		X	X			X		X	X	X	X
Dispense study cigarettes			X			X		X	X		
Smoke study cigarettes[5]			X[4]	X	X	X	X	X	X	X	
Collect study cigarette butts			X	X	X	X	X	X	X	X	
Return study cigarette butts						X		X	X	X	
Start 24h urine collection		X					X	X	X		
Return 24h urine			X					X	X	X	
Daily smoking diary		X	X	X	X	X	X	X	X	X	

[1]OBS= Observational Sample Collection Study Data collected during Study 1 will be used for Study 2; [2]BL=Baseline; [3]Demographics, Fagerstrom Test for Nicotine Dependence, NIAAA Alcohol Use Questionnaire; [4]Abbreviated questionnaires assessing changes from Study 1; [5]Participants will attend visit before noon and will begin smoking study cigarettes immediately after the clinic visit and before the last clinic visit for a full 7 days of smoking study cigarettes.

5.3 Individually Identifiable Health Information:

Identifiable health information will be collected at the University of Hawai'i. Identity of participants will not be shared with other investigators.

6.0 Data and Specimen Banking

6.1 Storage and Access:

Biomarker specimens will be collected and stored at the study site in Hawai'i until shipment to the Masonic Cancer Center's Drs. Stephen Hecht and Sharon Murphy laboratories for analysis or long term storage. Shipments may be requested at approximately quarterly intervals. Samples that are not used for the primary analysis of study biomarkers will be banked for future use. The banked samples will be stored until requested by a qualified investigator and used up or destroyed if it is determined they are no longer needed. The samples, including DNA or RNA, may be stored up to a maximum of 20 years from the study's end. A participant has the right to withdraw consent at any time by informing the Principal Investigator by following the instructions provided in the consent documents. If this occurs, any remaining identifiable research sample(s) will be destroyed.

6.2 Data:

Biomarker samples (blood components and urine) that are banked after the completion of the primary analyses will be stored at the Masonic Cancer Center Hecht and Murphy laboratories located at the Cancer and Cardiovascular Research Building for future use.

Local data will be stored at the study site at the University of Hawai'i Cancer Center. This data will be provided to the other Program Project Investigators as needed to conduct their study. Shared data will be de-identified and includes demographics, tobacco/nicotine exposure history and current use, health and medications, participative questionnaire data, and biomarkers analyses.

6.3 Release/Sharing:

We intend to share findings from this research through publications and presentations. Institutions and individuals wishing to access any resources or data must contact the Principal Investigator. Data will be available in two formats. One will be a summary of the data, with graphs and tables, posted as pdf files and as raw individual-level data for analysis. Data generated by this grant will be made to outside investigators, according to NIH Guidance. When data are shared, there will be no limits placed on how the data will be used, and co-authorship is not required as a condition for receiving data. Users will agree, however, that the recipient must not transfer the data to other users and that the data are only to be used for research purposes. A record of transfer of data and a copy of the dataset that was distributed will be kept by University of Minnesota.

7.0 Sharing of Results with Participants

7.1 Results of the study

No individual data will be shared with the participants.

8.0 Study Population

8.1 Inclusion Criteria:

a) Japanese American – one, but preferably 2 biological parents of Japanese descent;
b) Male or female 21 years or older (adolescents/young adults under the age of 21 will be excluded);
c) Daily smoker > 5 cigarettes for at least 3 months;
d) Eligible urinary ratios of total 3-HCOT to cotinine:
    - "Little or no-CYP2A6 activity" defined as a ratio of <0.6 or;
    - "Relatively high" CYP2A6 activity defined as a ratio of >3.0.
e) Stable and good physical health;
f) Stable and good mental health (e.g., not experiencing unstable or untreated psychiatric diagnosis, including substance abuse, within the past 3 months);
g) Provided written informed consent to participate in the study.

8.2 Exclusion Criteria:

a) Unwilling to avoid other nicotine containing products during the study and no use of any nicotine-containing products except cigarettes for 1 week prior to their study visits);
b) Currently taking any medications that affect relevant metabolic enzymes or anti-inflammatory medications such as ibuprofen (this will be reviewed by study investigators on a case-by-case basis);
c) Active infection (e.g., influenza, cold, respiratory infection, sinus infection) at the time of enrollment);
d) Experiencing medical conditions that might affect biomarkers of exposure and effect;
e) Pregnant or nursing or planning on becoming pregnant during the study;
f) Unable to read and understand English;
g) Unable to identify ancestry.

8.3 Screening:

Participants are initially screened in Study 1: Observational Study where urine and blood samples were collected and analyzed to determine rate of nicotine metabolism. During Study 1, Japanese American participants were informed of the opportunity to participate in Study 2 if they meet the eligibility criteria. When eligible participants are identified, they will be provided with detailed description of the study and attend a Study 2 Screening visit where the study will be described in detail, and informed consent will be obtained prior to any study procedures. Participants have completed several forms as part of Study 1 and this information will be shared with Study 2. As part of the Study 2 Screening visit participants will complete:

a) Brief Medical History

b) Concomitant medications
c) Tobacco Use Exposure and History Update
d) NIAAA Alcohol Use Questionnaire-short form
e) Fagerstrom Nicotine Dependence Questionnaire

Medical history will be reviewed by the study medical professional to determine eligibility.

9.0 Local Number of Participants

9.1 Local Number of Participants to be Consented:

Up to 50 participants will be consented in order to complete 20 participants. All participants will be recruited at the University of Hawai'i Cancer Center. No recruitment will occur at the University of Minnesota.

10.0 Recruitment Methods

10.1 Recruitment Process:

Participants are recruited from Study 1: Observation Study part of "Mechanisms of Ethnic/Racial Differences in Lung Cancer Due to Cigarette Smoking"

IRB ID: Study 00000335; University of Hawai'i IRB ID: 17649.

10.2 Identification of Potential Participants:

University of Hawai'i research staff will inform potential participants by phone that they are eligible to enroll in Study 2. These participants have already agreed to be contacted regarding the opportunity to participate in Study 2. Upon contact they can agree or decline further information and screening.

10.3 Recruitment Materials:

No additional recruitment materials will be used in this study beyond the Study 1 recruitment.

10.4 Payment:

Participants will receive $40 for time and $10 for travel for each of the 6 clinic visits ($300); $50 for each of the four 24 hour urine collections ($200); $10 for each of the 8 days of cigarette butt collections ($80) and a bonus of $250 for completing all study procedures. Payment is through the University of Hawai'i Cancer Center compensation method (gift card) to compensate for effort, time, and transportation for a total of $750.

11.0 Withdrawal of Participants

11.1 Withdrawal Circumstances:

If a participant experiences an adverse event or no longer meet eligibility criteria (e.g., quit smoking) they will be withdrawn.

11.2 Withdrawal Procedures: If a participant is no longer eligible, they will be informed at the visit and data collection will stop. If withdrawal is due to an adverse event, appropriate follow-up will be completed.

11.3 Termination Procedures: No additional procedures will be completed however, data already collected may be used.

12.0 Risks to Participants

12.1 Foreseeable Risks:

Potential risks: The potential risks for participants are minimal.

In this Clinical Study, the risks in this study include:

1) Loss of privacy due to a breach in confidentiality;

2) Risk of pain, bruising or infection at the site of the phlebotomy; and

3) Use of study cigarettes.

Protection against breach of confidentiality:
Participants will be told their participation in the project will be strictly confidential, that any identifying information will only be available to the site investigators, coordinating center study manager, an auditor for the Food and Drug Administration, study sponsor or University of Hawaii's institutional oversight. No identifying information concerning the data and results will be made known. Participants will have written assurance that while de-identified individual participant data may be available to other researchers for research purposes, only a summary of the results will ever be published or otherwise publicly released. They will also be informed that all raw data will be coded with numbers and any identifiers will be kept in locked file cabinets and only appropriate study personnel will have access.

All data will be de-identified and transferred through a secure, password-protected website that is only available to the investigators. All identifying information will be in a locked in a secure place and accessible only to relevant study staff.

Risk with phlebotomy:
Blood samples will be obtained by a trained phlebotomist with single-use, sterile blood collection supplies to minimize the risk associated with a blood draw.

Smoking Study Cigarettes:
Clinical Study participants will be smoking cigarettes containing deuterium labeled NNK, which has not led to any adverse events in the studies that we have completed (NCT01158456, NCT00691132). To minimize risk, participants will have a review of their medical history prior to entry into this study to screen for any potentially compromising medical condition. Participants will be under medical supervision throughout their

participation in the clinical study and adverse symptoms will be recorded at each clinic visit and monitored by the study's health professional.

The study cigarettes pose the same risks of conventional cigarettes. Because smoking causes a multitude of diseases, if at any time the smoker wants to quit use of all tobacco products, this decision will be encouraged and supported. At the end of study, participants will be strongly encouraged to stop use of all tobacco products and to set a quit date, and will be provided with a treatment resources and referral to local treatment options.

Participants will be informed the study cigarettes contain the same risks as smoking any combustible tobacco product. Precautions would be similar to other tobacco products including smoking related health risks, secondhand smoke exposure and fire danger. Participants will be advised to keep out of reach of children or pets.

Throughout their participation in the study, adverse symptoms will be recorded at each clinic visit and monitored by the PIs and study medical personnel. Participants who experience any significant adverse side effects will be examined by a physician, sent or accompanied to the emergency clinic or referred to their physician, depending on the nature and severity of the side effect or adverse event.

12.2 Reproduction Risks: No additional risk to the fetus than the risk associated with conventional cigarettes. We will conduct a pregnancy screen of child-bearing women prior to providing study cigarettes to verify status.

12.3 Risks to Others: No additional risk of study cigarettes; all cigarettes pose a risk of exposure to second-hand smoke.

13.0 Potential Benefits to Participants

13.1 Potential Benefits: None

14.0 Statistical Considerations

14.1 Data Analysis Plan:

In subjects with little CYP2A6 activity we expect the metabolism through NNAL to be significantly higher and metabolism by α-hydroxylation to be much lower than in smokers with "normal" CYP2A6 activity. The extent of NNK α-hydroxylation in the two groups will be described by a ratio of NNK metabolites, D_4- α-hydroxymethyl NNK Gluc (or other products of α-hydroxylation) to D_4-NNAL. The ratio is used as a measure of the pathway as a percent of dose. The values are predicted to be lower in the smokers with little or no CYP2A6-activity.

Statistical considerations: Our comparison will be between the means (or if the distribution is highly skewed the medians or geometric means) of the two groups (null versus average CYP2A6). The power of the test depends

on the size of the group difference compared to the within-group variability, i.e. to the standard deviation of NNK α-hydroxylation. Assuming that a normal distribution holds we will have 80 percent power to detect a difference in group means amounting to about 0.9 standard deviations of the distribution of NNK □- hydroxylation. In these analyses we will adjust for potential confounders including age, sex, race, total nicotine equivalents, BMI, etc.

We will also compare the NNK metabolic profiles of the two groups. The beauty of this new profiling method is that several pathway will be analyzed at once allowing the characterization of the overall flux of NNK through its major metabolic pathways. The aim is primarily descriptive (characterizing the NNK metabolic profile) and hypothesis generating. Data analysis will use multivariate methods such as cluster analysis, pathway mapping, heatmaps and other statistical applications to characterize metabolites variation and covariation of NNK, NNAL-N-oxide, NNAL-glucuronides, α-hydroxy glucuronides and other NNK metabolites identified in smokers receiving [Pyridine D_4]-NNK

15.0 Provisions to Monitor the Data to Ensure the Safety of Participants

15.1 Data Integrity Monitoring.

Monitoring will be accomplished internally by performing routine data quality checks through the Quality Control reports. In addition, the Project Manager will regularly receive reports that will assess key data variables including eligibility items, enrollment, Adverse Events, Serious Adverse Events and protocol deviations and violations. Consent forms and eligibility will be reviewed for completion, accuracy and compliance with protocol.

Increased monitoring activity will be implemented for cause

15.2 Data Safety Monitoring.

The study coordinator and the supervising physician (Charles Rosser, MD) at the site of data collection will be responsible for the daily oversight of participant safety. Entrance criteria will be reviewed following screening. For the Project 2 clinical study, medical history will be reviewed by the licensed medical professional and referred to the supervising licensed medical professional to identify any contraindications for smoking the study cigarettes. Participants will be under supervision while in the study and seen 4 of the 7 days on study cigarettes by research staff who will assess adverse events and vital signs and make appropriate referrals to the study physician.

The Project Manager will conduct a study initiation meeting and provide internal monitoring of participant safety. If any safety issues are identified, the investigator and staff at the University of Hawai'i will be contacted immediately.

A Data and Safety Monitoring Board will be convened and will be comprised of three experts in the areas of tobacco toxicants, clinical studies. The Board/Committee will include individuals with an MD, a PhD, and a statistician. The Data and Safety Monitoring Board will begin by reviewing the protocol and establishing guidelines for data and safety monitoring. This will include developing standard procedures for day-to-day monitoring by the internal monitors, investigators and study staff. This Board will meet at regular intervals (at least once a year) to evaluate the progress of the study, review data quality, patient recruitment, study retention, and examine other factors that may affect study outcome. They will also review the participant's ability to achieve the study requirements and the rates of adverse events to determine whether there has been any change in participant risk. Their review will ensure that participant risk does not outweigh the study benefits. A brief report will be generated from each of these meetings for the study record and forwarded to each of the study site's Institutional Review Boards (IRB). The DSMB will be available to convene outside of their regular meetings, if necessary, if concerns should arise regarding a particular participant, or any troublesome trends are observed in the participant experiences. They will make appropriate recommendations for changes in protocol, if needed. The UHCC also has a Cancer Protocol Review Committee that meets to review all cancer related protocols and this study will also be subjected to review by this committee on a yearly basis.

All study cigarette-related adverse events of a non-serious nature will be reported to each institution's IRB at the time of IRB renewal application submission. Reportable adverse events will be reported by telephone to the IRB within 3 days of our receipt of information regarding the event and written reports will be submitted within 10 days. The Data and Safety Monitoring Board will review all serious or unexpected adverse events and provide recommendations.

NIH will be informed of any significant action taken as a result of the Data and Monitoring Board's findings. We will inform the participants of any changes in risk.

At the time of IRB annual review, a copy of the submission will be provided to the UHCC and UMN Cancer Center Protocol Review Committee.

16.0 Provisions to Protect the Privacy Interests of Participants

16.1 Protecting Privacy:

For this study, most physiological and subjective measures will be noninvasive and should present no psychological or medical risk to the participant. Some questionnaires may be of a sensitive nature assessing participants' alcohol or drug use. Participants will be told that they may refuse to answer, however, refusal may

effect continued participation in the study. Participants will be told that their data will be kept confidential.

16.2 Access to Participants*:*

Participants will be referred from Study 1: Observation Study and may also be recruited locally through advertisements

17.0 Consent Process

Consent Process (when consent will be obtained): The consenting process will take place at the University of Hawai'i Cancer Center. The Japanese American participants will have been initially been briefed regarding the potential to be part of Study 2. They are told that after the analysis of the biomarker samples we will determine if they meet the eligibility criteria for Study 2 and if so, we would contact them by phone with information about the study. They can determine if they are interested in being screened for the study at that time. Only participants who agreed to be contacted regarding the second study will be recruited.

At the screening visit, the Research Assistant will present a PowerPoint that describes the study in detail. The participant will have ample time to read the consent form, ask questions and receive answers. The participant will respond to several questions regarding the purpose, procedures and risks of the study prior to signing the consent forms. Afterwards, the screening procedures can commence.

18.0 Setting

All recruitment activities will take place at the University of Hawai'i Cancer Center, 701 Ilalo Street, Honolulu, HI, 96813.

Biomarker specimens will be sent to the Sharon Murphy lab at the University of Minnesota, Masonic Cancer Center, 2231 6th Avenue, Room 2-127, Minneapolis, MN 55455.

19.0 Multi-Site Research

There is only one recruitment site, the University of Hawai'i Cancer Center. Dr. Hatsukami at the University of Minnesota is the PI for this study.

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