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"DECIPHERING GENETIC INFLUENCES: CYP1A1 AND CYP2A6 GENE ASSOCIATIONS WITH NICOTINE METABOLITES IN WOMEN BEEDI ROLLERS OCCUPATIONALLY EXPOSED TO TOBACCO"

Occupational Health

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ABSTRACT

Occupational tobacco exposure, particularly among beedi rollers, understanding the genetic influences on nicotine metabolism, mediated by cytochrome P450 enzymes such as CYP1A1 and CYP2A6, is crucial. This study aims to elucidate the complex interplay between genetic variations in CYP1A1 and CYP2A6 and nicotine metabolism pathways, with implications for mitigating tobacco-related harm in occupational tobacco exposure groups. A total of 600 individuals, aged 15-50 years, were involved in the study, comprising 320 tobacco exposed (TE) subjects and 280 tobacco non-exposed (TNE) subjects (control group). High-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was employed to quantify nicotine metabolites, including cotinine, nor nicotine, anabasin, NNN, and NNK, following established protocols. CYP1A1 (exon 7) and CYP2A6 (exon 5) genes were studied in both tobacco exposed (TE) and tobacco non-exposed (TNE) groups to identify single nucleotide polymorphisms (SNPs) in the exon region of each gene by sanger sequencing. Our study reveals significant association between nicotine metabolite levels and genotypic variations of CYP1A1 (A-G), CYP1A1 (C-T), and CYP2A6 (C-T) genes. In conclusion the findings contribute to our understanding of the genetic predispositions of CYP1A1 and CYP2A6 genes influencing nicotine metabolism among occupational tobacco exposure individuals, while CYP2A6 was negatively correlated with all the nicotine metabolites.

KEYWORDS

Tobacco exposure, Nicotine metabolites, CYP1A1, CYP2A6, Sanger sequencing.

INTRODUCTION

Tobacco exposure, particularly through beedi rolling, remains a widespread practice in various regions, particularly in South Asia (Jha et al., 2008). Beedi rollers, predominantly women from economically disadvantaged backgrounds, face heightened health risks due to prolonged exposure to tobacco constituents (Jha et al., 2008). Among these constituents, nicotine metabolites such as cotinine, nor-nicotine, anabasine, N-nitrosonornicotine (NNN), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) have been extensively studied for their association with tobacco-related health outcomes, including cancer (Hecht, 1999). Previous research has demonstrated the utility of cotinine as a biomarker for tobacco exposure, commonly measured in biological samples such as serum using LC-MS techniques (Jacob et al., 2017). Studies have also highlighted the importance of nornicotine and anabasine in understanding tobacco consumption patterns and associated health risks (Benowitz et al., 2009). Additionally, the carcinogenic nitrosamines NNN and NNK, formed during tobacco curing and fermentation processes, have been implicated in various cancers, including lung cancer (Hecht, 1999).

Understanding the levels of these metabolites in serum samples could provide insights into their exposure levels and associated health risks (Jacob et al., 2017). Furthermore, genetic studies focusing on the CYP2A6 and CYP1A1 gene polymorphisms are essential. CYP2A6 is the primary enzyme responsible for the metabolism of nicotine in the liver, while CYP1A1 is involved in the activation of procarcinogens found in tobacco smoke (Nebert & Russell, 2002). Variations in these genes can influence nicotine metabolism rates, tobacco consumption behaviors, and susceptibility to tobacco-related diseases (Nebert & Russell, 2002). Sanger sequencing is a widely used method for studying genetic variations, including single nucleotide polymorphisms (SNPs), within the CYP2A6 and CYP1A1 genes (Nebert & Russell, 2002).

In the context of occupational tobacco exposure, particularly among individuals engaged in beedi rolling, understanding the genetic determinants influencing nicotine metabolism is paramount. Cytochrome P450 enzymes, notably CYP1A1 and CYP2A6, play crucial roles in the biotransformation of nicotine and its metabolites, thus affecting individual susceptibility to tobacco exposure and response to smoking cessation interventions. However, comprehensive investigations into the associations between genetic variations in these enzymes and nicotine metabolism among beedi rollers remain limited. Therefore, this study aims to elucidate the intricate relationship between genetic factors and nicotine metabolism pathways by investigating serum levels of nicotine metabolites and CYP2A6 and CYP1A1 gene polymorphisms.

MATERIALS AND METHODS

Study Subjects

A total of 600 individuals, aged 15-50 years, were recruited for the study, comprising 320 women beedi rollers and 280 women non-beedi rollers (control group). Participants were selected based on stringent criteria to ensure homogeneity within the study groups, excluding individuals with underlying systemic illnesses, disorders, or those undergoing hormonal therapies. Prior to commencing the study, clearance was obtained from the Institutional Ethics Committee of Bhagwan Mahavir Medical Research Centre, Hyderabad. Structured questionnaires were used to collect detailed information on participants' tobacco exposure, including the duration of exposure in years and average daily hours of exposure. Additionally, demographic data such as age and socio-economic status were recorded to assess their potential influence on tobacco-related outcomes. Informed consent was obtained from each participant prior to data and blood sample collection.

Sample Collection

5ml of blood samples were collected from both the study groups and aliquoted into two separate vaccutainers for separation of serum for nicotine metabolites estimation and other vaccutainers containing

EDTA for molecular studies. Serum samples were promptly separated and stored at -80°C until further analysis to preserve sample integrity and minimize degradation.

Nicotine Metabolites Estimation

High-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was employed using Quattro premier XE (Waters Systems, USA) and Acquity UPLC (Waters Systems, USA) system as a Front End (LC) to quantify nicotine metabolites, including cotinine, nor nicotine, anabasin, NNN, and NNK, following established protocols. Calibration curves were meticulously prepared using standard solutions to ensure accurate and precise quantification of metabolite concentrations (Ying et al 2012).

Reagent Preparation

Reagents used in the study, including formic acid, dimethyl sulfoxide (DMSO), acetonitrile, methanol, and Milli-Q water. Standard reference materials, such as NNK, NNN, nornicotine, and verapamil, were obtained from Sigma Aldrich to ensure reliability and accuracy in the analysis.

Genomic DNA extraction and Polymerase Chain Reaction (PCR)

Whole genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) following the manufacturer's instructions. CYP1A1 and CYP2A6 genes were studied to identify single nucleotide polymorphisms (SNPs) in the exon region of each gene.

PCR was carried out to amplify targeted regions of DNA fragments. CYP1A1(Exon 7) primer details are forward: CTCCACTCACTTGACACTTCTG and reverse: CAGCCAGATCAGTGTCTATGAG. CYP2A6 (Exon 5) primers are forward: CTTCCCATCCTCTCTCTGCAAC, reverse: TGATTTCCCTCTGCCTGGCTT. The amplification of DNA was carried out using the Gradient PCR instrument (Takara, Model no: TP600, Japan). PCR conditions included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at optimized temperatures specific to gene for a duration of one minute, followed by an extension step at 72°C for one minute. The PCR products were analyzed for quality and quantity using agarose gel electrophoresis and BioPhotometer-D30 (Eppendorf, USA).

Sanger Sequencing Analysis

PCR products of all the genes were purified using a GSure® PCR product purification kit according to the manufacturer's instructions. Purified PCR samples were subjected to BigDye Terminator Cycle Sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit. Master mix for sequencing was prepared by combining purified PCR product, sequencing primer, BigDye Terminator reagent, and sequencing buffer. Thermal cycling conditions included denaturation, annealing, and extension steps. Sequencing reactions were loaded onto the ABI 3730XL sequencing machine (Applied Biosystems, USA), and data were analyzed using various bioinformatics tools for SNP identification and mutation analysis.

Statistical Analysis

Statistical tests, including chi-square tests, non-parametric t-tests, and correlations were conducted to compare metabolite levels between groups and assess associations with genetic variant and nicotine metabolites. Graphs were generated utilizing Graphpad Prism (Version 10). Data analysis was performed using SPSS (Version 28 and Graphpad Prism, Version 10) software packages, with significance set at $p < 0.05$.

RESULTS

The demographic profile of the study participants, as presented in Table 1, illustrates that the majority of individuals engaged in beedi rolling were aged between 21 and 30 years, with the next largest group falling within the 31-40 years age group. Notably, a subset of 10% of the sample population belonged to the 15-20 age group. In Table 2, the distribution of various nicotine metabolites in both tobacco-exposed (TE) and non-exposed (TNE) groups exhibits significant associations across all metabolites, including Anabasine, NNN, NNK, Nornicotine, and Cotinine. Regarding the association of CYP1A1 genotypes (A-G) in both TE and TNE groups, Table 3 reveals significant correlations, particularly with the AG and GG genotypes along with the G allele. Similarly, Table 4 and Table 5 demonstrate significant associations between CYP1A1 (C-T) and CYP2A6 (C-T) genotypes in both TE and TNE groups, particularly with the CT and TT genotypes and the T allele. Table 6 outlines the allele distribution of CYP1A1 (A/G), CYP1A1 (C/T), and CYP2A6 (C/T) in TE and TNE groups, indicating minor allele frequencies and significant adherence to Hardy-Weinberg Equilibrium.

The association study between nicotine metabolites and CYP1A1 (A-G) genotypes, as depicted in Table 7, showed significant associations between AA genotype and all metabolites, while AG genotype showed associations with all metabolites except NNK, GG genotype did not exhibit any notable associations with any of the metabolites. The results presented in the Table 8 on the association between nicotine metabolites and CYP1A1 (C-T) genotypes, showed significant association between CC and CT genotypes and all the metabolites, while TT genotype showed association primarily with Anabasine. Similarly, the analysis of the association between nicotine metabolites and CYP2A6 (C-T) genotypes in tobacco-exposed (TE) group revealed notable findings. Specifically, the CC genotype demonstrated significant associations with all metabolites examined. Conversely, the CT genotype exhibited significant associations solely with NNN, Nornicotine, and Cotinine. However, the TT genotype displayed significant associations with Anabasine, NNN, and Nornicotine. These observations are shown in Table 9.

The correlation analysis showed the strongest positive correlation between nor-nicotine and NNN with a coefficient of 0.52, and significant correlations with NNK and cotinine with 0.44 0.25 coefficient values, whereas NNN and NNK are correlated with a coefficient of about 0.38. NNK showed a correlation with Cotinine of 0.18. While CYP2A6 was negatively correlated with all metabolites in which nornicotine showed the strongest negative correlation with -0.010 coefficient values. When it comes to genes, CYP2A6 was significantly correlated with CYP1A1 (A-G) with a co-efficient value of -0.26 (Figures 1-4).

DISCUSSION

The cytochrome P450 (CYP) enzyme family, known for its hemoprotein structure, is recognized as one of the most prevalent and varied superfamilies in biological contexts (Nelson et al., 1996). Among these enzymes, human CYP enzymes are notably essential in Phase I drug metabolism, aiding in the metabolism of a wide array of pharmaceuticals (Zanger & Schwab, 2013). Among the human CYP isoforms, CYP1A emerges as a crucial subfamily involved in the metabolism of drugs, environmental toxins, and endogenous compounds (Nebert & Russell, 2002; Nebert et al., 2004). This subfamily comprises two functional genes, CYP1A1 and CYP1A2, both highly conserved across species (Nebert & Russell, 2002). In humans, these genes are positioned on chromosome 15 q24.1 (Nebert & Russell, 2002). Phylogenetic analysis suggests that CYP1A2 might have evolved from CYP1A1, sharing a common 5′-flanking region that contains bidirectional regulatory elements for both genes (Nebert & Russell, 2002). Despite their shared regulatory elements, the expression patterns of CYP1A1 and CYP1A2 differ significantly. For instance, while CYP1A2 exhibits constitutive and specific expression in the liver, CYP1A1 is predominantly expressed outside the liver (Nebert & Russell, 2002).

CYP1A enzymes catalyze hydroxylation and oxidation reactions of aromatic compounds, with CYP1A1 primarily metabolizing aromatic hydrocarbons and CYP1A2 showing preference for aromatic amines and heterocyclic compounds (Nebert & Russell, 2002). Functionally, CYP1A plays a crucial role in the metabolism of approximately 9% of clinical drugs, including analgesics, antipyretics, antipsychotics, antidepressants, and anti-inflammatory drugs (Nebert & Russell, 2002; Zanger & Schwab, 2013). Moreover, CYP1A contributes significantly to the biotransformation of environmental pollutants such as benzopyrene, aristolochic acid I, ellipticine, PhIP, and IQ (Nebert & Russell, 2002). CYP1A predominantly metabolizes a variety of substances in humans, including sex hormones (such as progestogens, androgens, and estrogens), retinol, melatonin, linoleic acid, phosphatidylcholine, and uroporphyrinogen, through various reactions such as hydroxylation, demethylation, epoxidation, oxidation, and quinol formation.

Polymorphic variants of the CYP1A1 gene are predominantly associated with tobacco-related cancers such as lung and esophageal cancer across diverse ethnic groups (Yang et al., 2005; Dong et al.,

2008; Lee et al., 2008). Specifically, four distinct sequence polymorphisms have been identified within the CYP1A1 gene, with the first known variant, termed CYP1A1*2, involving a T6235 to C transition in the 3' noncoding region (Jun et al., 2010). Single nucleotide polymorphisms and the inheritance of loss of both alleles are prevalent in these gene superfamilies, exhibiting varying frequencies among different population groups (Senthilkumar and Thirumurugan, 2012; Sharma et al., 2012; Zhou et al., 2012).

The biological consequences of CYP1A induction are twofold. Firstly, induction of CYP1A serves as a mechanism for regulating the chemical environment within cells by enhancing the metabolic clearance of substrates. However, since CYP1A1/2 is responsible for catalyzing the metabolic activation of polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic hydrocarbons (HHAs) into ultimate carcinogens, it is anticipated that enzyme induction may pose a risk to individuals exposed to high levels of PAHs and HHAs, such as those from cigarette smoke. Additionally, there are significant variations in the induction of these enzymes among human populations (Ma and Lu, 2003). Rajiv et al. (2013) conducted a study investigating clinical, cytogenetic, and CYP1A1 exon-1 gene mutation analysis among beedi workers in the Vellore region of Tamil Nadu. Their findings revealed a statistically significant increase in the frequencies of chromosomal aberrations in the exposed groups compared to their respective controls, and variations were observed in Exon 1 of the CYP1A1 gene. (Rajiv et al., 2013).

Cytochrome P450 2A6 (CYP2A6) plays a pivotal role in the metabolism of nicotine in humans, coordinating the conversion of nicotine into various metabolites. The primary pathway involves the oxidation of nicotine to cotinine, a major metabolite, through CYP2A6-mediated hydroxylation at the C-5 position. Additionally, CYP2A6 catalyzes the formation of nicotine N-oxide from nicotine, contributing to its metabolic profile. Furthermore, CYP2A6 facilitates the hydroxylation of cotinine at the 3'-position, leading to the formation of trans-3'-hydroxycotinine. These enzymatic transformations yield metabolites such as cotinine and trans-3'-hydroxycotinine, which are utilized as biomarkers for nicotine exposure due to their longer half-lives compared to nicotine itself. The intricate metabolic pathways mediated by CYP2A6 highlight its significance in nicotine metabolism and underscore its relevance in understanding nicotine addiction and smoking cessation efforts (Murphy, 2013; Messina & Tyndale, 2013).

Nornicotine undergoes metabolic conversion into NNN (N'-nitrosonornicotine) through a nitrosation reaction facilitated by nitrosating agents commonly present in tobacco smoke and the human digestive system (Hecht, 1998). This process involves the transformation of secondary amines, like nornicotine, into nitrosamines, exemplified by NNN. Enzymatic catalysis is pivotal in this pathway, with CYP2A6 playing a significant role. CYP2A6 effectively catalyzes the conversion of nornicotine to NNN in liver cells and exhibits activity in oral mucosa cells as well (Nakajima et al., 2006). This enzymatic activity is crucial in facilitating the formation of NNN in these physiological environments.

Analysis of metabolite data reveals strong correlations between nornicotine, NNN, and NNK (N'-nitrosoanatabine), suggesting their involvement in shared metabolic or biochemical pathways, thereby amplifying the carcinogenic risks associated with tobacco use (Hoffmann et al., 2001). The correlation between nornicotine and NNN implies a metabolic pathway wherein nornicotine serves as a precursor to NNN in the body, significantly contributing to the carcinogenic potential of tobacco use. Similarly, the observed correlations between nornicotine and NNK, as well as between NNN and NNK, suggest potential synergistic effects, augmenting the overall carcinogenic risk when these compounds are present at elevated levels.

The process of smoking cessation is impacted by genetic variations in CYP2A6, responsible for the inactivation of nicotine into cotinine and the subsequent metabolism of cotinine into 3′hydroxycotinine (Tanner et al., 2007; Hukkanen et al., 2005). The nicotine metabolite ratio (NMR), calculated as the ratio of 3′hydroxycotinine to cotinine, serves as a biomarker of CYP2A6 activity, with a higher NMR indicating faster nicotine clearance (St Helen et al., 2019). CYP2A6 activity is influenced by estrogen induction, leading to faster nicotine clearance particularly in post-pubertal pre-menopausal females compared to males (Benowitz et al., 2006). Chenoweth et al. (2021) conducted sex-stratified genome-wide association studies on NMR among European American (EA) and African American (AA) smokers. They found that AA smokers tend to have lower NMR compared to EA smokers, partly attributed to a higher prevalence of CYP2A6 decreased or loss-of-function variants in AA individuals (Chenoweth et al., 2021). Additionally, the higher prevalence of mentholated cigarette smoking among AA smokers may also contribute to the lower NMRs, as menthol has been shown to modestly inhibit CYP2A6 activity in vitro and reduce nicotine clearance in vivo (Benowitz et al., 2006; Ahijevych & Garrett, 2004; Yamanaka et al., 2007). Hence, factors such as sex, ancestry, and mentholated cigarette smoking can significantly influence CYP2A6 activity and subsequently affect NMR levels. Our study provides valuable insights into the correlation between nicotine metabolite levels and genotypic variations of CYP1A1 (A-G), CYP1A1 (C-T), and CYP2A6 (C-T) among tobacco exposure and tobacco non-exposure groups. Significant associations were observed, particularly with AG and GG genotypes of CYP1A1 (A-G), CT and TT genotypes of CYP1A1 (C-T), and CT and TT genotypes of CYP2A6 (C-T), along with the respective alleles. Moreover, positive correlations were noted between these genotypes and Anabasin levels, while negative correlations were evident with NNN, NNK, Nornicotine, and Cotinine levels, suggesting an inverse relationship.

In conclusion, our investigation sheds light on the intricate relationship between genetic variations and nicotine metabolism, particularly among occupational tobacco exposure group. Through comprehensive genotypic analysis of CYP1A1 (A-G), CYP1A1 (C-T), and CYP2A6 (C-T) genes, we have identified significant associations that underscore the influence of genetic predispositions on nicotine metabolite levels. Specifically, the observed associations between AG and GG genotypes of CYP1A1 (A-G), CT and TT genotypes of CYP1A1 (C-T), as well as CT and TT genotypes of CYP2A6 (C-T), along with their respective alleles, provide compelling evidence of genetic influences on nicotine metabolism among tobacco exposure group. These findings align with existing literature suggesting the involvement of these cytochrome P450 enzymes in the biotransformation of nicotine and its metabolites. Furthermore, the positive correlations between certain genotypes and Anabasin levels, coupled with the negative correlations with NNN, NNK, Nornicotine, and Cotinine levels, highlight the complex interplay between genetic factors and nicotine metabolism pathways. By elucidating the genetic underpinnings of nicotine metabolism in the context of beedi rolling, our study contributes valuable knowledge to the fields of pharmacogenetics and tobacco control. Further research is warranted to validate these findings and explore their clinical implications in larger, diverse cohorts of occupational tobacco exposure groups.

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Conflict of Interest: None to declare.
Data availability statement: Data is available with the corresponding author.

Table 1. General characteristics of tobacco exposed (TE) and tobacco non-exposed (TNE) groups.

Variable	TNE n=280 (%)	TE n =320 (%)	P value.
Age group (Years)			
15-20	28 (10)	5 (1.56)	<0.0001**
21-30	142 (50.71)	169 (52.8)	
31-40	65 (23.2)	94 (29.37)	
41-49	33 (11.78)	50 (15.62)	
50	12 (4.258)	2 (0.62)	
Marital Status			
Married	246 (87.86)	308 (96.25)	<0.0001**
Un Married	34 (12.14)	12(3.75)	
Food Habits			
Vegetarian	19(6.8)	10(3.1)	0.05*
Non-Vegetarian	261(93.2)	310(96.9)	

p value < 0.05 is significant.

Table 2. Distribution of Nicotine metabolites (Anabasine, NNN,

NNK, Nornictoine and Cotinine (ng/mL) in tobacco exposed (TE) and tobacco non-exposed (TNE) groups.

Nicotine Metabolites	TNE (n=280)		TE (n=320)		P Value
	Mean	SE	Mean	SE	
Anabasine (ng/mL)	1.72	0.14	7.29	0.44	< 0.0001***
NNN(ng/mL)	0.12	0.03	2.13	0.17	<0.0001***
NNK(ng/mL)	0.02	0.01	0.35	0.09	0.0019***
Nornicotine(ng/mL)	0.003	0.002	0.8700	0.09	< 0.0001***
Cotinine(ng/mL)	6.33	1.09	122.16	13.89	< 0.0001***

p value < 0.05 is significant.

Table 3. Association of CYP1A1 (A-G) in tobacco exposed (TE) and tobacco non-exposed (TNE) groups.

Genotype	TNE n=280 (%)	TE n=320 (%)	OR (95% CI)	P- value
AA	237 (84.6)	174 (54.4)	**1**	
AG	40 (14.3)	103 (32.2)	0.29 (0.19- 0.43)	<0.0001***
GG	3 (1.1)	43 (13.4)	0.05 (0.02- 0.17)	
Allele Frequency				
A	0.92	0.7	NA	1
G	0.08	0.3		<0.0001***

p value < 0.05 is significant.

Table 4. Association of CYP1A1 (C-T) genotypes in tobacco exposed (TE) and tobacco non-exposed (TNE) groups.

Genotype	TNE n=280 (%)	TE n=320 (%)	OR (95% CI)	P-value
CC	39 (12.2)	200(71.4)	1	<0.0001***
CT	259(80.9)	73 (26.1)	0.05 (0.04- 0.08)	
TT	22 (6.9)	7 (2.5)	0.06 (0.02- 0.16)	
Allele Frequency				
C	0.84	0.53	NA	1
T	0.16	0.47		<0.0001***

p value < 0.05 is significant.

Table 5. Association of CYP2A6 (C-T) genotypes in tobacco exposed (TE) and tobacco non-exposed (TNE) groups.

Genotype	TNE n=280 (%)	TE n=320 (%)	OR (95% CI)	P-value
CC	89 (31.8)	204 (63.8)	1	
CT	179(63.9)	52 (16.2)	0.29 (0.19- 0.43)	<0.0001***
TT	12 (4.3)	64 (20.0)	0.05 (0.02-0.17)	
Allele Frequency				
C	0.64	0.72	NA	1
T	0.36	0.28		0.006**

p value < 0.05 is significant.

Table 6. Association of CYP1A1 (A/G), CYP1A1(C/T) and CYP2A6 (C/T) Alleles distribution in tobacco exposed (TE) and tobacco non-exposed (TNE) groups.

Variants	Allele 1	Allele 2	Homozygous wild-type	Heterozygotes	Homozygous variant	MAF*	HWE** (p)
CYP1A1 (A/G)	0.7	0.3	0.54	0.33	0.13	0.3	<0.0001
CYP1A1 (C/T)	0.53	0.47	0.12	0.81	0.07	0.47	<0.0001
CYP2A6 (C/T)	0.72	0.28	0.64	0.16	0.2	0.28	<0.0001

Table 7. Association of nicotine metabolites in CYP1A1 (A-G) genotypes in tobacco exposed (TE) and tobacco non-exposed (TNE) groups.

Genotype	Nicotine metabolites (ng/mL)	Study groups		TE		TNE		P value
		TE n=320	TNE n=280					
				Mean	SE	Mean	SE	
AA	Anabasine	174	237	6.32	0.57	1.64	0.16	0.001**
	NNN			2.21	0.26	0.11	0.04	0.000***
	NNK			0.40	0.15	0.02	0.01	<0.001**
	Nornicotine			0.73	0.11	0.00	0.00	0.000***
	Cotinine			124.25	18.80	6.17	1.08	<0.001**
AG	Anabasine	103	40	8.37	0.81	1.95	0.40	<0.001**
	NNN			2.15	0.28	0.03	0.02	<0.001**
	NNK			0.37	0.15	0.08	0.06	0.291 ns
	Nornicotine			1.12	0.23	0.00	0.00	<0.001**
	Cotinine			115.00	20.28	7.52	4.28	0.004*
GG	Anabasine	43	3	8.66	1.24	5.47	2.00	0.80 ns
	NNN			1.78	0.42	1.70	1.70	0.86 ns
	NNK			0.134	0.09	0.00	0.00	0.86 ns
	Nornicotine			0.85	0.21	0.00	0.00	0.34 ns
	Cotinine			130.91	51.16	3.74	3.74	0.86 ns
Total		320	280					

a. p value < 0.05 is significant
b. Difference in Nicotine metabolites was tested by Mann-Whitney U test between the each genotype (AA,AG and GG) in two groups (TE and TNE) for "p" value

Table 8. Association of nicotine metabolites in CYP1A1 (C-T) genotypes in tobacco exposed (TE) and tobacco non-exposed (TNE) groups.

Genotype	Nicotine metabolites (ng/mL)	Study groups		TE		TNE		P value
		TE n=320	TNE n=280					
				Mean	SE	Mean	SE	
CC	Anabasine	39	200	6.44	1.15	1.49	0.15	0.001***
	NNN			2.85	0.53	0.13	0.05	0.001***
	NNK			0.63	0.48	0.03	0.01	0.09 ns
	Nornicotine			1.25	0.36	0.00	0.00	0.001***
	Cotinine			119.72	34.11	6.55	1.40	0.001***
CT	Anabasine	259	73	7.14	0.49	2.30	0.35	0.001***
	NNN			2.04	0.19	0.09	0.04	0.001***
	NNK			0.31	0.09	0.02	0.02	0.023*
	Nornicotine			0.83	0.11	0.00	0.00	0.001***
	Cotinine			127.08	16.12	5.87	1.68	0.001***
TT	Anabasine	22	7	10.58	1.76	2.29	1.08	0.048*
	NNN			1.98	0.73	0.28	0.28	0.11 ns
	NNK			0.36	0.36	0.00	0.00	0.57 ns
	Nornicotine			0.63	0.27	0.00	0.00	0.07 ns
	Cotinine			68.71	34.48	5.13	4.50	0.90 ns
Total		320	280					

a. p value < 0.05 is significant
b. Difference in Nicotine metabolites was tested by Mann-Whitney U test between the each genotype (AA,AG and GG) in two groups (TE and TNE) for "p" value

Table 9. Association of nicotine metabolites in CYP2A6 (C-T) genotypes in tobacco exposed (TE) and tobacco non-exposed (TNE) groups.

Genotype	Nicotine metabolites (ng/mL)	Study groups		TE		TNE		P value
		TE (n=320)	TNE (n=280)					
				Mean	SE	Mean	SE	
CC	Anabasine	204	89	7.50	0.54	1.80	0.26	0.001***
	NNN			2.03	0.20	0.18	0.10	0.001***
	NNK			0.41	0.12	0.01	0.01	0.024*
	Nornicotine			0.90	0.13	0.00	0.00	0.001***
	Cotinine			123.80	18.33	8.05	2.14	0.007**

CT	**Anabasine**	52	179	4.57	0.87	1.68	0.18	0.055^{ns}
	NNN			2.58	0.45	0.07	0.03	0.001**
	NNK			0.37	0.34	0.04	0.02	0.115^{ns}
	Nornicotine			1.27	0.28	0.00	0.00	0.001**
	Cotinine			148.99	36.33	5.80	1.30	0.001**
TT	**Anabasine**	64	12	8.86	1.11	1.91	0.81	0.016**
	NNN			2.09	0.49	0.48	0.42	0.034*
	NNK			0.16	0.07	0.00	0.00	0.233^{ns}
	Nornicotine			0.44	0.11	0.00	0.00	0.033*
	Cotinine			95.16	23.39	12.43	4.99	0.736^{ns}
Total		**320**	**280**					

a. p value < 0.05 is significant

b. Difference in Nicotine metabolites was tested by Mann-Whitney U test between the each genotype (AA,AG and GG) in two groups (TE and TNE) for "p" value

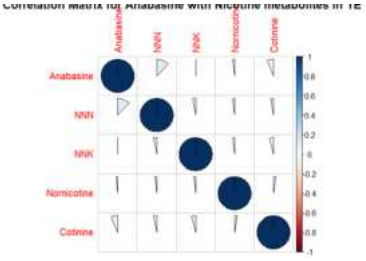

Figure 1. Correlation matrix for anabasine with nicotine metabolites in tobacco exposure.

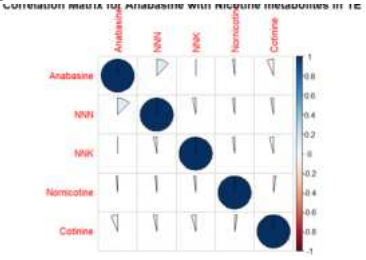

Figure 2. Correlation matrix for anabasine with nicotine metabolites in tobacco non exposure.

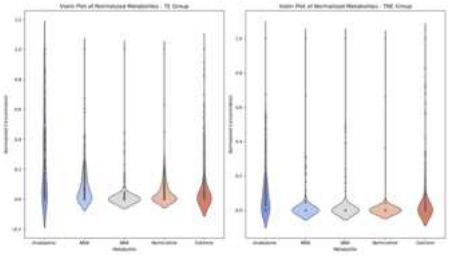

Figure 3. Violin plot of normalized metabolites in tobacco exposure and tobacco non exposure groups.

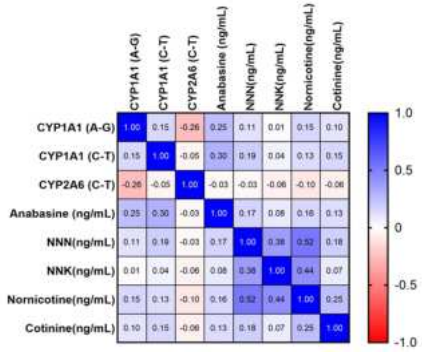

Figure 4. Correlation matrix between nicotine metabolites and CYP1A1 and CYP2A6 genotypes.

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