CYTOCHROME P450 ENZYMES AND OXIDATIVE STRESS IN TOBACCO/NICOTINE

MEDIATED HIV PATHOGENESIS

A DISSERTATION IN

Pharmacology

and

Pharmaceutical Sciences

Presented to the Faculty of the University
of Missouri-Kansas City in partial fulfillment of
the requirements for the degree

DOCTOR OF PHILOSOPHY

by

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B.S., Andhra University, 2010

Kansas City, Missouri

2016

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Anusha Ande, Candidate for the Doctor of Philosophy Degree

University of Missouri-Kansas City, 2016

ABSTRACT

In the US, the prevalence of cigarette smoking in the HIV-infected population is 50-70% compared to 15-20% in the general population, which further increases the risk of smoking-related health problems in this group. For example, HIV-infected smokers show decreased immune responses, poorer responses to antiretroviral therapy (ART), and greater risk of virological rebound, compared to HIV-infected non-smokers. Several *in vivo* and *in vitro* studies have shown that smoking/nicotine is associated with decreased immune responses, increased inflammation, increased oxidative stress, and increased numbers of opportunistic infections. Furthermore, *in vitro* studies have shown that smoking/nicotine enhances HIV replication in alveolar macrophages, microglia, and T cells. However, the mechanism by which smoking or nicotine increases HIV replication is largely unknown. As an exception, a report suggests that iron and oxidative stress are possible mechanisms of enhanced production of HIV by alveolar macrophages in cigarette smokers.

The role of CYP enzymes has not been studied in context with smoking/nicotine and HIV pathogenesis. However, there are several elegant studies that show the involvement of CYP2A6, CYP2A13, CYP1A1, and CYP1B1 in smoking/nicotine-mediated toxicity leading to various types of cancers and hepatic toxicity. The majority of tobacco constituents, including nicotine, are

metabolized and/or activated by CYP enzymes to generate reactive oxygen species (ROS) and/or reactive metabolites, NNK. Based on the fact that CYP pathways play a critical role in smoking-mediated cell/organ toxicity, there is a critical need to explore the involvement of CYP pathways in smoking/nicotine-mediated HIV pathogenesis. The present study was based on the central hypothesis that tobacco/nicotine, or its metabolites, enhance HIV replication in monocytes/macrophages through CYP pathway.

The key findings of our studies presented in this thesis indicate that (a) CYP2A6-mediated metabolism of nicotine increased the generation of ROS in HIV cell model, SVGA astrocytes, (b) Cigarette smoke condensate caused significant induction of CYP1A1, CYP2A6, ROS production, and cytotoxicity in U937 monocytes as well as enhanced HIV replication in HIV-infected primary macrophages, (c) Mild-to-moderate smoking increased viral load in HIV-infected individuals. Furthermore, our study suggested that smoking and HIV independently increase oxidative stress in the plasma as well as in monocytes. (d) There was a decrease in the level of nicotine and subsequent increase in the level of nicotine metabolites, suggesting an increase in nicotine metabolism in HIV-infected smokers compared with uninfected smokers.

In conclusion, our *in vitro* and *ex vivo* results are consistent with the hypothesis that CYP and CYP-mediated oxidative stress by tobacco/nicotine are associated with increased viral load by smoking/smoking constituents. This study has clinical implications in terms of targeting CYP and oxidative stress pathways to find potentially novel therapeutic interventions, as well as drug dose adjustment to treat HIV-infected smokers effectively.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of School of Graduate Studies have examined the dissertation titled "Cytochrome P450 enzymes and oxidative stress in tobacco/nicotine mediated HIV pathogenesis" presented by Anusha Ande, candidate for the Doctor of Philosophy degree, and hereby certify that in their opinion it is worthy of acceptance.

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Contents

TABLES

ILLUSTRATIONS

ABBREVIATIONS

AHR   Aryl hydrocarbon receptor

AIDS   Acquired immunodeficiency syndrome

ANOVA   Analysis of variance

AOE   Antioxidant enzymes

AP-1   Activator Protein -1

ARE   Antioxidant response element

ART   Antiretroviral therapy

ATCC   American type culture collection

BBB   Blood brain barrier

BCA   Bicinchoninic acid assay

BITC   Benzyl isothiocyanate

BSA   Bovine serum albumin

CAD   Collisionally activated dissociation

cAMP   Cyclic adenosine monophosphate

CC   Calibration curve

CCL   Chemokine ligand

CNS   Central nervous system

COPD   Chronic obstructive pulmonary disease

CPS   Counts per second

CSC   Cigarette smoke condensate

CV   Coefficient of variation

CYP1A1   Cytochrome P450 1A1

CYP2A6   Cytochrome P450 2A6

DCF	Dichlorofluorescein
DCFDA	Dichlorofluoroscein diacetate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal-regulated kinases
ESI	Electrospray ionization
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas chromatography – mass spectrometry
GST	Glutathione-sulfo-transferase
HAART	Highly active antiretroviral therapy
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HIVE	HIV encephalitis
HO-1	Hemoxygenase
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IL	Interleukin
IS	Internal standard
JNK	c-Jun N-terminal kinase

LC-MS	Liquid chromatography–mass spectrometry
LLOQ	Lower limit of quantitation
LOQ	Limit of quantitation
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MFI	Mean fluorescence intensity
MRM	Multiple reaction monitoring
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nAChRs	nicotinic acetylcholine receptors
NF-κB	Nuclear factor-kappa B
NIDA	National institute of drug abuse
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone
NNN	N-nitrosonornicotine
NNRTI	Non-nucleoside reverse trascriptase inhibitors
Nqo1	NADPH dehydrogenase quinone 1
Nrf-2	Nuclear factor (erythroid-derived 2)-like 2
PAH	Polycyclic aromatic hydrocarbon
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PEITC	Phenethyl isothiocyanate
PI	Protease inhibitors
PKC	Protein kinase C
PVDF	Polyvinylidene fluoride

QC	Quality control
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
S/N	Signal to noise ratio
SOD	Superoxide dismutase
SPE	Solid phase extraction
TB	Tuberculosis
TCE	Tobacco crude extract
TCID	Tissue culture infectious dose
TNF	Tumor necrosis factor
ULOQ	Upper limit of quantitation
WHO	World health organization
8e PGF2a	8-epi prostaglandin F2 alpha
8-OHdG	8-Oxo-2'-deoxyguanosine

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Santosh Kumar for mentoring me throughout my graduate career and providing me this great opportunity to work in his laboratory. I am thankful for his support in both professional and personal aspects of my life since the first day of my Ph.D. journey. I would like to thank Dr. Anil Kumar, Chair of the Division, for not only serving as my supervisory committee member but also for his direct guidance in my research project. I am very thankful for his generous support in letting me work in his lab facilities. I wholeheartedly thank both of them for mentoring me and for their support in all aspects of my life since I joined Ph.D. at UMKC.

I would like to thank, Dr. Carole McArthur for collaborating with us and providing us the most precious human samples for my ex vivo project. I also thank her for serving as a member of my supervisory committee. I thank Dr. Russell Melchert and Dr. Kun Cheng for serving on my committee and guiding me thoroughly in the process.

I thank Dr. Mengyao Jin, Dr. Ankit Shah, and Dr. Ravinder Earla for teaching me several research techniques early in my Ph.D. career. I would like to specially thank all the administrative assistants of pharmacology/pharmaceutical sciences divisions for their support. I would like to thank all the graduate students from pharmacology division for their love and encouragement. My special thanks to UMKC women's council, school of pharmacy, school of graduate studies for bestowing me with awards.

I am very thankful to my husband Dr. Sandeep Putty for his great love, support, and motivation. I am very thankful to my dearest friends, Dr. Ramya Vadlapatla, Anantha Ram Nookala, Dr. Aswani Dutt Vadlapudi, Dr. Nanda Mandava, and Naveen Gowda for their love and support towards me. Without all their encouragement it would have been impossible to make it

DEDICATION

I would like to dedicate this dissertation to my family, especially to my dad, "Satyanarayana Ande" for his inspiration and encouragement. His quest for knowledge always motivated me in pursuing higher levels of education……….as the journey continues!!!!!!!!!!!!!!

CHAPTER 1

TOBACCO SMOKING MEDIATED OXIDATIVE STRESS IN ORGAN TOXICITIES:

ROLE OF CYTOCHROME P450 SYSTEMS

1.1 Prevalence, diseases, and lifestyles

According to WHO, tobacco continues to kill nearly 6 million people annually, including more than 600,000 non-smokers who are exposed to tobacco smoke[1]. Further, WHO predicts that the smoking related deaths will reach approximately 8 million annually by 2030[2]. Cigarette smoking is associated with three major diseases; lung cancer, chronic obstructive pulmonary disease (COPD), and cardiovascular diseases, which are responsible for the highest rate of mortality in the US [3]. Another study has recently suggested that cigarette smoking is the cause of 30% of all deaths by cancers, 10% of deaths by cardiovascular diseases, 9% of deaths by respiratory diseases, and 7% of deaths by digestive diseases[4]. Numerous literatures suggest that smoking-mediated adverse effects of human health, including premature death, occur not only because of nicotine, but through other cigarette smoke constituents[5]. Tobacco smoke contains 61 known carcinogens, which increase the risk of cancers including lung, oro-pharyngeal, pancreatic, and renal cancers[6]. Chemicals found in tobacco smoke, including carcinogens, can cross the placenta[7] and cause chromosomal damage to the fetus[8]. Further, animal studies have shown that the developing brain is much more likely to develop tumors as a result of exposure to neurocarcinogens in utero than later in life[9]. Therefore, it is necessary to assess the biological pathways responsible for tobacco-mediated toxic effects on disease in order to identify potential targets for future drug development and improve the health outcomes of tobacco users.

1.2. General mechanisms of tobacco-mediated toxicities

In addition to carcinogens, cigarette smoke (CS) contains over 4000 different chemical substances. Some of the mainstream cigarette smoke constituents are ammonium hydroxide, benz[A]anthracene (B[a]A), benzo[A]pyrene (B[a]P), carbon monoxide (CO), diammonium phosphate, 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), and N-nitrosonornicotine (NNN), "tar", acetaldehyde, acrolein, acrylonitrile, benzene, formaldehyde, and hydrogen cyanide[10, 11]. Tobacco constituents have shown to be both proliferative [12-15] and apoptotic or cytostatic[16]. It has been shown that tobacco induces cell death via nitric oxide and through the formation of ROS [12]. It has also been reported that many tobacco constituents and nicotine-derived nitrosamine ketones (NNK) induce mutations through DNA damage. In a study, Elias et al. have shown that tobacco crude extract (TCE) induces apoptosis (dose-dependent) in oral squamous carcinoma cell line (OSCC-3) via caspase activation, which was accompanied by DNA fragmentation. This suggests that despite tobacco's role in tumor formation, it may have some component that is useful in fighting cancer [17]. However, additional work is needed to further explore its beneficial use in cancer treatment.

When oral mucosa cells are exposed to lead and benzo[A]pyrene, another cigarette constituents, caspase-3 activity and 8-epi prostaglandin F2 alpha (8e PGF2a) are increased, while 3-nitrotyrosine levels are increased with chronic exposure to lead [18]. 8e PGF2a is of non-enzymatic origin, which is produced by random oxidation of tissue phospholipids. In other words, these cigarette constituents may increase oxidative and nitrosative stress.

Nicotine is the major addictive and abundant (95%) substance present in tobacco smoke. Nicotine is metabolized by liver CYP2A6 through 5' oxidation to its major metabolite cotinine (75%) and many other metabolites (25%) [19]. CYP2A6 is known to further

metabolize these compounds into procarcinogenic compounds, including nicotine-derived nitrosamine ketone (NNK). CYP2B6 enzyme is also known to be involved in the formation of cotinine from nicotine in those who lack CYP2A6 enzyme [20]. NNK, the major tobacco specific carcinogen is metabolically activated by 2A6, 1A2, 3A4, and 2B6 CYP isoforms. These enzymes are involved in α-hydroxylation of NNK forming unstable diazohydroxides and aldehydes [20]. These diazohydroxides then form carcinogenic methyl or pyridyloxobutyl adducts by reacting with DNA. CYP2A13, a lung specific enzyme, is also known to metabolize nicotine and cotinine as well as activate NNK [21, 22]. NNK is also known to cause increase in the levels of reactive oxygen and nitrogen species in mouse microglia cell line leading to neuronal damage [23]. Several reports from literature have shown that NNK is responsible for generating oxidative stress in neurons and white blood cells [24, 25].

Smoking frequency may be determined by the polymorphisms in CYP2A6. In some individuals with low expression of CYP2A6, the half-life of nicotine is increased making these individuals crave for nicotine less often, lowering cigarette consumption per day [26]. In contrast, smokers with relatively high expression of CYP2A6 have increased nicotine metabolism and metabolism of other constituents, which leads to increased smoking frequency in these individuals. These individuals would also produce increased ROS, carcinogens, and reactive metabolites, which will make them more susceptible to cancers and organ toxicities.

1.3. Respiratory tract disorders

Lung cancer is the leading cause of cancer deaths and about 90% of the lung cancer cases are attributed to cigarette smoking [27]. In addition to nicotine metabolism, CYP enzymes are also responsible for the oxidation of aromatic moieties and olefins of tobacco constituents in the lungs tissues. Further, CYP enzymes are known to activate NNK, a tobacco

specific carcinogen, by α hydroxylation leading to the formation of ROS and carcinogenic DNA adducts in the lungs tissues. Further, ROS-mediated lipid peroxidation products may enhance the inflammation through activation of stress kinases (JNK, MAPK, p38) and redox sensitive transcription factors such as AP-1 [28]. The overexpression of the Wnt5a gene, followed by PKC activation, has been implicated in cigarette smoke-mediated lung carcinogenesis [29]. One study has shown the chemopreventive effects of isothiocyanates (ITC) by dual mechanisms involving the inhibition of CYP enzymes and induction of Phase II enzymes [27]. The inhibition of CYP enzymes leads to reduced activation of procarcinogens e.g. NNK, and increased expression of Phase II enzymes eliminates the toxic metabolites generated by CYP enzymes.

Glutathione peroxidase-1 (gpx-1) is a detoxifying enzyme that may protect lungs from ROS-induced protein denaturation, lipid peroxidation, and DNA damage. Gpx-1 converts two molecules of glutathione (GSH) and one molecule of H_2O_2 into oxidized glutathione (GSSG) and two molecules of water. One study has shown that gpx-1 knockout mice exposed to cigarette smoke has enhanced bronchoalveolar lavage fluid (BALF) neutrophils, macrophages, proteolytic burden, and whole lung IL-17A compared to WT mice. Ebselen, a gpx-1 mimetic, inhibits the established BALF inflammation when administered therapeutically. Thus, it is suggested that gpx-1 protects against cigarette smoke-induced lung inflammation, and gpx-1 mimetics may have therapeutic use in inflammatory lung diseases in smokers [30].

Manganese SOD2 eliminates anion superoxide free radicals, and is a major determinant of antioxidants in matrix mitochondria. One study isolated leukocyte cells of 20 lung cancer patients, and compared the SOD2 activity to 50 healthy smokers and 50 non-

smokers. They found that SOD2 activity is lower in lung cancer patients, and plasma carbonyl levels are elevated. Free radical production in lung cancer patients thus appeared high. Smokers also tended to exhibit lower SOD2 and higher carbonyl radicals compared to non-smokers, thus suggesting a strong role of oxidative stress in lung cancers [31].

1.4. Cardiovascular diseases

Women who smoke are 2.4-fold more likely to have sudden cardiac death than non-smoking women; this risk subsides linearly, with 20 years smoking cessation completely abolishing the risk [32]. Cigarette smoke particulate matter-treated human vascular epithelial cells has been shown to increase osteopontin (OP), a matrix protein found in bone, which is implicated in vascular disease. This effect is diminished following treatment with ascorbate, an antioxidant [33]. Increased oxidative stress may activate NFκB and AP-1, transcription factors that regulate pro-inflammatory cytokines. Inflammation of vascular endothelial cells may lead to serious cardiovascular events, especially in the presence of an atherosclerotic plaque.

A recent study conducted in mice highlights the negative effects of short-term CS exposure (STCSE) on several organ systems. They have shown that STCSE enhances the generation of ROS and causes increased lipid peroxidation in heart, liver, and kidneys [34]. On the other hand, it has been shown that the increase in SOD activity counteracts the damaging effects of ROS. This is a protective mechanism in acute exposure of CS but in case of chronic smoking the antioxidant defense system is largely impaired leading to ROS induced damage.

1.5. Reproductive system

Cigarette smoke has been found to significantly affect the reproductive system of treated mice via oxidative stress [35]. This was confirmed by upregulation of peroxiredoxin,

decreased glutathione levels, and increased 8-OHdG in the epithelial cells of the epididymis. Structural proteins involved in normal epididymal cell motility, such as Odf2 and Actg1, were down-regulated post-treatment [36]. Cigarette smoke has also been associated with reduced fertility and possibly the health of newborns. One study has confirmed the role of cigarette smoke-induced ROS generation in genotoxicity by exposing mouse embryos to cigarette smoke condensate that showed increased oxidative stress and telomere shortening and loss. The administration of an antioxidant, N-acetyl-L-cysteine, greatly reduced this toxicity, implicating the role of oxidative stress in cigarette-smoke induced genotoxicity [37].

Furthermore, a study has shown the role of the metabolism of benzo[A]pyrene (BAP) into (+)-7,8-Diol-9,10-epoxide, which may subsequently form deoxyguanosine BAP adducts, on ovotoxicity. They found significantly increased levels of mitochondrial ROS, lipid peroxidation, and severely reduced sperm-egg binding in both low and high dose treatments. This suggests that BAP is partially responsible for the effects of cigarette smoke on follicular development and subfertility [38].

Inhaled cigarette smoke is known to contain trace amounts of cadmium [39], which can traverse the placental barrier and exert toxic effects on the fetus. Gestational cadmium exposure has been found to delay puberty. In the ovary cadmium-treated rats show an increase in peroxide and lactoperoxidase (LPO) activities, while a decrease in SOD activity [40]. Since LPO is known to oxidize estrogenic hormones, it is implicated in the initiation of breast cancer[41]. Lipid peroxidation and other oxidative stress markers were reduced in cadmium-treated rats upon treatment with diallyl tetrasulfide, a CYP inhibitor [42]. Overall, these studies suggest that constituents of cigarette smoke disrupt normal reproductive system via CYP pathway-mediated oxidative stress.

1.6. Neurodegenerative diseases

Several studies have reported the regulation of CYP enzymes by nicotine in the brain. CYP2B6 is expressed in a region specific manner in the brain and is also found in astrocytes and neurons [43]. CYP2B6 is known to metabolize several centrally acting drugs and endogenous hormones. Smoking and alcoholism are known to induce CYP2B6 protein in different regions of brain which interfere with the metabolism of centrally acting drugs leading to neurotoxicity [43]. Since smoking is known to increase CYP2B6 levels, studies were done to examine the effect of nicotine on the induction of CYP2B6. Nicotine binds to nicotinic acetylcholine receptors (nAChRs) and exerts its pharmacological effects, however, it was shown that the induction of CYP2B6 by nicotine is not mediated through nAChRs [44]. Other studies also reported the induction of CYP2B6 in astrocytes and neurons of African green monkeys upon chronic nicotine treatment without affecting their hepatic levels [45]. Together these studies suggest that smoking-mediated induction of CYP enzymes may have altered therapeutic response leading to neurotoxicity.

Several studies have shown that nicotine and NNK cause oxidative stress in many other cells such as neurons, microglia, and white blood cells [23-25]. These cell types are very important for HIV pathogenesis and neuroAIDS which will be discussed in detail in chapter 2.

1.7. Clinical implications

Selective inhibition of CYP2A6 and CYP2A13 is considered as a potential therapeutic approach to treat nicotine dependence and tobacco-mediated cancers and organ toxicities. In addition, isothiocyanate dietary supplements can be used to prevent tobacco-mediated lung carcinogenesis. The use of benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC), which are present in high levels in cruciferous vegetables, such as watercress,

gardencress, cabbage, cauliflower, and broccoli, have been extensively studied to prevent CYP-mediated lung cancer[46]. Our findings involving the role of CYP2A6 in nicotine-mediated oxidative stress suggest the potential use of CYP2A6 inhibitors in treating smoking related diseases. Furthermore, since our recent findings have shown that CYP2A6 is induced by oxidative stress-mediated PKC/MEK/Nrf-2 pathway in monocytes and astrocytes, this pathway may also be targeted for the development of potentially novel pharmaceuticals for smoking-mediated neurodegenerative diseases. Furthermore, CYP2A6, oxidative stress, and PKC/JNK/Nrf-2 pathways can also be utilized for developing novel pharmaceuticals for HIV-infected smokers.
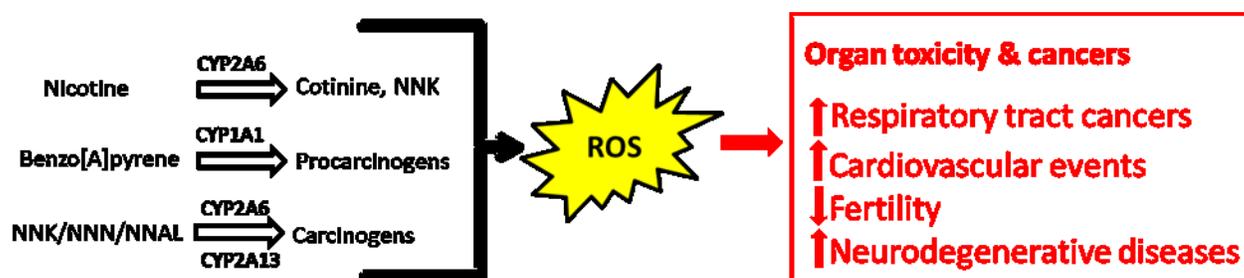
Figure 1. Tobacco associated organ toxicity

Tobacco constituents are metabolized/activated by CYP isozymes, leading to the formation of ROS and reactive metabolites that cause cancers and organ toxicity. CYP, cytochrome P450; ROS, reactive oxygen species; NNK, nicotine-derived nitrosamine ketone or 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone; NNN, N'-nitrosonornicotine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

CHAPTER 2

TOBACCO SMOKING EFFECT ON HIV PATHOGENESIS: ROLE OF

CYTOCHROME P450 ISOZYMES

2.1. Introduction

HIV infection is a global pandemic that affects 34 million people worldwide, among which, sub-Saharan Africa accounts for 67% of this population [47]. Recent reports reveal that the HIV infection is highly prevalent among pregnant women in Africa [48], and this increases pediatric infections due to mother-to-child transmission [49]. However, the majority of African populations don't have access to effective treatment to circumvent the increase in transmission. The co-infection by hepatitis C virus (HCV) in HIV- infected individuals is highly prevalent in the US (25% co-infected individuals)[50]. Similarly, HIV infection is also known to be associated with an increased risk of acquiring *Mycobacteria tuberculosis* (TB) infection in sub-Saharan Africa[51]. The advent of highly active antiretroviral therapy (HAART) has not only decreased the mortality rates substantially among individuals living with AIDS [52], but has also significantly decreased the incidence of HIV-associated dementia (HAD) [53]. However, the incidence of HIV-associated neurocognitive disorders (HAND), especially the milder form, has actually increased, perhaps due to central nervous system (CNS) toxicities mediated by HAART [54, 55]. Consumption of drugs of abuse, such as alcohol, tobacco, cocaine, methamphetamine, and marijuana, has also been shown to exacerbate HIV pathogenesis by accelerating the incidence and progression of HAND/HAD [56].

Tobacco-related deaths are the highest among addictive substances, and tobacco-associated deaths are estimated to increase from 3.0 million in 1990 to 8.4 million in 2020

[57]. Further, according to the World Health Organization (WHO) predictions, smoking related deaths though are not as severe as predicted by National Institute of Drug Abuse (NIDA), but will account for about 8 million annually by 2030 [2]. Furthermore, tobacco use is projected to account for 9% of global disability-adjusted life years by 2020, which would make it the world's single largest health problem [58]. In the US, the prevalence of cigarette smoking in the HIV-infected population is 50-70% [59] compared to 15-20% in the general population [60], which further increases the risk of smoking-related health problems in this group. For example, HIV-infected smokers show decreased immune responses, poorer responses to antiretroviral therapy (ART), and greater risk of virological rebound, compared to HIV-infected non-smokers [61]. Several *in vivo* and *in vitro* studies have shown that smoking/nicotine is associated with decreased immune responses [62-64], increased inflammation [62, 65, 66], increased oxidative stress [67, 68], and increased numbers of opportunistic infections [66]. Several *in vitro* studies have shown that smoking/nicotine enhances HIV replication in alveolar macrophages [69], microglia [70], and T cells [71]. However, the mechanism by which smoking or nicotine increases HIV replication is largely unknown. As an exception, a report suggests that iron and oxidative stress are possible mechanisms of enhanced production of HIV by alveolar macrophages in cigarette smokers [72].

Tobacco constituents are known to be metabolized by different isozymes of cytochrome P450 (CYP), and this leads to the generation of reactive metabolites and reactive oxygen species (ROS) in various organ systems such as, liver, lung, esophagus, and brain [22, 23, 73]. In view of the fact that ROS is known to play an important role in HIV pathogenesis,

the authors propose a novel pathway involving the role of CYP enzymes in oxidative stress-mediated HIV pathogenesis in tobacco users.

In this chapter, we discuss the possible role of CYP pathways in tobacco/nicotine-mediated effects in HIV/AIDS with respect to: 1) the effect of smoking on HIV pathogenesis, 2) the general mechanism of smoking-mediated oxidative stress and toxicity, 3) the potential role of the CYP pathway in tobacco/nicotine-mediated oxidative stress in HIV model systems, 4) conclusions, and 5) an expert opinion on this subject.

2.2. Role of smoking on HIV pathogenesis

A recent study conducted on the assessment of the mortality rates among AIDS patients has revealed that the risk of death is twice as high among smokers in the HIV-infected population than the non-HIV-infected population[74]. In part, this could be because smoking is known to increase the prevalence of other viral infections such as human papilloma virus (HPV) leading to the risk of cervical cancer in HIV seropositive women [75]. Similarly, the incidence of emphysema is also reported to occur earlier and at an increased level among HIV-infected smokers than in non-HIV-infected smokers [76]. There is an evidence from the literature suggesting that smoking is associated with decline in CD4 cell counts in the HIV-infected population, despite of the higher baseline CD4 cell counts observed among smokers than non-smokers [77]. However, the role of smoking in the progression to AIDS is unknown. Recent reports from the literature have also demonstrated the toxic effects of the tobacco constituents, nicotine and nicotine-derived nitrosamine ketones (NNK) in neurons, microglia, and white blood cells, which are exposed to the virus or viral proteins [23, 24]. For example, the treatment with NNK to mouse microglial cells and *in vivo* injection of mouse with NNK showed an increase in ROS, as well as an increase in the levels of proinflammatory cytokines [23]. In addition, a multisite longitudinal study for up to 7.9 years using 924 women has shown

11

that tobacco smoking negates the effect of HAART medication in relation to CD4 recovery [61]. However, the mechanism of tobacco-mediated toxicity and decreased efficacy of HAART is largely unknown.

Limited studies have addressed the effects of smoking on cognitive function and neurological disorders in the HIV-infected individuals with controversial findings [78]. Of relevance, it has been reported that chronic nicotine exposure can lead to the disruption of the blood brain barrier (BBB) integrity, which may enhance the exposure to HIV-infected monocytes, tobacco constituents, and antiretroviral drugs [79]. The exposure of these agents to CNS might exacerbate neuroAIDS and neurodegenerative diseases [79]. Another report from an observational cross-sectional study conducted in 56 women (36 HIV-infected and 20 non-HIV-infected) suggests that there is no correlation between smoking and cognitive dysfunction [78] in the HIV-infected population. Collectively, the available evidence suggests that there is an increase in smoking-mediated HIV pathogenesis among individuals with HIV/AIDS and patients may benefit from the development of novel therapeutic agents and/or drug dose adjustments that can be used to treat HIV-infected smokers effectively.

2.3. Mechanism of tobacco smoking-mediated toxicity: Role of cytochrome P450

The role of CYP enzymes has not been studied in context with smoking/nicotine and HIV pathogenesis. However, there are several elegant studies that show the involvement of CYP2A6, CYP2A13, CYP1A1, and CYP1B1 in smoking/nicotine-mediated toxicity leading to various types of cancers and hepatic toxicity [19, 80]. The majority of tobacco constituents, including nicotine, are metabolized and/or activated by CYP enzymes to generate reactive oxygen species (ROS) and/or reactive metabolites, NNK [81]. ROS and reactive metabolites are known to cause DNA damage, lipid peroxidation, and protein oxidation that leads to cell toxicity [82]. Nicotine, the major constituent of tobacco, is predominantly metabolized by

liver CYP2A6 and lung-specific CYP2A13 to cotinine (75%) and other metabolites (15%) [22, 80, 83, 84]. In addition, CYP2A6 and CYP2A13 are involved in the metabolic activation of tobacco-specific nitrosamines into reactive compounds that lead to liver and lungs toxicities [21]. Other tobacco constituents, such as polyarylhydrocarbons (PAH) are mainly activated by CYP1A1, and CYP1B1[85, 86] that leads to the formation of recative metabolites, which has been shown to be associated with oral toxicity [86]. Furthermore, a study comprising 600 patients has demonstrated that CYP1A1 is a risk factor in oral cancer among smokers [85]. Based on the fact that CYP pathways play a critical role in smoking-mediated cell/organ toxicity, there is a critical need to explore the involvement of CYP pathways in smoking/nicotine-mediated HIV pathogenesis.

2.4. Potential role of cytochrome P450 in tobacco/nicotine-mediated oxidative stress and toxicity in HIV systems

In the following sections we discuss whether CYP enzymes, especially those that are related to tobacco/nicotine, are expressed in monocytes/macrophages, lymphocytes, astrocytes, and neurons (Figure 2). We then discuss the role of CYPs in tobacco/nicotine-mediated oxidative stress in these cells, and their possible link to HIV pathogenesis and neuroAIDS. Finally, we identify the future course of investigation in this area of research.
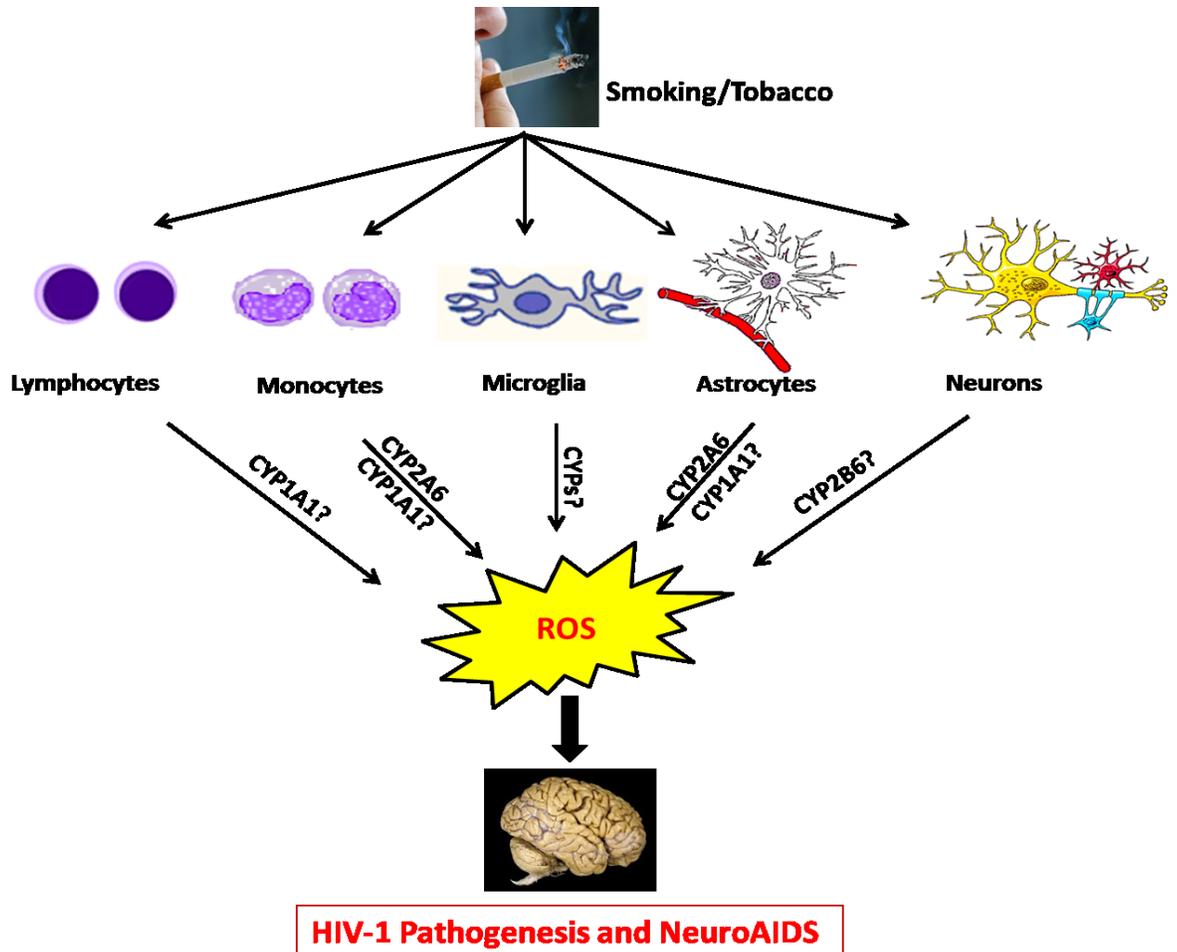
Figure 2: Smoking mediated Organ toxicity

Cells or organs that are involved in tobacco/nicotine metabolism or activation in context to HIV infection. The metabolism and/or activation of tobacco/nicotine may be involved in the toxicity of lymphocytes, monocytes/macrophages, microglia, astrocytes, and neurons causing HIV pathogenesis and neuroAIDS.

2.5. Monocytes/Macrophages

Monocytes/Macrophages are considered to be one of the major cellular targets of HIV and are thought to function as important viral reservoir [87]. Compared to activated CD4+ T lymphocytes, macrophages are more resistant to the cytopathic effect of HIV and survive HIV infection for extended periods of time [88, 89]. Furthermore, infiltration of macrophages into the brain results in spreading of the virus to resident glia, including perivascular macrophages, microglia, and astrocytes [90-92]. Migration of HIV-infected monocytes through the BBB is thought to be one of the major mechanisms responsible for the neuropathogenic effects of HIV, such as HAD and HIV encephalitis (HIVE)[93, 94]. Investigations to study the effect of tobacco/nicotine in monocytes/macrophages *in vivo* are therefore clinically relevant.

The major nicotine-metabolizing CYP2A6 mRNA and protein are expressed in U937 monocytic cell line [95]. A comparison between the levels of CYP2A6 mRNA in U937 cells and previously reported hepatocytic cells demonstrated that the level of CYP2A6 mRNA is similar in both the cells [96-98]. More importantly, the relative levels of the mRNA of CYP2A6 are much higher than the other CYP enzymes, including the mRNA of CYP1A1 and CYP1A2 [95, 96]. A relatively high abundance of CYP2A6 in U937 cells is intriguing and suggests further investigation on its role in HIV-infected smokers. Other investigations in macrophages derived from the U937 monocyte cell line have shown that nicotine is metabolized to cotinine and NNK by CYP2A6, a finding that was later confirmed using a CYP2A6 selective inhibitor, tryptamine [99]. In addition, it has been demonstrated that the metabolism of nicotine by CYP2A6 results in the generation of ROS [99]. Since oxidative stress has been linked to increased HIV replication in human primary macrophages *in vitro* [100], we hypothesize that CYP2A6-mediated nicotine metabolism and resultant oxidative stress may be a mechanism for the increase in viral replication among HIV-infected smokers.

This hypothesis is also supported by the fact that alcohol-mediated oxidative stress through CYP2E1 pathway increases the replication of another deadly virus, hepatitis C virus (HCV)[101, 102]. In one of these studies, it has been shown that CYP2E1-mediated alcohol metabolism, which produces ROS, increases the replication of HCV in hepatic cells and decreases the antiviral action of interferon [101]. The study involving the role of CYP2A6 and other tobacco/nicotine-metabolizing CYPs in oxidative stress and HIV pathogenesis in monocytes/macrophages of HIV-infected smokers and non-smokers is needed.

In addition to CYP2A6, CYP2E1 and CYP3A4 are abundantly expressed in U937 monocytic cell lines [96]. Furthermore, CYP2E1 and CYP3A4 (mRNA and protein) are both induced by alcohol by approximately 2-fold in U937 cells [96]. CYP3A4 is mainly involved in the metabolism of ART drugs, non-nucleoside reverse trascriptase inhibitors (NNRTI) and protease inhibitors (PI) [103]. Thus an altered level of CYP3A4 in the HIV model systems by agents, such as alcohol and other medications, is expected to affect the response to ART drugs, especially NNRTI and PI.

2.6. Lymphocytes

Smoking has been shown to be associated with a reduction in CD4+ T cells [77] and an increase in mortality in HIV-infected individuals [74, 104], but the mechanism for smoking-mediated effects on lymphocytes is unknown. From these studies it appears that tobacco constituents, especially nicotine, decrease immune response possibly by altering cytokines and chemokines in these cells. In addition, other studies have shown that nicotine toxicity in lymphocytes is mediated through superoxide anion formation [105]. It has been further shown that the ROS generated in lymphocytes, by smoking, results in the oxidation of lipids, protein, and DNA. It also decreases the levels of AOE (GST and SOD) [105]. This finding is further supported by the fact that DNA fragmentation in lymphocytes is mediated

16

by oxidative stress resulting from nicotine [106]. Earlier study using quantitative RT-PCR showed that CYP1A1 mRNA is present at very low levels in human blood lymphocytes [107]. Furthermore, this study demonstrated that CYP1A1 mRNA is induced 20-fold by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in lymphocytes. Since the residual levels of tobacco/nicotine-metabolizing CYP enzymes (CYP1A1, CYP1A2, and CYP2A6) are extremely low in lymphocytes, a study involving recombinant expression of these enzymes was performed to examine the role of these enzymes in lipid peroxidation through CYP pathways[108]. The results showed that these CYP enzymes increased lipid peroxidations by 2.1-fold, suggesting their role in oxidative stress. Further, a recent report showed that CYP2A6 is significantly induced in peripheral blood lymphocytes of lung cancer patients, suggesting that the expression of CYP2A6 could potentially be used to predict environment-induced disease and toxicity in humans[109]. However, there is nothing known about the expression of CYP2A6 or other tobacco/nicotine-metabolizing CYPs in the lymphocytes of HIV-infected smokers or non-smokers. Studies are underway to investigate the role of CYP pathway in smoking-mediated HIV pathogenesis in peripheral blood lymphocytes.

2.7. Astrocytes

Despite of the fact that HAART therapy increases the life span of HIV-infected individuals, the neurological complications, such as HAND, continue to be a source of significant morbidity. This is increasingly important, because since HIV-infected individuals live longer, this trend is expected to continue [110]. Astrocytes, the most abundant glial cells in the brain, which are important to protect the integrity as well as nourishment of neurons, play a major role in the pathology of HAND [111]. Astrocytes are also known to be infected by HIV, though to a lesser extent compared to microglia [112]. In addition, viral proteins and cytokines released by HIV-infected microglia are known to damage astrocytes [111], which

may ultimately lead to HAND. It has been shown that HIV protein gp120 induces proinflammatory cytokines/chemokines such as IL-6, IL-8 and CCL5 in SVGA astrocytic cell lines and primary astrocytes [113, 114]. The expression of these cytokines/chemokines is regulated through the nuclear factor-kappa B-dependent (NF-κB) pathway [113, 114]. In these studies, the involvement of NF-κB in the regulation of the inflamatory cytokines/chemokines was shown using specific chemical inhibitors, as well as siRNA of NF-κB pathway.

Nicotine has been shown to increase BBB permeability by modulating tight junction proteins [115]. Since BBB integrity is critical to prevent the development of HAND, smoking could enhance the development and progression of these disorders through the effect of nicotine on the BBB. It has been reported that a decreased uptake of methyllycaconitine, (a selective antagonist for the nicotinic acetylcholine receptor alpha7 subtype), in chronic nicotine exposed rodent models, is due to diminished blood–brain passive diffusion of compounds with very low extraction rates [116]. Hence it is vital to understand the interactions of smoking and HAART with the BBB, which may affect the pharmacokinetic profile of these drugs leading to decreased efficacy and increased toxicity.

2.8. Neurons

There is very limited information on the effects of smoking on HAND. A recent report has shown that genetically expressed HIV viral proteins in rats attenuate nicotine-induced behavioral sensitization, which is associated with altered cAMP response element binding protein and extracellular regulated kinase signaling pathway in mesocorticolimbic system [117]. *In vitro* and *in vivo* studies reveal that NNK is known to cause neuroinflammation in brain [23]. NNK activates microglia and astrocytes to induce proinflammatory cytokines/chemokines with subsequent neuronal damage [23]. In addition, NNK treatment

increases phosphorylation of inflammatory signaling molecules such as NF-κB, ERKs, JNK, and p38 MAPK in the brains of BALB/c mice [23]. Since HIV gp120, a major viral protein, induces proinflammatory cytokines in astrocytes [113, 114], NNK-mediated neuroinflammation could further aggravate HAND.

Another nicotine metabolizing enzyme, CYP2B6, is expressed in both astrocytes and neurons [43]. An earlier report has shown that rat CYP2B1 (homologous to human 2B6) is induced by nicotine in the brain, however, in this case, induction is not mediated through nicotinic acetyl choline receptors (nAChRs) [44]. Similarly, the level of CYP2B6 is increased in the brains of smokers compared to non-smokers, specifically in cerebellar purkinje cells and hippocampal pyramidal neurons [43]. Similarly, a study in African green monkeys shows the induction of CYP2B6 in the brain, but not in hepatic cells following chronic nicotine treatment [45]. An increased level of CYP2B6 in smokers could lead to an altered metabolism of CYP2B6 substrates. For example, CYP2B6 metabolizes nicotine and also activates NNK, which may lead to increased oxidative stress and damage [118]. In addition, CYP2B6 metabolizes an important HAART drug, efavirenz, which may cause tobacco-HAART interaction. Further studies are needed to examine the role of CYP enzymes in tobacco/nicotine-mediated neuropathogenesis and tobacco-HAART interactions in neurons.

2.9. Conclusions

Although smoking is highly prevalent in HIV-infected individuals and it is known to increase mortality [74, 104], studies related to the effects of smoking on HIV pathogenesis are very limited. Therefore, there is an urgent need to investigate the complexities of these pathways that are responsible for decreased immune responses, increased HIV replication, and increased AIDS related conditions in HIV-infected smokers. Recent findings suggest that cytochrome P450 plays an important role in smoking-mediated effects on HIV model systems,

such as monocytes/macrophages, lymphocytes, astrocytes, and neurons. In addition, it is known that smoking decreases the efficacy of HAART, especially NNRTI and PI, which may also involve the CYP pathway. CYPs are the major drug metabolizing enzymes for HAART, nicotine, and drugs used to treat opportunistic infections such as tuberculosis. A differential expression of CYP enzymes may alter the pharmacokinetic profiles of these drugs, including efficacy and toxicity. Therefore, it is imperative not only to examine the role of smoking on the CYP pathways, but also the impact of smoking on drug-drug interactions. Future studies in the following areas are necessary: 1) investigation of CYP pathway in HIV-infected smokers, especially the CYP enzymes by which tobacco-mediated oxidative stress enhances HIV replication in macrophages, astrocytes, and neurons, 2) a similar study with women and diverse ethnic groups who have CYP2A6 and CYP2B6 polymorphisms, which would provide an opportunity for personalized medicine in the future, 3) an investigation in HIV-infected patients co-infected with TB/hepatitis who receive HAART and/or anti-tubercular drugs that are metabolized through the CYP pathway, 4) studies to determine the role of smoking on the immune system in HIV infection, 5) clinical trials to identify potent CYP inhibitors as therapeutic agents that block nicotine-mediated HIV replication, 6) investigation of the role of the CYP pathway in the combined effect of alcohol and nicotine on oxidative stress-mediated HIV replication because alcohol consumption is common among smokers and appears to follow a similar CYP pathway (CYP2E1)[95].

2.10. Central hypothesis

Smoking is common among HIV-infected individuals and smoking/nicotine has been shown to enhance HIV replication in alveolar macrophages [69], microglial cells [70], and T cells [71]. Monocytes are considered to be one of the major cellular targets for HIV and also found to be the crucial viral reservoirs [87]. Infiltration of HIV infected monocytes into the

brain through blood brain barrier is found to be one of the major mechanism responsible for the development of HIV associated dementia (HAD) and HIV encephalitis (HIVE). These infiltrated macrophages carry the virus into the brain and spread the virus to perivascular macrophages, microglia and astrocytes [90-92]. Astocytes are the most abundant glial cells in the brain providing support and protection to neurons. Studies have shown that HIV affects astrocytes leading to induction of neuroinflammatory responses [119]. Similarly HIV proteins such as gp120, Tat, Nef transfection in SVGA astrocytes have induced proinflammatory cytokine release [113, 120, 121] with implications in neuroAIDS. As neurons don't get infected, the disruption of their supportive astrocytes might be responsible for overall neuron damage. The major nicotine metabolizing enzyme CYP2A6 is found to be predominantly expressed in monocyte-derived-macrophages (MDM's) as well as astrocytes. Therefore, it is critical to study the role of CYP2A6 in the metabolism of nicotine and resultant oxidative stress in HIV replication in monocytes/macrophages, as well as in astrocytes. Furthermore, oxidative stress is known to enhance nicotine-mediated HIV replication in alveolar macrophages and monocyte-derived macrophages [72, 100]. However, little is known about the mechanism/pathways by which nicotine or other constituents of smoking exert its effect on these cells. Our approach explores the *in vitro* effect of nicotine/tobacco on monocytes/macrophages, and astrocytes as well as an *ex vivo* study involving monocytes/macrophages of peripheral blood mononuclear cells (PBMC) obtained from HIV-infected smokers.

We propose that tobacco/nicotine, or its metabolites, enhance HIV replication in monocytes/macrophages through CYP pathway (Figure 3). This is supported by the fact that CYP2A6 induces oxidative stress in monocytes/macrophages upon nicotine metabolism [99].

These findings suggest a fruitful new area of study to explore the role of CYP2A6 in nicotine metabolism and oxidative stress in HIV replication in HIV-infected smokers. Furthermore, it provides an opportunity to explore the use of selective inhibitors to prevent nicotine/tobacco-mediated oxidative stress and reduce HIV replication. In addition to CYP2A6, several CYP enzymes, such as CYP1A1 and CYP2A13, are expected to metabolize or activate other tobacco constituents (PAH, NNK, benzopyrene) into procarcinogens and produce oxidative stress in monocytes/macrophages, lymphocytes, astrocytes, and neurons (Figure 2). Therefore, examination of the role of these CYP enzymes would further aid in development of novel pharmaceuticals using selective inhibitors of these CYP enzymes. It is possible that specific anti-oxidants, such as vitamin C, vitamin E, melatonin, and taurine, which are very effective in alleviating oxidative stress-mediated damage in alcoholic liver disease [122], could also reduce nicotine/tobacco-mediated HIV pathogenesis.
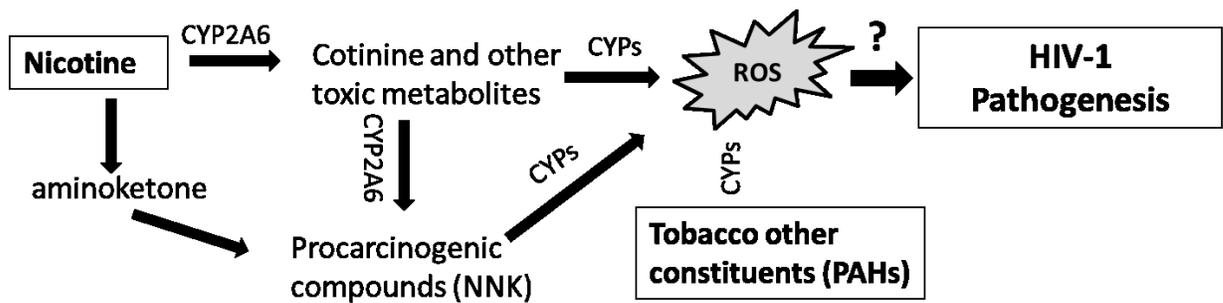
Figure 3: Role of CYP enzymes in nicotine metabolism

The proposed mechanistic pathway involving the role of CYP enzymes in nicotine metabolism and/or activation of tobacco constituents, especially nicotine, which may lead to HIV pathogenesis.

Tobacco/nicotine causes CYP-mediated oxidative stress [99] as well as inflammation through the release of inflammatory cytokines in monocytes/macrophages and astrocytes [123].These findings suggest that there is a cross talk between CYP and cytokines in HIV-infected patients, which may occur through a common pathway in these cells. This hypothesis is strengthened by the fact that nicotine activates CYP1A1 in macrophages, which releases cytokines that further activate T-lymphocytes leading to chronic inflammation [123]. Furthermore, IL-6, an important proinflammatory cytokine, has been shown to be involved in the regulation of CYP3A4 [124]. Thus, the cross talk between CYP and cytokines is very important to study within the context of HIV pathogenesis.

In conclusions, smoke extract/nicotine is known to enhance HIV replication in both T cells and macrophages, and therefore it would be expected to exacerbate HIV pathogenesis leading to accelerated disease progression. This chapter puts forward a novel hypothesis where CYP enzymes would be involved in increased oxidative stress that will in turn accelerate HIV replication. Since CYP enzymes are not abundantly present in T-cells, we believe that further studies using monocytes from *in vitro, in vivo*/*ex vivo* human samples from HIV-infected non-smokers and smokers will substantiate the role of CYP pathway in HIV pathogenesis and neuroAIDS. These studies, if successful, will provide a novel and additional target for drug development for effective treatment of HIV infected smokers.   "The central hypothesis of this project is that, in monocytes and astrocytes, CYP and oxidative stress pathways play important role in tobacco-mediated cell toxicity and/or HIV replication. The hypothesis will be tested using the following four specific aims.

2.11. Specific aims

In order to study our central hypothesis, we designed the following specific aims:

Specific Aim 1: To determine the role of CYP2A6 in nicotine metabolism in HIV cell models, SVGA astrocytes (chapter 4).

Specific Aim 2: To study CSC-mediated oxidative stress and cell death in U937 monocytes and HIV replication in primary macrophages (chapter 5)

Specific Aim 3: To determine the levels of CYP enzymes, antioxidant enzymes (AOE), oxidative stress, cytokines, and HIV replication in blood plasma/monocytes isolated from a cohort of patients (Healthy, smokers, HIV and HIV smokers) (chapter 6)

Specific Aim 4: To study the nicotine metabolism in plasma samples of smokers and HIV Smokers (chapter 7)

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1. Chemicals

RPMI 1640, DMEM, gentamicin and FBS were all purchased from Mediatech Inc. (Manassas, VA). Qiagen RNeasy kit for RNA extraction and all prep DNA/RNA/Protein kit were brought from Qiagen (Valencia, CA). The dynabeads flowcomp human CD14 kit was purchased from Invitrogen (Grand Island, New York). Ficoll hypaque plus was obtained from GE Healthcare (Piscataway, NJ). Bicinchoninic Acid (BCA) assay kit was obtained from Thermo Scientific (Rockford, IL). Protease inhibitor cocktail, tryptamine, ritonavir, nicotine, ascorbic acid (vitamin C), were bought from Sigma-Aldrich (St. Louis, MO). Gene expression kits and primer probes (CYP1A1, Hs01054794_m1; Catalase, Hs00156308_m1; SOD1, Hs00533490_m1; CYP2A6, Hs0071162_m1; CYP2E1, Hs00559367_m1; CYP3A4, Hs00430021_m1; AHR, Hs00169233_m1; CYP1A2, Hs01070369_m1; CYP2A13, Hs00426372_m1; CYP2B6, Hs03044636_m1; CYP2C9, Hs00426397_m1; CYP2C19, Hs00426387_m1; CYP2D6, Hs_00164385_m1; CYP3A5, Hs0024417_m1; Nrf2, Hs00232352_m1; GAPDH 4333764F) were obtained from Applied Biosystems (Foster City, CA). Radioimmunoprecipitation assay (RIPA) buffer was obtained from Boston BioProducts, (Ashland, MA). All primary and secondary antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Luminata™ Crescendo western HRP substrate was obtained from EMD Millipore Corporation (Billerica, MA). Cigarette smoke condensate (CSC) was obtained from Murty Pharmaceuticals (Lexington, KY). HIV type 1 p24 antigen ELISA kit was bought from ZeptoMetrix Corporation (Buffalo, NY). Multiplex cytokine assay kit was purchased from Bio-Rad (Hercules, CA). Oxiselect oxidative DNA damage ELISA kit was

purchased from Cell Biolabs (San Diego, CA). EpiQuik™ 8-OHdG DNA Damage Quantification Direct Kit (Fluorometric) was obtained from Epigentek (Farmingdale, NY). Human-mCSF was obtained from Peprotech (Rockyhill, NJ). Dichlorofluoroscein diacetate (DCFDA) was purchased from Life Technologies (Grand Island, NY). Cotinine and NNK were bought from Toronto Research Chemicals (North York, On, Canada). Annexin V PE apoptosis detection kit was purchased from BD Bioscience (San Jose, CA). MTT salt was purchased from Sigma-Aldrich (St. Louis, MO). MTT reagent was prepared by dissolving MTT in cell culture medium to a final concentration of 0.1 mg/ml which was used to treat cells. AhR inhibitor CH223191 was purchased from Tocris (Minneapolis, MN). Nicotine, cotinine, trans-3-hydroxycotinine, nornicotine, norcotinine, nicotine-d4, and cotinine-d3 were purchased from Cerilliant Analytical Reference Standards (Round Rock, TX). HPLC-grade methanol, acetonitrile, ammonia solution, and formic acid were procured from Fisher Scientific (New Brunswick, NJ). An Exterra HPLC reverse phase MS C18 column was obtained from Waters Corporation (Milford, MA) and strong cation SPE cartridges from Agility DVB; Orochem Technologies (Lombard, IL).

3.2. Cell culture and treatments

All experiments were conducted in U937 human monocytic and SVG human astrocytic cell lines. U937 cell line was obtained from ATCC (Manassas, VA), and maintained in Roswell Park Memorial Institute (RPMI) 1640 media containing 10% fetal bovine serum (FBS) and 1% gentamicin. Cells were cultured at an initial concentration of 2×10^5 cells per ml and media was refreshed every two days. The human SVGA astrocytic cell line was generously provided by Dr. Avindra Nath. SVGA astrocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 50 µg/ml gentamycin. SVGA cells were cultured in 10 ml media in a 75 mm^2 flask with an initial

amount of 0.8×10^6 cells and passaged every other day. Both U937 and SVGA cells were incubated at 37°C in the incubator containing 5% CO2, and passaged 2 to 3 times before using them for experiments. For treatment purposes, U937 cells were plated at a cell density of 0.8 $\times 10^6$ per mL and SVGA cells were seeded at 0.25×10^6 per mL in a 12-well plate the night before the treatment. Astrocytes were treated with 1 μM nicotine in triplicate in a 6-well plate containing 2 ml of medium. Each experiment was repeated 2-3 times. The nicotine concentration was optimized by using various concentrations of nicotine ranging from 0.25 – 10 μM. The final concentration of nicotine that was used was 1 μM for subsequent experiments. This concentration was selected based on our preliminary observations that ≤0.5 μM nicotine did not show significant induction of CYP1A1 and CYP2A6, whereas concentrations ≥2.5 μM were unable to further enhance CYP1A1 and CYP2A6 expression levels. In addition, nicotine treatments at ≤0.5 μM showed a very low amount of cotinine and NNK formation in SVGA astrocytes. For inhibition experiments, the cells were preincubated with 20 μM of tryptamine, a specific inhibitor of CYP2A6, for 30 min prior to nicotine treatment. U937 cells were treated with 50 μg/ml CSC (40mg/ml stock solution in DMSO) in triplicate in a 12-well plate containing 1 ml of medium. DMSO Vehicle control was employed to compare with effects of CSC treatment. For inhibition experiments, the cells were preincubated with 50 μM of AHR antagonist, 100 μM of Vit-C for one hour prior to CSC treatment.

3.3. RNA extraction and quantitative reverse transcriptase-polymerase chain reaction

RNA was extracted from nicotine treated astrocytes using Qiagen RNeasy Kit as per manufacturer's protocols. RNA (120 ng) from each sample was used for quantitative reverse transcription polymerase reaction (qRT-PCR) using a two-step TaqMan_Gene Expression Kit in an iCycler iQ system (Bio-Rad Laboratories, Hercules, CA). Relative fold expression of

the genes was calculated using the 2-ΔΔCt method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control.

3.4. Western blot analysis

Total cell lysates were prepared in RIPA Buffer containing protease inhibitor and protein concentration was measured using a Pierce BCA protein assay kit. Protein (20 μg) was loaded on an acrylamide gel and electrophoresed, followed by transfer to a PVDF membrane. After blocking the membrane in 5% non-fat dry milk, it was incubated with primary antibody (1:1000 dilution) followed by secondary antibody (1:2000 dilution). The blot was visualized by LuminataTM crescendo western HRP substrate using the Alpha Innotech FluorChem HD2 gel documentation system (Alpha Innotech, San Leandro, CA), and the densitometric data was analyzed using AlphaEase FC StandAlone software (version 6.0.0.14; Alpha Innotech). β-Actin was used as an internal loading control to normalize the expression of proteins.

3.5. Measurement of Reactive Oxygen Species (ROS)

The production of ROS was measured by flow cytometry using dichlorofluoroscein diacetate (DCFDA). Briefly, the cells were treated with nicotine or CSC for different times in a 12-well plate. After treatment the cells were washed with PBS and incubated with 10 μM DCFDA in PBS for 30 min at 37°C. Then the cells were harvested and dissolved in 1 ml PBS to measure the DCF emission at 525 ± 20 nm by flow cytometer (BD Biosciences, San Jose, CA), and Mean Fluorescence Intensity (MFI) was measured and analyzed.

3.6. MTT cell viability assay

Cell viability test was performed in 12-well plates in triplicate, using MTT assay [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] in U937 monocytes. Cells from each well were centrifuged at 2000 rpm for 5 min to remove treatment medium. After washing once with PBS, cells of each well were incubated for 4 h in 5 ml flow tubes with 500

29

µl 0.2 mg/ml MTT solution in fresh media. After incubation the tubes were spun down and the formazan crystals formed were dissolved in 300 µl of DMSO. Then absorbance was measured at 570/630 nm on Biorad Benchmark Plus microplate spectrophotometer (Biorad, Hercules, CA) using the Microplate Manager 5.2.1 software.

3.7. Annexin V apoptosis assay

Annexin V apoptosis assay was performed in U937 monocytes to measure the effect of CSC on apoptosis. Briefly, media was removed and cells of each well were suspended in binding solution at a final concentration of 1×10^6 cells per ml, 100 µl of which was transferred into a 5 ml tube. Then, 5 µl PE and 5 µl 7-AAD were added to the 100 µl cell solution, followed by 15 min incubation at room temperature in darkness. For setting up compensation, the following controls were used: unstained, PE stained, 7-AAD stained tubes. After incubation, 400 µl binding solution was added to each tube and fluorescence was detected using a flow cytometer (BD Biosciences, San Jose, CA). Mean fluorescence intensity (MFI) was measured and analyzed.

3.8. Preparation of standard stocks and spike solutions

For measurement of nicotine, cotinine and NNK in astrocytes, calibration curve and quality control dilutions were prepared and processed as follows: calibration curve standards (0.33-663.14 ng/mL for nicotine and 0.11-663.14 ng/mL for both cotinine and NNK) and quality control standards (0.33-435.26 ng/mL for nicotine and 0.11-435.26 ng/mL for cotinine and NNK). Spike standards dilutions 0.33-663.14ng/mL for nicotine and 0.11-663.14 ng/mL for both cotinine and NNK were made in astrocyte cell matrix samples from untreated cells. The internal standard, ritonavir (15 µg/mL) was prepared in 20% methanol. All the spike standards were stored in a freezer at -80^0C until further analysis.

For measurement of nicotine and its metabolites in plasma samples of HIV-negative smokers and HIV-positive smokers, nicotine, cotinine, trans-3-hydroxycotinine, nornicotine, norcotinine, and the internal standards (ISs) (deuterated nicotine-d4 and cotinine-d3) were dissolved at a concentration of 1 mg/ml in 80% methanol (v/v). To prepare the standard curve, 20 ml of a working solution at different concentrations (12.60 to 5.04, 2.02, 0.76, 0.25, 0.076, 0.027, and 0.013 mg/ml) was spiked into 500 ml plasma from a nonsmoker (blank) to obtain calibration

standards of decreasing concentrations (504.0, 201.6, 80.6, 30.6, 10.1, 3.03, 1.06, and 0.53 ng/ml). Similarly, the quality control (QC) samples were independently prepared at four concentrations (504.0, 80.6, 3.03, and 1.06 ng/ml) in plasma from nonsmokers.

3.9. Determination of CD4 count and viral load

Whole blood was drawn in EDTA tubes from each subject who qualified for the study followed by confirmation of HIV status and a CD4 count by flow cytometry (Becton Dickenson, San Jose, CA). The viral load of HIV-infected individuals was determined in plasma using quantitative reverse transcriptase polymerase chain reaction (RTPCR) (Roche Amplicor System, Biocentric) at Pasteur Institute, Yaounde, Cameroon. The remaining sample of plasma from each patient was frozen immediately and shipped on dry ice to the University of Missouri-Kansas City for analysis of cytokines and determination of markers of oxidative stress and CYP enzymes.

3.10. Preparation of peripheral blood mononuclear cells and monocytes

For the peripheral blood mononuclear cells (PBMC) isolation, gradient centrifugation technique was employed using ficoll hypaque plus. From the PBMCs, monocytes were isolated using dynabeads flowcomp human CD14 kit (Invitrogen, Grand Island, New York). The monocytes were immediately lysed using RLT buffer provided in the All prep

DNA/RNA/Protein kit (Qiagen, Valencia, CA). The lysed samples were frozen and shipped to the University of Missouri-Kansas City for further studies.

3.11. CSC treatment and HIV replication in primary macrophages

Primary monocytes were differentiated into macrophages and infected with HIV, prior to treatment with CSC, using previously reported protocol [125, 126]. Briefly, PBMCs were plated in T-75 flask and non-adherent cells were removed after 1 h at 37 °C. Cells were cultured for 6-8 days in media containing human mCSF to facilitate macrophage differentiation. Mature macrophages were collected and activated by treating with polybrene (2 µg/ml) for 30 min before HIV infection. Macrophages were infected with TCID50 of HIV for 2 h and cultured in 6-well plates (1 million/well) for 6 days. Cell supernatant was collected on days 0, 3, and 6 to analyze p24 levels. Starting day 7, HIV-infected macrophages were treated, once daily, with 25 ug/ml CSC or equivalent amount of DMSO. During the treatment period, one-half milliliters of fresh media was added daily to each well to avoid cytotoxicity. Supernatant collected on day 11 (24 h after last treatment) was analyzed for viral load using the HIV type 1 p24 antigen ELISA.

3.12. Multiplex cytokine assay

The protein levels of various pro-inflammatory cytokines/chemokines; RANTES, IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1), IL1-β, and TNF-α were measured using multiplex cytokine assay kit (Bio-Rad, CA, USA) as per the manufacturer's protocol. Briefly, plasma samples were centrifuged at 3000 g for 10 min and the supernatants were diluted with three volumes of sample diluent. Next, 50 µl of each sample and standards were mixed with magnetic beads and incubated on a shaker at room temperature for 30 min. The beads were washed and 25 µl of detection antibody was added to each well followed by incubation for 30 min at room temperature. Furthermore, the samples were washed and incubated with 50 µl

streptavidin-PE conjugate for 10 min. Finally, 125 µl of the assay buffer was added and the samples were analyzed using Biorad Bioplex HTS (Bio-Rad, CA, USA). The concentrations of the cytokines were determined with Bio-plex manager 5 using 5-PL statistics using standard curve.

3.13. Quantitation of 8-hydroxy-2′-deoxyguanosine content

The concentration of 8-Hydroxy-2′-deoxyguanosine (8-OHdG) was determined in the plasma samples using oxiselect oxidative DNA damage ELISA kit according to manufacturer's instructions. Briefly, after diluting the plasma 50:50, the samples along with the 8-OHdG standards provided in the kit were added to an 8-OHdG/BSA conjugate on a preabsorbed EIA plate. After incubation for an hour, an anti-8-OHdG primary antibody was added, followed by an HRP conjugated secondary antibody. After incubation with substrate solution for an hour, the reaction was terminated using the assay "stop solution" and the absorbance was measured at 450 nm using a plate spectrophotometer. The 8-OHdG content of the DNA in monocytes was estimated using EpiQuik™ 8-OHdG DNA Damage Quantification Direct Kit (Fluorometric) following manufacturer's instructions. It is a highly sensitive fluorometric technique to detect 8-OHdG by using as low as 300 ng of unmodified DNA. Initially, DNA is bound to the wells that have a high DNA binding affinity. Then the 8-OHdG present in the samples is detected by using capture and detection antibodies. The enhancer solution is used to enhance the signal followed by quantification by reading the plate in a fluorescence microplate reader equipped with an excitation filter of 530 nm and emission filter of 590 nm. The amount of 8-OHdG in each sample is calculated by using a standard curve generated from a series of standards.

3.14. DNA/RNA/protein isolation

Isolation of DNA, RNA, and protein was done from the lysed samples using the All prep DNA/RNA/Protein QIAGEN Kit by following the manufacturer's instructions. Briefly, the lysed sample was homogenized and transferred to the DNA column and the flow through was transferred to RNA column for obtaining RNA. The flow through from RNA column was used to precipitate protein using APP buffer. The RNA and DNA were quantified using UV spectrophotometer by measuring their absorbance at 260nm. The protein was quantified using BCA protein assay kit (Thermo scientific, Rockford, IL). However, the quantity of protein from the some samples, especially HIV positive smokers and HIV positive nonsmokers, were very low and insufficient for complete analysis.

3.15. Statistical analysis

Statistical analysis for *in vitro* studies was performed to determine mean ± SD and a student t-test was applied to determine p values. A p value of ≤ 0.05 was considered significant. For *ex vivo* study, demographic variables (age, sex ratio) were summarized using descriptive statistics. All outcome variables were summarized as the mean plus or minus the standard error. Comparisons among the four groups were conducted using one way ANOVA with no corrections for multiple comparisons. All tests were two-sided and results that were statistically borderline significant at p≤0.1 (#) and significant at p ≤0.05 (*) and ≤0.01 (**). A two-way ANOVA was done to determine whether they interact synergistically or additively in HIV positive smokers. All analyses were performed using IBM SPSS version 21.

CHAPTER 4

AN LC-MS/MS METHOD FOR CONCURRENT DETERMINATION OF NICOTINE

METABOLITES AND THE ROLE OF CYP2A6 IN NICOTINE METABOLITE

MEDIATED OXIDATIVE STRESS IN SVGA ASTROCYTES

4.1. Introduction

Recent studies by Jin et al have shown that CYP2A6 is the most abundantly expressed CYP (36% of the total CYPs) in the U937 macrophage cell line [96]. Since macrophages are critical HIV reservoirs and migration of these cells into the brain may result in neuroAIDS [90], Jin studied the role of CYP2A6 in nicotine metabolism and nicotine-mediated oxidative stress in U937 macrophages [128]. Results from those studies clearly demonstrate a role for CYP2A6 in nicotine metabolism and associated oxidative stress in these cells. Nicotine can cross the blood-brain barrier and exert its toxic effects on various brain cells, including astrocytes. Therefore, in the present study we examined the role of CYP2A6 in nicotine metabolism in astrocytes and its involvement in oxidative stress. However, in order to study nicotine metabolism in astrocytes, it was necessary to develop an extremely sensitive analytical technique to measure nicotine metabolites. In recent years, tandem triple quadrupole linear ion Qtrap mass spectrometry (LC-MS/MS) method has been increasingly used for the measurement of nicotine and its metabolites in urine samples and other cell lysates [128-132]. Since the level of CYP2A6 enzyme is much lower in astrocytes than most other cells, we developed a relatively more sensitive LC-MS/MS method to determine nicotine metabolites.

Nicotine, the major bioactive constituent of tobacco, is metabolized into cotinine and other less abundant metabolites, primarily by liver CYP2A6 and to some extent by CYP2B6

and CYP2D6 [19]. Some of the nicotine and cotinine metabolites are further metabolized by CYP2A6 into procarcinogenic compounds, including nicotine-derived nitrosamine ketones (NNK). For example, nicotine is directly metabolized by 2'hydroxylation to aminoketone, which is converted into the carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [127]. In addition, nasal and lung CYP2A13 are involved in the biotransformation of NNK into a carcinogen [19, 21, 128]. CYP2A6-mediated nicotine metabolism and the subsequent activation of reactive metabolites into procarcinogens and formation of reactive oxygen species (ROS) have been shown to be involved in liver damage and lung/pancreatic cancers [19, 128-130]. Nicotine and NNK have also been demonstrated to generate oxidative stress in microglia [23], neurons [25], and white blood cells [24]. However, the mechanism by which nicotine induces oxidative stress in these cells has not been determined. We hypothesize that CYP2A6 plays a critical role in nicotine metabolism-mediated oxidative stress in SVGA astrocytes. Therefore, our specific aim is to determine the role of CYP2A6 in nicotine metabolism-mediated oxidative stress in SVGA astrocytes.

4.2. Development of an analytic method for the measurement of nicotine, cotinine, and NNK in SVGA astrocytes

4.2.1. Extraction procedures and sample preparation

The liquid-liquid extraction technique was optimized for method development and subsequently used for SVGA astrocyte sample analysis. Astrocyte samples were thawed and 200 µL was aliquoted followed by addition of 25 µL of 15.0 µg/mL freshly prepared internal standard stock. Then, 30 µL of 30 % v/v ammonia solution was added to the samples and vortexed for 1 minute. The samples were extracted by adding 1000 µL of ethyl acetate then vortexed for 2 minutes followed by centrifugation at 12,000 rpm at 40C for 45 min. Subsequently, 850 µL of the organic layer was withdrawn and evaporated to dryness using a

Speed-Vac at 40C for 60 minutes. The dried samples were redissolved in 200 µL of mobile phase and vortexed for 1 minute. To prepare samples for LC-MS/MS analysis 200 µL calibrator, control, and nicotine-treated astrocytes, 25 µL working internal standard, and 30 µL of 30% ammonium hydroxide were mixed and evaporated to dryness at 40°C and the samples were reconstituted in 0.2 mL of mobile phase for chromatography. The final concentration of working internal standard was 1.5 µg/mL.

4.2.2. HPLC and mass spectrometry

HPLC and mass spectrometry were performed with modifications of our previously described method [131]. Briefly, complete column separation was performed on a Hybrid particle organosiloxane Xterra reverse phase carbon 18 HPLC column (50 mm x 4.6 mm (i.d.); 5µm bead size; 50-Å pore size; Waters Corporation) using a mobile phase consisting of 250 ml HPLC water, 750 mL acetonitrile and 1 mL formic acid (88%). Sample injection volume used was 15 µL and the total analytical run time was 4 min at a flow rate 0.3 mL/min. The HPLC system consisted of a high-pressure liquid pump and autosampler (LC-20AD and SIL-20AST, Shimadzu USA) and a tandem mass spectrometer (API 3200; AB Sciex, Sciex, Faster City, CA). The HPLC column was connected directly to the heated nebulizer source of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system and the experiment was performed under conditions as described earlier [131].

The mass spectrometry parameters for LC-MS/MS were optimized as follows: curtain and CAD gases (20 psi), gas 1 (45 psi), gas 2 (50 psi), ion spray voltage (4800 V), ionization source temperature (400oC), and dwell time (200 milliseconds). The de-clustering potentials for nicotine, cotinine, and NNK were fixed to 80, 40, and 40 V, respectively, while collision energies for nicotine, cotinine, and NNK were adjusted to 26, 28, and 15 V, respectively. The

optimization of MS/MS mass spectra for nicotine, cotinine, NNK, and ritonavir was performed by MRM positive mode using ion mass transitions of m/z 163.2/130.1 for nicotine, m/z 177.4/98.3 for cotinine, m/z 208.4/122.1 for NNK, and 721.6/296.4 for ritonavir (Table 1).

Table 1: LCMS/MS method calibration in SVGA astrocytes

Compounds	MRM Transitions (m/z)	Retention time (min)	Correlation coefficient equation (y=mx+c)	Coefficient of determination	Calibration curve range (ng/ml)	LOQ (ng/ml)
Nicotine	163.2→130.1	1.79	y = 175.98x +140.36	$R^2 = 0.9998$	0.33 - 663.14	0.33
Cotinine	177.4→98.3	1.89	y = 511.19x - 16.834	$R^2 = 0.9997$	0.11 - 663.14	0.11
NNK	208.4→122.1	2.1	y = 215.68x +21.742	$R^2 = 0.9935$	0.11 - 663.14	0.11

4.2.3. Quantification

The concentration of each analyte was determined by calculating the ratio of each analyte peak response relative to its respective internal standard peak response. Results from calibration analyses were used to create a calibration curve using simple linear regression analysis. The slopes and intercepts from the resulting calibration equations were used to calculate control and specimen results (Table 1). The calibration curves were linear within the concentration range of 0.11-663.1 ng/ml for cotinine ($r2 = 0.9998$) and NNK ($r2 = 0.9935$) and 0.3-663.1 ng/ml for nicotine ($r2 = 0.9963$).

The LC-MS/MS MRM chromatograms of nicotine, cotinine, NNK, and ritonavir in SVGA astrocytes showed no interference in the blank at the retention time of nicotine, cotinine, NNK, and ritonavir (Fig. 4). Similarly, there was a small base line peak at the lower level of quantification of all the analytes, suggesting a high signal to noise ratio (S/N). In

addition, the results suggested that this LC-MS/MS method is extremely sensitive, rapid, and robust for quantifying these analytes at very low concentration levels (0.3 ng/mL for nicotine and 0.11 ng/mL for both cotinine and NNK). Finally, the peak response was directly proportional and uniform to concentrations ranging from the lower limit to the upper limit of quantification for all the analytes, demonstrating that the results are highly reproducible (Figure 4).

Figure 4: Chromatograms of extracted cotinine samples
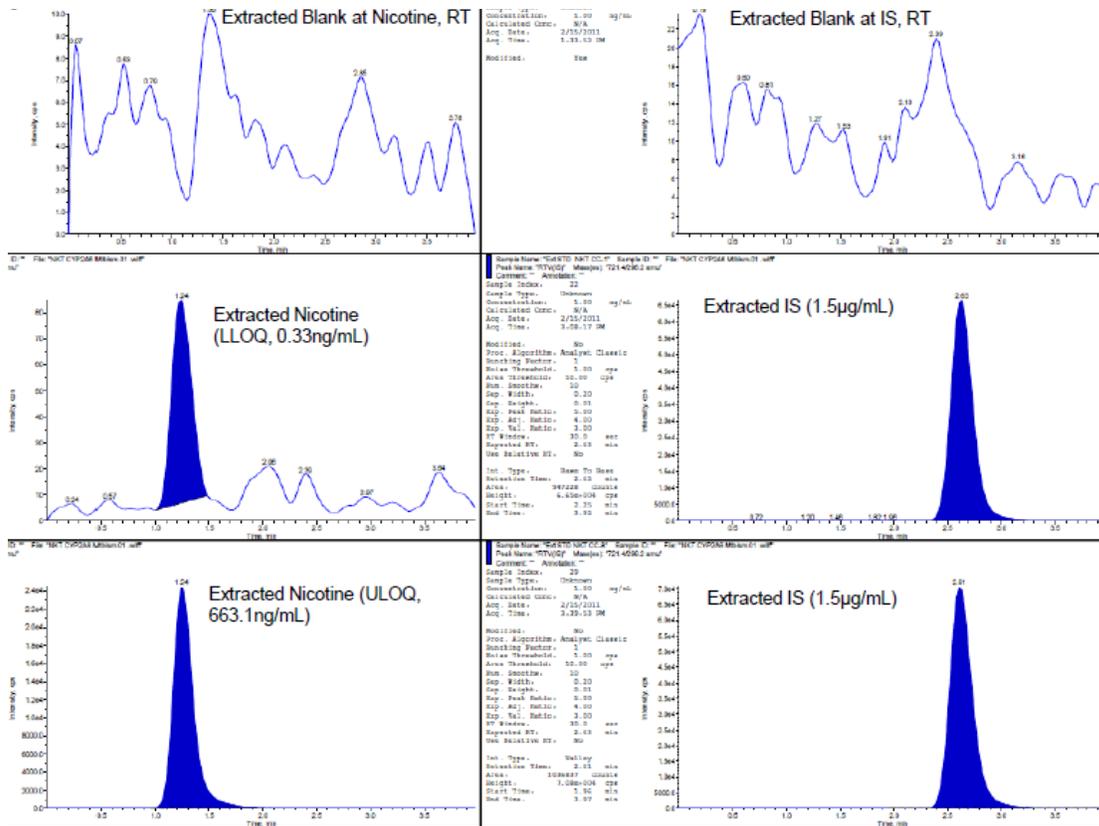
Figure 5: Chromatograms of extracted nicotine samples
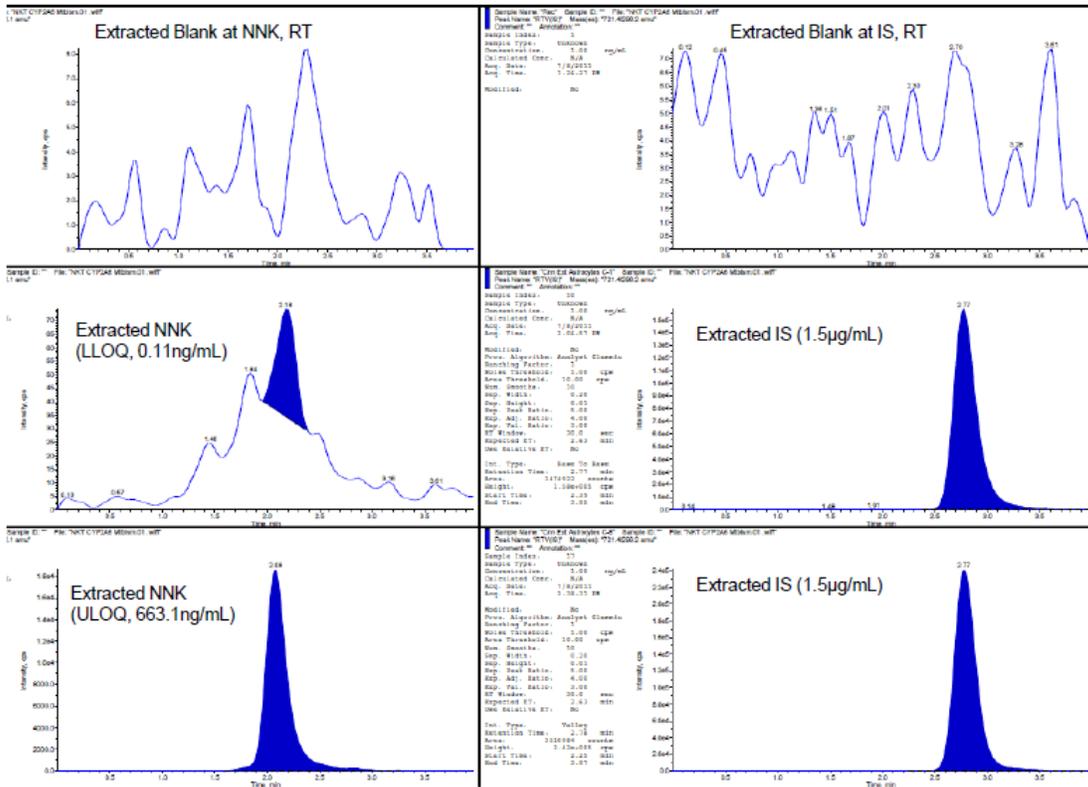
Figure 6: Chromatograms of extracted NNK samples

LC-MS/MS MRM chromatograms of cotinine, nicotine, and NNK along with the internal standard ritonavir in SVGA astrocytes. The level of the blank, the lower limit of quantification, and upper limit of quantification for nicotine (top three panels), cotinine (middle three panels), and NNK (bottom three panels) are shown in the left panels. Ritonavir is shown in the right column except for the blank for each compound. The intensity (cps) is presented in the Y-axis and time (min) is presented in the X-axis. The standard linear curve of nicotine, cotinine, and NNK are shown from top to bottom in the right side of the chromatograms.

4.2.4. Recovery, precision, and accuracy

Recovery of samples was analyzed by preparing six replicates of each of two standard concentrations in human astrocytes. Recovery was performed and reported as the percentage difference between extracted and non-extracted samples as previously described [131, 132]. Precision and accuracy were evaluated by analyzing calibration curve and quality control (QC) samples. Accuracy was determined by analyzing six replicates of each QC sample. Accuracy was reported as the percentage difference between the mean and the nominal concentrations divided by the nominal concentration. Precision was reported using the coefficient of variation (CV) (standard deviation/mean concentration) multiplied by 100. The limit of quantitation was optimized for each analyte, 0.33 ng/mL for nicotine and 0.11 ng/ml for both cotinine and NNK. The recoveries, accuracy, precision, and coefficient of variation (CV) of nicotine, cotinine, and NNK in astrocytes are presented in Table 2. The recoveries for nicotine, cotinine, and NNK were 97.5%, 85.9%, and 78.4%, respectively. The accuracies for nicotine, cotinine, and NNK were in the range of 82-114%, 89-118%, and 86-117%, respectively. The coefficients of variation for nicotine, cotinine, and NNK were within 14.6%, 11.9%, and 17.0%, respectively. The limits of quantitation accuracy were 115.2% for nicotine, 96.5 for cotinine and 83.3 for NNK and precision were within 14.9% for nicotine, 4.5% for cotinine and 9.3% for NNK (Table 2).

Table 2: Validation and Stability Experiment in SVGA astrocytes

	Nominal Conc (ng/ml)	Nicotine		Cotinine		Nitrosamine ketone (NNK)	
		Calculated Conc (ng/ml)	% CV (±)	Calculated Conc (ng/ml)	% CV (±)	Calculated Conc (ng/ml)	% CV (±)
CC-1	0.11			0.105 ± 0.005	4.5	0.091 ± 0.016	17.3
CC-2	0.33	0.28±0.041	14.9	0.32 ± 0.0091	2.8	0.29 ± 0.043	11.9
CC-3	1.09	1.12 ± 0.03	2.1	1.18 ± 0.09	7.6	1.25 ± 0.16	14
CC-4	10.86	12.0 ±1.135	9.4	12.02 ± 1.10	9.6	11.02 ± 0.05	0.5
CC-5	36.21	31.98 ±3.89	12.5	35.65 ± 0.89	2.5	32.65 ± 3.51	10.7
CC-6	103.45	119.21 ±17.1	14.1	111.21 ± 7.30	6.5	121.21 ± 19.3	16
CC-7	258.62	278.34 ±18.63	6.6	240.34 ± 19.3	8	235.43 ± 22.92	9.9
CC-8	431.04	420.59 ±14.1	3.4	430.57 ± 5.0	1.1	475.59 ± 50.10	10.2
CC-9	663.14	689.92 ±21.1	3	642.36 ± 5.97	8.7	672.39 ± 95.59	14.3
QCV-1	0.11			0.091 ±0.016	17.3	0.091±0.0082	9
QCV-2	0.33	0.28 ±0.041	9.6	11.7 ± 1.9	9	12.2 ± 2.6	1.2
QCV-3	10.97	11.03 ± 0.06	0.6	10.4 ± 0.1	8.2	10.49 ±0.63	9.9
QCV-4	36.56	37.59 ± 4.1	13.8	340.9 ± 6.6	9.8	35.9 ± 21.7	9.3
QCV-5	104.46	120.9 ± 13.1	10.5	118.3 ± 17.5	14.7	119.3 ± 23.5	19.6
QCV-6	435.26	470.8 ± 19.7	4.1	479.7 ± 34.1	7.2	467.7 ± 35.1	7.4
STQC-1	0.11			0.091 ±0.016	17.3	0.095 ± 0.0082	8.6
STQC-2	0.33	0.28±0.041	9.6	0.27 ± 0.04	18.5	0.36 ± 0.016	4.4
STQC--3	10.97	12.2 ± 2.6	1.2	10.4 ± 0.1	0.96	10.49 ± 0.63	9.9
STQC--4	36.56	31.98±3.89	12.4	34.9 ± 4.6	13.4	35.9 ± 1.7	5.9
STQC--5	104.46	124.9 ± 23.1	18.5	100.4 ± 0.1	1.2	96.3 ± 13.5	14.03
STQC--6	435.26	465.8 ± 17.9	3.8	479.7 ± 34.1	7.5	473.7 ± 31.1	6.5

4.2.5. Stability analysis

In order to evaluate the stability of nicotine, cotinine, and NNK in astrocytes, we prepared six individual specimens of SVGA astrocytes containing nicotine, cotinine, and NNK of each of six concentration levels and we assessed analyte stability at different temperatures (ambient, 4°C, and -80°C), after 34 days. The mean results from six replicates for each replicate, temperature, and concentrations were compared with the standard calibration values and expressed as percentages of accuracy and coefficient of variation (CV). The results are shown in Table 2.

4.2.6. Statistical analysis

Statistical analysis was performed to determine mean ± SD and a student t-test was applied to determine p values. A p value of ≤ 0.05 was considered significant.

4.3. Results

4.3.1. Induction of CYP1A1 and CYP2A6 by nicotine in SVGA astrocytes

First, the basal levels of mRNA of CYP enzymes were determined in the SVGA astrocyte cell line. Interestingly, the relative mRNA expression levels of CYP2A6 and CYP1A1 were 56% and 43% of the total CYPs' mRNA expression in SVGA. The remaining 1% accounted for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5. Furthermore, exposure of astrocytes to nicotine resulted in significant up regulation of CYP1A1 mRNA at 1h (150%) and CYP2A6 mRNA between 1 and 6 h (~200%) (Figure 7). Although there was no change in CYP1A1 mRNA level at later time points, CYP2A6 mRNA was down regulated at 12 h nicotine treatment. To ensure that the mRNA induction was consistent with the protein induction, we measured levels of CYP1A1 and CYP2A6 proteins (Fig. 8). Nicotine induced levels of CYP1A1 that were 170% relative to that of control CYP1A1 at 3 h, as well as levels of CYP2A6 that were 140% and 230% relative to that of control at 1 h and 3 h, respectively. Similar to CYP2A6 mRNA, nicotine down regulated CYP2A6 protein at 12 h. Our findings that CYP2A6 is the most abundant CYP in astrocytes and that even higher levels of expression are induced by nicotine, suggest its important role in nicotine metabolism and nicotine metabolite-associated oxidative stress in astrocytes.
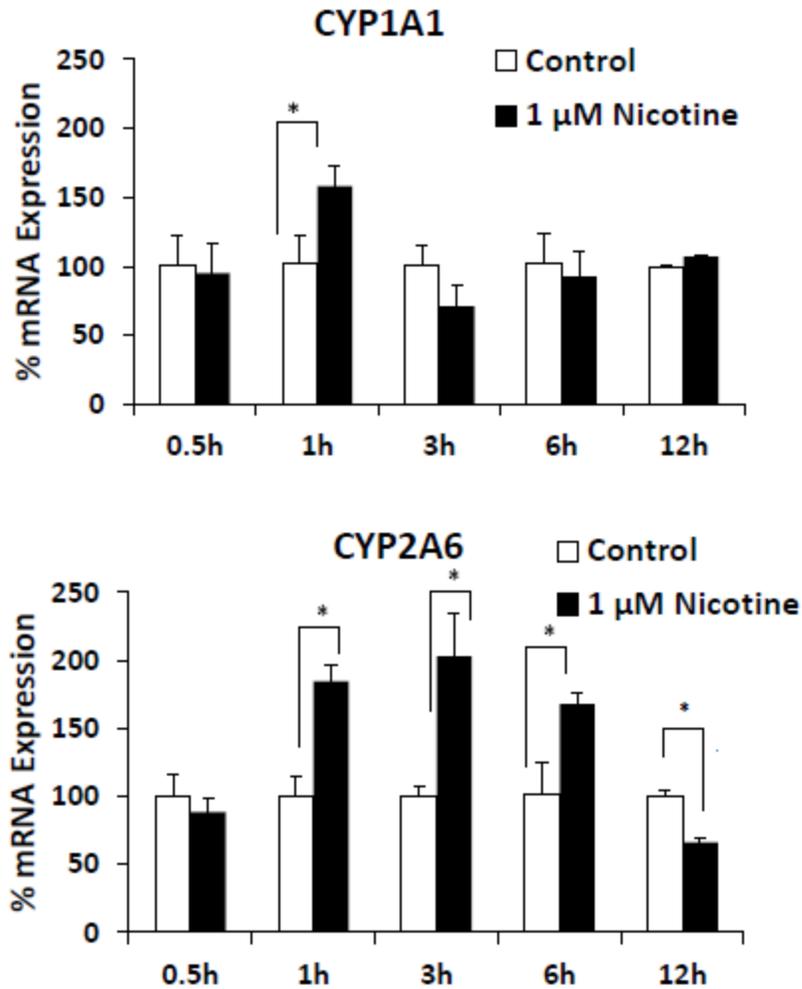
Figure 7: Effect of nicotine on mRNA expression of CYP1A1 and CYP2A6 in SVGA astrocytes

The SVGA astrocytes were treated with 1 µM nicotine for 0.5, 1, 3, 6, and 12 h. The percentage mRNA levels were calculated using qRT-PCR, with 100% expression normalized for the control at every time point. Expression of both the CYP genes was normalized to glyceraldehyde 3-phosphate dehydrogenase. In the above figures, the X-axis represents treatment time (hours) and Y-axis represents % mRNA expression. * Represents $p \leq 0.05$ compared to respective controls.
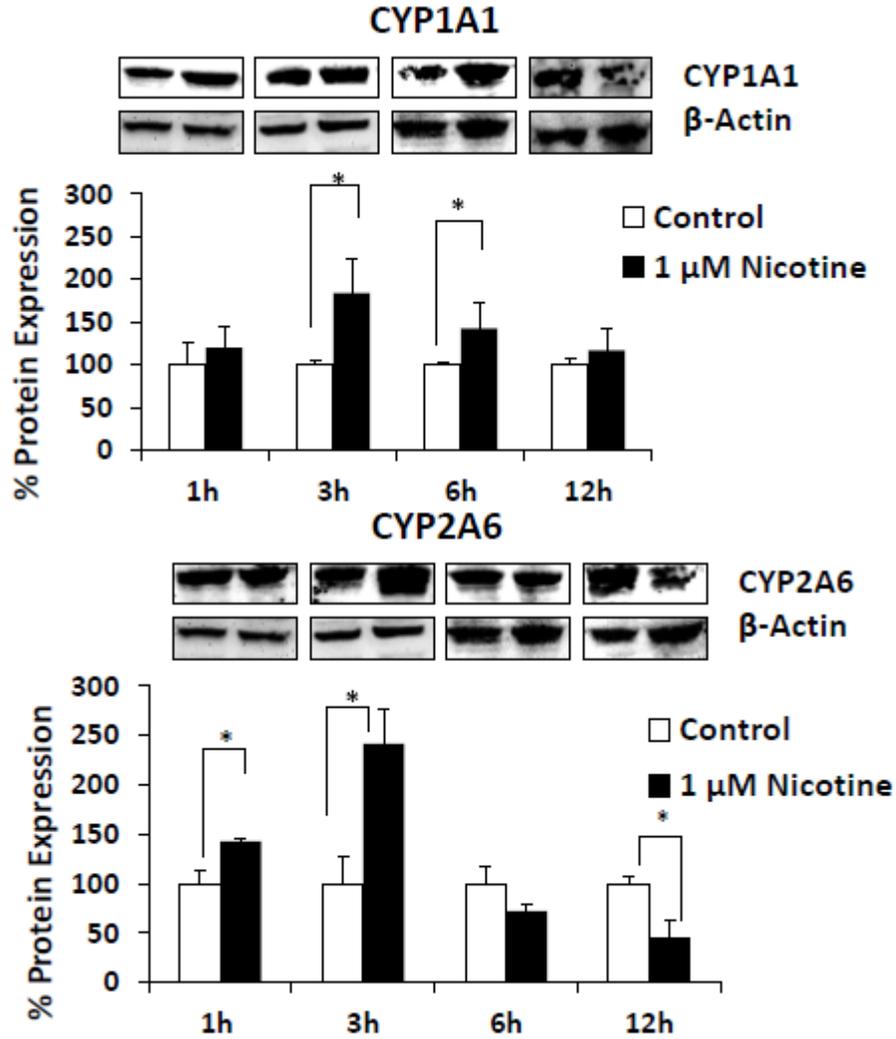
Figure 8: Effect of nicotine on levels of protein expression of CYP1A1 and CYP2A6

The SVGA astrocytes were treated with 1 µM nicotine for 0.5, 1, 3, 6, and 12h and the protein was isolated. The percentage protein expression was calculated by quantifying the immunoblots, with 100% expression normalized to the untreated cells (control) at every time points. Expression of CYP1A1 and CYP2A6 was normalized against β-actin. In the above figures, X-axis represents treatment time (hours) and Y-axis represents % protein expression. * Represents $p \leq 0.05$ compared to respective controls.

4.3.2. Metabolism of nicotine by CYP2A6 in SVGA astrocytes

The metabolism of nicotine in SVGA astrocytes was analyzed using the LC-MS/MS method as described in Materials and Methods and depicted in Figure 9. Figure 10 shows representative MRM chromatogram profiles of nicotine, cotinine, and NNK. Figure 11 shows the kinetic profile of cotinine and NNK formation. A very low amount of cotinine (5-7 ng/ml) and NNK (1-3 ng/ml) was detected in astrocytes. To ensure that CYP2A6 is involved in nicotine-metabolism, cotinine and NNK levels were measured in the presence of tryptamine, a CYP2A6 specific inhibitor [133]. Tryptamine decreased cotinine formation by approximately 80% at both 1 h and 2 h, whereas NNK formation was reduced by 60% at 1h and was almost completely abolished at 2 h (Fig. 12).
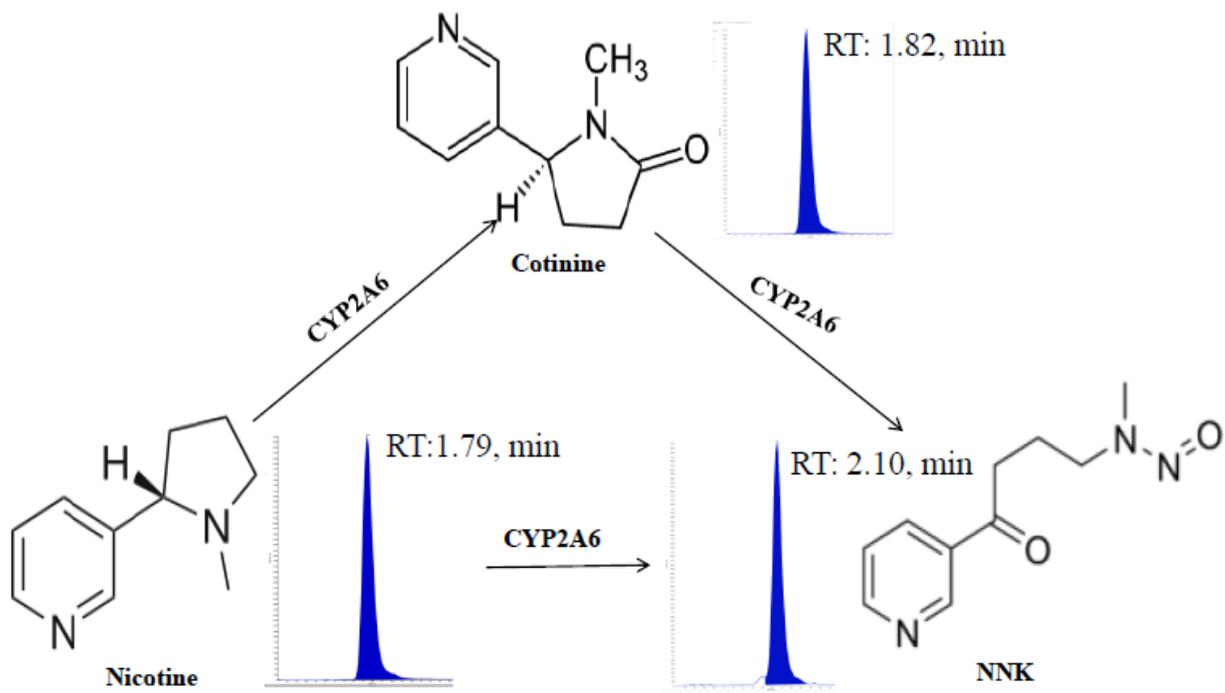
Figure 9: Schematic of nicotine metabolism by CYP2A6
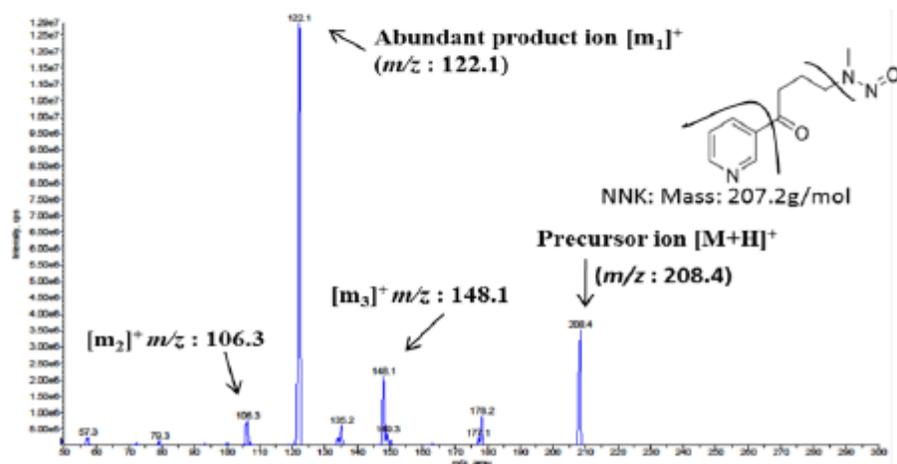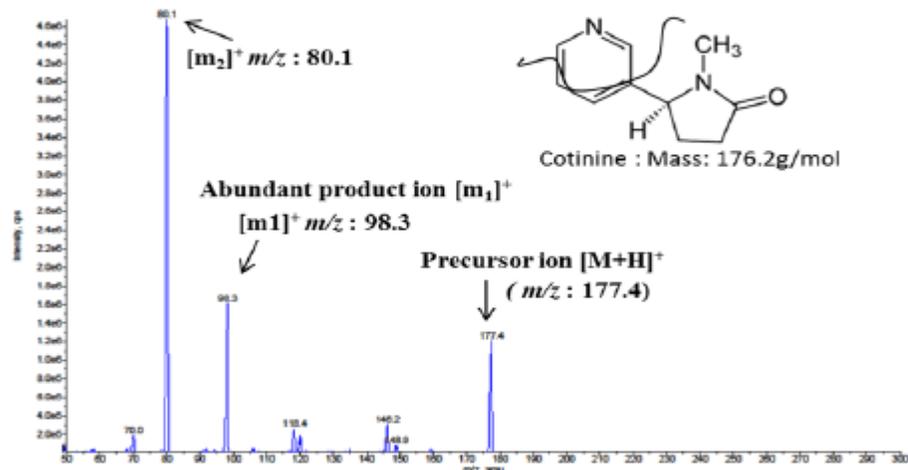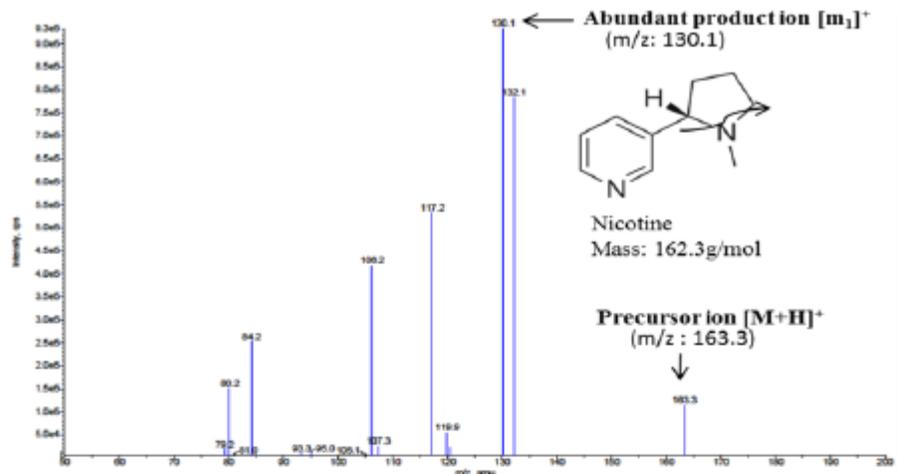
Figure 10: Representative MRM chromatogram profiles of nicotine, cotinine, and NNK
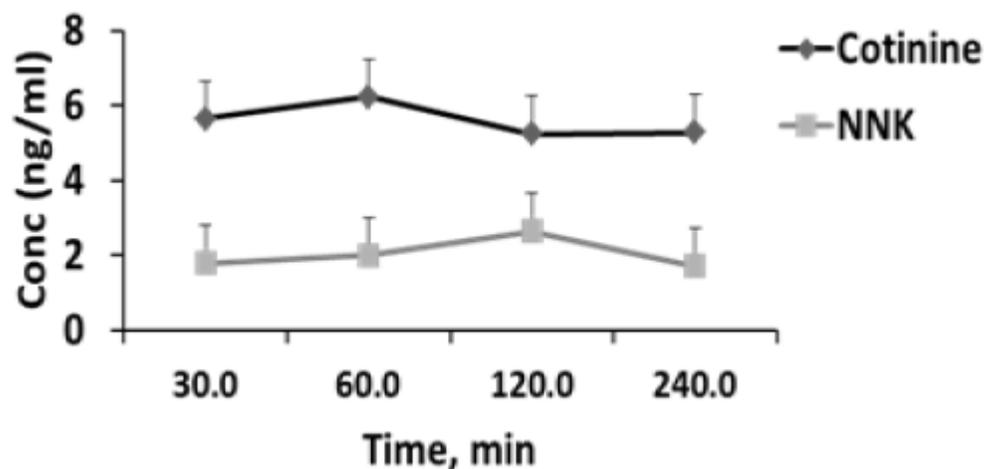
Figure 11: Kinetic profile of cotinine and NNK formation

Metabolism of nicotine in SVGA astrocytes. The cells were incubated with 1 µM nicotine for 0.5, 1, 2, and 4h and cotinine and NNK levels were measured using the LC-MS/MS method. A. Diagram depicting the metabolism of nicotine and the metabolic products cotinine and NNK. B. Representative MS2 profiles of nicotine, cotinine, and NNK with parent molecular ion transition and its fragmentation pattern in MS2 of nicotine, cotinine, and NNK. C. Kinetic profiles of cotinine and NNK formation. In the figures, X-axis represents the treatment time (hours) and Y-axis represents concentration of cotinine and NNK. The graphs were plotted as mean ± SD using three replicates for each time point.
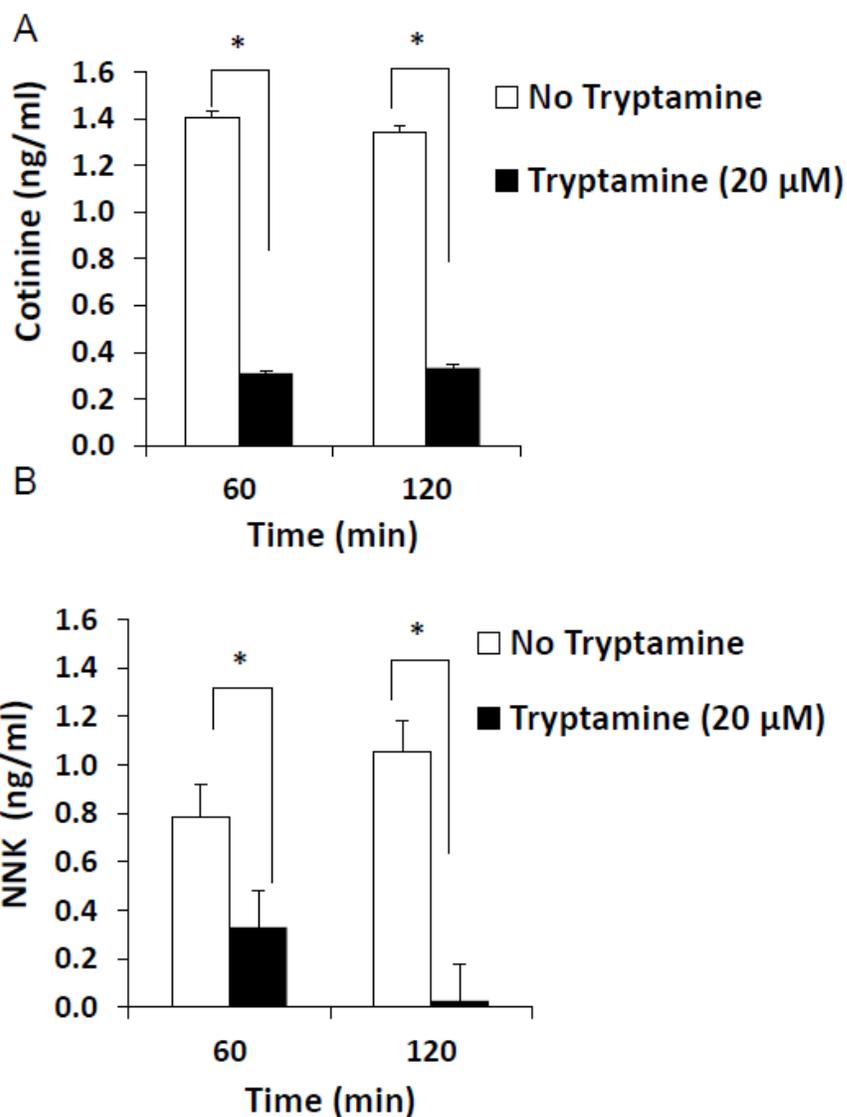
Figure 12: Effect of Tryptamine on Cotinine and NNK formation

Effect of tryptamine (20 µM), a CYP2A6 selective inhibitor, on the formation of cotinine and NNK in 1 µM nicotine treated SVGA astrocytes. In the figures, percent of cotinine and NNK formation are presented in Y-axis. The time points of nicotine treatment are presented on the X-axis. The graphs were plotted as mean ± SD using three replicates for each time point. * Represents $p \leq 0.05$ compared to respective controls.

4.3.3. Formation of ROS by CYP2A6-mediated nicotine metabolism in SVGA astrocytes

We measured the generation of ROS in nicotine-treated SVGA astrocytes using flow cytometry. Figure 13 shows the mean fluorescence intensity (MFI) of ROS formation in nicotine-treated astrocytes from 15 - 120 min. The results showed a slight decrease in MFI at 15 min, a peak at 30 min, and then a decrease at 90 and 120 min. To ensure that ROS is formed as a result of nicotine metabolism by CYP2A6, we pre-incubated astrocytes with CYP2A6-specific inhibitor tryptamine. The results showed that tryptamine significantly quenched the formation of ROS, suggesting the role of CYP2A6-mediated nicotine metabolism in ROS formation. Since our results showed a decrease in the levels of ROS at later time points (Fig. 13), we determined the expression level of the antioxidant enzymes superoxide dismutase (SOD) and catalase at 1, 3, 6, and 12 h (Fig. 14). The results showed that nicotine significantly induced SOD, especially at 3 h (~200%). In contrast, nicotine significantly reduced the expression of catalase at 1 and 12 h.
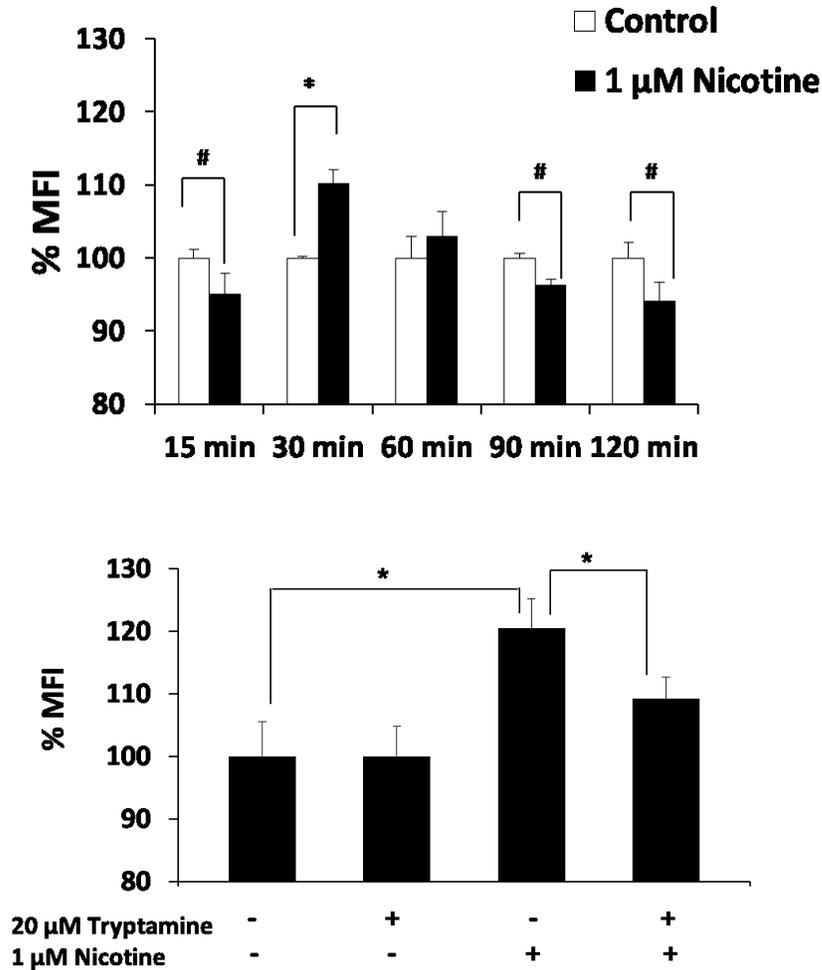
Figure 13: Role of CYP2A6 in nicotine mediated oxidative stress in SVGA astrocytes

The ROS production was measured using a flow cytometer from untreated and 1 μM nicotine treated cells from 15, 30, 60, 90, and 120 min. A. Bar graphs representing mean fluorescence intensity (MFI) from control and nicotine-treated cells. MFI is presented in Y-axis and the time points of nicotine treatment are presented in X-axis. The graphs were plotted as mean ± SD from three replicates. B. Effect of 20 μM tryptamine, a CYP2A6 selective inhibitor on the formation of oxidant contents in untreated and 1 μM nicotine treated cells for 30 min. * Represents p value ≤ 0.05 and # represents p value ≤ 0.1.
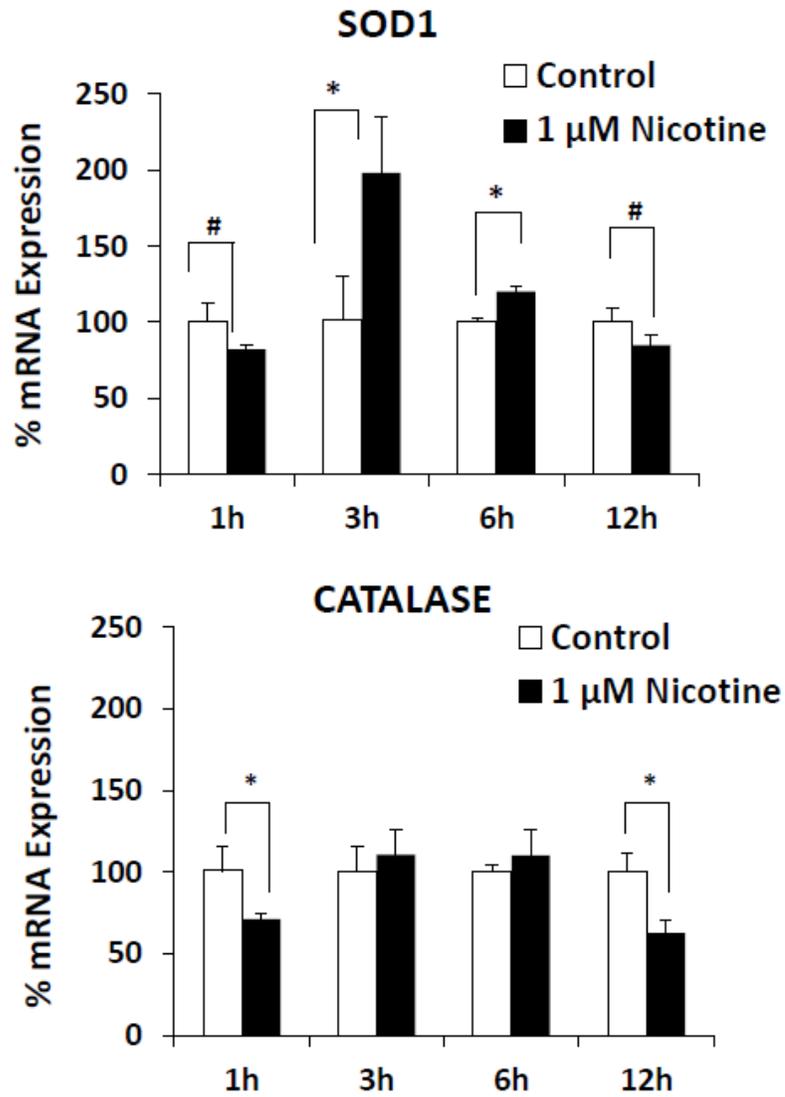
Figure 14: Effect of 1 μM nicotine on the mRNA expression of SOD and catalase in SVGA astrocytes

The SVGA astrocytes were treated with 1 μM nicotine for 1, 3, 6, and 12h. The percentage mRNA levels of both the enzymes were calculated using qRT-PCR, with 100% expression normalized to the control. Expression of both the genes was normalized against glyceraldehyde 3-phosphate dehydrogenase. X-axis represents the treatment time and Y-axis represents % mRNA expression. * Represents p value ≤ 0.05 and # represents p value ≤ 0.1.

4.4. Discussion

4.4.1. Abundance of CYP1A1 and CYP2A6 in SVGA astrocytes

The current study demonstrates very high levels of CYP1A1 (43%) and CYP2A6 (56%) in SVGA astrocytes, which account for only 3% and 0.1% of the total CYPs, respectively, in the liver [134]. Similarly, our previous studies revealed that CYP2A6 is the second most abundant (30%) CYP enzyme in the U937 macrophage cell line. [96]. It should be noted that, in many respects, macrophages are similar to microglia in the CNS. Although CYP2A6 is the most abundant CYP in SVGA astrocytes, its expression level in SVGA astrocytes is approximately 10-fold lower than in U937 macrophages. However, the level of CYP1A1 mRNA in astrocytes was comparable to that of U937 macrophages. This is the first report that demonstrates the presence of CYP2A6 and CYP1A1 in SVGA astrocytes. CYP2A6 is the major enzyme involved in the metabolism of nicotine, whereas CYP1A1 is responsible for the metabolism/activation of other tobacco constituents, such as polycyclic aromatic hydrocarbons (PAH) [135].

4.4.2. Induction of CYP2A6 by nicotine in SVGA astrocytes

This is the first report that demonstrates the induction of CYP1A1 and CY2A6 by acute nicotine treatment. To our knowledge, there is no report in the literature that demonstrates the induction of CYP2A6 and CYP1A1 by nicotine in hepatocytes or lungs. Similarly, our recent study did not show an induction of CYP1A1 and CYP2A6 by nicotine in U937 macrophages [131]. It is possible that CYP1A1 and CYP2A6 are inducible in astrocytes but not in hepatocytes or macrophages because their levels in astrocytes are much lower than that of hepatocytes [134] and macrophages [96]. Thus, CYP2A6 and CYP1A1 in macrophages and liver may already be at maximal expression levels. Alternatively, the inducibility of CYP1A1 and CYP2A6 is a tissue-specific phenomenon. This is quite possible

as other CYP's have been demonstrated to be inducible in a tissue-specific manner. An earlier study showed an induction of rat CYP2B1 in certain regions of the brain upon chronic nicotine treatment [136]. Similarly, a recent study showed that while nicotine does not induce hepatic CYP2B1, it induces CYP2B1 in the frontal cortex, brain stem, and olfactory tubercle [44]. Furthermore, chronic nicotine treatment in African green monkeys increased CYP2B6 (homologous to rat CYP2B1) in astrocytes and neurons (frontal cortex, caudate, thalamus) whereas hepatic levels of CYP2B6 were unchanged [45]. These reports are consistent with another finding that smokers have higher levels of CYP2B6 in the brain [43], which may increase the metabolism of certain drugs such as bupropion and propofol, and lead to a decreased response to these drugs.

We speculate that nicotine induces CYP1A1 and CYP2A6 in astrocytes through nicotine metabolism-mediated oxidative stress. Our speculation is based on a recent report which demonstrated that increased CYP2A6 expression is associated with increased CYP2E1-mediated alcohol metabolism and oxidative stress in the liver [137]. Additional observations suggest that oxidative stress mediates CYP2A6 induction by alcohol in U937 cell line [95]. Although the mechanism by which nicotine induces CYP2B1 or CYP2B6 is not clear, it has been shown that nicotine induces CYP2B1/CYP2B6 through transcriptional regulation, and in this way enhances its own metabolism [44]. In contrast to CYP2B6 induction, another study suggests that a non-transcriptional mechanism, perhaps a post-translational modification, is responsible for the induction of CYP2D6 and CYP2E1 by chronic nicotine treatment [138].

4.4.3. Development of LC-MS/MS method for concurrent measurements of nicotine, cotinine, and NNK in SVGA astrocyte

We have demonstrated a simple, rapid, highly sensitive and reproducible LC-MS/MS analytical technique for concurrent determination of nicotine, cotinine, and NNK in SVGA astrocytes. Nicotine, cotinine, and NNK achieved requisite recovery by a simple liquid-liquid extraction procedure. This extraction method is efficient, simple, fast, sensitive, and cost-effective and has been developed for the first time for astrocytes. We and other investigators have previously described   LC-MS/MS methods in other cell types that because of low sensitivity were inadequate to determine the intracellular concentrations of nicotine, cotinine, and NNK in SVGA astrocytes [131, 139-142]. Our previously reported LC-MS/MS method for nicotine, cotinine, and NNK in U937 macrophages [131] was not sufficiently sensitive to estimate these analytes in SVGA astrocytes, because astrocytes express a much lower level of CYP2A6 than macrophages. Nicotine metabolism in astrocytes is much lower than the macrophages, thereby the conversions of nicotine to cotinine and NNK levels are very low in SVGA astrocytes. The current method has been successfully applied for determination of intracellular levels of nicotine, cotinine and NNK in astrocytes. This is a very important step towards the determination of very low levels of cotinine and NNK in brain samples, especially in smokers (ex-vivo study), because these compounds may be associated with increased oxidative stress and astrogliosis leading to neuronal damage.

4.4.4. Nicotine metabolism by CYP2A6 in SVGA astrocytes

The nicotine metabolic profile obtained from SVGA astrocytes is different from those obtained with U937 macrophages, in which cotinine and NNK formation was relatively high and achieved a peak at 2-4h [131]. In addition, nicotine was metabolized rapidly (within 30 min) in macrophages [131], whereas the nicotine concentration was decreased by only 25%

in 4h in astrocytes (data not shown). These results are consistent with the fact that astrocytes express much lower level of CYP2A6 than do macrophages.

Several CYP enzymes such as CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP2F1 and CYP4B1 [143] are known to metabolize nicotine to cotinine. However, CYP2A6 plays a major role in metabolizing nicotine to cotinine (~80%) and NNK, as well as to some extent in activating NNK into procarcinogens [144, 145]. CYP2A13 is another isoform of the CYP2A family which is known to activate NNK into procarcinogens, and is expressed at very high levels in nasal mucosa, lungs, and trachea [146]. However CYP2A13 was not detected in astrocytes and other nicotine-metabolizing CYPs were present at very low levels (data not shown). CYP2A5, a homologue of human CYP2A6, is the major enzyme involved in nicotine metabolism in mouse [147]. Similarly, CYP2B1 and CYP2B6 are present at high levels in brain and they are further induced by nicotine [44, 45], which also correlates with a higher CYP2B6 level in the brains of smokers [43]. However, there has been no characterization of the role of CYP enzymes in nicotine metabolism in the brain (astrocytes or neurons). Using SVGA astrocytes as a model cell line, our study provides the first evidence of nicotine metabolism by CYP2A6 in the brain.

4.4.5. Nicotine metabolism-mediated oxidative stress in SVGA astrocytes

The increase in nicotine metabolism-mediated ROS formation in SVGA astrocytes is relatively lower than in U937 macrophages [131], which is consistent with the relatively low level of CYP2A6 in astrocytes compared to macrophages. Our results suggest that acute nicotine metabolism-mediated oxidative stress is ameliorated by the increase of the antioxidant enzyme SOD. However, the chronic exposure of nicotine in smokers, especially in astrocytes, would produce high levels of ROS, which can activate astrocytes. Astrocytes are the major non-neuronal cell type which are situated in close association with neurons, and

provide the neurons with structural, metabolic and trophic support [148]. Activation of astrocytes as a result of increased oxidative stress has been shown to disrupt the support to neurons [149]. Our study would help better understand the role of astrocytes in neurodegenerative diseases. Increased oxidative stress is known to occur in various chronic neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis and is associated with neuronal cell death [150].

The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway has been shown to be activated in astrocytes, which protects the neurons from oxidative insult [150, 151]. Nrf2 is a transcription factor which is translocated into nucleus when cells are subjected to oxidative stress. In the nucleus, Nrf2 binds to an antioxidant response element and leads to transcriptional activation of antioxidant genes (e.g. SOD and phase II detoxifying enzymes). Recently, HIV gp120 has been shown to induce Nrf2 in astrocytes, which is associated with stimulation of antioxidant genes such as hemoxygenase (HO-1) and NADPH dehydrogenase quinone 1 (Nqo1) [152]. A recent report also demonstrates transcriptional regulation of CYP2A6 by the Nrf2 pathway in human hepatocytes [153]. Our unpublished observations suggest a similar role for Nrf2 in the induction of CYP2A6 by alcohol-mediated oxidative stress in monocytes. Our study opens the way to test the hypothesis that CYPA6-mediated nicotine metabolism facilitates the oxidative stress induced by HIV in astrocytes. Our hypothesis is consistent with the findings that chronic exposure of nicotine/smoke increases the expression of other CYP enzymes, including CYP2A6 in the brain [43, 45]. Nicotine-induced brain CYP enzymes have relevance to several neurodegenerative diseases, including Parkinson's disease [154].

4.5. Clinical relevance

In the U.S. cigarette smoking is about three times more prevalent in HIV-infected people than in the uninfected population [59]. Nicotine, the major constituent of tobacco, is known to enhance HIV replication in alveolar macrophages [69] and microglia [70]. Although the mechanism is not known, studies have suggested that oxidative stress plays a role in increased viral replication in smokers [72, 155]. A study has also correlated smoking with increased infection, decreased immune response, poor response to antiretroviral drugs, and enhanced risk of virological rebound [61, 156]. Our recent study with macrophages [96], and the current study with astrocytes, suggest an important role for CYP2A6 in nicotine/smoking-mediated oxidative stress and HIV replication in monocytes and astrocytes. Since HIV protein gp120 is known to stimulate oxidative stress pathways [152], we speculate that nicotine/smoking in the presence of HIV infection may further enhance oxidative stress in a synergistic manner leading to increased HIV replication. Inhibition of the CYP2A6-mediated oxidative stress pathway has the potential to decrease viral replication and increase the response to ART that would reduce the progression of AIDS in smokers.

4.6. Conclusion

In conclusion, this is the first report on the expression levels of CYP1A1 and CYP2A6 in SVGA astrocytes, and their further induction by nicotine. We also developed a highly sensitive LC-MS/MS method for simultaneous determination of nicotine, cotinine, and NNK in SVGA astrocytes, which allowed us to determine the role of CYP2A6 in nicotine metabolism in astrocytes. Furthermore, the role of CYP2A6 in nicotine metabolism-mediated oxidative stress was analyzed in these cells. These findings have clinical relevance because smoking among HIV-infected individuals is highly prevalent and these individuals show increased HIV replication. Since our previous study in monocytes (Jin et al) specifically used

nicotine, one of the major constituents of tobacco smoke, our further goal is to study the net effect of cigarette smoke in U937 monocytes by treating these cells with CSC.

CHAPTER 5

CIGARETTE SMOKE CONDENSATE (CSC) MEDIATED OXIDATIVE STRESS AND

CYTOTOXICITY IN U937 MONOCYTES

5.1. Introduction

Cigarette smoke contains 4000 different chemical constituents; of which 60 are identified as known carcinogens [157]. CSC contains several other constituents along with nicotine including dioxins, halogenated and nonhalogenated polycyclic aromatic hydrocarbons (PAHs), among which benzo[a] pyrene and semiquinones are few examples [158, 159]. CSC constituents are known to induce oxidative stress leading to alterations in gene expression in various cell and tissue type [160, 161]. For instance, CSC treatment in oral cancer cells resulted in upregulation of genes including CYPs and keto reductases that are responsible for metabolism of PAHs [162]. Most of these gene expressions are known to be modulated through activation of AHR by its agonists present in CSC [163]. Similarly, reactive oxygen species (ROS) produced by CSC can also activate nuclear factor-erythroid2-related factor 2 (Nrf2), the master regulator of oxidative stress which in turn transcriptionally regulates genes driven by antioxidant response elements (AREs).

In our earlier studies we explored the role of nicotine (major addictive constituent of tobacco) alone in ROS formation and CYP2A6 regulation in U937 monocytes and SVGA astrocytes. Since CSC contains most of the cigarette smoke constituents, it represents a better smoking model for *in vitro* use. Our hypothesis for this chapter is that CSC induces CYPs, oxidative stress, cytotoxicity and HIV replication in monocytes/macrophages. Our specific aim is to study the CSC-mediated CYP induction, oxidative stress, and cell death in U937

monocytes, and HIV replication in HIV-infected primary macrophages. In the current chapter, we discussed the effects of CSC on AHR-mediated CYP1A1 induction, ROS formation and cytotoxicity in U937 cells. We also determined the effects of CSC treatment on viral replication in HIV-infected primary macrophages.

5.2. Results

5.2.1. Induction of CYP1A1 and CYP2A6 by CSC in U937 monocytes

CSC treatment at 50 µg/ml in U937 monocytes resulted in significant up regulation of CYP1A1 mRNA from 1H to 96H. CSC treatment significantly induced CYP2A6 mRNA at 48H and 72H that was quenched at 96H (Fig. 15). To ensure that the mRNA induction was consistent with the protein induction, we measured levels of CYP1A1 and CYP2A6 proteins (Fig. 16 & 17). Although much lower than the respective mRNA expression, CSC also induced CYP1A1 protein levels from 3H to 12H, as well as CYP2A6 at later time points of 12H and 24H. Our findings that CYP1A1 and CYP2A6 expression are induced by CSC, suggest their important role in PAH and nicotine metabolism, respectively, and metabolite-associated oxidative stress in monocytes.
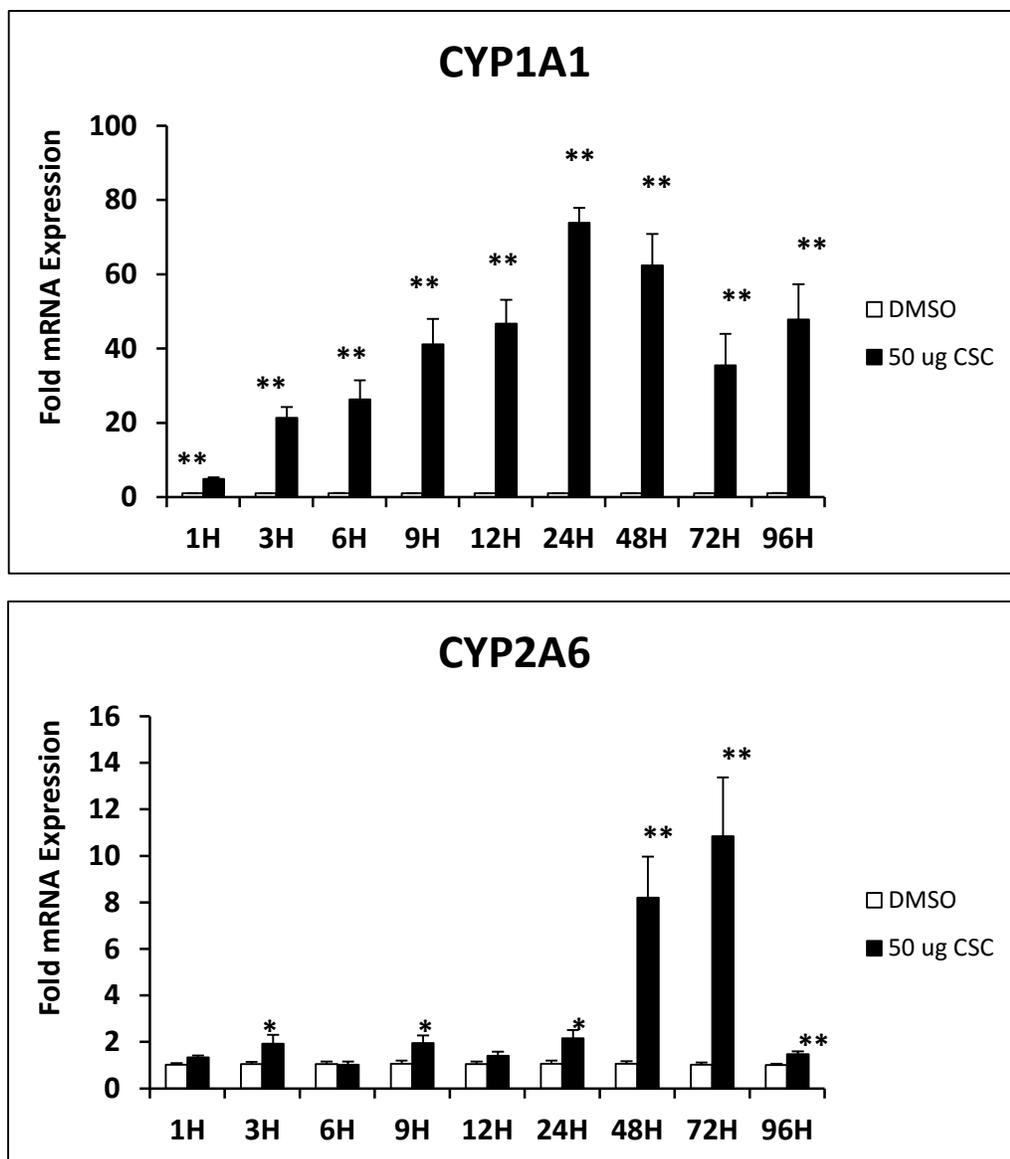
Figure 15: Effect of CSC on mRNA expression of CYP1A1 and CYP2A6 in U937 monocytes

The U937 monocytes were treated with 50 μg/ml CSC for 1, 3, 6, 9, 12, 24, 48, 72, and 96 H. The percentage mRNA levels were calculated using qRT-PCR, with 100% expression normalized for the control at every time point. Expression of both the CYP genes was normalized to glyceraldehyde 3-phosphate dehydrogenase. In the above figures, the X-axis represents treatment time (hours) and Y-axis represents % mRNA expression. * Represents p ≤ 0.05 compared to respective controls.
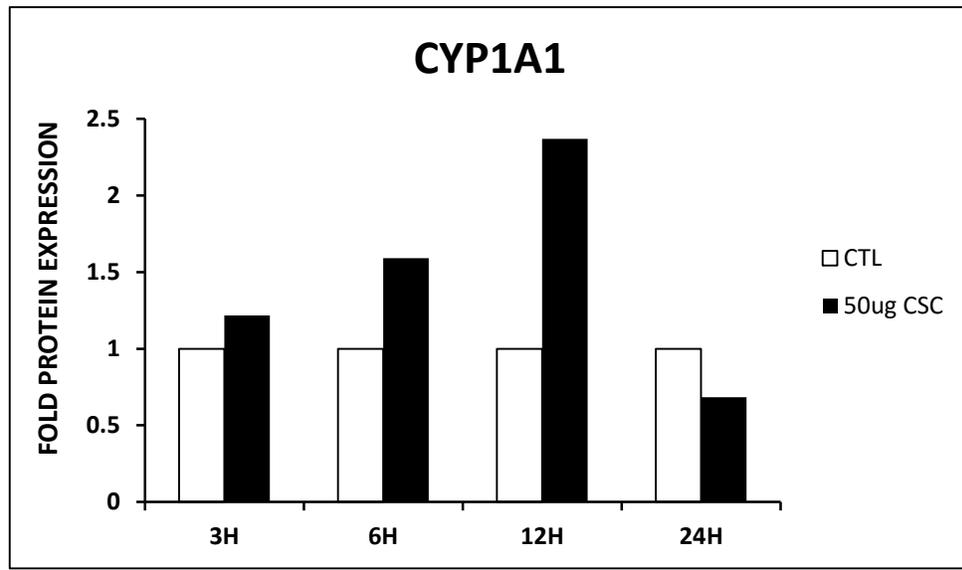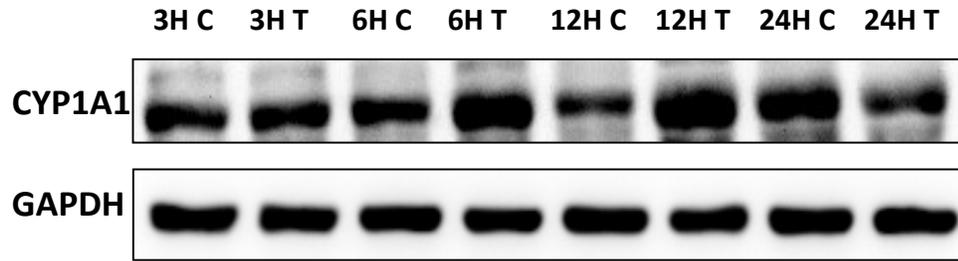
Figure 16: Effect of CSC on protein expression levels of CYP1A1
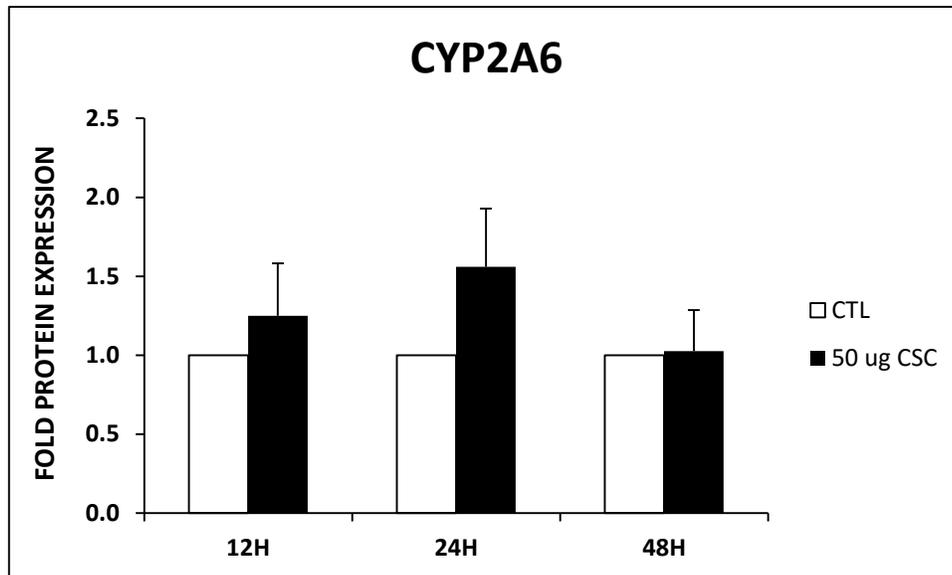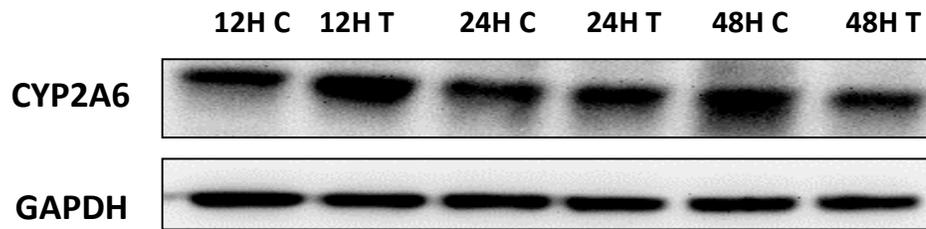
Figure 17: Effect of CSC on protein expression levels of CYP2A6

The U937 monocytes were treated with 50 µg/ml CSC for 1, 3, 6, 9, 12, 24, 48, 72, and 96 H and the protein was isolated. The percentage protein expression was calculated by quantifying the immunoblots, with 100% expression normalized to the untreated cells (control) at every time points. Expression of CYP1A1 and CYP2A6 was normalized against GAPDH. In the above figures, X-axis represents treatment time (hours) and Y-axis represents % protein expression.

5.2.2. CSC mediated changes in CYP1A1 gene expression are AHR dependent

CSC is comprised of PAHs which are known to activate AHR pathway. To determine whether CSC-mediated CYP1A1 mRNA induction involved AHR signaling, we used a specific AHR antagonist, CH223191, to block its effects. We selected CH223191 because of it competitive binding to AHR receptor thus preventing its translocation to the nucleus without demonstrating any agonistic action. We treated monocytes with 50 µM CH223191 followed by 50 µg/mL CSC for 3 H and examined CYP1A1 mRNA expression level by q-RTPCR. As shown in Fig. 18, pretreatment with the AHR antagonist significantly attenuated the CSC-mediated CYP1A1 mRNA induction.
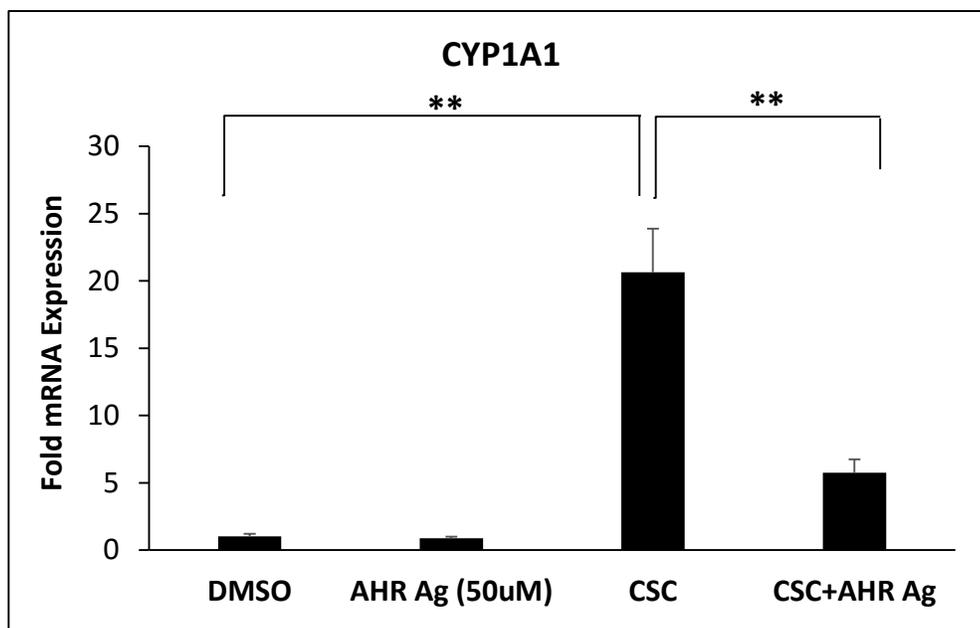
Figure 18: Effect of AHR antagonist on CYP1A1 gene expression

5.2.3. Time kinetics of CSC induced ROS formation

We measured the generation of ROS in 50 µg/mL CSC treated U937 monocytes using flow cytometry. Figure 19 shows the mean fluorescence intensity (MFI) of ROS formation in nicotine-treated monocytes from 1 - 48 hours. CSC treatment showed a time dependent increase in ROS until 6H, where the peak ROS formation was observed and then declined to basal level at 48H. Furthermore, our studies showed significant reduction in CSC induced ROS formation upon 1H pretreatment with 100 µM Vitamin-C (Vit-C), a potent antioxidant compound (Fig. 20). The results suggest that a general antioxidant is not capable of completely quenching the ROS induce by CSC.
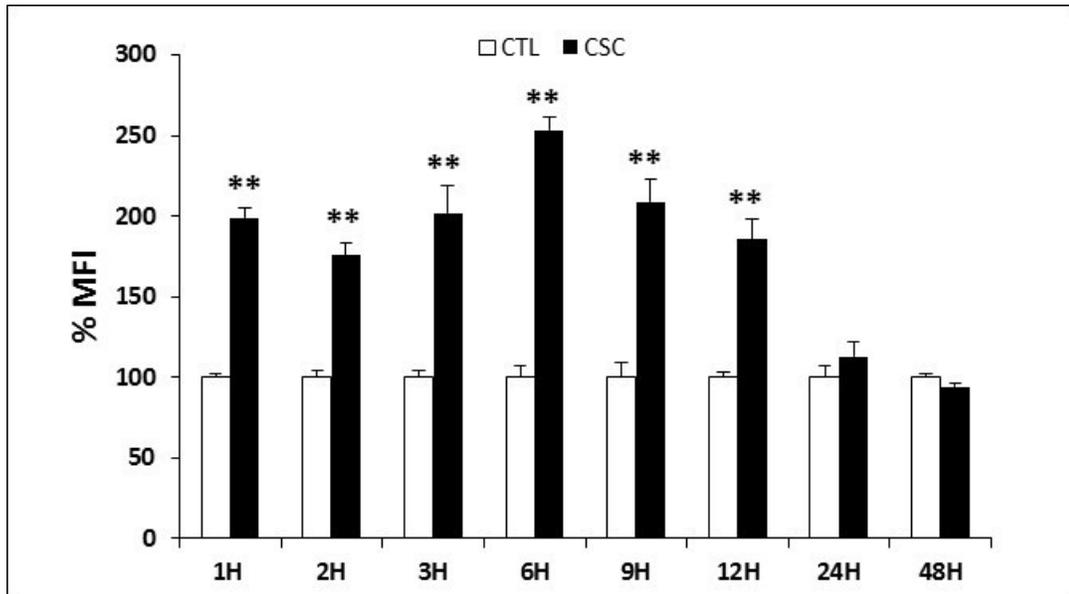
Figure 19: Effect of CSC on oxidative stress in U937 monocytes

The ROS production was measured using a flow cytometer from untreated and 50 µg/ml CSC treated cells from 1H to 48H. Bar graphs representing mean fluorescence intensity (MFI) from control and CSC-treated cells. MFI is presented in Y-axis and the time points of CSC treatment are presented in X-axis. The graphs were plotted as mean ± SD from three replicates.
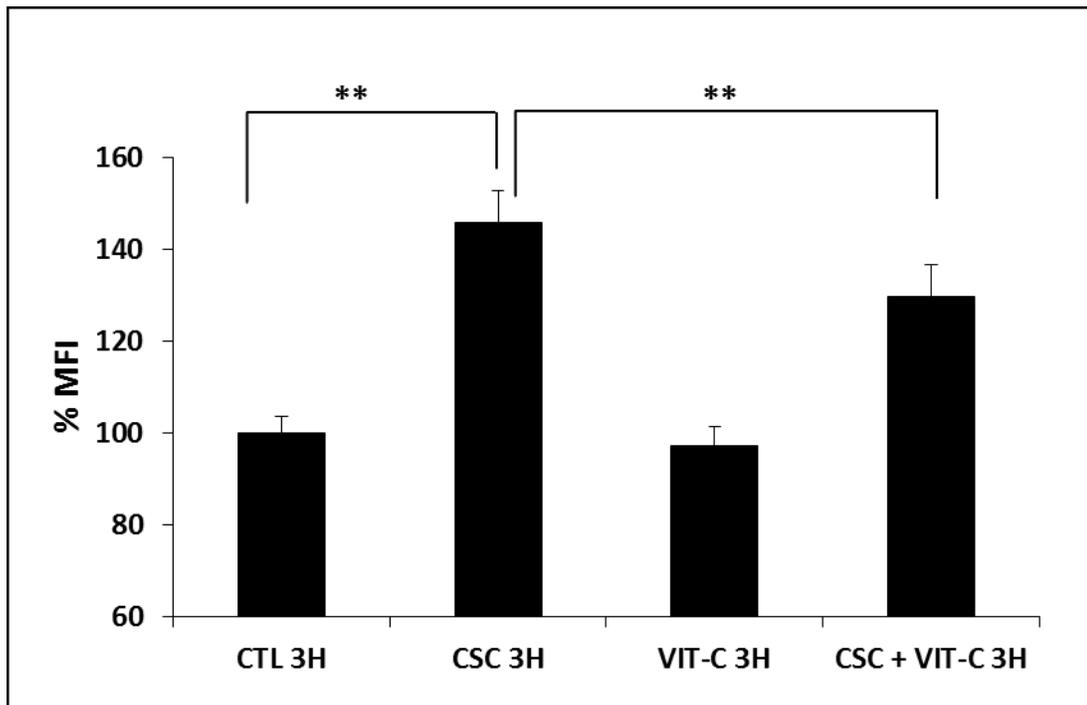
Figure 20: Effect of Vitamin-C pretreatment on CSC induced ROS formation in U937 monocytes.

The ROS production was measured using a flow cytometer from untreated and 50 µg/ml CSC treated cells for 3H upon 1H pretreatment with 100 µM Vitamin-C. Bar graphs representing mean fluorescence intensity (MFI) from control and CSC-treated cells. MFI is presented in Y-axis and the time points of CSC treatment are presented in X-axis. The graphs were plotted as mean ± SD from three replicates.

5.2.4. CSC induced cell death in U937 monocytes

To determine if CSC treatment is leading to cell death in U937 monocytes, the cells were treated with 50 µg/mL CSC for 48 H and MTT assay was performed. Annexin staining was also performed to determine the apoptotic cells. As shown in Fig. 21, CSC treatment caused significant increase in apoptosis in these cells. To find out if CSC-mediated apoptosis involves caspase-3 activation, caspase-3 activity was measured upon CSC treatment for 24H. The results showed that CSC significantly increased caspase-3 activity by 2 fold (Fig. 22 A). Cleaved caspase-3 protein levels are also increased as determined by western blot (Fig.22 B).
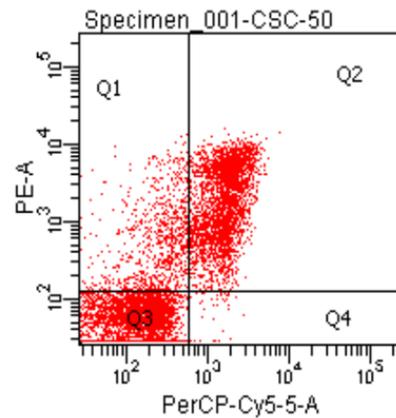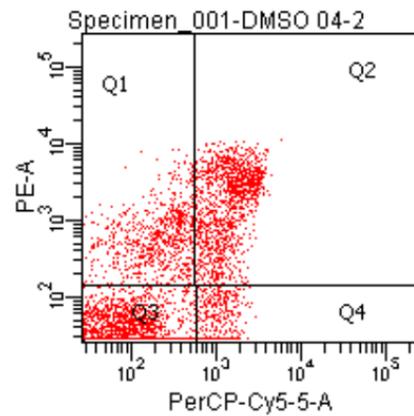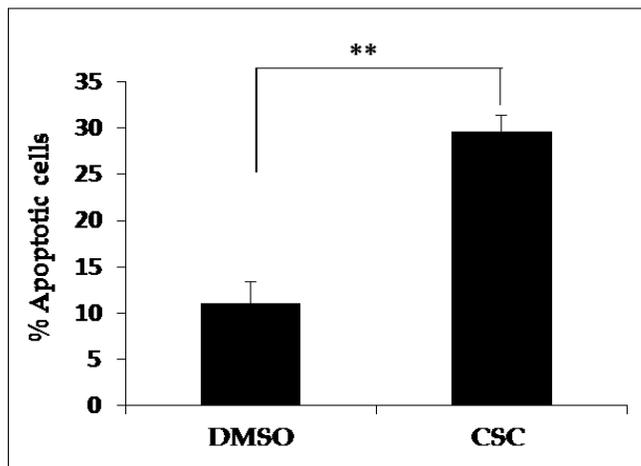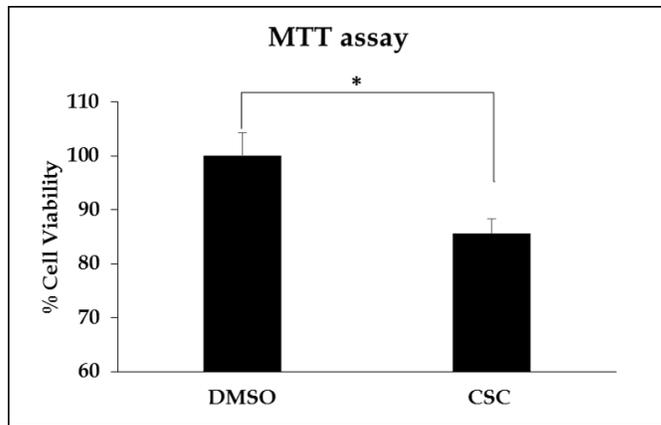
Figure 21

Cell death was measured using MTT assay from vehicle control and 50 µg/ml CSC treated cells for 48H. Apoptosis was measured using a flow cytometer from vehicle control and 50 µg/ml CSC treated cells for 48H upon staining with Annexin-PE reagents. Bar graphs representing % apoptotic cells in Q2 from control and CSC-treated cells. The graphs were plotted as mean ± SD from three replicates.
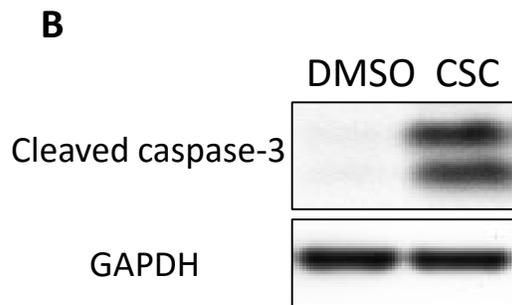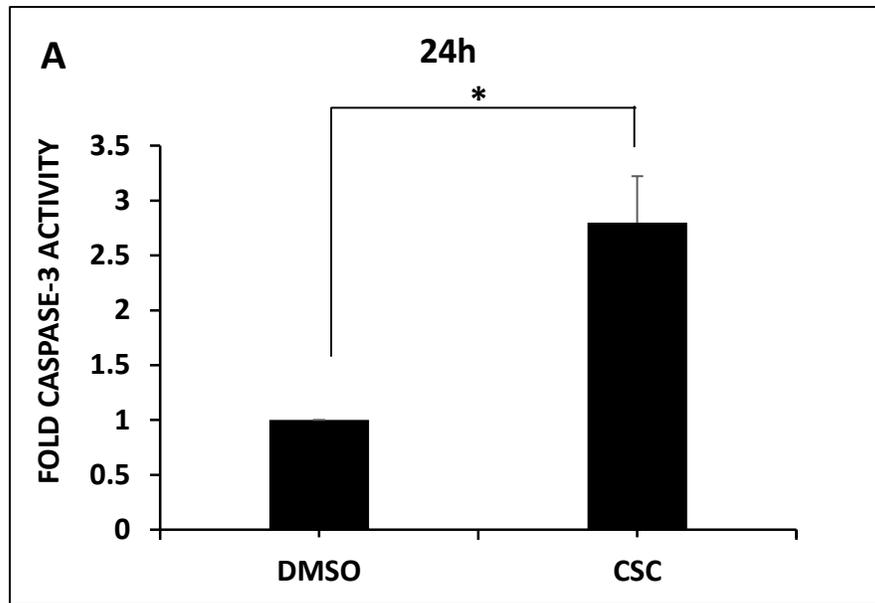
Caspase-3 activity is measured in cell lysates obtained from vehicle control and 50 µg/ml CSC treated cells for 24H using a colorimetric assay kit. (A) Bar graphs representing fold caspase-3 activity in DMSO and CSC-treated cells. The graphs were plotted as mean ± SD from three replicates. (B) Western blot stained for cleaved caspase-3 antibody and GAPDH in DMSO and 50 µg/ml CSC treated cells for 24H.

5.2.5. Effect of CSC on HIV replication in primary macrophages

To determine the effect of CSC treatment on HIV replication in monocytes, we treated HIV-infected primary macrophages with CSC for four days (once-daily). The results showed that the amount of p24 antigen in cell free supernatant was found to be significantly higher in CSC treated HIV-infected macrophages compared to DMSO treated HIV-infected macrophages (Figure 23).
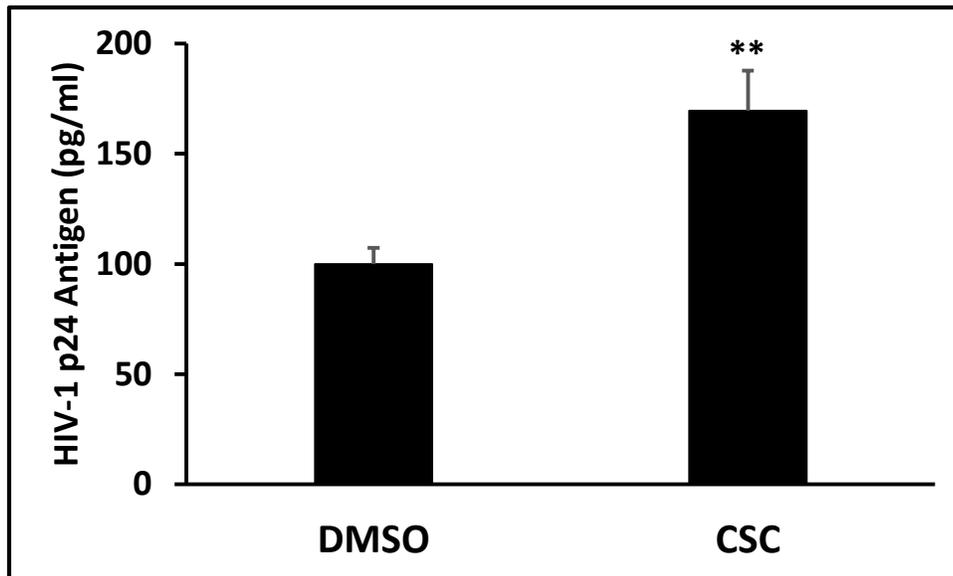
Figure 23: Determination of p24 antigen levels in DMSO and CSC treated HIV-infected macrophages

The viral load in macrophages was determined by measuring p24 antigen levels using ELISA. The data for the above assay represents mean of multiple analysis of p24 titer obtained from HIV-infected macrophages from three different donors. The p values (** represent $p \leq 0.01$) are calculated using one way-ANOVA and presented in the graph.

5.3. Discussion

In our previous studies, nicotine treatment alone didn't cause significant induction of CYP1A1 and CYP2A6 levels in U937 monocytes. However in the current study, CSC caused AHR-mediated CYP1A1 induction as well as CYP2A6 induction, which is significantly higher than the effect of nicotine. CSC treatment also caused significantly higher production of ROS compared to our earlier study with nicotine. It is possible that since CSC comprises of PAHs, the amount of ROS produced was very high compared to that of nicotine alone. CSC also caused significant apoptosis as measured by caspase-3 activity as well as cleaved caspase-3 formation. This may occur both directly, by genotoxicity of CSC constituents, and indirectly through generation of reactive oxygen species. Future studies will be conducted to determine the role of ROS in CSC-mediated cell death by using general as well as specific antioxidants that quench the formation of ROS. Further studies also need to be performed to decipher the involvement of CYP1A1 in the generation of ROS as well as oxidative stress-mediated cell death. We conducted few studies using nonspecific CYP1A1 inhibitors which didn't yield conclusive findings portraying the need to design novel inhibitors targeted against CYP1A1 specifically. In addition, in HIV-infected mature macrophages, HIV replication was found to be significantly higher in CSC treated macrophages compared to control cells supporting the effects of smoking on HIV pathogenesis. Although this study did not decipher the molecular mechanism of CSC-mediated induction of ROS and HIV-replication, it led the foundation that CSC increases HIV replication and apoptosis, which is consistent with increased induction of CYP enzymes and ROS.

5.4. Conclusion

In conclusion, as rationalized, in comparison to nicotine alone, the total extract of cigarette represented by CSC had a pronounced and significantly higher impact on expression

of CYPs and OS in monocytes. These dramatic changes induced by CSC translated into enhanced viral replication in primary macrophages. Since our in vitro findings thus far have shown CSC-mediated induction of CYPs, ROS formation, enhanced HIV replication, and the involvement of CYP2A6 in nicotine metabolism-mediated OS, further goal is to examine underlying mechanism, and to confirm the above findings in an *ex vivo* study using human smoker subjects.

CHAPTER 6

EFFECT OF MILD-TO-MODERATE SMOKING ON VIRAL LOAD, CYTOKINES,

OXIDATIVE STRESS, AND CYTOCHROME P450 ENZYMES IN HIV-INFECTED

INDIVIDUALS

6.1. Introduction

The specific effects of mild-to-moderate smoking on the components of HIV pathogenesis such as inflammatory cytokines and oxidative stress, is poorly studied. Cytokines/chemokines play a crucial role in HIV pathogenesis by regulating HIV replication and immune responses [164, 165]. Several studies have shown that cytokine dysregulation contributes to HIV disease progression [166, 167]. For example, cytokines have shown to play an important role in development of viral latency and maintenance of latently infected CD4+ T cells during antiretroviral therapy (ART) [167]. Furthermore, the myriad of pro-inflammatory cytokines including tumor necrosis factor (TNF-α), interleukin-2 (IL-2), and IL-18, present in the plasma and lymphoid tissues, induce HIV replication even after prolonged ART. Similarly, oxidative stress is known to play a significant role in HIV pathogenesis [100, 168, 169]. More specifically, oxidative stress has been linked to HIV replication in monocytes/macrophages [100, 170]. In addition, a report suggested the role of iron and oxidative stress in smoking-mediated HIV replication in alveolar macrophages [72]. Of importance, the pathway leading oxidative stress, especially in monocytes, in HIV-infected smokers is unknown.

Our recent *in vitro* studies have also shown that acute nicotine exposure causes increased production of reactive oxygen species (ROS) through CYP2A6-mediated pathway in monocytic and astrocytic cell lines [95, 99, 171]. Since it is not feasible to conduct an *ex*

vivo study in astrocytes, we will be performing our ex vivo studies in peripheral monocytes isolated from human subjects. CYP3A4 metabolizes approximately 50% of commercially available drugs including ART in the liver [172] and results in the production of ROS [173, 174]. However, the functional role of CYP3A4 is unknown in monocytes, which is an important site of viral infection and major viral reservoir. Our previous studies have demonstrated predominant expression of CYP2A6, CYP2E1, and CYP3A4 in monocytic cell lines [96]. The hypothesis in this aim is that smoking increases HIV replication, which is associated with increase in the expression of CYP enzymes and oxidative stress. Our specific aim is to determine the levels of CYP2A6, CYP2E1, CYP3A4, and antioxidant enzymes (AOE), oxidative stress, cytokines, and HIV replication in blood plasma/monocytes isolated from a cohort of patients belonging to uninfected non-smokers (Healthy), uninfected smokers (smokers), HIV-infected non-smokers (HIV) and HIV-infected smokers (HIV smokers). In the current study, we sought to determine whether smoking increases viral replication in clinical samples of HIV-infected smokers. Furthermore, we sought to determine whether there is a relationship between viral load, cytokines/chemokines, oxidative stress, and CYP pathways in HIV-infected smokers.

6.2. Experimental design

6.2.1. Study population

Thirty two human subjects were recruited and assigned to four different cohorts as follows: a) healthy HIV negative control subjects who reported that they were non-smokers (HIV negative non-smokers), b) HIV positive non-smokers, c) HIV negative mild-to-moderate smokers, and d) HIV positive mild-to-moderate smokers. Participants were recruited in Cameroon, Africa, from within a long standing reference population of more than 2000 known HIV positive subjects. The participants provided their written informed consent to participate in this study. The signed consent form from the participants was stored as hard copy as well as electronically on a password protected computer. The consent procedure was approved by Institutional Review Board (IRB) from the University of Missouri-Kansas City, Kansas City, MO and IRB/Institutional Ethic Committee (IEC) from the Provincial Regional Hospital, Ministry of Public Health, Bamenda, Cameroon. We performed power analysis using "Power and Precision" software by considering the magnitude of the known effects of smoking on HIV replication [70, 72] and oxidative stress [99, 171]. The results showed that 6-8 samples from each group would provide a power of ≥ 0.80. Participants between the ages of 21-65 years were recruited, because individuals <13 years and >65 years of age generally have altered expression of metabolic (CYP) and to some extent antioxidant enzymes (AOEs) [25, 26]. For those individuals in the HIV negative non-smoker category, we recruited individuals who reported no history of "cough" and physical examination although some may have had malaria in the past (*Plasmodium falciparum* is indigenous in Cameroon). With respect to the HIV negative and positive smokers, mild-to-moderate smokers with a smoking history of ≤ 20 pack years (a pack year is defined as smoking at least one pack a day for one year), were enrolled in the study. The documentation of smoking was determined by a HIV

84

counselor and the phlebotomist following a personal interview. With respect to the HIV positive category, individuals with CD4+ counts <600 cells/µl were enrolled. Participants were recruited using the following strict exclusion criteria: 1) Pregnant or lactating women were excluded since they show increased metabolism of nicotine and cotinine [175] (smoking is rare among Cameroonian women); 2). Liver damage and lung disease since these alter the metabolism of tobacco constituents such as nicotine; 3) Individuals with other infectious diseases, such as documented malaria, tuberculosis, and hepatitis B since hepatitis and active TB are prevalent in HIV-infected population, and some of these diseases are known to interact with HIV [176]; 4) Individuals who were receiving ART. ART drugs or other medications are expected to interfere with tobacco constituents [99, 177]. Sufficient numbers of HIV-infected individuals who were not already receiving ART or other medication were available among village populations in Cameroon, because medications or access to CD4 counting is frequently not available in these areas (WHO, Cameroon 2004-2005 report http://www.who.int/hiv/HIVCP_CMR.pdf). In order to qualify for free therapy provided by the Cameroonian Government, an adult must obtain a CD4 count and that result must be under 350cells/µl. The test to determine CD4 count is costly and not available in rural areas. Second, the local recommendation during the period of the study was to initiate therapy when the CD4 count was <350 cell/µl. 5) Individuals who consume other recreational substances of abuse, e.g. methamphetamine, cocaine, or marijuana. These drugs are expected to interfere with HIV pathogenesis and/or oxidative stress [178, 179]. Applying these inclusion and exclusion criteria was challenging but subjects were recruited upon personal interview, analysis of their history, and following clinical screening for HIV, malaria, and hepatitis B. The clinical screening tests for malaria and hepatitis were conducted using standard procedures as

described [180] and patients with "cough" suggestive of TB received an AFB or were excluded.

6.2.2. Statistical analysis

Demographic variables (age, sex ratio) were summarized using descriptive statistics. All outcome variables were summarized as the mean plus or minus the standard error. Comparisons among the four groups were conducted using one way ANOVA with no corrections for multiple comparisons. All tests were two-sided and results that were statistically borderline significant at $p \leq 0.1$ (#) and significant at $p \leq 0.05$ (*) and ≤ 0.01 (**). A two-way ANOVA was done to determine whether they interact synergistically or additively in HIV positive smokers. All analyses were performed using IBM SPSS version 21.

6.3. Results

6.3.1. Characteristics of the study population

A total of 32 subjects were selected upon screening approximately 2000 HIV positive individuals attending the HIV clinic at the Regional Hospital, Mezam Polyclinic HIV/AIDS Treatment Center, or at surrounding village health centers. The recruitment was very difficult and time consuming owing to strict exclusion criteria, paucity of smokers who also do not also drink alcohol and are not yet receiving ART or other medications. Furthermore, smoking was rare among women in this region, especially in HIV positive population in which it is discouraged. The subjects with such inclusion criteria are nearly impossible to recruit in the USA, because HIV-infected individuals receive ART promptly after diagnosis and most receive other medications and/or abuse illegal drugs. The recruitment of subjects who were not receiving ART was critical to determine the specific effects of mild-to-moderate smoking in HIV-infected individuals without an interference by drugs on the results of the study. The number of individuals in each group (HIV negative non-smoker, HIV negative smoker, HIV positive non-smoker, and HIV positive smoker) along with the age-range, median age, and male/female ratio of all the subjects are presented in Table 3. The median age of all the groups was 35-45. The male/female ratio varied in each group, with relatively low ratio in HIV positive group but high ratio in all smokers because of the low incidence of smoking among women with the strict exclusion criteria. Our analysis showed that the subject-to-subject variation in CD4 counts, viral load, cytokine production, and oxidative stress was not significant on the basis of age and gender differences (data not shown). This analysis was performed using HIV negative non-smokers and HIV negative smokers, in which, the number of subjects were sufficient to perform such analysis.

87

Table 3: Demography and clinical outcomes (CD4) of the subjects

Subjects		HIV negative non-smokers	HIV positive non-smoker	HIV negative smokers	HIV positive smokers
Number, age (years), male/female ratio	#	11	6	11	4
	Age range	20-64	23-42	31-60	38-57
	Median age	45	34.5	45.5	42
	Male/female	1.2	0.5	1.75	3
CD4 count (cells/µL)	Range	631-1086	256-551	500-1488	13-584
	Mean ± SE	865 ± 45	374 ± 43	1037 ± 100	387 ± 130

6.3.2. Effect of smoking and HIV on CD4 and viral load in plasma

The mean CD4 count (cells/µL) and mean viral load (log copies/mL) of each cohort are presented in Table 3 and Figure 24. As expected, the CD4 count was lower in HIV positive non-smokers (374 ± 43) and HIV positive smokers (387 ± 130) than HIV negative non-smokers (865 ± 45) or smokers (1037 ± 100). There was a significant increase in the CD4 count in HIV negative smokers compared with non-smokers (1037 ± 100 vs. 865 ± 45). Our results showed that the viral load is significantly increased in HIV positive smokers (4.5 ± 0.7) compared to the HIV positive non-smokers (3.1 ± 0.5) (Figure 24). These results suggest that smoking is associated with increased viral replication.
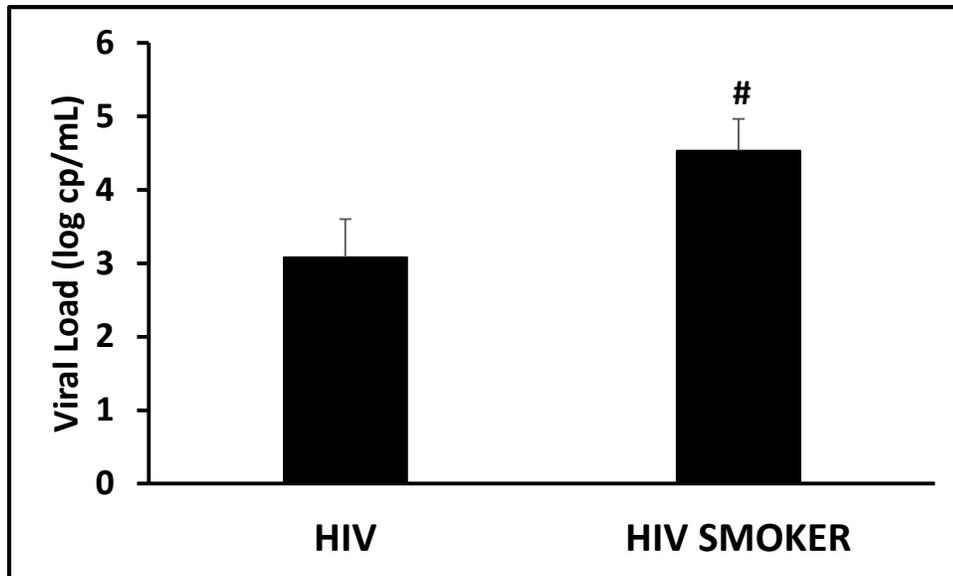
Figure 24: Determination of Viral load in plasma of HIV-infected and HIV-infected smoker subjects

The viral loads in the plasma of human subjects were determined by analyzing HIV RNA using q-RTPCR. The p values (# and ** represent p≤0.1 and p≤0.01, respectively) are calculated using one way-ANOVA and presented in the graph.

6.3.3. Effect of smoking and HIV on cytokine/chemokine production in plasma

We determined the levels of various pro-inflammatory cytokines, IL-1β, IL-6, IL-8, RANTES, TNF-α and MCP-1 in all plasma samples. The levels of IL-1β were not detectable in any plasma sample within the detection limit of the assay (2 pg/mL). The concentrations of cytokines in each group showed significant subject-to-subject variations, and therefore, we presented these results in box plot as shown in Figure 25. The concentrations of most of the cytokines were in the range of 10-80 pg/mL, while the concentration of RANTES ranged from 500-12,000 pg/mL. The concentrations of MCP-1 and IL-8 were significantly lower in HIV positive non-smokers and HIV negative smokers and marginally lower in HIV positive smokers than HIV negative non-smokers (Figure 25). The concentration of RANTES was marginally higher in HIV positive non-smokers and smokers than HIV negative non-smokers and smokers. However, there were no significant differences in the levels of IL-6 and TNF-α between the test and control groups (Figure 25).
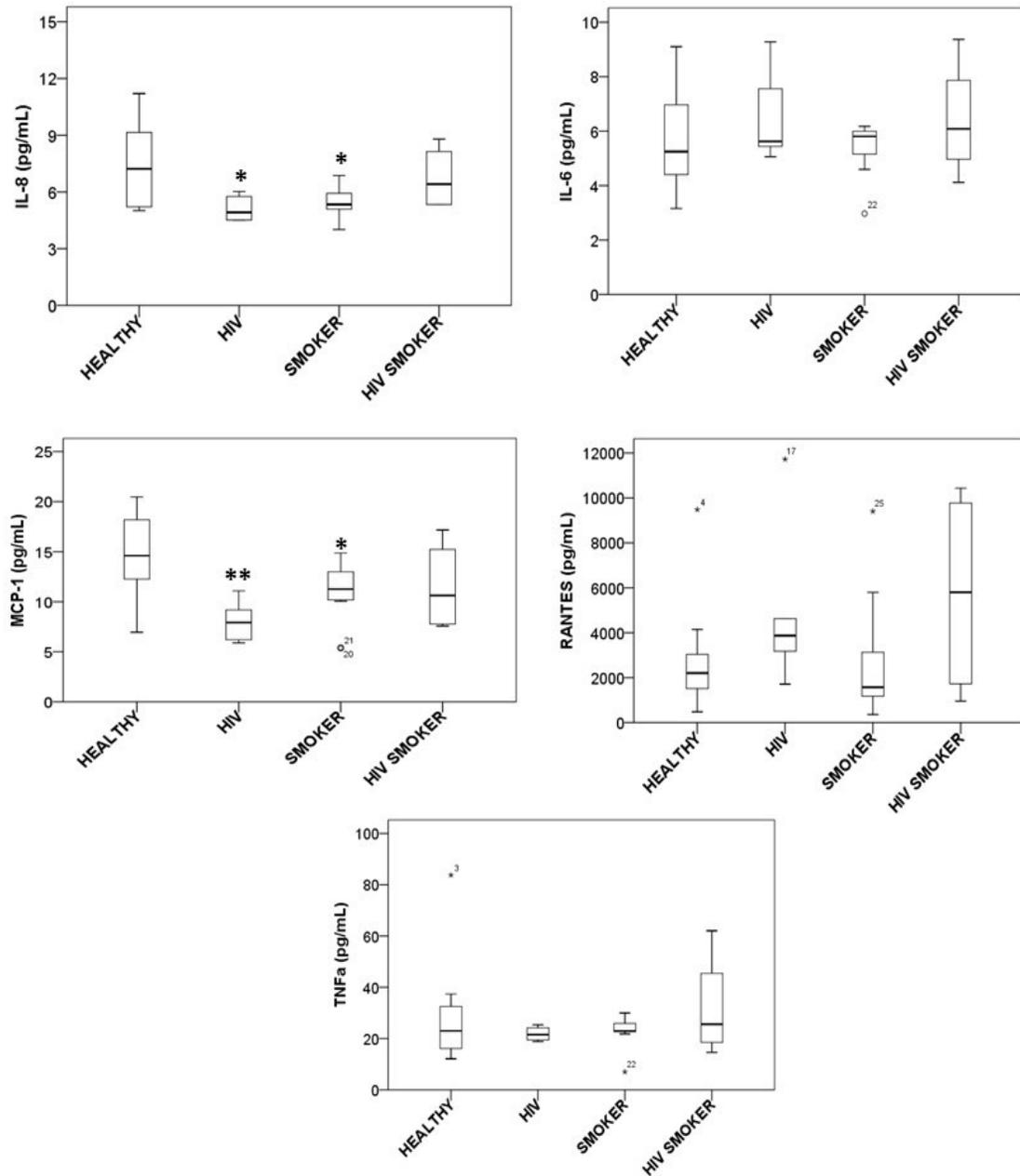
Figure 25: Box plots of Cytokine levels in plasma samples

Box and whisker plots of cytokine/chemokine levels in plasma of HIV negative non-smokers (HEALTHY), HIV positive non-smokers (HIV), HIV negative smokers (SMOKER), and HIV positive smokers (HIV SMOKER) groups. The box represents the 25th-75th quartile, the whiskers represent the range of values, the median is presented as a line inside the box, and the out of range values are presented as circles or stars above and below the whiskers. The p

values (* and ** represent p≤0.05 and p≤0.01, respectively) are calculated using one way-ANOVA and presented above the bars in the graph. The concentrations of RANTES, IL-6, IL-8, MCP-1 and TNF-α were shown in the figure.

6.3.4. Effect of smoking and HIV on oxidative stress in plasma and monocytes

To evaluate oxidative stress in clinical samples, we determined the oxidative DNA damage by measuring 8-OHdG content. The results showed that the oxidative damage is significantly increased in plasma from HIV negative smokers (2-fold), and HIV positive non-smokers (2.5-fold) groups compared with HIV negative non-smokers (Figure 26A). Furthermore, there was an additive increase in the levels of oxidative damage in HIV positive smokers (4-fold) compared to HIV positive non-smokers and HIV negative smokers (two-way ANOVA for testing the interaction gave p=0.39 suggesting the absence of synergy). Similarly, we determined the oxidative DNA damage in the monocytes of all the cohorts. Our results demonstrated an increase in 8-OHdG levels in HIV negative smokers (~30%) and HIV positive non-smokers (~40%) compared to HIV negative non-smokers (Figure 26B). An additive increase was also observed in 8-OHdG levels in the DNA of HIV positive smokers (~75%) compared to HIV positive non-smokers and HIV negative smokers. Overall, the results suggest an increase in oxidative stress as a result of HIV infection and smoking.
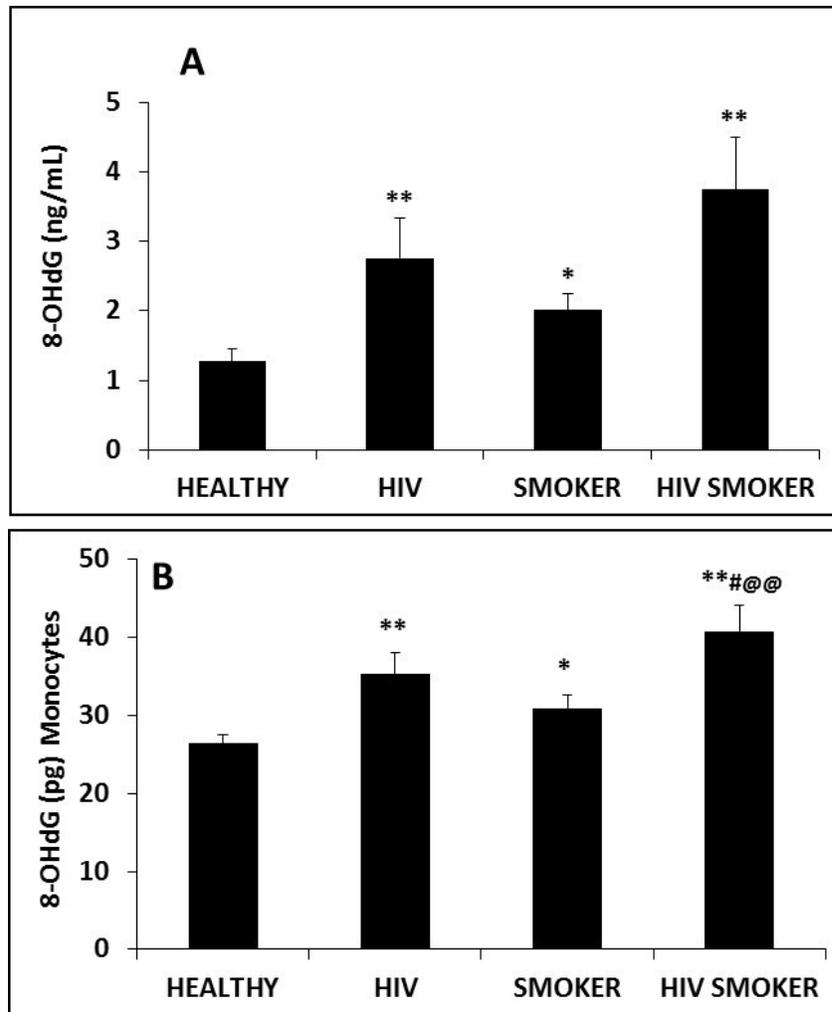
Figure 26: 8-OHdG levels in plasma and monocyte samples

Measurement of oxidative stress in plasma (A) and monocyte (B) samples of HIV negative non-smokers (HEALTHY), HIV positive non-smokers (HIV), HIV negative smokers (SMOKER), and HIV positive smokers (HIV SMOKER) groups. The 8-OHdG contents are plotted as a bar graph for each category, and the p values ≤ 0.05 and ≤ 0.01 are represented as * and **, respectively. * represents the significance with respect to HEALTHY, # represents the significance with respect to HIV and @ represents the significance with respect to SMOKER in HIV SMOKER group.

6.3.5. Effect of smoking and HIV on the expression of antioxidants in monocytes

The expression of antioxidant enzymes (AOEs) are generally induced to combat increased oxidative stress triggered by an agent. Therefore we measured the levels of antioxidant genes; SOD1, SOD2, catalase, and Nrf2 in our four cohorts. Our results demonstrated that the levels of mRNA of the most antioxidant genes are not altered in HIV positive and/or smoker groups compared to HIV negative non-smokers (Figure 27). However, we found a 2-fold increase in the level of Nrf2 in HIV negative smokers compared to the HIV negative non-smoker. In general, lack of induction of the most AOEs suggests their inability to counterbalance oxidative stress generated by smoking and HIV infection.
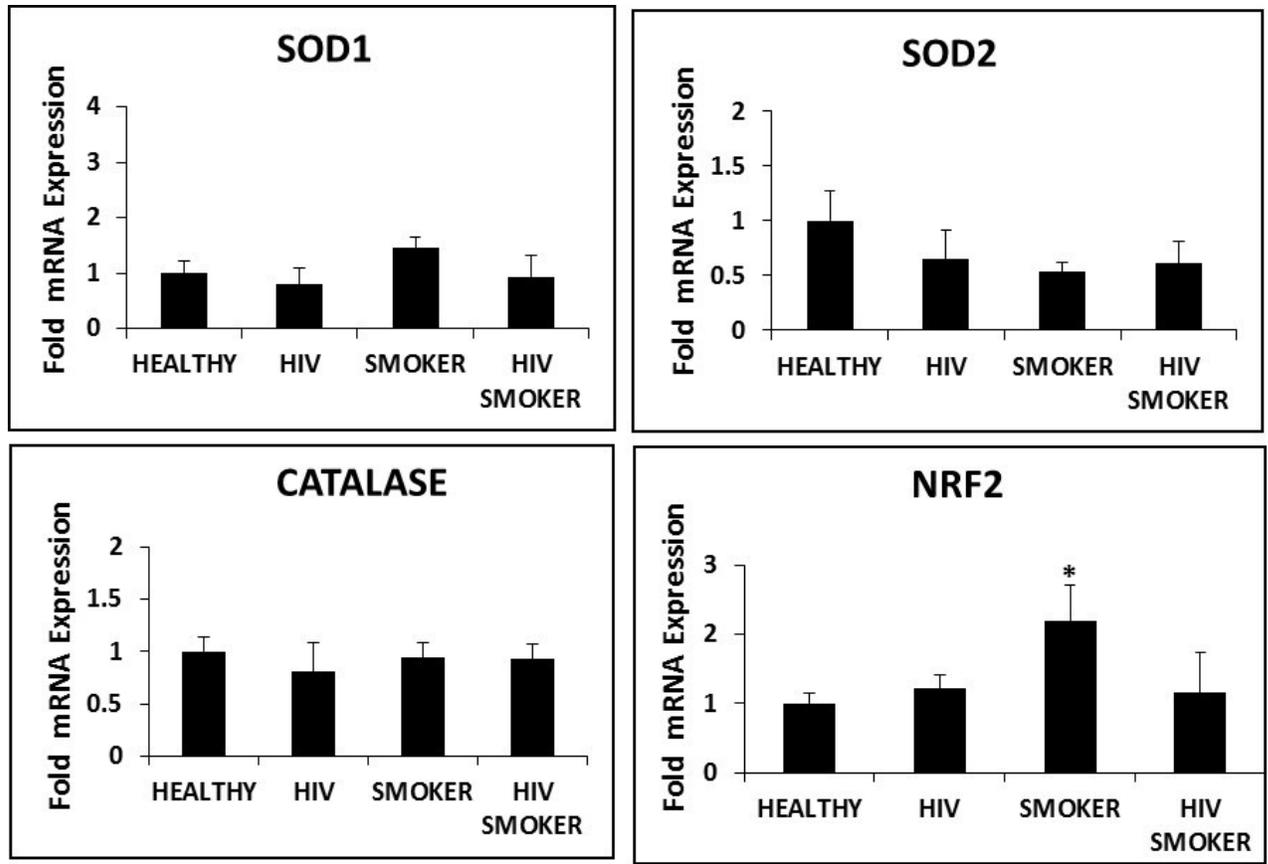
Figure 27: Antioxidant gene expression levels in monocytes from human cohorts

Determination of antioxidant gene expression in HIV negative non-smokers (HEALTHY), HIV positive non-smokers (HIV), HIV negative smokers (SMOKER), and HIV positive smokers (HIV SMOKER) groups. The mRNA expressions of SOD1, SOD2, catalase, and Nrf2 were measured by qRTPCR. A one way-ANOVA was employed to calculate the p value (* represent $p \leq 0.05$) with respect to HEALTHY in all the groups.

6.3.6. Effect of smoking and HIV on the levels of CYP enzymes in monocytes

CYP enzymes play important role in generating oxidative stress by generating superoxide and peroxide as a result of P450-mediated reaction cycle. Therefore, we measured the levels of important CYP enzymes (CYP2A6, CYP2E1, and CYP3A4), which are known to produce oxidative stress by metabolizing endogenous compounds or xenobiotics such as tobacco constituents and marketed drugs. As expected, the results showed that the levels of CYP2A6 mRNA is increased ($p=0.07$; borderline significance) in HIV negative smokers (1.5-fold) compared to HIV negative non-smokers (Figure 28). However, the level of CYP2A6 mRNA was only marginally increased in HIV positive non-smokers and HIV positive smokers compared to HIV negative non-smokers. Interestingly, the level of CYP3A4 was increased in HIV positive non-smokers and HIV positive smokers by approximately 5-fold compared to HIV negative non-smokers (Fig 28). However, its level was not significantly increased in the HIV negative smokers. As expected, there was no significant increase in the level of CYP2E1 in HIV positive and/or smoker cohorts, suggesting that CYP2E1, which is induced by alcohol, does not play role in inducing oxidative stress in these cohorts.
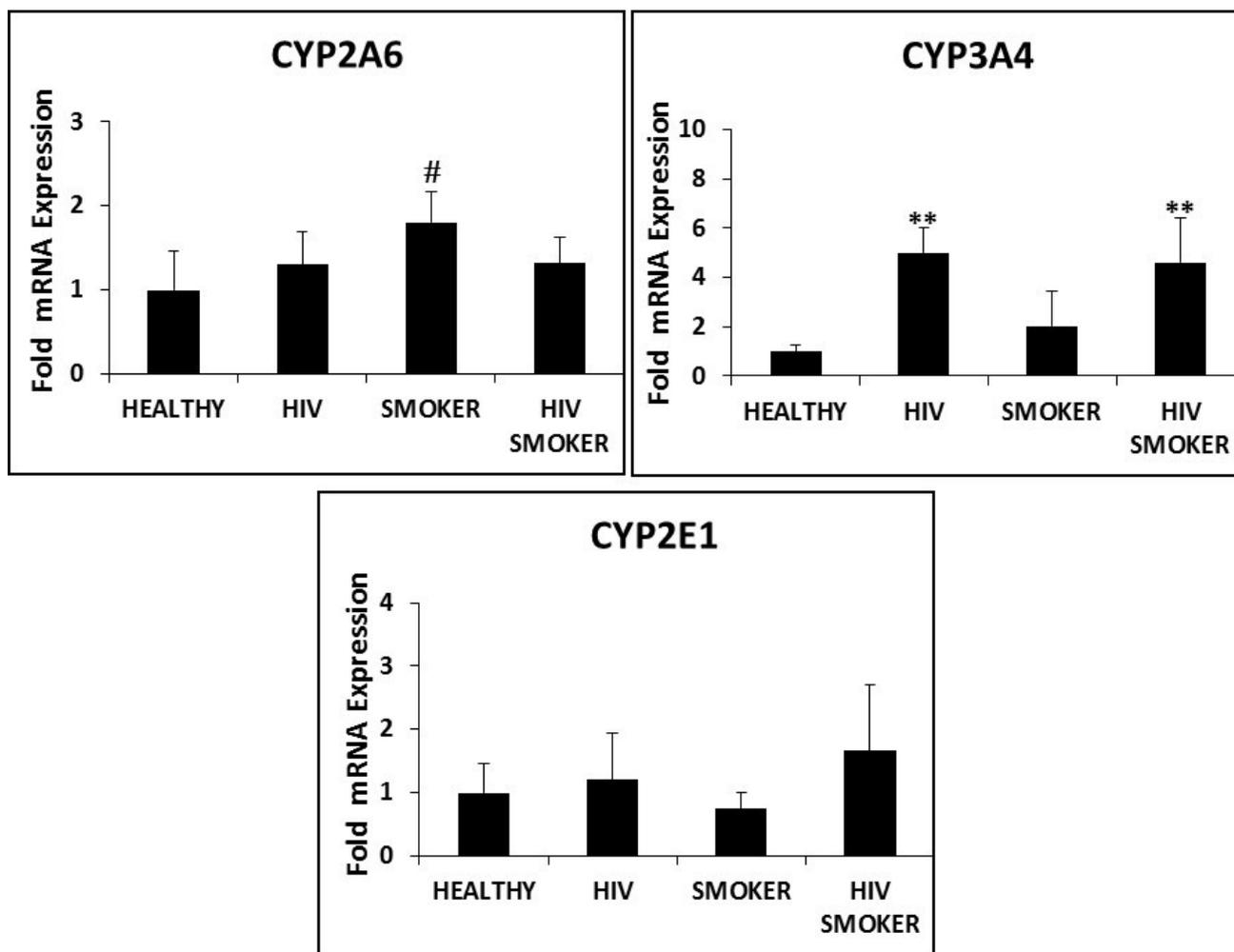
Figure 28: CYP gene expression levels in monocytes from human cohorts

Determination of CYP levels in HIV negative non-smokers (HEALTHY), HIV positive non-smokers (HIV), HIV negative smokers (SMOKER), and HIV positive smokers (HIV SMOKER) groups. The expression levels of CYP2A6, CYP3A4, and CYP2E1 mRNA were determined by qRTPCR in all the groups. A one way-ANOVA was employed to calculate the p value (** represent $p \leq 0.01$; # represents $p \leq 0.1$, borderline significance) with respect to HEALTHY in all the groups.

6.4. Discussion

Nicotine/tobacco smoking has shown to increase HIV replication in *in vitro* studies [69, 70]. *In vitro* studies have also shown an association between oxidative stress and HIV replication [72, 100, 168]. However, there is no clear evidence from the clinical samples of HIV-infected smokers, especially in ART-naïve patients. Similarly, to date, there is no clinical evidence of the involvement of CYP and oxidative stress pathways in HIV and/or smoking in HIV-infected individuals. We have recently shown the involvement of CYP enzymes in nicotine metabolism and oxidative stress in *in vitro* in HIV monocyte and astrocyte model systems (Chapter 4). Furthermore, we have shown an association between induction of CYP/ROS and HIV replication by CSC in monocytes (Chapter 5). In the current study, we used clinical samples from HIV-infected smokers to establish possible involvement of CYP and oxidative stress pathways in smoking-mediated HIV pathogenesis. This is the first clinical evidence of an increase in viral load in HIV-infected smokers, as well as, possible involvement of CYP and oxidative stress pathways in HIV positive smokers and non-smokers.

Several studies have shown that mild-to-moderate smoking leads to increased CD4 cell count and CD4/CD8 ratio [181]. In contrast, another study reported that heavy smoking caused a decline in CD4 cell count [182]. Our findings are generally consistent with the former literature in which smoking showed an increase in the CD4 counts. Our study also suggest an increase in HIV replication by tobacco smoking, which is consistent with previous *in vitro* findings [69, 71]. However, this is the first report on clinical samples obtained from HIV-infected smokers. An earlier study in HIV-seropositive women has shown an association of cigarette smoking with viral immune and cognitive function [78]. However, the underlying mechanism of the effects of smoking on viral immune, cognitive function, and HIV pathogenesis is largely unknown.

HIV infection is known to increase the expression of pro-inflammatory cytokines including IL-1, IL-6, RANTES, MCP-1 and TNF-α [183, 184]. In contrast, our results did not show an increase in the levels of these cytokines in HIV positive cohorts, rather there were significant decrease in the levels of IL-8 and MCP-1. There is a report demonstrating that the levels of TNF-α, IL-1β and IL-8 are significantly higher in asymptomatic HIV-infected African women than women living with AIDS, suggesting the role of cytokines in early phase of HIV infection [185]. Thus, it is likely that unaltered/decrease in cytokines levels in our study is because the HIV-infected patients had in many cases already progressed to full-blown AIDS. Likewise, cigarette smoking is associated with an increased production of pro-inflammatory cytokines, especially IL-1β and IL-1RA in bronchoalveolar lavage [186], and IL-6, IL-1β and TNF-α in the serum [187, 188]. In contrast, in our study, smoking was not associated with an increase in the levels of pro-inflammatory cytokines, except RANTES, rather it showed a decrease in the levels of IL-8 and MCP-1.

Tobacco smoking has been shown to affect cytokine network balance and thereby alter the host immune responses in the pathogenesis of both periodontal disease and cardiovascular disease. For instance, both nicotine and CSC treatment to human endothelial cells had significantly reduced MCP-1 levels in those cells compared to control [189]. Similarly, a study conducted in smokers with computed tomography (CT) detected emphysema and without airway obstruction had demonstrated decreased plasma levels of cytokines including EGF, IL-8, IL-15, and IL-1RA than the smokers without CT detected emphysema [190]. In contrast, patients with COPD usually exhibit higher IL-8 levels. Therefore these studies suggest that the plasma cytokine profiles of smokers widely vary in individuals with either emphysema or COPD. This also suggests that smoking could differentially affect the cytokine levels based

on their respiratory disease conditions which may be responsible for the decreased levels of cytokines observed in our smokers and HIV positive smokers. Moreover, it is well known that HIV infection alters the levels of cytokine production in vitro as well as in vivo [191]. Nonetheless, there is no report till date about correlation between smoking associated increased viral load and cytokine levels. Therefore an extensive longitudinal study that monitors the clinical status of smokers can provide us with a better understanding of the relation between smoking prompted viral load and their cytokine profiles. In general, we acknowledge that our data show contrasting findings, which needs to be investigated further. However, it can be noted that the population demography in our study is different from other studies in terms of strict exclusion criteria of the subjects (non-HIV medication as well as use of other substances of abuse).

An increase in the oxidative stress in both plasma and monocytes in smokers and HIV-infected individuals is consistent with the previous reports, especially with monocytes [72, 100, 168, 192]. However, this is the first evidence of increased oxidative DNA damage in the plasma and monocytes using clinical samples from HIV-infected smokers. It is noteworthy that while a previous report has shown higher serum 8-OHdG levels in males versus females [193], no gender difference in 8-OHdG level was observed in our study, at least in HIV negative non-smokers and HIV positive smokers. Further, relatively higher levels of oxidative DNA damage in plasma compared with monocyte samples in all the groups suggests that there may be increased oxidative stress in other tissues draining into plasma. While the DNA damage in plasma may be as a result of ROS generated by liver and other blood cells including monocytes, DNA damage in monocytes is expected to entirely come from increased ROS in monocytes.

An optimal level of ROS is required for cellular functions. However, when the level of ROS is elevated, defense mechanism may counterbalance the increased ROS to protect the cells. As one of the major defense mechanism, the Nrf2 signaling pathway is activated, which results in the transcription of a myriad of AOEs such as SOD1 and catalase to protect the cells from oxidative insult [194]. However, if the ROS reaches a threshold level, the AOEs pathway is compromised leading to cellular death. On the other hand, a defect in the defense system may cause either a decrease or no change in the levels of these AOEs leading to increased level of ROS. Since there is no significant increase in the levels of AOEs determined in smokers, we suggest that the levels of ROS has reached the threshold level, or the defense system through the Nrf2 pathway may be compromised in these individuals.

The role of oxidative stress as a result of production of ROS, including nitric oxides has been implicated in HIV pathogenesis [100, 168, 169, 195, 196]. Similarly, previous literature [69-71, 99, 171, 177] and our current findings demonstrate that smoking and oxidative stress both are independently associated with increased HIV replication. Thus, we suggest that smoking-mediated oxidative stress may be responsible for increased HIV replication. Although several mechanisms have been implicated in the production of oxidative stress in the HIV systems, smoking-mediated production of ROS through CYP pathway may play an important role in HIV pathogenesis. Since CYP-mediated metabolism of tobacco constituents is known to produce reactive oxygen species in the liver [197], it is reasonable to conclude that CYP-mediated pathways contributes to the DNA damage in both plasma and monocytes. Our results relatively low levels of DNA damage in monocytes suggest that CYP-mediated metabolic pathway also exists in monocytes. Our speculation is consistent with our earlier observation that although monocytes contain relatively low levels of CYP enzymes

compared to the liver, the relative level of CYP2A6 in monocytes is much higher that other CYP enzymes [198]. Therefore, we suggest that although CYP2A6 in smokers and HIV positive non-smokers are not induced, the basal level of CYP2A6 is sufficient to metabolize tobacco constituents and endogenous compounds and produce ROS. Furthermore, our observation is consistent with the recent finding that CYP2A6-mediated metabolism of nicotine produces ROS in monocytes and astrocytes [99], which may be responsible for increased DNA damage in monocytes. Another recent study from our laboratory, which demonstrated an increased metabolism of nicotine in HIV positive smokers compared to HIV negative smokers, is also consistent with this observation [199]. It is also possible that the increased metabolism is due to increased activity of enzyme rather than the actual protein level. An increase in enzyme activity through substrate-mediated enhance stability of enzyme is known in the P450 system [200].

The CYP pathway is also known to produce oxidative stress through the metabolism of endogenous compounds and xenobiotics such as marketed drugs [201]. The major drug-metabolizing enzyme CYP3A4, which is also known to metabolize many endogenous compounds and is present at relatively much higher level than other CYP enzymes in the liver, is expected to produce oxidative stress. A significant increase in the level of CYP3A4 in HIV-infected non-smokers as well as in HIV-infected smokers suggests an important role of CYP3A4 in HIV pathogenesis. It is possible that induction of CYP3A4 by HIV pathogenesis increases the metabolism of many endogenous compounds as well as xenobiotics, which in turn leads to increased oxidative stress. Since oxidative stress is also linked with HIV replication, the induction of CYP3A4 would further increase HIV replication. In addition to its role in oxidative stress, CYP3A4 may play significant role in HIV patients who are

receiving ART, especially non-nucleoside reverse transcriptase inhibitor (NNRTIs), protease inhibitors (PIs), and more recently integrase inhibitors. CYP3A4 metabolizes majority of NNRTIs, integrase inhibitors, and all the PIs, which are an essential component of ART [174]. Thus, increased level of CYP3A4 in HIV positive smokers and HIV positive non-smokers would increase the metabolism of NNRTIs, PIs, and integrase inhibitors in these individuals. An increased metabolism of ART would subsequently decrease the bioavailability of these ARTs ultimately leading to decreased response to these drugs and increased toxicity. Therefore, further investigation to clarify the role of CYP3A4 in HIV-infected individuals over time is necessary.

6.5. Conclusion

In conclusion, the current study suggests that mild-to-moderate smoking increases viral load in HIV-infected individuals, thereby confirming our *in vitro* earlier findings in HIV-infected macrophages. Furthermore, our study suggests that smoking and HIV independently increase oxidative stress in the plasma as well as in monocytes. An increase in oxidative stress could be as a result of both, CYP-mediated hepatic metabolism of smoking constituents and/or endogenous compounds and lack of induction of AOEs. Although we suggest a possible association of CYP and oxidative stress pathways in HIV replication, there is a lack of evidence supporting changes in metabolism of cigarette constituents in HIV-infected individuals. Hence, our next goal was to report the rate of metabolism of chief cigarette constituent, nicotine, in HIV-infected individuals.

Patient consent

Declared none.

Human/Animal Rights

Declared none.

CHAPTER 7

ENHANCED NICOTINE METABOLISM IN HIV-POSITIVE SMOKERS

COMPARED TO HIV-NEGATIVE SMOKERS: SIMULTANEOUS

DETERMINATION OF NICOTINE AND ITS FOUR METABOLITES IN THEIR

PLASMA USING A SIMPLE AND SENSITIVE ESI-LC-MS/MS TECHNIQUE

7.1. Introduction

Our findings from the *ex vivo* human samples demonstrated an increase in the oxidative stress in both plasma and monocytes in smokers and HIV-infected individuals. Since our recent *in vitro* findings in monocytes by Jin et al and astrocytes showed the role of nicotine metabolism by CYP2A6 in ROS production [99, 202], we sought to determine the plasma levels of nicotine and four of its metabolites that are formed by CYP2A6 in HIV-positive smokers subjects from the above *ex vivo* study. Our hypothesis is that HIV infection enhances nicotine metabolism in HIV-infected smokers. Therefore our specific aim for this chapter is to develop a sensitive and rapid method for simultaneous determination of plasma levels of nicotine and its major metabolites, and determine the levels of nicotine and its metabolites in plasma sample of the human subjects.

Previously nicotine and its metabolites have primarily been studied in urine and to some extent in other biological matrices such as serum, plasma, hair, and nails using immunoassay, HPLC, GC-MS, and LC-MS with protein precipitation, liquid extraction methods, and SPE methods [141, 203, 204]. However, usefulness of these methods has been limited by factors like requirement of high sample volume, lengthy extraction process among other impediments. Generally, the plasma concentration of nicotine and its metabolites is much lower than that in urine [205]. It is therefore necessary to have a more sensitive

analytical technique using an LC-MS/MS instrument with a triple quadrupole linear ion trap, which is commonly employed for the determination of low levels of nicotine and its metabolites. A previous study has shown the application of the LC-MS/MS method for quantifying only cotinine at a sensitivity of 0.5 ng/mL [206, 207]. Subsequently, another LC-MS/MS method using solid phase extraction (SPE) was developed that was able to quantitate two analytes, nicotine and cotinine with a sensitivity of 2 ng/mL [141]. Recently, several LC-MS/MS analytical techniques have been reported to determine nicotine, cotinine, and *trans*-3'-hydroxycotinine but also required a large volume of serum to achieve a sensitivity of 1 ng/mL. Although these methods had a sensitivity of 1-2 ng/mL, the sample preparation and extraction methods were time-consuming and tedious, required high sample volume, and were limited to 2-3 analytes [140, 208]. Therefore, our objective in this study was to develop a sensitive, selective, and simple ESI-LC-MS/MS technique using a SPE cartridge for the simultaneous determination of nicotine and its four metabolites in plasma. In this communication, we report the concentrations of nicotine and its metabolites detected in plasma from HIV-positve and HIV-negative smokers using the above approach.

7.2. Development of an analytic method for the measurement of nicotine and its metabolites in plasma

7.2.1. Mass spectrometry optimization

The mass spectrometer (3200 QTRAP LC-MS/MS system, AB Sciex) was optimized for detection of nicotine and its metabolites along with IS by using 200 ng/mL. Mass spectrometry data of each compound was first acquired in full scan mode from the range between 50-300 Da to identify their precursor ions. The most suitable proton adduct in the positive mode $[M+H]^+$ precursor ions was determined for nicotine (163.3), cotinine (177.5), *trans* 3'-hydroxycotinine (193.2), nornicotine (149.5), norcotinine (163.4), nicotine-d4

107

(167.3), and cotinine-d3 (180.3) (Fig. 29 and Table 4). These precursor ions were optimized by setting the curtain gas, declustering potential, ion spray voltage, and source gas 1.

7.2.2. Tandem (MS/MS) mass spectrometry conditions

The proton adduct m/z [M+H]$^+$ precursor ions of nicotine and its metabolites along with IS were selected in positive mode for collision cell quadrupole 2 (MS2). Precursor ions were fragmented by applying collisionally-activated dissociation gas and collision energy to obtain their most abundant and stable product ions. The product ions for nicotine (117.1), cotinine (80.3), *trans* 3'-hydroxycotinine (80.1), nornicotine (132.3), norcotinine (80.3), nicotine-d4 (121.4), and cotinine-d3 (101.2) were optimized by adjusting collision energy, curtain gas, entrance potentials, and source gas 2 (Fig. 29 and Table 4). The multiple reactions monitoring (MRM) transitions (m/z) [M+H]$^+$, (Q$_1$→Q$_3$) selected for quantitative analyses were: 163.3→117.1 for nicotine, 177.5→80.3 for cotinine, 193.2→80.1 for *trans* 3'-hydroxycotinine, 149.5→132.3 for nornicotine, 163.4→80.3 for norcotinine, 167.3→121.4 for nicotine-d4, and 180.3→101.2 for cotinine-d3 (Fig. 29 and Table 4). A dwell time of 500 ms and a source temperature of 400^0C were employed for all the analyte determinations.

Table 4: MRM parameters for nicotine and its metabolites

Analyte	Precursor ion Q1 (MS1)	Product ions Q2 (MS2)	DP (V) (MS1)	EP (V) (MS1)	CEP (V) (MS1)	MRM transition [Q1→Q3]	CE (V)
Nicotine	163.3	84.1, 106.0, **117.1**, 130.3, 132.4	36.0	5.5	10.0	163.3→ 117.1	33.0
Nornicotine	149.5	80.1,117.1, 130.1, **132.3**	36.0	5.0	11.0	149.5→ 132.3	17.1
Nicotine-d4(IS)	167.3	84.3, 110.5, **121.4**, 134.2, 136.4	36.0	7.0	9.5	167.3→ 121.4	35.0
Cotinine	177.5	**80.3**, 98.1, 146.2	46.0	7.0	12.0	177.5→ 80.3	23.0
Norcotinine	163.4	**80.3**, 84.1, 135.2, 146.3	51.0	6.5	10.0	163.4→ 80.3	25.0
Trans 3'-hydroxy cotinine	193.2	**80.1**, 134.3, 149.2	51.0	5.5	11.5	193.2→ 80.1	25.0
Cotinine-d3(IS)	180.3	80.3, **101.2**, 118.1, 146.0	46.0	7.5	12.0	180.3→ 101.2	29.0

MS- mass spectrometry; **m/z**- mass-to- charge ratio; **DP**- declustering potential; **CE**- collision energy; **CEP**- Cell exit potential; **CAD**- collisionally activated dissociation gas : 3.0 psi; **MRM**- multiple monitoring reactions; **Dwell time**- 500 ms; **Source gas (GS1)**- 30.0 psi; **Source gas (GS2)**- 30.0 psi; **Source temperature (TEM)-** 400^0C ; **Ion spray voltage (IS)**- 5500 Volts; **Interface heater**- on and **Analytical total run time**- 4.0 min;
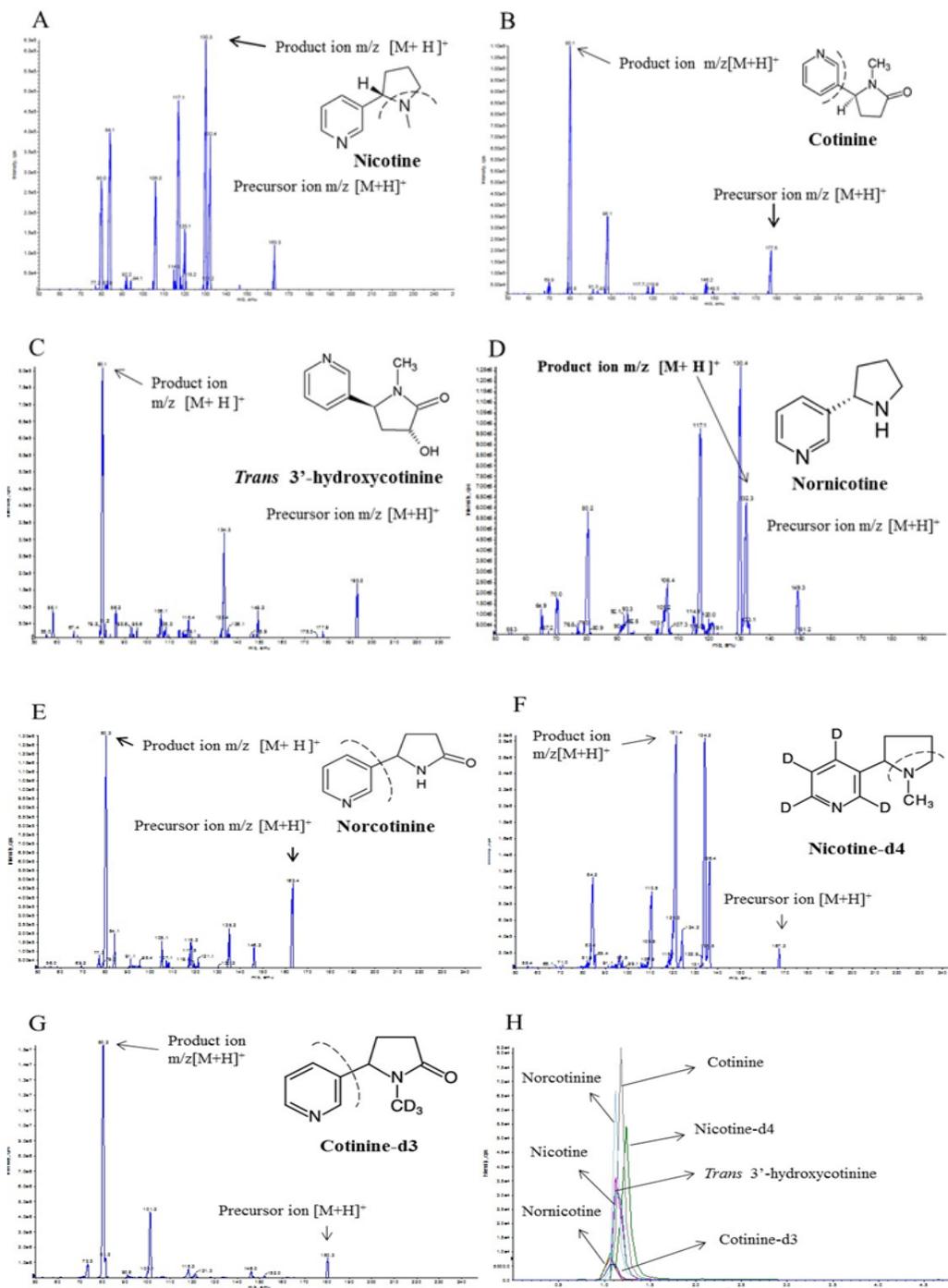
Figure 1

Figure 29: MS/MS spectra of nicotine and its metabolites with proton adducts in ESI positive mode

7.2.3. LC-MS/MS chromatographic separation

An LC-MS/MS chromatographic separation was achieved by a reverse phase Xterra MS C 18 column (50 x 4.6 mm, i.d, 5µm) using UFLC Shimadzu LC-20AD HPLC (California, USA). An isocratic mobile phase composed of 55% acetonitrile in water containing 0.05% of formic acid at a flow rate of 0.4 mL/min was used. The samples were reconstituted in a 500 µL of acetonitrile-water-formic acid (70:30:0.05) solution. A 15 µL aliquot of each sample was injected into LC-MS/MS for quantitative analysis for 5 min. The LC-MS/MS acquired MRM data was processed with Analyst software (version 1.4.2, AB Sciex).

7.2.4. Sample preparation and extraction

A simple SPE technique was used for sample extraction. Five hundred microliters of plasma from a non-smoker was aliquoted and to this 20 µL of 3µg/mL IS (final concentration of ~0.1 µg/mL) was added. The mixture was vortex-mixed for 30 sec followed by addition of 25 µL of an aqueous 0.6% formic acid solution, which was again vortex-mixed for 1 min prior to SPE. The strong cation SPE columns (SCX 30 mg, 1 mL cartridge) were preconditioned with 1 mL of methanol and equilibrated with 1mL of 0.6% formic acid in water. The plasma samples were loaded on the SPE cartridge and drained slowly by applying positive pressure at 15 psi with a 48 well plate Positive Vacuum Manifold. The SPE columns were washed with 1 mL of 0.6% formic acid followed by 1 mL each of water and methanol. Next, the SPE columns were air dried under positive vacuum at 20 psi for 3 minutes, and analytes were eluted with 1 mL of a 7% ammonium hydroxide in methanol. After elution, the pH of the eluate was neutralized with 100 µL of 6% of formic acid in methanol and vortex-mixed prior to evaporation using a speed vacuum at 35^0C for 60 min. Samples were re-dissolved in 500 µL of reconstitution solution.

7.2.5. Specificity and selectivity

The specificity and selectivity were tested by analyzing six blank plasma samples from HIV-negative non-smokers. These blank matrices were used for method standardization, which did not show measurable interference at analyte peak of interests for nicotine and its metabolites. Lower limit of quantitation (LLOQ, 0.53 ng/mL) of six samples were processed in order to assess the blank plasma interference at the analyte peak of interest (Fig. 30-35). The percentage of interference determined in the blank was calculated by comparing the mean peak area of LLOQ of the analyte with the peak response obtained from the blank samples. The peak areas of blanks co-eluting with the analytes were required to be less than 5 times of the mean peak area at the LLOQ.
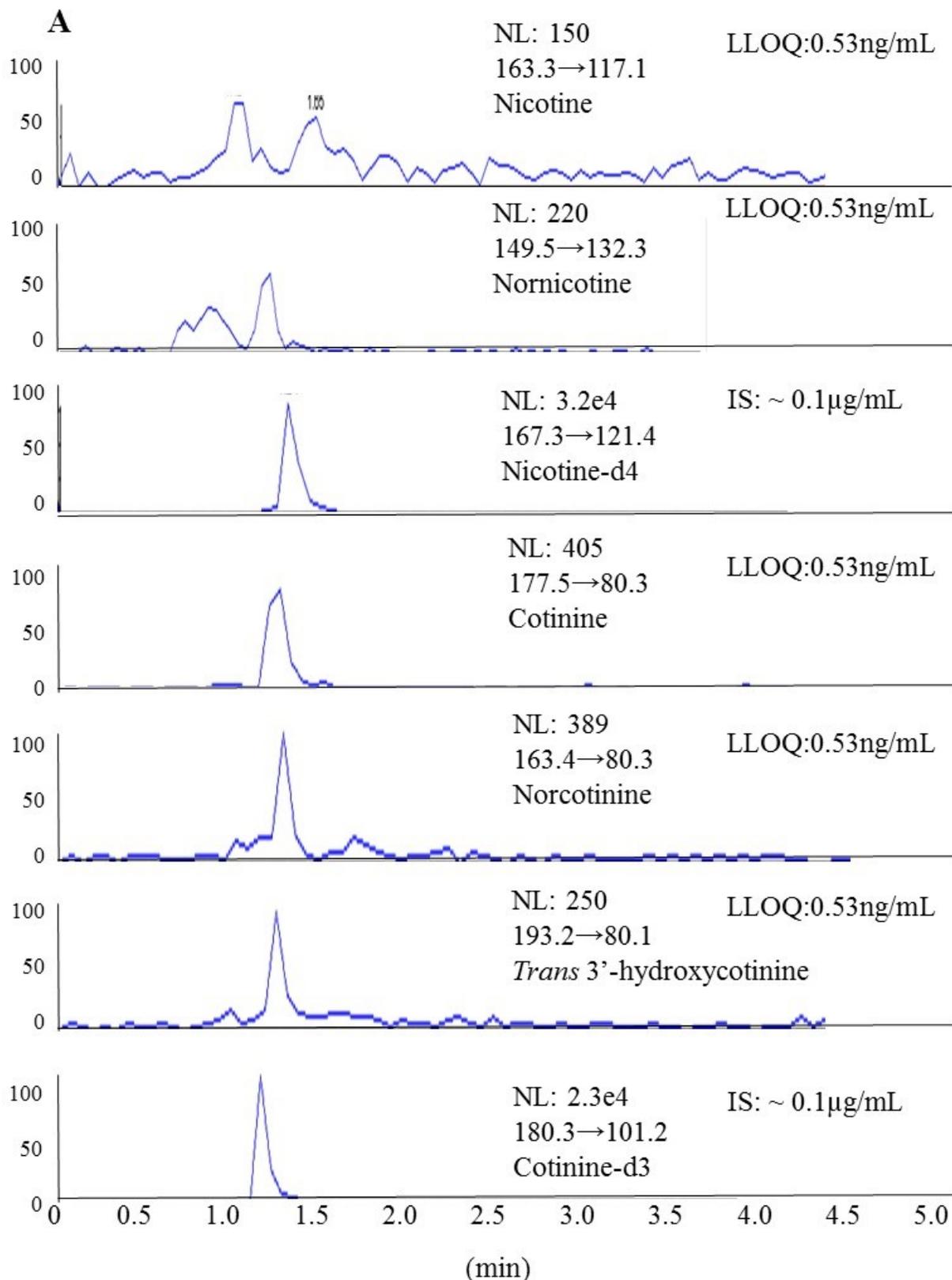
Figure 30: LC-MS/MS-MRM chromatograms of nicotine, its metabolites, and IS for extracted lower limit of quantitation (LLOQ, 0.53 ng/mL)

B

NL: 3.2e4
163.3→117.1
Nicotine

ULOQ: 504 ng/mL

NL: 1.2e4
149.5→132.3
Nornicotine

ULOQ: 504 ng/mL

NL: 3.6e4
167.3→121.4
Nicotine-d4

IS: ~ 0.1µg/mL

NL: 1.0e5
177.5→80.3
Cotinine

ULOQ: 504ng/mL

NL: 5.2e4
163.4→80.3
Norcotinine

ULOQ: 504ng/mL

NL: 8.8e4
193.2→80.1
Trans 3'-hydroxycotinine

ULOQ: 504 ng/mL

NL: 2.6e4
180.3→101.2
Cotinine-d3

IS: ~ 0.1µg/mL

(min)
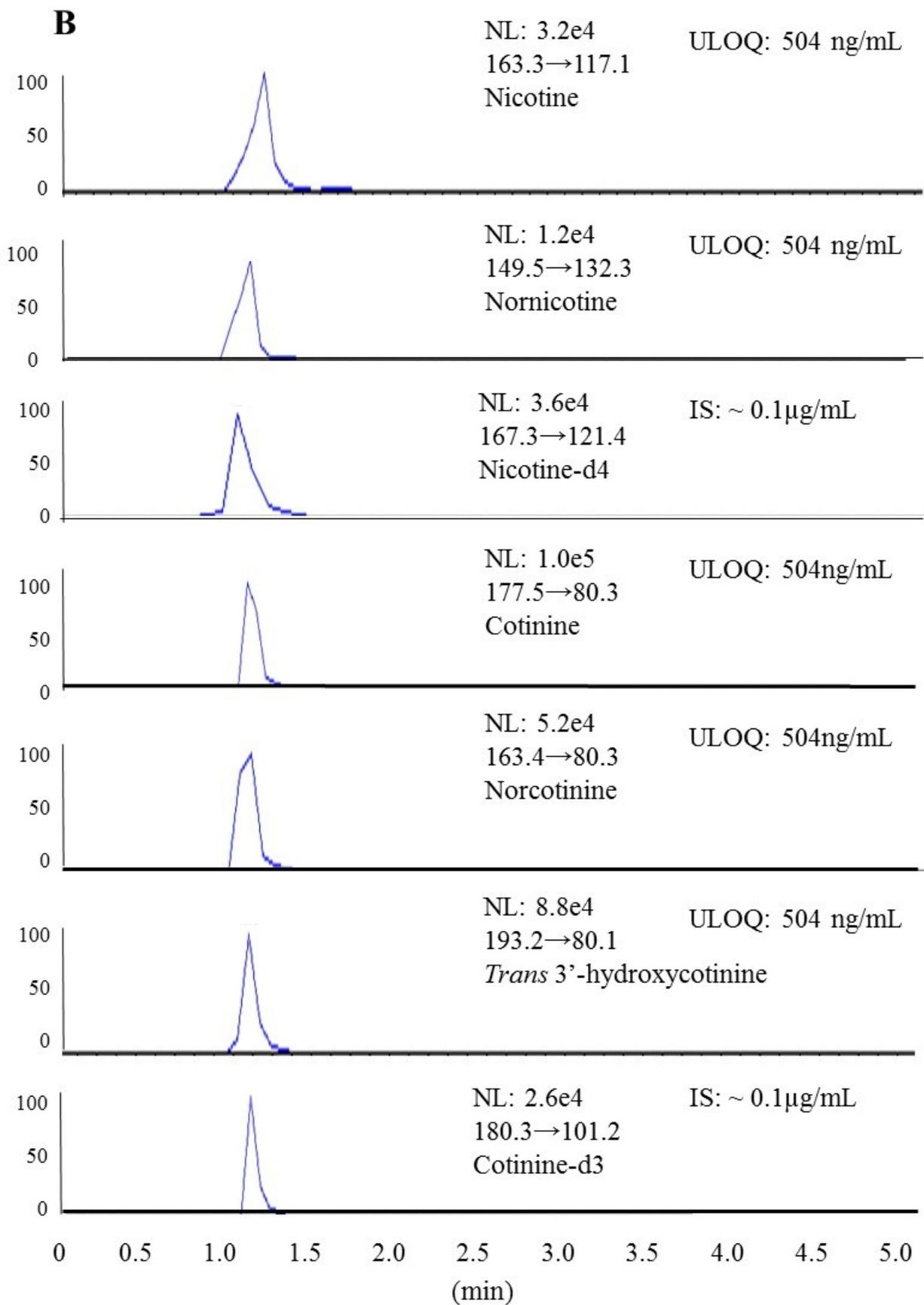
Figure 31: LC-MS/MS-MRM chromatograms of nicotine, its metabolites, and IS for extracted upper limit of quantitation (ULOQ, 504 ng/mL)
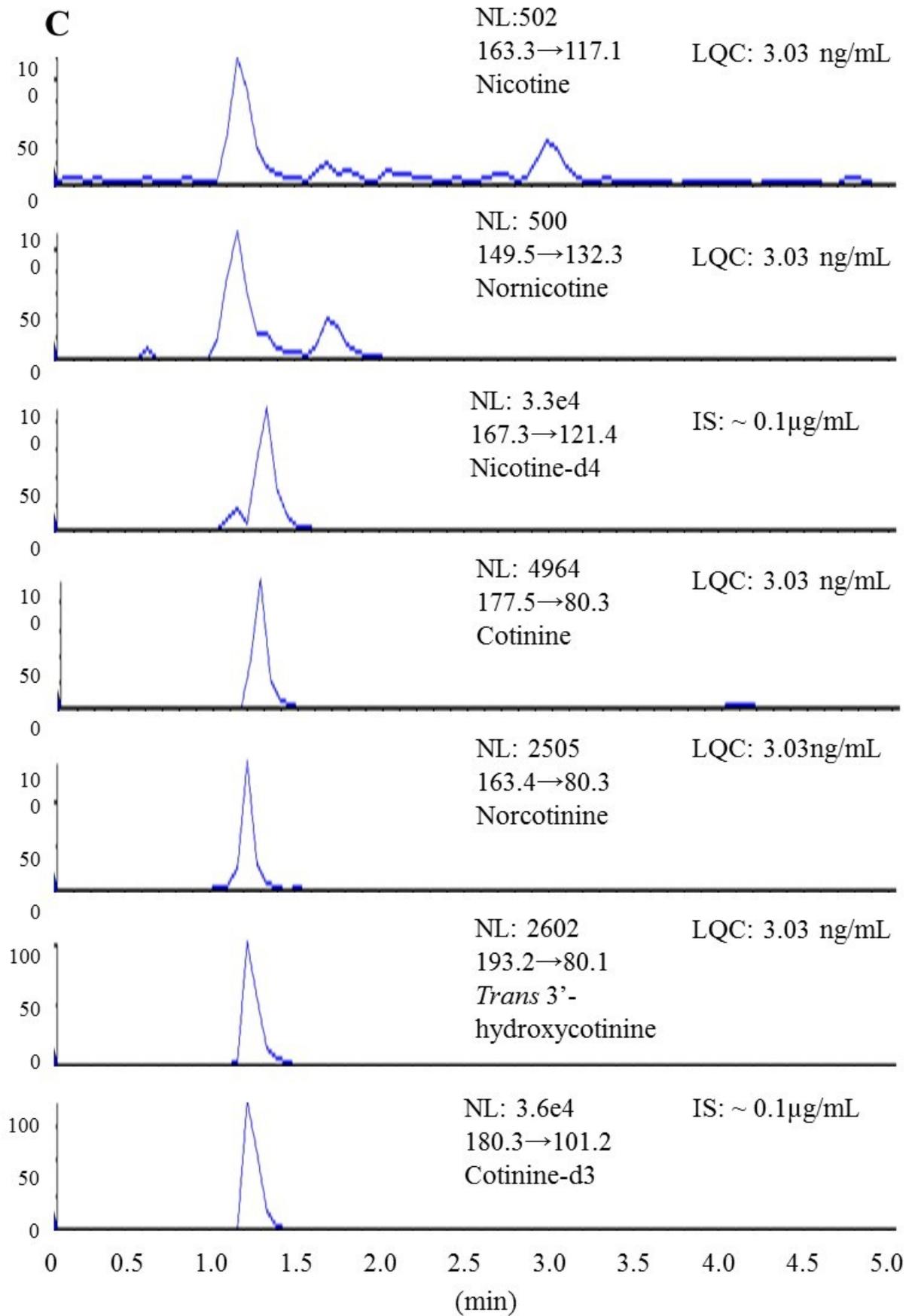
Figure 32: LC-MS/MS-MRM chromatograms of nicotine, its metabolites, and IS for low quality control (LQC, 3.03 ng/mL) standard
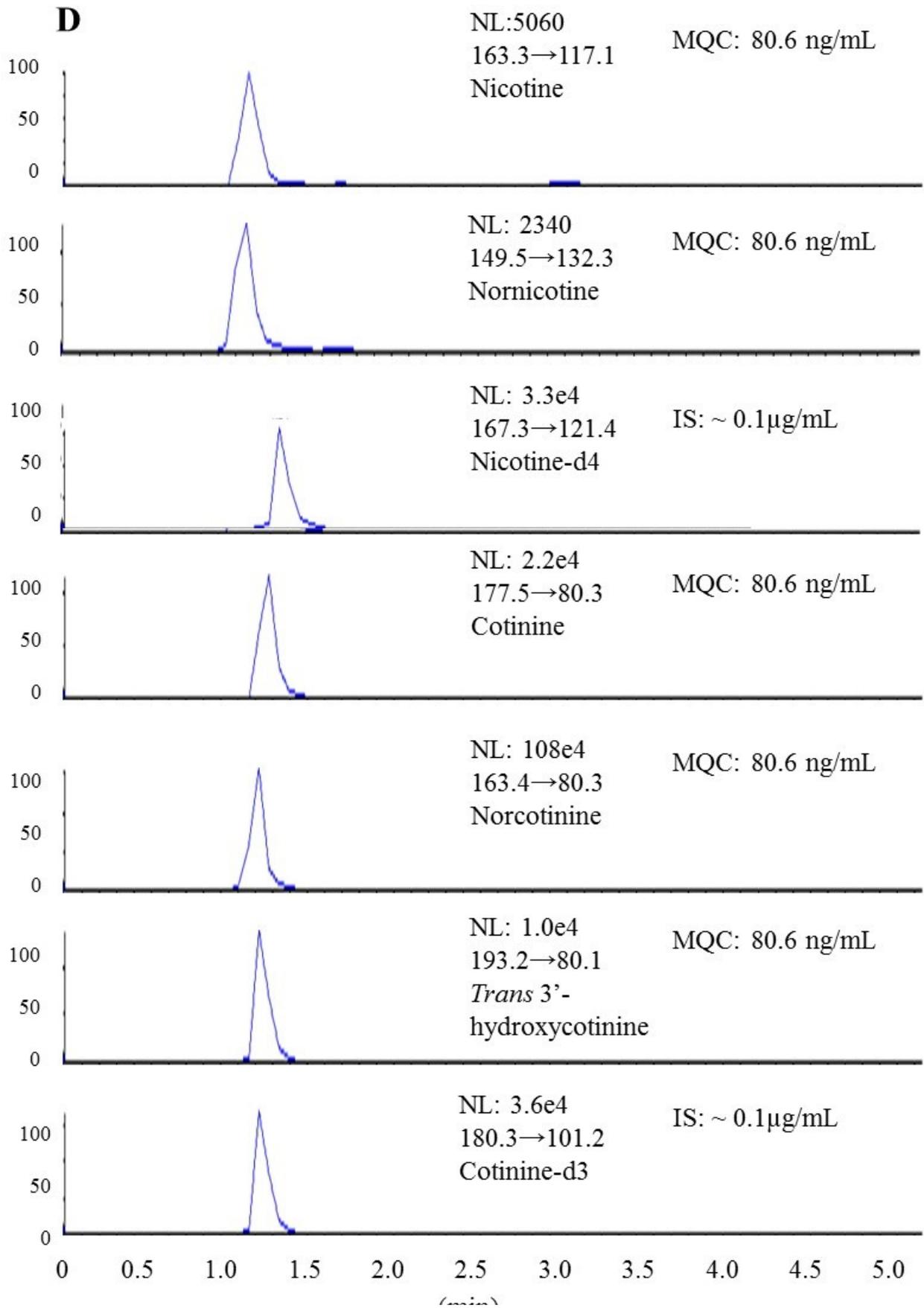
Figure 33: LC-MS/MS-MRM chromatograms of nicotine, its metabolites, and IS for extracted middle quality control (MQC, 80.6 ng/mL)

E

NL:3.4e4
163.3→117.1
Nicotine
HQC: 504 ng/mL

NL: 1.2e4
149.5→132.3
Nornicotine
HQC: 504 ng/mL

NL: 8.5e4
177.5→80.3
Cotinine
HQC: 504 ng/mL

NL: 5.8e4
163.4→80.3
Norcotinine
HQC: 504 ng/mL

NL: 7.7e4
193.2→80.1
Trans 3'-hydroxycotinine
HQC: 504 ng/mL

NL: 2.9e4
180.3→101.2
Cotinine-d3
IS: ~ 0.1μg/mL
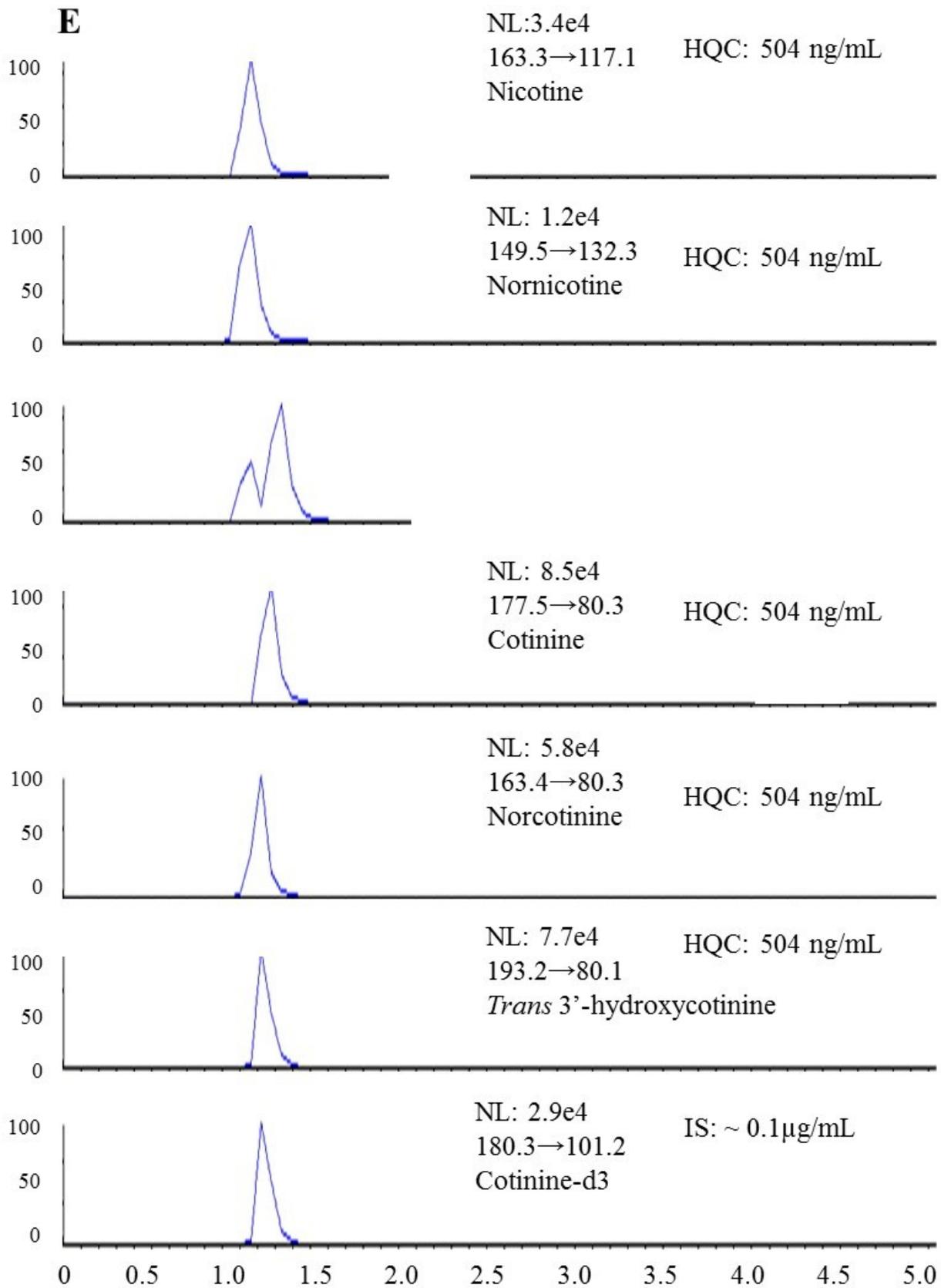
Figure 34: LC-MS/MS-MRM chromatograms of nicotine, its metabolites, and IS for extracted high quality control (HQC, 504 ng/mL)

LC-MS/MS-MRM chromatograms of nicotine, its metabolites, and IS: **30**. extracted lower limit of quantitation (LLOQ, 0.53 ng/mL) with IS, **31**. Extracted upper limit of quantification (ULOQ, 504 ng/mL) with IS, **32**. extracted low quality control (LQC, 3.03 ng/mL) standard, **33**. extracted middle quality control (MQC, 80.6 ng/mL) and **34**. extracted high quality control (HQC, 504 ng/mL) from non-smokers plasma.
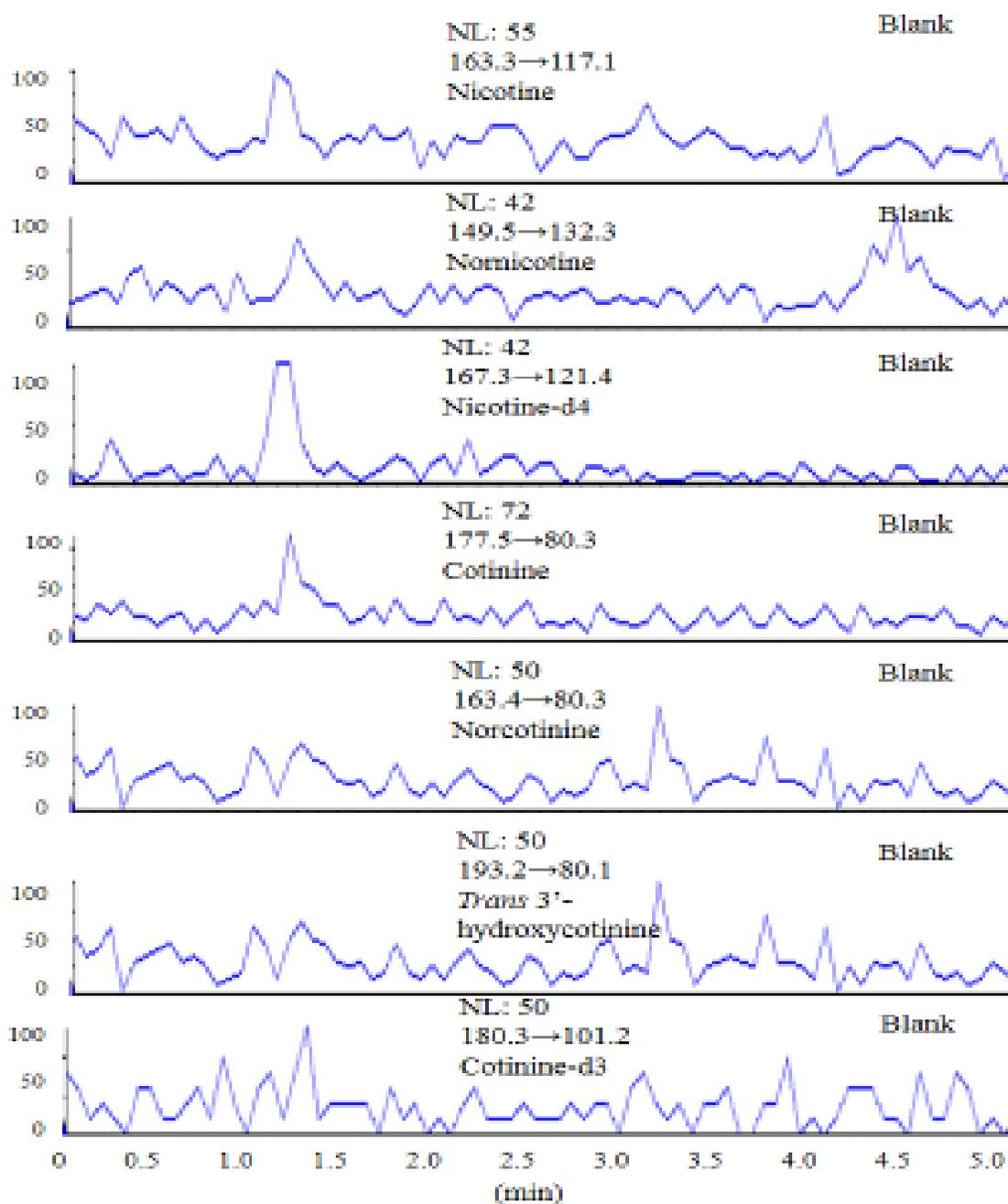
Figure 35: Extracted blank plasma for nicotinine, cotinine, trans-3-hydroxycotinine, nornicotine and norcotinine

7.2.6. Precision and accuracy

Within-assay & between-assay, precision & accuracy experiments were performed by analyzing eight extracted calibration and four levels of QC standards (Table 5). The pooled blank plasma samples from HIV-negative non-smokers were used to prepare a calibration curve and QC standards. The standard samples were prepared based on the procedure described earlier [132]. Precision was determined by analyzing six replicates at each of the four levels of QC standards. Accuracy was reported as the percentage difference between the mean concentrations divided by the nominal concentration, multiplied by 100. Accuracy was required to be ±15% of the nominal value of all the standards, except at LLOQ level where an accuracy of ±20% was accepted according to *the guidance for industry bioanalytical method validation* in Food and Drug Administration guidelines published in May 2001 (www.fda.gov). Precision was calculated using the CV (standard deviation/mean concentration) multiplied by 100. Precision of the method was required to be less than ≤15% of the nominal concentration, except in the LLOQ where ≤20% was an accepted CV.

Table 5: Precision and Accuracy of CC and QC standards for nicotine and its metabolites in pooled blank human plasma

Analyte	Nominal Conc (ng/mL)	Within-day: CC (n=4), QC (n=6)			Between-day : CC (n=6), QC(n=6)		
		Calculated Conc (ng/mL)	Precision (% CV)	Accuracy (%)	Calculated Conc (ng/mL)	Precision (% CV)	Accuracy (%)
Nicotine							
CC std-1	504.00	476.57	7.6	94.6	543.9	3.8	107.9
CC std-2	201.60	175.18	8.1	86.7	179.0	6.5	88.6
CC std-3	80.64	78.57	5.5	97.5	91.6	9.1	113.7
CC std-4	30.64	32.63	5.5	106.6	32.3	5.9	105.7
CC std-5	10.11	11.24	12.6	114.7	9.9	15.4	100.6
CC std-6	3.03	3.18	12.4	102.6	2.9	1.7	92.6
CC std-7	1.06	1.07	5.1	97.3	1.1	4.9	102.2
CC std-8	0.53	0.51	3.8	96.3	0.47	10.3	88.7
High QC	504.00	483.33	5.3	95.9	483.3	5.3	95.9
Middle	80.64	70.71	13.5	87.7	71.7	13.6	88.9
Low QC	3.03	2.64	23.3	87.0	2.82	16.3	93.2
Lower	1.06	1.22	31.5	114.8	1.16	27.8	109.1
Nornicotine							
CC std-1	504.00	544.00	3.8	107.9	527.2	7.3	104.6
CC std-2	201.60	179.10	6.5	88.8	179.2	6.4	88.9
CC std-3	80.64	90.38	8.9	112.1	90.7	9.3	112.4
CC std-4	30.64	32.27	5.9	105.3	32.5	5.9	105.8
CC std-5	10.11	10.19	14.4	100.7	10.3	13.1	102.3
CC std-6	3.03	2.99	2.2	98.6	3.0	1.6	99.1
CC std-7	1.06	1.08	6.2	101.8	1.08	6.2	101.8
CC std-8	0.53	0.55	23.4	103.4	0.57	22.5	106.6
High QC	504.00	483.33	5.3	95.9	483.33	5.3	95.9
Middle	80.64	70.71	13.5	87.7	71.44	14.8	88.6
Low QC	3.03	2.82	16.3	93.2	2.82	16.3	93.2
Lower	1.06	1.17	18.4	110.7	1.24	22.4	117.0
Cotinine							
CC std-1	504.00	518.83	7.3	102.9	504.83	8.7	100.2
CC std-2	201.60	179.60	6.4	89.1	181.43	6.7	90.0
CC std-3	80.64	90.55	9.2	112.2	90.11	10.0	111.7
CC std-4	30.64	31.94	7.5	104.2	31.61	6.9	103.2
CC std-5	10.11	10.69	11.2	105.6	10.85	10.6	107.3
CC std-6	3.03	2.98	2.1	98.4	2.99	2.7	98.8
CC std-7	1.06	1.06	6.8	100.3	1.09	8.4	102.5
CC std-8	0.53	1.49	14.8	81.7	1.49	148.0	281.8

Analyte	Nominal Conc (ng/mL)	Calculated Conc (ng/mL)	Precision (%CV)	Accuracy (%)	Calculated Conc (ng/mL)	Precision (%CV)	Accuracy (%)
High QC	504.00	480.83	5.1	95.4	478.00	4.6	94.8
Middle	80.64	70.61	12.4	87.6	72.44	7.4	89.8
Low QC	3.03	2.84	14.6	93.9	2.84	14.5	93.7
Lower	1.06	1.19	18.7	112.3	1.21	17.6	114.5

Norcotinine

CC std-1	504.00	545.67	3.8	108.3	545.67	3.8	108.3
CC std-2	201.60	180.60	6.0	89.6	180.60	6.0	89.6
CC std-3	80.64	92.61	8.0	114.8	93.94	8.0	116.5
CC std-4	30.64	32.44	6.6	105.9	31.44	6.7	102.6
CC std-5	10.11	10.19	14.4	100.7	10.35	14.5	102.4
CC std-6	3.03	2.98	2.0	98.2	2.99	1.6	98.8
CC std-7	1.06	0.99	7.3	93.1	1.02	11.2	96.2
CC std-8	0.53	0.64	19.9	119.8	0.62	17.9	117.3
High QC	504.00	478.00	4.7	94.8	484.50	3.7	96.1
Middle	80.64	74.27	6.1	92.1	77.27	8.6	95.8
Low QC	3.03	2.95	4.0	97.5	3.00	1.9	99.1
Lower	1.06	1.19	18.7	112.3	1.17	15.9	110.7

Trans 3'-hydroxy cotinine

CC std-1	504.00	511.67	7.4	101.5	529.17	7.8	105.0
CC std-2	201.60	183.10	6.0	90.8	182.43	6.1	90.5
CC std-3	80.64	89.61	9.4	111.1	90.27	10.2	111.9
CC std-4	30.64	30.94	7.4	101.0	31.44	6.7	102.6
CC strd-	10.11	11.02	9.8	109.0	10.69	12.7	105.7
CC std-6	3.03	2.98	2.9	98.2	3.01	2.2	99.3
CC std-7	1.06	1.05	7.4	99.4	1.07	10.9	100.9
CC std-8	0.53	2.39	116.8	451.6	0.61	19.3	115.1
High QC	504.00	484.17	3.7	96.1	484.17	3.7	96.1
Middle	80.64	73.61	7.4	91.3	75.44	10.7	93.6
Low QC	3.03	3.02	0.4	99.8	3.00	1.9	98.9
Lower	1.06	1.12	9.2	106.0	1.11	10.1	105.0

CC-calibration curve; **QC**- quality control; **LOQ**- lower limit of quantitation

7.2.7. Recovery, matrix effect, and stability of analytes in plasma from non-smokers

Recovery of nicotine and its metabolites was estimated by analyses of two sets of six replicates of extracted plasma at low, middle, and high QC standards and post-spiked (represent 100% recovery) samples along with IS (Table 6). The three levels of recovered QC

standards were prepared in blank extracted plasma from non-smokers. The aqueous dilutions were post-spiked in non-smoker blank plasma sample to get the same concentrations (504.0, 80.6, 3.03 ng/mL). An overall extraction recovery was determined by comparing the mean peak area ratios of the analytes with the IS obtained from the extracted QC (matrix samples from non-smokers versus the unextracted standards).

Matrix effect of nicotine and its metabolites along with IS was evaluated by analyzing 2 sets of six replicates of each low, middle, and high QC standards from post-spiked (extracted blank plasma samples), and spiked standards in aqueous solutions (represent no matrix effect) (Table 6). Ninety blank matrix samples from a non-smoker and 18 samples of each analyte were processed and extracted as described above. The aqueous stock QC dilutions (low, middle, and high) were spiked in the extracted blank samples to obtain the QC standards (504.0, 80.6, and 3.03 ng/mL). Similarly, these QC standards were prepared by spiking analytes in reconstitution solution to obtain the same concentrations. Matrix ion suppression was calculated by comparing the mean peak area ratios of each analyte and IS generated from the post-spiked QC standards from plasma samples from non-smokers with reconstituted spiked QC standards. A relative matrix effect was estimated by comparing the mean peak area ratios of the analytes to IS obtained from the post-spiked QC.

Six replicates of stability samples at concentrations of 504.0, 80.64, and 3.03 ng/mL were prepared for each analyte in pooled plasma from the non-smokers (Table 7). They were stored at -80^{0}C for several weeks to estimate the degradation of analyte in the matrix. Stability QC samples were extracted along with freshly prepared calibration standards in pooled plasma from the non-smokers. The stability samples were frozen and stored for 6 months at -80^{0}C. Stability QC samples were freeze-thawed for three cycles, and analyzed with freshly prepared

123

calibration curve standards. The linearity of the analysis was determined using freshly spiked calibration standards, which were analyzed in duplicate along with stability samples (*bench top*, *freeze-thaw* stability) processed as described previously [132].

Table 6: Recovery and matrix effect of QC standards of nicotine and its metabolites in pooled blank human plasma

Analyte	Nominal conc (ng/mL)	Recovery (n=6)		Matrix effect (n=6)	
		% CV	% Recovery	% CV	%Matrix effect
Nicotine					
High QC	504.00	5.6	97.9	5.7	97.6
Middle QC	80.64	8.4	102.9	8.3	102.5
Low QC	3.03	24.4	95.7	27.2	91.6
Nornicotine					
High QC	504.00	8.6	98.5	9.8	100.9
Middle QC	80.64	8.3	102.5	8.9	101.1
Low QC	3.03	22.9	86.7	23.6	86.1
Cotinine					
High QC	504.00	9.7	100.9	5.6	97.9
Middle QC	80.64	9.1	99.5	8.4	102.9
Low QC	3.03	29.3	89.6	24.4	95.7
Norcotinine					
High QC	504.00	9.7	100.9	9.6	101.2
Middle QC	80.64	9.1	99.5	8.7	100.2
Low QC	3.03	29.3	89.6	21.0	83.2
***Trans* 3'-hydroxy cotinine**					
High QC	504.00	8.7	104.2	9.7	103.2
Middle QC	80.64	11.3	102.8	9.0	106.2
Low QC	3.03	23.5	86.6	15.7	90.5

CC- calibration curve; QC- quality control; CV- coefficient of variation

Table 7: Benchtop and freeze thaw stability of QC standards of nicotine and its metabolites in pooled blank human plasma

Analyte	Nominal conc.(ng/mL)	Bench top (n=6)			Freeze-thaw (n=6)		
		Calculated conc.(ng/mL)	% CV	% stability	Calculated conc.(ng/mL)	% CV	% stability
Nicotine							
High QC	504.00	511.71	9.3	101.5	512.01	9.2	101.6
Middle QC	80.64	83.19	7.1	103.2	82.89	6.8	102.8
Low QC	3.03	2.56	13.3	88.6	2.68	15.2	93.5
Nornicotine							
High QC	504.00	564.28	22.8	112.0	574.01	20.3	113.9
Middle QC	80.64	79.67	14.2	98.9	78.26	15.7	97.1
Low QC	3.03	3.00	10.3	101.9	2.78	15.0	96.5
Cotinine							
High QC	504.00	523.86	8.4	103.9	562.01	19.8	111.5
Middle QC	80.64	81.87	6.6	101.6	76.36	14.8	94.8
Low QC	3.03	2.49	14.7	87.6	2.67	14.7	94.0
Norcotinine							
High QC	504.00	528.38	13.8	104.8	577.01	19.7	114.5
Middle QC	80.64	83.19	14.2	103.2	81.36	14.4	101.0
Low QC	3.03	2.69	13.2	94.5	3.00	14.5	104.8
***Trans* 3'-hydroxy cotinine**							
High QC	504.00	507.28	9.9	100.7	520.56	9.6	103.3
Middle QC	80.64	82.37	7.3	102.3	78.28	13.5	97.1
Low QC	3.03	2.76	15.2	95.5	2.69	14.0	94.5

CC- calibration curve; **QC**- quality control; **LOQ**- lower limit of quantitation; **%CV**- percentage of coefficient of variation.

7.2.8. Patient recruitment

Patient recruitment has been previously described [209]. Briefly, following Institutional Review Board approval from the University of Missouri-Kansas City and Cameroonian Ministry of Health Regional Hospital in Bamenda, Cameroon, we recruited our subjects in Cameroon, Africa using strict inclusion and exclusion criteria. For Smokers category, we recruited 11 volunteers (7 males, 4 females) who reported a smoking history of 1-2 packs per day at least for the past 3 years. For HIV- positive smoker category, we recruited 4 individuals (3 males and 1 female) with CD4 counts ranging between 100-500 cells/μL with a similar smoking history. The demographics in both groups including packs per day and duration of smoking are similar, except for diagnostic dates of the infection for HIV-positive smoker category. The male to female ratio is relatively high in both the categories owing to the low prevalence of smoking among women in Cameroon, especially in HIV-infected population. Our exclusion criteria include: pregnant females, individuals under 21 years or over 65 years (smoking is illegal in the US among youth and individuals >65 have an altered metabolism of tobacco constituents), patients with either hepatitis B, malaria, and TB, a CD4 count <100 or >500 CD4 lymphocytes/μL for HIV-positive group, and those receiving either antiretroviral drugs, traditional medicines, or over the counter drugs. We conducted the clinical screening tests for malaria, hepatitis, and TB infections using standard procedures as described [180, 210-212]. After recruitment, 60 ml of blood was drawn from each individual followed by determination of their CD4 counts (Table 3) using Becton Dickenson instrument in Provincial Regional Hospital, Bamenda, Cameroon. The viral load of HIV-infected smokers was determined in plasma using real time reverse transcriptase polymerase chain reaction by Roche Amplicor System (Biocentric) at Pasteur center, Bamenda, Cameroon. The remaining plasma samples were shipped to our lab (Kansas City, USA) and stored at -80ºC

until further analysis. Six control samples were also collected from non-smoker healthy volunteers at the University of Missouri-Kansas city (4 males, 2 females) ranging in age from 25-45 years for optimizing the method. HIV-positive and HIV-negative smoker plasma samples were processed and extracted according to the sample preparation and extraction protocols described above.

7.2.9. Statistical analysis

The concentration of all analytes in the plasma from subjects was calculated by Analyst software (AB Sciex, Foster City, CA). The statistical significance (p-values) was calculated using one-way analysis of variance and the mean and standard deviations were calculated using Microsoft excel software. The LC-MS/MS data of nicotine, cotinine, *trans* 3'-hydroxycotinine, nornicotine, and norcotinine obtained from HIV-positive and HIV-negative smokers was analyzed using boxplot by SPSS IBM statistical software.

7.3. Results

7.3.1. System suitability and carry-over tests

The *system suitability test* resulted in <2% variation for nicotine and its metabolites. The %CV for nicotine and nornicotine with nicotine-d4 as IS were 0.72 and 1.97, respectively (Table 8). Similarly, the %CV for cotinine, *trans* 3'-hydroxycotinine, and norcotinine with their IS cotinine-d3 were 1.01, 0.77, and 0.31, respectively. The *carry-over test* for nicotine and its metabolites at the highest calibrator did not show any carry over to the blank sample.

Table 8: System suitability test for all analytes compared to IS represented in % CV

Nicotine	Nicotine-d4 (IS)	Area ratio (Analyte/IS)	Nornicotine	Nicotine-d4 (IS)	Area ratio (Analyte/IS)
1141682	1897707	0.5911	2185983	1897707	1.1519
1149019	1931564	0.5949	2142384	1931564	1.1091
1171239	1943119	0.6028	2162761	1943119	1.1130
1148919	1939139	0.5925	2173457	1939139	1.0980
1161239	1943519	0.5975	2152861	1943519	1.1077
1184409	1979449	0.5984	2153761	1979449	1.0881
Mean		**0.596198**			**1.11130**
SD		**0.0042781**			**0.021844**
%CV		**0.72**			**1.97**
Cotinine	Cotinine-d3 (IS)	Area ratio (Analyte/IS)	Norcotinine	Cotinine-d3 (IS)	Area ratio (Analyte/IS)
2999425	2273971	1.3190	3771038	2273971	1.6583
3060224	2316229	1.3212	3804754	2316229	1.6427
3134471	2313156	1.3551	3815535	2313156	1.6495
3033583	2290737	1.3243	3781464	2290737	1.6508
3100583	2390728	1.3243	3790161	2390728	1.6508
3103501	2298747	1.3243	3879214	2298747	1.6508
Mean		**1.32803**			**1.65048**
SD		**0.013436**			**0.004958**
%CV		**1.01**			**0.31**
Trans 3'hydroxy cotinine	Cotinine-d3 (IS)	Area ratio (Analyte/IS)			
3479011	2273971	1.5299			
3549332	2316229	1.5324			
3589533	2313156	1.5518			
3485113	2290737	1.5214			
3525243	2390728	1.5214			
3486313	2298747	1.5214			
Mean		1.52971			
SD		0.011852			
%CV		0.77			

7.3.2. Specificity, selectivity, limit of quantification, and linearity of calibration standards

Nicotine and its metabolites, as well as IS were separated from endogenous interference peaks of the blank plasma matrix (Figure 35). Figure 30, 31 shows the extracted chromatogram peaks for LLOQ and ULOQ with IS. The LLOQ for nicotine and its metabolites in plasma was obtained at 0.53 ng/mL. The mean LLOQ peak areas for nicotine, cotinine, *trans* 3'-hydroxycotinine, nornicotine, and norcotinine were 1254, 4902, 941, 481, and 1689, respectively. Similarly, the mean ULOQ peak area for nicotine, cotinine, *trans* 3'-hydroxycotinine, nornicotine, and norcotinine were 153522, 1293454, 523735, 134858, and 575084, respectively. Figure 32-34 represents an example of a QC extracted sample validation of LQC (3.03 ng/mL), MQC (80.64 ng/mL), and HQC (504 ng/mL) chromatogram peak responses of nicotine, its metabolites, and IS in control plasma. The coefficient of determination (R^2) for nicotine and its metabolites ranged from 0.9963-0.9994 (Figure 36).
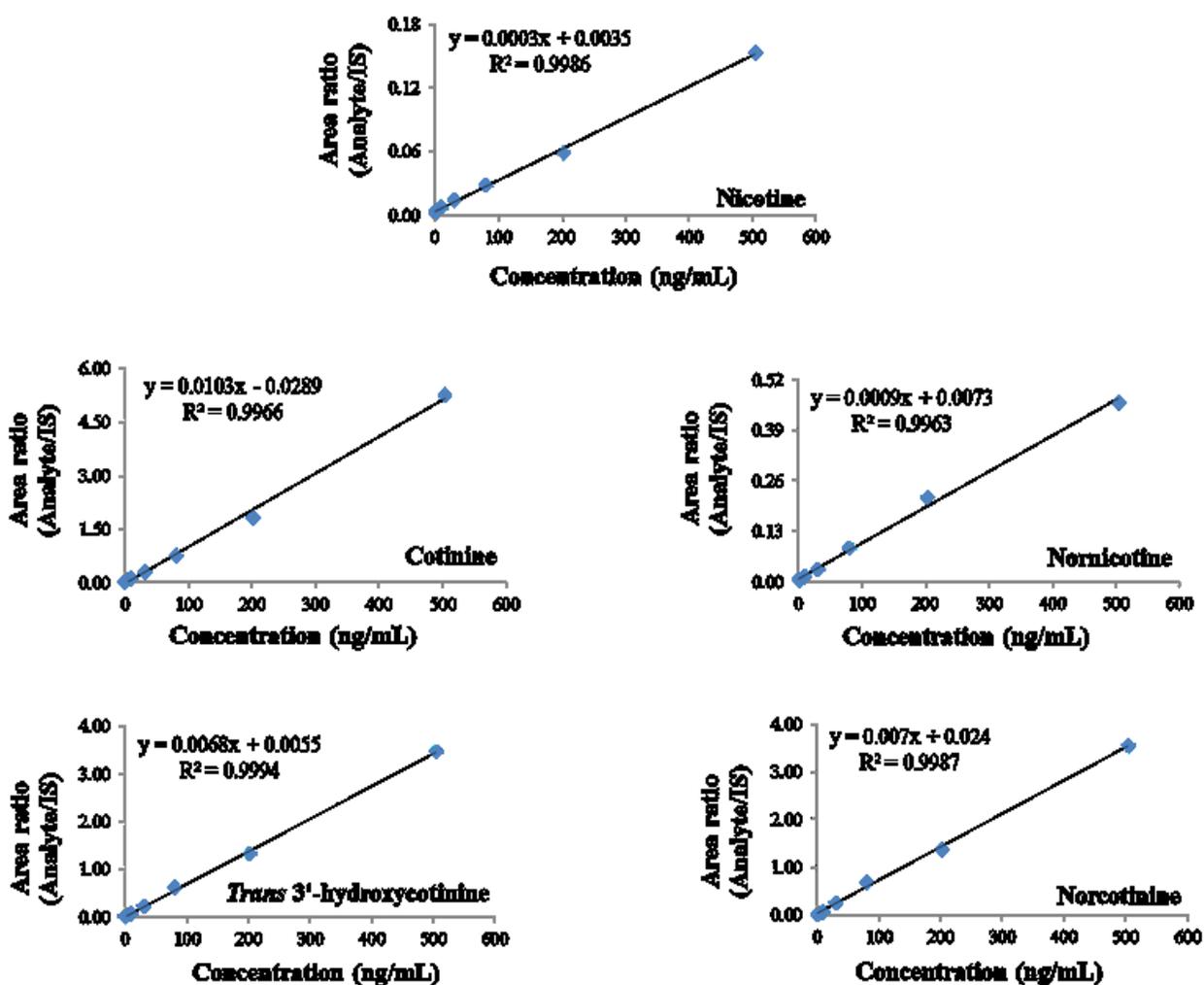
Figure 36: Linear regression analysis of CC standards of nicotine and its metabolites in pooled blank human plasma

7.3.3. Accuracy and precision

The best linear fit and least-square residuals for the standards were achieved with a 1/X weighing factor that yielded a mean linear regression equation for the calibration curve. The assay was linear over the range of 0.53-504 ng/mL with $R^2 \geq 0.996$ (n=6). A regression equation and coefficient of determination were obtained as follows (Figure 35): nicotine: $y = 0.0003x + 0.0035$, *trans* 3'-hydroxycotinine: $y = 0.0068x + 0.0055$, nornicotine: $y = 0.0009x + 0.0073$, cotinine: $y = 0.0103x - 0.0289$, and norcotinine: $y = 0.007x + 0.024$. The % accuracy at LLOQ between the days ranged as follows: 109.1-114.8 % for nicotine, 110.7-117% for nornicotine, 112.3-114.5% for cotinine, 110.7-112.3 % for norcotinine, and 105-106 % for *trans* 3'-hydroxycotinine (Table 8).

7.3.4. Recovery, matrix effect, and stability of analytes in plasma from non-smokers

The percentage recovery of nicotine, cotinine, *trans* 3'-hydroxycotinine, nornicotine, and norcotinine ranged from 95.7-102.9%, 89.6-100.9%, 86.6-104.2%, 86.7-102.5%, and 89.6-100.9%, respectively (Table 6). Six replicates of LQC, MQC, and HQC were tested for a matrix effect. The % matrix effects were between 91.6-102.5%, 95.7-102.9%, 90.5-106.2%, 86.1-101.1%, and 83.2-101.2% for nicotine, cotinine, *trans* 3'-hydroxycotinine, nornicotine, and norcotinine, respectively (Table 6). The stability results, which are presented as percentage of stability (Table 7), yielded bench top stability of <13% and freeze-thaw stability of ~14% for nicotine and its metabolites. The degradation of nicotine and its metabolites were determined to be <15% at LQC, MQC and HQC levels.

7.3.5. Quantitative estimation of nicotine and its metabolites in plasma

Nicotine and its metabolites cotinine, *trans* 3'-hydroxycotinine, nornicotine, and norcotinine were determined at ng/mL levels in the plasma of both HIV-positive and HIV-negative smokers (Table 9). Nicotine concentration in the HIV-negative smokers ranged from

10.87 to 61.59 ng/mL, while nicotine concentration in the HIV-positive smokers ranged from 1.9 to 12.3 ng/mL. The concentrations of cotinine, *trans* 3'-hydroxycotine, nornicotine, and norcotinine ranged 2.3-123.38 ng/mL, 1.5-94.19 ng/mL, 1.09-4.10 ng/mL, and 0.63-3.87 ng/mL, respectively in HIV-negative smokers. However, the concentrations of cotinine, *trans* 3'-hydroxycotinine, nornicotine, and norcotinine concentrations ranged 56.00-95.1 ng/mL, 12.20-34.1 ng/mL, 4.50-8.40 ng/mL and 1.30-2.20 ng/mL, respectively in HIV-positive smokers (Table 9). There were no significant differences in the concentration of nicotine and its metabolites between male and female subjects when analyzed for HIV-negative smokers (data not shown). The mean nicotine concentration in the plasma of HIV-positive smokers (6.98 ± 4.26 ng/mL) was 5-fold lower than HIV-negative smokers (33.38 ± 16.41 ng/mL) (Figure 37, Table 9). The mean concentration of nicotine metabolite nornicotine was 3-fold higher in HIV-positive smokers (5.83 ± 1.8 ng/mL) than HIV-negative smokers (2.58 ± 1.01 ng/mL). Although not statistically significant, the mean concentration of cotinine was also higher in HIV-positive smokers (78.08 ± 16.67 ng/mL) than HIV-negative smokers (77.15 ± 40.52 ng/mL). However, the mean concentrations of other nicotine metabolites were not altered (Figure 37, Table 9).

Table 9: Nicotine and its metabolites levels in plasma from HIV-positive and HIV-negative smokers

HIV-negative smokers

| Patient #	CD4 Count	Nicotine (ng/mL)	Cotinine (ng/mL)	*Trans* 3'-hydroxycotinine (ng/mL)	Nornicotine (ng/mL)	Norcotinine (ng/mL)
1	1416	10.87	2.33	2.28	4.10	0.88
2	938	33.07	115.55	68.19	3.97	2.45
3	1488	14.85	123.38	24.33	2.15	1.61
4	1289	27.49	84.93	4.86	1.65	1.19
5	742	28.96	79.51	94.19	3.61	3.28
6	936	53.06	65.49	3.43	2.91	0.63
7	1438	61.59	119.04	71.65	1.91	3.87
8	1039	51.45	98.37	33.28	1.64	2.42
9	500	17.10	23.59	1.50	2.46	0.87
10	700	31.86	38.45	18.86	2.94	1.17
11	921	36.91	98.02	20.12	1.09	1.15
Mean		33.38	77.15	31.15	2.58	1.77
SD		16.41	40.52	32.38	1.01	1.08
HIV-positive smokers						
2	584	6.50	56.00	34.10	4.70	1.60
3	13	1.90	75.80	12.20	4.50	1.30
4	540	12.30	85.40	14.00	5.70	1.40
5	412	7.20	95.10	22.50	8.40	2.20
Mean		6.98	78.08	20.70	5.83	1.63
SD		4.26	16.67	10.00	1.8	0.40
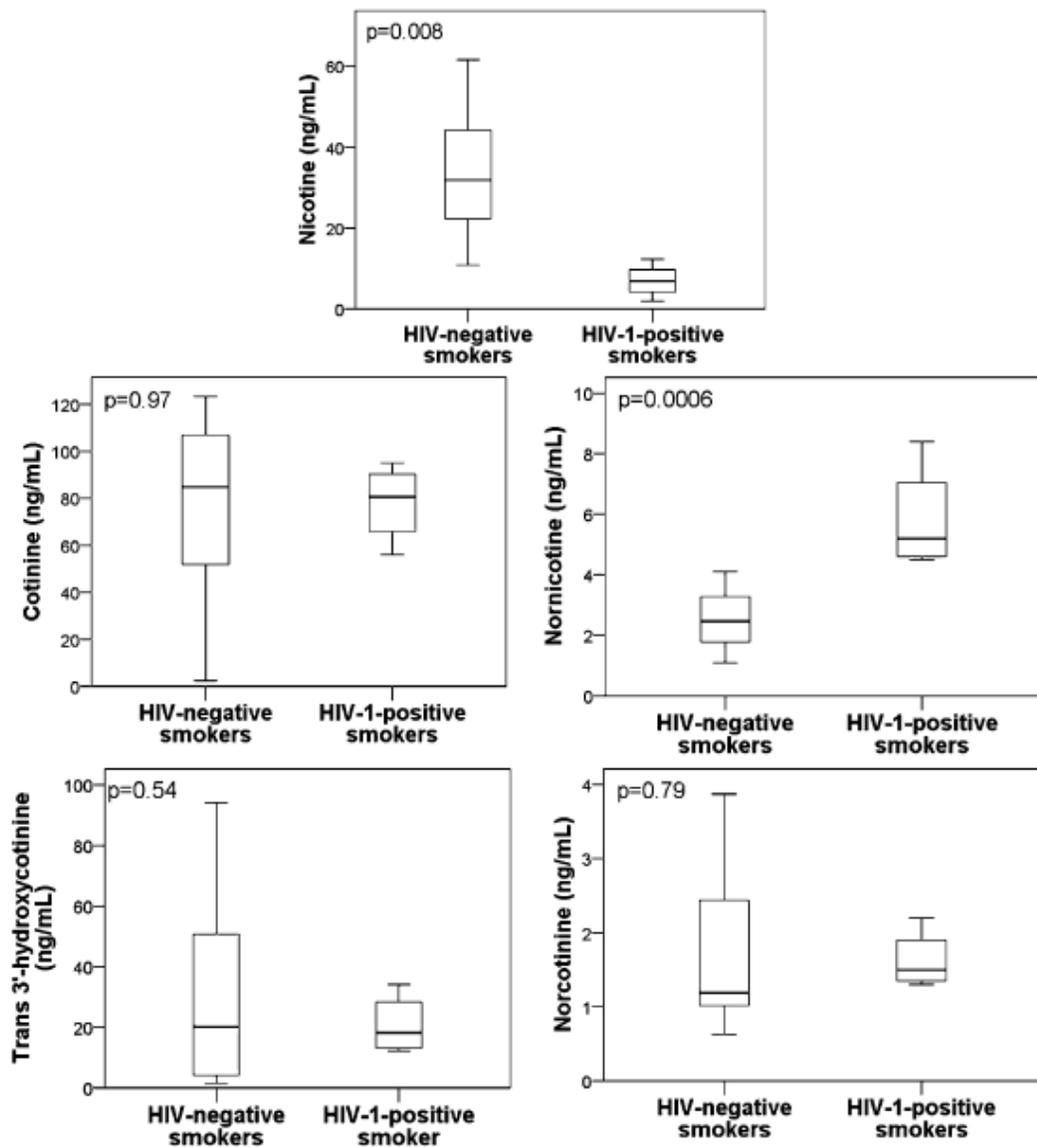
Figure 37: Box plots representing the levels of nicotine and its metabolites in plasma of HIV-negative and HIV-postive smokers

Boxplot shows distribution of all individuals, median, first quartile below the median, third quartiles above the median and outliers in both HIV-positive smokers and HIV-negative smokers plasma.

7.4. Discussion

In this study, we have developed a simple, fast, and sensitive ESI-LC-MS/MS analytical method to simultaneously determine the concentrations of nicotine and four of its metabolites in plasma samples. This method enables the determination of concentrations of nicotine and its metabolites in HIV-positive and HIV-negative smokers. To our knowledge, this is the first report demonstrating the application of an ESI-LC-MS/MS method for concurrent determination of the concentrations of nicotine and four of its metabolites with a sensitivity of <1 ng/mL. Of importance, this is also the first report to show the difference in the concentrations of nicotine and its metabolites in HIV-positive from HIV-negative smokers.

In general, LC-MS/MS bioanalytical results are frequently inconsistent because of ineffective sample preparation and extraction methods. These problems, however, can be eliminated by modifying sample extraction techniques and mass spectrometry parameters including MRM transitions. Liquid-liquid and solid-phase extractions are generally the most effective approach. However, they are expensive and time consuming [213]. The elimination of unwanted water-soluble compounds, such as phosphates and sulfates from plasma, are important in electrospray ionization technique. Zinc sulfate and phosphate present in the plasma has been shown to cause ion suppression of analytes leading to inconsistent results during the ESI analysis [213-215]. In addition to extraction and sample preparation during the LC-MS/MS analysis, pH of the reconstitution solution and mobile phase are important factors to achieve optimal chromatographic separation, peak resolution, reproducibility, and reliability. Furthermore, reconstitution of the solution also plays an important role in the ionization of analytes in the electrospray ionization method. By considering all the above array of parameters, we have developed a simple and rapid ESI-LC-MS/MS technique to

simultaneously estimate nicotine and its four metabolites in plasma by using strong cation exchange SPE. In this technique, the use of formic acid and ammoniated methanolic solutions for SPE cartridge conditioning and elution, respectively, enabled the generation of clear and reliable samples. We found an increased ionization and peak response upon reconstituting in acetonitrile: water: formic acid mixture compared to other reconstitution solutions such as acetonitrile, methanol, and its mixtures.

The *system suitability test* results showed that the method is consistent and reproducible for all analytes. *Carry-over test* results showed that there is no significant carry-over to confound results of the next samples analyzed. The assay conditions had adequate specificity for nicotine and its metabolites, and no interfering peaks were observed with retention times coinciding with the peak of interest. The *signal*-to-*noise ratio* of all analytes was measured at LLOQ level and found to be >5-fold that of the extracted blank peak area. High *signal*-to-*noise* ratio suggests a relatively high quality of extraction efficiency and selectivity, with minimal plasma endogenous interference. The chromatogram peak response and intensity of nicotine and its metabolites were proportional to the concentrations from LLOQ to ULOQ with two IS, which strongly suggests that the method is reproducible and robust. Moreover, this method was relatively more sensitive than the other methods, because The LLOQ (0.53 ng/mL) for nicotine and its metabolites including cotinine, *trans* 3'-hydroxycotinine, nornicotine and norcotinine, was 2- to 5-fold higher than other reported methods (LLOQ: 2-10 ng/mL) [140, 141, 203, 216]. More importantly, this level of sensitivity was obtained with five analytes simultaneously as opposed to the reported methods, in which, the methods were developed with 2-3 analytes simultaneously. Generally, the optimization of

LLOQ and extraction are difficult using the plasma matrix as opposed to other matrices because plasma has more endogenous interferences than many other matrices.

The results of the *accuracy* and *precision* chromatograms suggest the method is robust, precise, and accurate for the analysis of nicotine and its metabolites in the plasma. The calibration curve showed good linearity for nicotine and its metabolites over the entire calibration range. All previously reported LC-MS/MS methods have used ammonium acetate or ammonium formate buffers in their mobile phase systems with a proton adducts in negative and positive modes [140, 215]. Such ammoniated buffer mobile phase systems may enhance ionization of analytes and clog the peak tubes, pump seals, and precipitate in the flow line of the mobile phase. This would lead to an increase in backup pressure in the HPLC column causing leakage of the samples. Therefore, the optimization of nicotine and its metabolites in a formic acid mobile phase system with a proton adduct $[M+H]^+$ in the positive ion mode is considered superior than the other mobile phase. Formic acid-containing mobile phases have the advantage over the ammoniated buffer mobile phase systems, because the former mobile phase system never becomes obstructed, and maintains a consistent pressure in the HPLC column [213]. Second, this mobile phase system is easy to clean owing to lack of precipitation. The nicotine and its metabolites were all eluted from the column within the same retention time (~2 min) showing a sharp resolution peak. The analytes were separated and quantified based on their mass and charge ratio (*m/z*), which is relatively fast and efficient compared with other reported methods [140, 217].

The *recovery efficiency* of this method is higher than the previous methods [99, 171], and the peak response is also reproducible. Previously, we have developed a liquid-liquid extraction method for nicotine and its metabolites in astrocytic and monocytic cell lines [99,

202]. However, in the present method we used strong cation exchange SPE cartridges for plasma sample analysis. Plasma has numerous complex endogenous interfering components compared to astrocytes and monocytes matrices. During optimization of the plasma extraction, we tested several other SPE techniques using different solvent conditions and elution methods such as strong anion SPE and lipophilic-hydrophilic balance SPE. The current method is more sensitive than other reported methods [139, 140], and is able to quantify nicotine and its metabolites at LLOQ level without any interference, with high reproducibility and high accuracy. In the currently described sample preparation technique, all plasma samples including controls, standards, and test samples were pretreated with aqueous formic acid solution to make them more acidic prior to SPE. Under acidic conditions we found the results were consistent without any interference. The nicotine and its four metabolites were stable for 48 weeks with three freeze-thaw cycles at -80^0C and for 5 hours at ambient temperature.

Using our newly developed LC-MS/MS technique, we analyzed nicotine and its metabolites in the plasma of HIV-positive and HIV-negative smokers. These individuals were recruited with strict exclusion criteria to reduce the possibility of confounding by other drugs or co-morbidities. The exclusion criteria were critical to determine the specific effects of mild-to-moderate smoking in HIV-positive patients without any interference by antiretroviral therapy, other medications, or other substances of abuse. The nicotine level is ~5-fold lower in HIV-positive smokers than HIV-negative smokers strongly suggesting that HIV infection increases the metabolism of nicotine (Fig. 38). This hypothesis is further strengthened by the findings that the levels of nicotine metabolites, cotinine, and nornicotine are relatively higher in HIV-positive than HIV-negative smokers. CYP2A6 is known to metabolize nicotine into

the major metabolite cotinine and other minor metabolites including nornicotine [218]. Cotinine is further metabolized into *trans* 3'-hydroxycotinine, norcotinine, and other minor metabolites by CYP2A6 [127, 219].
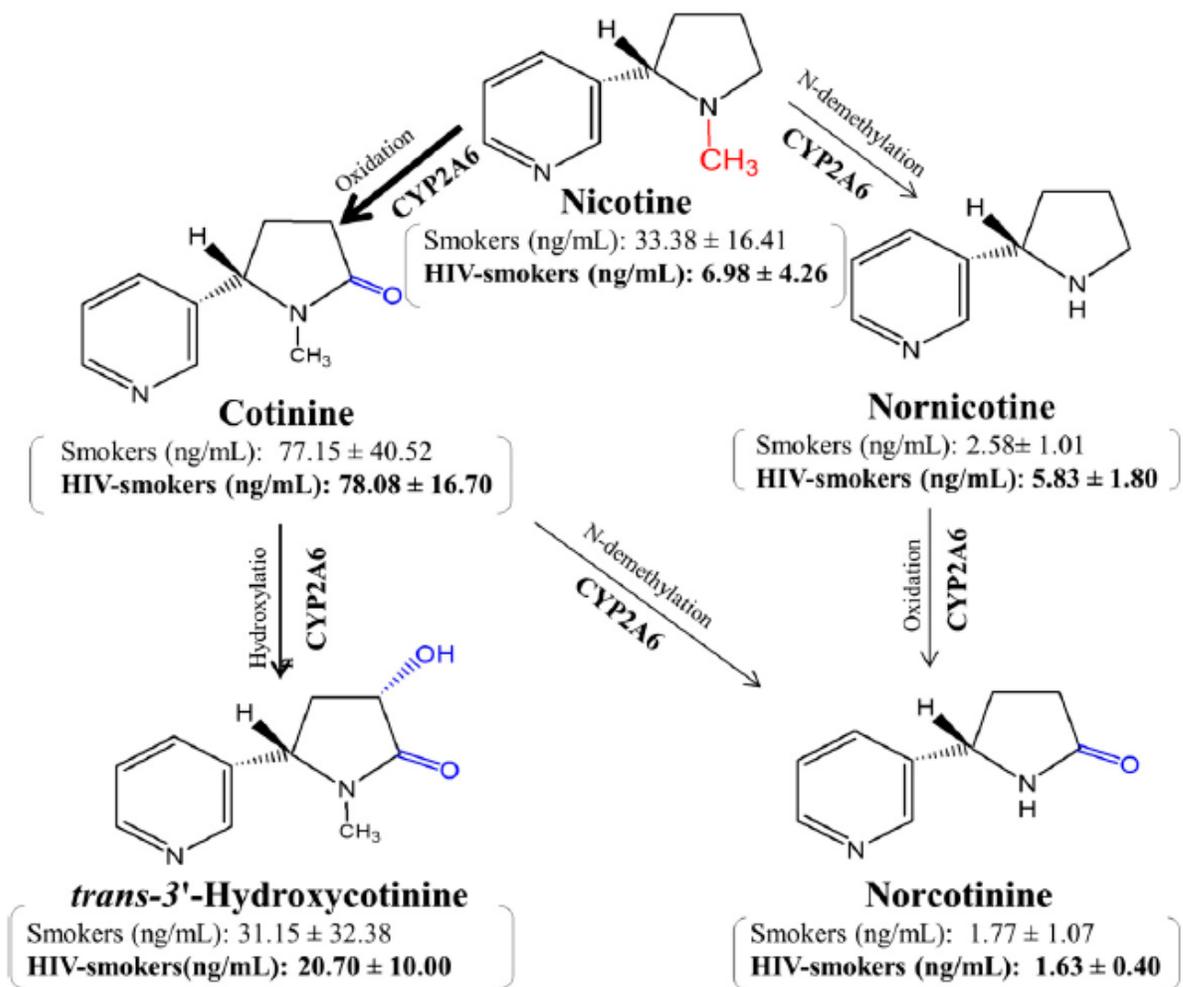
Figure 38: Scheme of nicotine metabolism representing the concentrations of nicotine and its metabolites

The remaining mean relative amounts ± SD (ng/mL) of nicotine, cotinine, nornicotine, norcotinine and trans 3'-hydroxycotinine found in the plasma of HIV-positive smokers (n=4) and HIV-negative smokers plasma (n=11) are shown in parenthesis. The intensity of arrows suggests relative contribution and amounts of CYP2A6-mediated pathways for nicotine metabolism. The nicotine is mainly metabolized to cotinine followed by trans 3'-hydroxycotinine. In addition, nicotine is also metabolized by other pathways to nornicotine and norcotinine.

Collectively, these results suggest that nicotine metabolism is enhanced significantly in HIV-positive smokers compared with HIV-negative smokers. Enhanced nicotine metabolism is known to increase ROS and reactive metabolites leading to increased cell toxicity and cancer of the lungs, esophagus, and liver [177]. This may lead to an increased risk of cancer in HIV-positive smokers compared to HIV-negative smokers. Indeed, our recently published data from these patients showed an increase in oxidative DNA damage in the plasma of HIV-positive smokers compared to HIV-negative smokers [209]. We also reported enhanced oxidative stress that was correlated with an increase in the viral load in HIV-positive smokers compared with HIV-positive non-smokers [209]. Previous reports also suggest a role of smoking in increased viral replication and the possible association with increased oxidative stress [72, 100, 168].

7.5. Conclusion

In conclusion, since the prevalence of smoking is higher in HIV-positive individuals than the general population [59] and in turn smoking is associated with increased viral replication [69-71], it is important to understand the underlying mechanism of smoking/nicotine-mediated HIV replication. This report suggests a possible role of nicotine metabolism in oxidative stress-mediated increase in viral replication in HIV-positive smokers. However, further studies are necessary to elucidate this mechanism to confirm the role of CYP2A6-mediated nicotine metabolism, especially in monocytes/macrophages, in HIV replication.

CHAPTER 8

SUMMARY AND FUTURE DIRECTIONS

8.1. Summary and significance

Assessment of the mortality rates among AIDS patients has revealed that the risk of death is twice as high among smokers in the HIV-infected population compared to the non-HIV-infected population. There is an evidence from the literature suggesting that smoking is associated with decline in CD4 cell counts in the HIV-infected population, despite of the higher baseline CD4 cell counts observed among smokers than non-smokers [77]. However, the role of smoking in the progression to AIDS is unknown. Recent reports from the literature have also demonstrated the toxic effects of the tobacco constituents, nicotine and nicotine-derived nitrosamine ketones (NNK) in neurons, microglia, and white blood cells, which are exposed to the virus or viral proteins [23, 24]. For example, the treatment with NNK to mouse microglial cells and *in vivo* injection of mouse with NNK showed an increase in ROS, as well as an increase in the levels of proinflammatory cytokines [23]. However, the mechanism of tobacco-mediated toxicity and decreased efficacy of HAART is largely unknown.

The present study investigated the role of CYPs in tobacco/nicotine-mediated oxidative stress in HIV cell models, and their possible link to HIV pathogenesis and neuroAIDS. The experiments in specific aim 1 were designed to determine the role of CYP2A6 in nicotine metabolism in HIV cell models, SVGA astrocytes (chapter 4). It can be noted that the role of CYP2A6 in nicotine metabolism in other HIV model, U937 monocytes were earlier performed by Jin et al. 2012. In Specific Aim 2, CSC-mediated CYP induction, oxidative stress, and cell death in U937 monocytes and HIV replication in primary macrophages were investigated (chapter 5). In Specific Aim 3, the levels of CYP2A6 and

antioxidant enzymes (AOE) were determined in blood monocytes isolated from a cohort of patients (Healthy, smokers, HIV and HIV smokers) using a cross-sectional *ex vivo* study design (chapter 6). In Specific Aim 4, upon developing a sensitive and robust LC-MS/MS method for nicotine and its metabolites, their levels were determined in plasma samples of smokers and HIV Smokers from *ex vivo* study (chapter 7).

Results from Specific aim 1 showed that nicotine induced CYP1A1 and CYP2A6 at both mRNA and protein levels in SVGA astrocytes. Nicotine also increased the production of ROS in these cells. The role of CYP2A6 in nicotine metabolism-mediated oxidative stress were further demonstrated using a specific CYP2A6 inhibitor, tryptamine. Results from Specific aim 2 demonstrated that CSC increases CYP1A1 induction and oxidative stress, which were significantly higher than that caused by nicotine. CSC treatment also induced caspase-3-mediated apoptosis in U937 monocytes. Finally, CSC treatment enhanced HIV replication in HIV-infected primary macrophages as determined by p24 antigen levels. Results from Specific aim 3 demonstrated that mild-to-moderate smoking increases viral load in HIV-infected smokers in ART naïve subjects. In addition, smoking and HIV both independently increased oxidative DNA damage in plasma and monocytes. Higher oxidative stress was further observed in HIV-infected smokers when compared to other cohorts independently. Our results demonstrated that the levels of mRNA of the most antioxidant genes are not altered in HIV positive and/or smoker groups compared to HIV-negative non-smokers. As expected, the results showed that the levels of CYP2A6 mRNA is increased in HIV negative smokers compared to HIV-negative non-smokers However, the level of CYP2A6 mRNA was only marginally increased in HIV-positive non-smokers and HIV positive smokers compared to HIV-negative non-smokers. Results from Specific aim 4 showed that the nicotine level is ~5-

fold lower in HIV-positive smokers than HIV-negative smokers strongly suggesting that HIV infection increases the metabolism of nicotine. This hypothesis is further strengthened by the findings that the levels of nicotine metabolites, cotinine, and nornicotine are relatively higher in HIV-positive than HIV-negative smoker.

8.2. Conclusions and future directions

The role of oxidative stress as a result of production of ROS, including nitric oxides has been implicated in HIV pathogenesis. Similarly, previous literature and our current findings demonstrate that smoking and oxidative stress both are independently associated with increased HIV replication. Thus, we suggest that smoking-mediated oxidative stress may be responsible for increased HIV replication. Since CYP-mediated metabolism of tobacco constituents is known to produce reactive oxygen species in the liver, it is reasonable to conclude that CYP-mediated pathways contribute to the DNA damage in both plasma and monocytes. Our results showing relatively low levels of DNA damage in monocytes suggest that CYP-mediated metabolic pathway also exists in monocytes. Our speculation is consistent with our earlier observation that although monocytes contain relatively low levels of CYP enzymes compared to the liver, the relative level of CYP2A6 in monocytes is much higher that other CYP enzymes [190]. Therefore, we suggest that although CYP2A6 in smokers and HIV positive non-smokers are not induced, the basal level of CYP2A6 is sufficient to metabolize tobacco constituents and endogenous compounds and produce ROS. Furthermore, our observation is consistent with the recent finding that CYP2A6-mediated metabolism of nicotine produces ROS in monocytes and astrocytes [99], which may be responsible for increased DNA damage in monocytes. Our studies also demonstrated an increased metabolism of nicotine in HIV positive smokers compared to HIV negative smokers. It is also possible that the increased metabolism is due to increased activity of enzyme rather than the actual

protein level. An increase in enzyme activity through substrate-mediated enhance stability of enzyme is known in the P450 system [192].

An increase in oxidative stress could be as a result of both, CYP-mediated hepatic metabolism of smoking constituents and/or endogenous compounds and lack of induction of AOEs. Although we suggest a possible association of CYP and oxidative stress pathways in HIV replication, other pathways cannot be excluded. Therefore, there is a need to further investigate CYP or alternative pathways in smoking-mediated HIV pathogenesis up on treatment with CSC as well as the active constituents of CSC (for e.g. PAH, benzyl naphthalene, phenanthrene) in *in vitro* HIV-infected macrophages as well as in an *in vivo* HIV-infected humanized mice model.

In summary, our *in vitro* and *ex vivo* studies have shown the phenomenon of enhanced oxidative damage, nicotine metabolism, and HIV replication in HIV-infected smokers. Since we employed a cross sectional study design in delineating the above phenomenon, furthermore there is an urge to investigate the mechanism delineating the role of CYP-mediated oxidative stress on cytotoxicity and viral replication. Therefore, an *ex vivo* study following longitudinal design using large number of individuals would help in identifying specific targets for CYP pathway to alleviate smoking-mediated HIV pathogenesis and drug interactions.

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VITA

Anusha Ande was born on July 15, 1989, in Bhimavaram, India. She was educated at public schools in Bhimavaram and graduated from high school by securing the first highest GPA in the district. She attended Andhra University, college of Pharmaceutical sciences, the second oldest Pharmacy school in India. She obtained her Bachelor's degree of Science in Pharmacy in 2010 with distinction. After graduation from college, Ms. Ande joined the interdisciplinary Ph.D. program at University of Missouri-Kansas City (UMKC) in the fall of 2010, majoring in Pharmacology and Pharmaceutical Sciences as co-discipline. Her keen interest and progress in research led her to publish 8 manuscripts in peer reviewed journals. She maintained a GPA of 4.0/4.0 with an outstanding performance in coursework. She also presented her work at various international conferences by poster presentations as well as a podium talk upon receiving Early Career Investigator Travel Award twice from the conference committee. During her graduate study at UMKC, Ms. Ande was bestowed with several awards and scholarships, including Best Student Award (2011), Dean's Scholar Award (2012), Women Council Graduate Assistant Fund Award (2012, 2013, 2014, 2015), Preparing Future Faculty Award (2013-2014) (awarded to the six outstanding graduate students among all the graduate students from the university), and Robert C. Lanman Graduate Pharmacology Scholarship (2014).

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