ABSTRACT

MANAVI, KIANO REZA. Effect of Blood Serum Cotinine Concentration on Blood Serum Vitamin D Concentration Among Women with Different Ethnic Backgrounds in the United States. (Under the direction of Dr. Brenda Alston-Mills and Dr. Jonathan C. Allen).

The suffering from lung cancer as the disease becoming the usual cause of cancer mortality has been on the rise rapidly in the world since the 20th century. Results from epidemiological investigations have correlated cigarette smoking with the causation of lung cancer in the 1950s. Over time, increasing evidence has shown that smoking is an inevitable cause of many health concerns, such as chronic obstructive pulmonary disease (COPD) including chronic bronchitis and emphysema, lung cancer, and several other serious health problems. Besides those epidemiological studies that have shown the relationship between cigarette smoking and lung cancer, there are other epidemiological chances as observed in lung cancer, including but not limited to the narrowing of the gap between men and women affected by the disease, where women who are not smokers are affected by the disease. Tobacco usage kills more than 8 million people a year. Approximately 7 million of those deaths are the result of direct tobacco use, while approximately 1.2 million are the result of non-smokers being exposed to second-hand smoke. Nicotine from smoking tobacco, specifically its metabolite cotinine, has negative effects on human health causing lung cancer, COPD and non-respiratory problems.

Over a billion people worldwide are vitamin D deficient or insufficient, which is prevalent across all age-groups, geographic regions, and sunlight exposure. With the discovery of vitamin D in 1919, a new chapter in the prevention of rickets was introduced opening the door to its therapeutic properties for other diseases. Since 1919,
There have been many clinical and epidemiological studies performed globally on the effect of the vitamin on prevention of other diseases, including but not limited to, cancer, autoimmune disorders, cardiovascular diseases, and osteoporosis.

There have been few epidemiological and experimental studies in the search for effect of tobacco smoking on vitamin D, and the therapeutic effects of vitamin D on Lung Cancer, Chronic Obstructive Pulmonary Disease (COPD) and other diseases. Yet, the area of research in this field is relatively new compared to other studies such as breast and prostate cancers.

In this study, nutrition epidemiology investigations used NHANES 2001-2014 data to test the hypothesis that blood serum cotinine concentrations are correlated with vitamin D concentrations (vitamin D$_3$, vitamin D$_2$ and total vitamin D “D$_2$+D$_3$) in addition to other previously investigated and known factors such as gender, ethnicity, dietary supplement intake and sun exposure among women. A weak negative correlation was observed between serum cotinine concentration and serum vitamin D concentration among non-smokers, light/passive smokers and heavy smokers; therefore, it was not statistically significant. The sample size for this investigation was small, therefore a larger sample size would be beneficial for better understanding of this correlation.

The in-vitro investigation with a cell culture model system indicated that cotinine may induce CYP2R1 and CYP3A4 expression in the liver microsomes. The increased activity of CYP3A4 may accelerate vitamin D catabolism and may contribute to vitamin D deficiency.

Further research is necessary to better understand the potential correlation between serum cotinine concentration and serum vitamin D concentration, including
further investigation to demonstrate the mechanistic link between the effect of cotinine on the CYP2R1 and CYP3A4 to further clarify the impact of smoking on vitamin D status.
Effect of Blood Serum Cotinine Concentration on Blood Serum Vitamin D Concentration Among Women with Different Ethnic Backgrounds in the United States

by
Kiano Reza Manavi

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Nutrition

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APPROVED BY:

_______________________________
Dr. Brenda Alston-Mills
Co-Chair of Advisory Committee

_______________________________
Dr. Jonathan C. Allen
Co-Chair of Advisory Committee

_______________________________
Dr. Marvin P. Thompson
External Member

_______________________________
Dr. DeYu Xie

_______________________________
Dr. Slavko Komarnytsky
DEDICATION

To my father, thank you for always believing in me and inspiring me. To my mother, thank you for teaching me the true meaning of unconditional love. Thank you both for all the sacrifices you made in life for our education and success.

To my wife, Dr. Sarvenaz Dabiri, thank you for never leaving my side, no matter how tough things got. Your continued support and encouragement kept me moving forward.

To my sister Kathy, and brother-in-law Ryan who never left me alone during this journey. And to my smart and loving nieces and nephews, Sarah, Kamran, Leon, Sophia and Isaac who have given me the joy of being an uncle.
BIOGRAPHY

Kiano Reza Manavi was born on September 16th, 1975 to father Ghasem Manavi and mother Masoumeh Miralinaghi in Tehran, Iran. He lived in Iran and migrated to Canada and the United States from his motherland after the revolution in Iran with his parents and two siblings to pursue new life and for better education and future.

Next, he attended St. Augustine’s College in Raleigh, North Carolina and completed his first Bachelor of Science degree in Computer Science in 2000 and Master of Science from Capitol Technology University in Laurel, Maryland in 2004. After working few years in the technology industry, he completed his second Bachelor of Science degree in Botany at North Carolina State University in 2006 with a minor in Environmental Toxicology to pursue a new career path in life sciences industry, with focus on human health.
ACKNOWLEDGMENTS

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I am especially grateful for my committee chair Dr. Alston-Mills, for her unwavering belief that I can successfully reach my academic goals; her continued patience, support, and encouragement along my journey, and for all the experiences and skills I gained by working with her. For me, she is a great role model as a kind, respectful, open-minded, dedicated, and compassionate educator, researcher, and mentor. I really appreciate the time and energy she has given to me; I will be forever thankful to her.

Dr. Thompson and Dr. Xie both have played an important role in my education path as they were both my undergraduate advisors, and I am always grateful for their encouragement to pursue the science path further.

Dr. Allen, for all his challenges to make me a stronger person and allowing me to use his lab and staff.

Dr. Komarnytsky for all his openness to my project, allowing me to use his lab and staff.

I also want to thank the individuals at the Department of Statistics who helped me with data analysis in my research.
While I had lots of reasons for seeking a PhD degree at NCSU, the greatest thing I had not counted on was making forever friends.

I am thankful to the lord that put all these great people in the path of my success.
“The knowledge of anything, since all things have causes, is not acquired or complete unless it is known by its causes”.

Persian Physician & Philosopher
Avicenna
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CHAPTER 1

Literature Review

The History of Tobacco, Smoking, Women and Vitamin D

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Nicotiana

Nicotiana also known as Paka, Tabac, Tabaco or the more familiar name Tobacco, is one of the members of Solanaceae family (Table 1) from a Dicot (two seed leaves) group. Other known members of this family are the plants such as potato, tomato, eggplant, belladonna, and nightshade. Some of the characteristics of this plant are such as, it may be either annual or perennial in the form of shrubs or small trees, with five lobed colorful flowers in white, yellow, greenish-yellow or red colors, attached to a large tubular and wide terminal panicles, surrounded by hairy and sticky leaves with smooth edges. The Nicotiana genus has been a renowned plant native to the continent of Americas, which adopted its name from the Swedish botanist Carlous Lennaeus in the honor of the French Ambassador, Jean Nicot. The genus hosts approximately fifty (50) different species with many varieties such as, Nicotiana rustica, Nicotiana acuminate, Nicotiana alata, Nicotiana attenuate, Nicotiana clevelandii, Nicotiana excelsior, Nicotiana forgetiana, Nicotiana glauca, Nicotiana glutinosa and few others. Among all the different species, nonetheless, the Nicotiana tabacum, is well known for its smoking purpose in the ancient Latin America, which is the main ingredient of nearly all commercial tobaccos today. The Nicotiana tabacum has been the product of human cultivation since it has a low production yield naturally [1].
Table 1: Classification of Kingdom Plantae Down to Genus Nicotiana L. – Cultivated Tobacco Nicotiana tabacum L.

<table>
<thead>
<tr>
<th>Kingdom: Plantae – Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subkingdom: Tracheobionta – Vascular plants</td>
</tr>
<tr>
<td>Superdivision: Spermatophyta – Seed plants</td>
</tr>
<tr>
<td>Division: Magnoliophyta – Flowering plants</td>
</tr>
<tr>
<td>Class: Magnoliopsida – Dicotyledons</td>
</tr>
<tr>
<td>Subclass: Asteridae</td>
</tr>
<tr>
<td>Order: Solanales</td>
</tr>
<tr>
<td>Family: Solanaceae – Potato family</td>
</tr>
<tr>
<td>Genus: Nicotiana L. – tobacco</td>
</tr>
</tbody>
</table>

The poisonous nature of tobacco plant is known to all parts of the plant, but the specific nature of the toxin depends on the alkaloids substance specificities of Nicotiana. For instance, Green Tobacco Sickness “GTS” was the nicotine poisoning in farmers during the harvest and handling of the green leaves due to the nicotine toxin derived from Nicotiana tabacum, when their clothing or skin became saturated with tobacco [2].

History of Tobacco – From Mayans to Europeans

The earliest relics of the Mayan civilization in Mexico have exposed the history of tobacco use in the distant past of the Latin Americas. The use of tobacco was part of their custom in their ceremonies such as offerings to their gods, medical remedies as well as for peace and friendship. The Mayans harvested tobacco leaves, then dried and grounded up to be used for smoking, chewing, and snifing. Further to the east from Mexico, in Cuba, the Taino tribes not only smoked tobacco in the form of pipes and cigars for rituals and medicine, but also, they made tobacco cigarettes from palm leaves and corn husk. A combination of tobacco and coca-leaf dust, and chewed tobacco was widely used to relieve fatigue and as stimulant in the South America. It is believed that the European explorers experienced smoking tobacco when they landed in the New
World in 1492 when tobacco leaves were given as gifts by Native Americans and were thrown away by Europeans. Columbus in his personal diaries referenced the use of the tobacco plant for ceremonial religious purposes, as well as smoking it by the natives. Rodrigo de Jerez, is believed to be the first European who was sent by Christopher Columbus on an exploration of Cuba’s inlands. Upon his return to Spain when he smoked tobacco in public, he was sentenced to prison for the reason of “the Inquisition for being in league with the devil for puffing away” [3,4]. As time passed, the European physicians revealed the medicinal properties of the tobacco, and it became popular in the region. To the extent that in 1571 the Spanish physician Nicolás Bautista Monardes in his work of “Segunda parte del libro des las cosas…do se trata del Tabaco…” referred to tobacco as cure for common cold, cancer, stomach pain, joint pain, toothache and snakebites amongst few other healing recommendations [5].

At the end of the expedition journey, the sailors brought tobacco back to Europe with them, and it was in 1558 when tobacco was first introduced in Portugal. Then toward the end of 1570 the tobacco cultivation was expanded into Belgium, Spain, Italy, Switzerland, and England, later by 1600, it was expanded to the Philippines, India, Java, Japan, West Africa, and China. Despite its popular properties in Europe at the time, there was still resistance to its use. For instance, in the book of “Counterblast to Tobacco” in 1604 by King James-I of England, the King denounced smoking. In the same era in 1606, King Phillip-III of Spain issued a decree, limiting the cultivation of tobacco in Spain and his Spanish colonies. In France tobacco was only allowed by prescription, and in other countries such as Russia to have a ban on tobacco use, the smokers were exiled to Siberia, but Peter the Great in 1700 granted the permission of
smoking in Russia; But in China and Turkey the punishment was death. Despite all these reactions, tobacco use nevertheless became widely popular, and tobacco cultivation became a profitable business; To the extent that in 1776 “tobacco was used as collateral for loans to help finance the American Revolutionary Wars” [6,7].

The Rise of Tobacco in North America

The *Nicotiana rustica* grown by the native Indians was not up to the European’s standard for its taste. William Strachey, the first secretary to the Virginia Colony described that “the leaf was found to be poore and weake and of byting tast”. In order to produce a better-quality tobacco, John Rolfe in 1612, mixed seeds of *Nicotiana* grown in Trinidad and Venezuela known for their sweeter and more flavorful properties with the native plants grown in Jamestown and resulted in darker leaf comparable in quality to its Latin American ancestor. During the early years of settlement and exploration, the most popular form of tobacco use in America was pipe-smoking. But snuffing into the nose had been developed into an elaborated ritual by the French, and by the mid of the eighteenth century, the snuffbox had become the mark of a gentleman in Virginia. The origin of cigarette travels back to the *cigarito* in the Spanish colonies of the New World, and it had become popular in the early years of the nineteenth century. Cigarettes’ popularity was largely a 20th century phenomenon, created by the habit of World War I soldiers in the trenches of Europe. During the war sending cigarettes to soldiers was considered a patriotic act. After the war was over and up on the return of the soldiers, cigarette smoking quickly spread through the middle class, making it more respectable [8,9].
Women, Tobacco and Cigarette Smoking Epidemiology

In the early years smoking was considered an unacceptable manner for women. In most cultures and societies, it was observed as an improper and unfeminine behavior. The consumption of tobacco by women began to draw serious attention around 1880 in Europe. However, the women's association with smoking went back at least to the 1600s in Africa and India, where women smoked pipes, and it was acceptable. From the 1880 to 1908, smoking was limited mainly to patrician women, who smoked secretly at social gatherings held by the other upper-class members and for the society. As the liberation of women grew stronger in North America and Europe, smoking eventually became increasingly acceptable as well throughout years. When women started smoking in public in the 1920, they took it as a sign of liberation and parity; in the 1930s it became a fashionable illustration among urban women, then by 1935, 18.1% of American women were smokers. Women played a major role during World War II as a contributor to the war effort by working with freedom, liberation, and patriotism, therefore women smoking became more relevant. When the war ended the smoking frequency among the British women was 40%, 30% among Australian women, and 25% among American women [10].

Since beginning of 1908 to 1919, women smokers publicly challenged the authorities against smoking that applied to them alone. Throughout this period, no restaurants in America allowed women to smoke officially on premises, but most did by 1919, because of the pressure placed by women on the institutions. Despite all these changes, smoking could and did land several women in jail. During 1919 to 1927 the smoking was practiced by women in American higher education institutions, yet none...
allowed women to smoke anywhere within its jurisdictions and outside of it in 1920. But around 1925 and 1926 most did, although conditions were often imposed. Smoking by women had come a long way by 1927, and it had all taken place without any cigarette advertising aimed directly at women. In 1919, when the 18th Amendment of the U.S. Constitution, Prohibition was ratified by the states, in which it remained the law of the lands until 1933 when the 21st Amendment to the Constitution, the repeal of the 18th Amendment, was ratified by the states. Ratification and then enactment of Prohibition gave a big psychological impetus to smoking opponents to continue the fight against women smoking [11].

From 1927 to 1950, cigarette industry advertisers targeted women more, since opposition movements had faded away, and the last of the laws that barred adults from buying cigarettes was repealed. In 1930 per capita consumption of cigarettes was more than double the level in 1925, owing in part to the growing number of women smokers, enjoying the fruits of freedom and the loosening of moral standards. During 1930s, as the incident of lung cancer increased, scientists and physicians became more obliged to study the smoking and the cancer relationship more closely. After a long struggle, women smokers had gained full equality with men smokers by 1950, when the cigarette smokers and smoking were widely accepted as part of the America’s social norm, and a companionable thing to do, a habit indulged in by men and women. Yet, smoking by women did not take place in any public places [12].

In 1964 when the first Surgeon General’s Report was released, “the prevalence of smoking amongst women has declined much more slowly than amongst men”. The lung cancer mortality rates have increased with a steady trend amongst women, and the
public health impact of smoking on this trend has been observed. In the 1950s and 1960s, the cigarette industry developed the “filter” and the “low-tar” elements as the safety “guarantee” and were introduced as “light cigarettes” to the consumers [13,14].

Throughout 1965 to 1993, a survey monitored the smoking behavior of all smokers at the age of 18 years and older, with a 100 cigarettes requirement as the minimum annual consumption of in the U.S. The survey showed that in women the number of cigarettes smoked was reduced from 8 cigarettes per day in the mid-1970s, to 5 cigarettes per day by 1995 [15,16]. Up until 1990s the smoking epidemiologic spread was the correlation of two factors, the consumption of manufactured cigarettes increases, and the increase in cigarette consumption in gender and at different countries [17]. The World Health Organization (WHO) report on the global tobacco epidemic has indicated that the epidemic killed 100 million people worldwide in the 20th century with the potential of killing one billion during the 21st century, and by 2030 there will be more than 8 million deaths every year (Figure 1) [18].

In fast growing and more populated countries, the most threatening factor of the smoking epidemic’s growth is observed to be the smoking amongst younger women. The tobacco industry markets to women by “advertising, promotion and sponsorship, including charitable donations to women’s causes”, and in some cases employing fashion industry models to advertise smoking more aggressively than before. Some of the advertising and marketing approaches that were used in marketing tobacco in the U.S. and other western countries for quite some time, now has threatened women in the other developing world as well. Several studies in the North Americas correlated one
factor associated with smoking amongst girls was that girls had lower self-esteem than boys [19].

**Figure 1:** *Tobacco will kill over 175 million people worldwide between now and the year 2030.* Tobacco Cumulative Related Deaths, From 2005 to 2030.
Lung Cancer Statistics in the United States and Worldwide

In the U.S., based on the population data (from 1999 to 2017) from the Centers for Disease Control and Prevention (CDC), the annual rate of new lung and bronchus cancers are on declining trends in both men and women (Figure 2).

![Graph showing declining cancer rates](image)


**Figure 2: Annual Rates of New Cancers, 1999-2017.** Lung and Bronchus new cancer rates in the United States.

But the most recent data from 2013-2017 showed that among women, whites had the highest rate (53.0) of new lung and bronchus cancers per 100,000 people in the nation compared to blacks (46.9), American Indians/Alaska Natives (38.5), Asian/Pacific Islanders (27.9) and Hispanics (24.2) but all groups were lower compared to their male counterparts (Figure 3).
Figure 3: Rate of new cancers by Gender and Ethnicity, 2013-2017. Lung and Bronchus new cancer rates in the United States.

The number of new lung and bronchus cancers in women increased between 2013-2017 among whites (457,968) more than blacks (53,339), American Indians/Alaska Natives (3,353), Asian/Pacific Islanders (13,932) and Hispanics (22,314) compared to their men counterparts (Figure 4).
**Figure 4:** Number of new cancers by Gender and Ethnicity, 2013-2017. Lung and Bronchus new cancer numbers in the United States.
Also, the number of women in the nation who have died from the cancer was the highest among whites (301,794) relative to blacks (34,671), American Indians/Alaska Natives (1,837), Asian/Pacific Islanders (8,205) and Hispanics (11,291) but less compared to their male counterparts between 2013 through 2017 (Figure 5).

![Figure 5: Number of Cancer Deaths by Gender and Ethnicity, 2013-2017. Lung and Bronchus cancer deaths numbers in the United States.](image)

Smoking tobacco is the main reason for lung cancer, and cigarettes are the main transportation factor of delivering tobacco smoke into the body. There is evidence from some countries that lung cancer rates in women may be stabilizing, but the situation in most countries continues to increase in both the incidence and mortality. Compared to the U.S. most of the European countries, except the U.K., Ireland, and Denmark are still
substantially lower in incidence rates and death statistics. In several other European
countries, including France, Switzerland, Germany and Italy tobacco smoking has
become more common amongst women in their young to middle-aged, yet overall
national mortality rates have been relatively low compared to the U.S., although
noticeable upward trends have been observed over the last twenty years. Regardless of
the statistics in the U.S. and other countries, the role of tobacco smoking is well
understood in causing lung disease over the past 50 years; yet, “90% of lung cancer
may be avoidable simply through avoidance of cigarette smoking” [20]. It has been quite
a while since 1964, when the U.S. Surgeon General announced that smoking causes
lung disease. Although, lung cancer is the most well-known smoking incidence lung
disease, smoking also causes other diseases as well such as Chronic Obstructive
Pulmonary Disease (COPD), asthma, respiratory infections, pulmonary hemorrhage,
pulmonary metastatic disease among women with breast cancer amongst other health
complications. Women smokers have a higher risk of perishing from the cancer that is
about 12 fold more than non-smoking women, whereas in men it is 22 folds more than
non-smokers (Figure 6) [21].

![Figure 6: Lung Cancer risk for men and women smokers, compared to people who never smoked.](image-url)
Passive Smoking

Passive smoking is an inclining apprehension because of more awareness of tobacco smoking harmful consequences on health. Urgent consideration in further risks of passive smoking have been the examination of environmental tobacco smoke and its carcinogenicity. There are two components to the environmental tobacco smoke; one is the cigarette smoke exhaled by the smoker and the other is the smoke produced from the burning end of the cigarette. The evaluation of the chemical composition both smokes have suggested that the toxic and carcinogenic effects of both were qualitatively similar; however, some greater amounts of many of the organic components of smoke, including some carcinogens, are found in the smoke produced from the burning end of the cigarette. Therefore, passive smoker women are at the same risk as smoker women. Several studies have indicated that there is a 20-50% greater risk of developing lung cancer for women who are non-smokers but are exposed to partner smokes than those whose partner does not smoke. (“The National Research Council report for 1986 stated that at least 2,500 of the 12,000 deaths from lung cancer among non-smokers in the United States could be attributed to passive smoking. The total number of deaths from lung cancer in the United States in 1986 was about 136,000”). Another form of exposure to smoking is “Environmental tobacco smoke (ETS)”, second-hand or passive smoking. ETS contains nearly 5,000 compounds, including over 40 chemicals that met the criteria of a known human or animal carcinogen. Adults who are exposed to ETS are also associated with an increased danger of several cancers, notably lung cancer and diseases. Second-hand smoking is a serious and avoidable risk factor for respiratory disease among children because they
are especially sensitive to smoke. Since the second report of the U.S. Surgeon General (1971), there has been a growing interest in further investigation on the health consequences of ETS. Passive tobacco smoking (PTS) was recently emphasized as etiology for diseases in second-hand smokers, particularly lung cancer risk in women non-smokers. The eating pattern (diet) of smokers also had a habit of to be less adequate than the non-smokers. Exposure to tobacco smoke modifies the sense of taste and smell, leading to changes in food preferences and food intake, and eventually to a more imbalanced diet [22,23].

**Nicotine and Its Metabolism**

Nicotine (IUPAC Name: 3-[(2S)-1-methylpyrrolidin-2-yl] pyridine; 3′-pyridyl-2-N-methylpyrrolidin “C_{10}H_{14}N_{2}”) is the natural secondary metabolite of tobacco that acts as botanical insecticide. It is a weak base with pKₐ of 8.0 that is synthesized mainly in the roots of the plant but is found in the leaves at its highest concentration. It has an alkaloid structure that contains two nitrogenous cyclic structures with different pKₐ values of 3.12 and 8.02, and hybridization that underwent both carbon and nitrogen oxidation in biological systems. The most alkaloids (or Nicotinoids) in the plant are 3-pyridyl derivatives, and the pyridine ring of nicotine is formed from nicotinic acid (a precursor). Based on the amounts of alkaloid accumulation in leaves of the plant, nicotine (principal alkaloid in commercial tobacco; 50 to 60% of the species), nornicotine (main alkaloid in 19 out of 65 species), anatabine (only in *N. otophora, N. tomentosa,* and *N. tomentosiformis*), and anabasine (the third most important alkaloid) are the major alkaloids present in the genus [24,25].
After nicotine is carried to the lungs via smoke, the lung’s alveoli cells function as a pipe to extract nicotine quickly and condense it into the pulmonary vein in a concentrated dose sometimes called the bolus or lump. Further, the concentrated dose of nicotine moves from the lungs to the left ventricle of the heart, where it is pumped through the arteries throughout the body. The small size of nicotine molecules along with their high degree of solubility in both water and fat, enables nicotine to pass quickly through the membrane’s blood-brain-barrier. Nicotine is absorbed more slowly when it is delivered by way of the mouth instead of the lungs, where the absorption is rapid regardless of pH [26]. The absorbed nicotine further is received by the nicotinic acetylcholine receptor (nAChR), which is the prototype ligand-gated channel (LGLC) that comprise an α- and β- subunit with many variants and is mostly found in brain and central nervous system (CNS) [27].

Metabolism of nicotine takes place in the liver, where the molecule is degraded into few metabolites, and cotinine being the major form in mammalian species. In human body, approximately 70 to 80% of nicotine is formed into cotinine, and the pathway involves two steps. CYP2A6 initiates the first step of the metabolism by producing nicotine-Δ^1(5')-iminium ion, which is in balance with 5'-hydroxynicotine. The second step is an oxidation process by a cytoplasmic aldehyde oxidase (Figure 7).
There is no further metabolization of nicotine N’-oxide to any other metabolites, but it is believed that it is reduced back to nicotine in the intestine that may lead to recycling nicotine in the body. Besides the oxidation of the pyrrolidine ring, nicotine is also metabolized by two nonoxidative pathways, methylation of the pyridine nitrogen called N-methylnicotinium ion, and by glucuronidation. Cotinine goes through a further metabolization and forms 3’-hydroxycotinine, which is detected in smokers’ urine [28]. Cotinine is considered as the best biomarker for tobacco smoking and exposure measurements, and it can be measured in urine, saliva, hair, nails and blood serum, since it persists longer in blood serum due to its longer half-life of approximately 16-hours, compared to the nicotine’s shorter half-life (3-5hrs), it is the body fluid used for further investigations, but its further metabolism is slower than nicotine [29]. There are many factors that the metabolism of nicotine is influenced by, such as diet, age, pharmacokinetics of nicotine, gender, pregnancy and menstrual cycle, kidney disease,
medications interactions (such as inhibitors and inducers), smoking habits, ethnicity differences [30].

**Early History of Rickets, Vitamin D and Its Discovery**

Rickets is a bowed legs disorder of bones caused by soft bone that is mostly observed in toddlers living in urban or in northern latitudes. In 1650, an English physician, Francis Glisson described rickets “the English Disease”, as it was most common in children from the age of six months to two and a half years young, which became more common in England during the late 1700s with the start of industrial revolution.

Later, in 1800s Armand Trousseau the French physician, suggested that cold, sunless climates or poor diet were the causes of the disorder. During same era time in Poland, Jedrzej Sniadecki noticed that children in Warsaw were more likely to suffer from the bone disorders than those in the countryside. He believed that the lack of sunshine was the cause of bowed legs condition. Theobald Palm, one of the Edinburgh Missionary Medical Society physicians also noticed in countries, where abundant sunshine was rare, rickets was less obvious, but common in large cities. Finally, in 1890, based on the answers to his questionnaires sent to his fellow missionaries in China and Tibet, he concluded that the toddlers raised in filthy homes or consumed poor food did not develop rickets, if they were breastfed and spent time outside in the sun. The conclusion of all these observations at different places during different times, led to search for the cause of rickets and was the path to the discovery of vitamin D [31].
Therefore, in 1919 the investigation of Sir Edward Mellanby eventually led to the acknowledge of nutrient deficiency disorder, rickets. In 1922, McCollum and his team found that cod fish oil was almost identical to the prior discovered fat-soluble vitamin A, except that the oil maintained its anti-ricket particularity after degradation of the vitamin A by heating and aeration. Consequently, including the vitamin A, the oil also was comprised of a new fat-soluble vitamin that in 1925 it was named as, vitamin D. During the same period, Zucker and his colleagues unveiled that vitamin D was present in the unsaponifiable fraction of the cod liver oil, and proposed that it was closely related to cholesterol, which led to an recognizing the function of vitamin D from the discovery of its biologically active metabolite [32].

There are two kinds of vitamin D, one is cholecalciferol that is formed in skin and is noted as vitamin D\textsubscript{3}; and the other form is ergocalciferol that is the product of ultraviolet irradiation in mushrooms also known as vitamin D\textsubscript{2} (Figure 8).

![Figure 8: Nutritional forms of Vitamin D.](image)
The structure of the vitamin is considered seco-steroid, where the B-ring is cleaved and the A-ring is rotated. Both forms have same affinity to bind to the calcitriol receptors (also known as Vitamin D Receptor “VDR”) in target tissues [33].

**Photosynthesis of Vitamin D**

Skin is the organ where the vitamin D₃ synthesis occurs. It is in the epidermis layer of skin that the 7-dehydrocholesterol is formed through a chemical, non-enzymatic reaction by the absorption of sunlight radiation on conjugated 6,7-diene in the B-ring, resulting the ring to open at the C9-C10 conjugation to form the pro-vitamin D₃ at the wavelength of 290-315nm. Since the pro-vitamin D₃ is biologically inactive and thermodynamically unstable, it undergoes isomerization to form the pre-vitamin D₃. Between the epidermis and the dermis, the pro-vitamin D₃ distribution is identical, but since sunlight penetrates the epidermis surface first nearly ninety-percent (90%) of the total pre-vitamin D₃ is made in the epidermis during a single extended exposure time to the sun. Usually in Malpighian layer the synthesis of pre-vitamin D₃ reaches a plateau at about 10 to 15% of the initial 7-dehydrocholesterol concentration. At this point, the pre-vitamin instead of converting to vitamin D₃ undergoes photo-isomerization that concludes in formation of two biologically inactive products, lumisterol and trachysterol ([Figure 9]) [34].
Metabolism, Regulation and Catabolism of Vitamin D

The metabolism of vitamin D$_3$ is a series of hydroxylation phases, in which one product is more polar than the former hydrophobic substrate. The initiation happens in the liver, where most of the vitamin taken up by the organ via Vitamin D Binding Protein (DBP) is hydroxylated by the presence of enzyme 25-hydroxylase that acts on Carbon-25 and yields 25-hydroxy-vitamin D or calcidiol “25-OH-D$_3$”. The calcidiol is the main circulating metabolite in plasma, as well as the best indicator of the vitamin’s level. Previous investigations have shown that several enzymes including CYP2R1, CYP27A1, CYP3A4, CYP2D25 are thus far the 25-hydroxylases responsible for 25-hydroxylation of vitamin D$_3$ (Figure 10).
The CYP2R1 (subcellular location: microsomal) is believed to play the major responsibility in the hydroxylation of vitamin D₃ in liver [35]. The enzyme CYP27A that has a Km of 10⁻⁵ M, catalyzes the hydroxylation of cholecalciferol twice as fast as ergocalciferol, and requires ferredoxin and ferredoxin reductase for activity, whereas the enzyme CYP2D25 with a Km of 10⁻⁷ M only acts on cholecalciferol, and not ergocalciferol. The second step of the metabolism takes place in kidneys, where the 25-OH-D₃ goes through the next hydroxylation at the carbon-1 of the A-ring with presence of the enzyme 1α-hydroxylase, which the CYP27B1 (subcellular location: mitochondrial) is believed to be the enzyme responsible for this phase of hydroxylation. Upon successful processing, the 1α,25-dihydroxy-vitamin D₃ or calcitriol (1α,25(OH)₂D₃) is
formed, which is known as the active metabolite of vitamin D$_3$ (or the hormone). During all the phases the CYPs function dependently to the presence of electron transport chains [36]. The 25-hydroxylase activity appears to be only poorly regulated, primarily by the hepatic concentration of vitamin D$_3$, with little or no inhibition by 25-OH-D$_3$.

Vitamin D$_3$ regulation is affected by tight regulator of the activity of the 1-hydroxylase by several factors such as 1α,25(OH)$_2$D$_3$, parathyroid hormone (PTH), calcitonin, circulating calcium and phosphate as well as some hormonal regulation by estrogen. It is increased by inducers of CYP and is inhibited by isoniazid. The catabolism of vitamin D$_3$ (further regulated by PTH, serum phosphate and other factors) continues further via more hydroxylation, to the extent that there are 947 derivatives of both the D$_2$ and D$_3$.

The active metabolite of vitamin D$_3$ is the one with therapeutic properties and performs its biological effects via genomic pathway. On target tissues the 1α,25(OH)$_2$D$_3$ binds to a specific nuclear receptor, also known as VDR that is found in a wide variety of different cell types. Once entered into a target cell, with the presence of Retinoid X Receptor “RXR” it binds to Vitamin D Response Element “VDREs” inside the cell, and this is how the genomic function of the active metabolite is further continued [37,38].

**Smoking, Vitamin D and Health**

There have been few epidemiological and experimental studies in the search for effect of tobacco smoking on vitamin D, and the therapeutic effects of vitamin D$_3$ on Lung Cancer, Chronic Obstructive Pulmonary Disease (COPD) and other diseases. Yet, the area of research in this field is relatively new compared to other studies such as breast and prostate cancers. A cross sectional study conducted in Denmark on the smoking effect on serum vitamin D on healthy women in the ages of 45 to 58 years-old
with different smoking lifestyles, revealed that the levels of 25-OH-D₃ significantly had reduced in smokers, and the smokers and non-smokers showed no difference in serum ionized calcium [39]. Another cross-sectional on investigation in China on 348 patients undergoing coronary angiography, showed that the smoking and the severity of Coronary Heart Disease (CHD) appeared to be substantially stronger among patients with severe hypo-vitaminosis D, as compared with those with higher vitamin D₃ levels. It suggested vitamin D₃ sufficiency may have a shielding effect against the damaging effects of smoking on coronary arteries [40]. Another cohort study on 626 Normative men between the years of 1984 and 2003 indicated that lower lung function was associated with vitamin D₃ deficiency, and smokers with a longer history of smoking had more rapid lung function declined in this cohort of elderly men. This study suggested that vitamin D₃ sufficiency may have a protective effect against the damaging effects of smoking on lung function [41]. Another study conducted in Brazil on healthy adult women, classified as smokers, ex-smokers and never smokers indicated that the smoking habit of the subjects was associated with cardiac remodeling, and excess of vitamin D₃ was associated with the action of smoking on cardiac variables. Thus, higher vitamin D₃ values have a deleterious effect on the heart in this model [42]. Another study in Korea on the effects of smoking on vitamin D₃, showed that the Cigarette Smoking Extract (CSE) had the inhibition capability on vitamin D-induced receptor translocation, but mitogen activated protein kinase (MAPKs) was not activated in the inhibition. The result of this study showed that after five-minutes treatment of 1α,25(OH)₂D₃ induced translocation of VDR from nucleus to microsomes by a dose-dependent manner. CSE inhibited 1α,25(OH)₂D₃ induced translocation of VDR in both
concentrations of 10% and 20%. All MAPKs inhibitors did not suppress the inhibitory effects of CSE on the $1\alpha,25$(OH)$_2$D$_3$ induced translocation of VDR [43]. Another study in China on 194 male patients with ischemic stroke suggested that the depression observed in the patients may be due to the lower vitamin D$_3$ caused by smoking [44].

**Issues and Topics for Research**

As the debate over tobacco with industry supporters, tobacco lawyers, tobacco smokers, and anti-tobacco groups continues, the issues and questions surrounding tobacco smoking and its health concerns have become increasingly clear. One question regarding further research interests has been around the cause and effect of smoking. Granting smoking cigarettes cause various lung illnesses, it does not follow that smoking cigarettes is the sole cause of that illness. Thus, as Philip Morris (PM) executives have argued, “cancer victims suing PM should have to prove that cigarettes, rather than any other possible cause, were the proximate cause of their disease” [45]. In an investigation performed by Boffetta et al., it was demonstrated that the cotinine level in blood serum is an indicator of lung cancer risk among smokers, and the carcinogenicity of smoking does not differentiate between the genders [46].
Conclusion

Throughout the history it has been shown that tobacco has been part of the human civilizations from ancient Mayans to modern Europeans. From cultural practices to social events smoking tobacco has been adopted into our lifestyle. As part of the further development and discoveries in science and medicine it has been also shown that smoking tobacco has negative effect on human health and wellbeing, which can cause serious diseases including lung cancer and COPD.

The discovery of vitamin D₃ in 1919 also introduced a new chapter in prevention of rickets and opened a new door to its therapeutic properties for other diseases, including cancers. Vitamin D₃ deficiency is also a serious global epidemic concern as well. Since 1919 up until now there have been many clinical and epidemiolocal studies performed globally on the effect of the vitamin on prevention of other diseases including but not limited to breast cancer, prostate cancer, colon cancer, autoimmune disorders, cardiovascular diseases, osteoporosis, calcium metabolism and potentially other disorders, yet there are not enough research has been done on the effect of vitamin D₃ on lung cancer, or the effect of smoking on the vitamin D₃ levels.

The goal of this dissertation topic is to further investigate how cigarette smoking may affect vitamin D₃ status amongst women with diverse ethnic backgrounds.
References


CHAPTER 2

NUTRITION EPIDEMIOLOGY Part - I

Effect of Serum Cotinine on Serum 25-hydroxyvitamin D₃ Using

NHANES 2001 – 2006 Datasets

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Introduction

The first major epidemiological study that took place in the United Kingdom (U.K.), conducted by Doll and Hill in 1950, showed that the tobacco smoke has carcinogenic effect [1]. In the United States (U.S.) the statistical data in 2018 published by Centers for Disease Control and Prevention (CDC) showed that in adults aged 18 years or older (13.7%), 14 out of every 100 Americans currently smoked cigarettes. According to the national data, 12% were adult women about 12 of every 100 adult women, and the smoking was more prevalent among the ages of 25 to 44 years-old (16.5%), and ages 45 to 64 years-old (16.3%). Nearly 15 of every 100 smokers were non-Hispanic blacks (15%), 15 of every 100 smokers were non-Hispanic whites (15%), nearly 10 of every 100 Hispanics (9.8%), nearly 23 of every 100 non-Hispanic American Indians and Alaska natives (22.6%), about 19 of every 100 non-Hispanic multiple race individuals (19.1%), and about 7 of every 100 non-Hispanic Asians (does not include Native Hawaiians or Other Pacific Islanders) (7.1%) [2].

According to the American Cancer Society 2020 surveillance research on Cancer Prevention and Early Detection Facts and Figures, the current cigarette smoking percentage among women has been in the age group of 25-44 (14%) and 45-64 years old (14%), with 14% being whites, 12% being blacks and 7% being Hispanics. Although since 1990 the percentage of cigarette smoking trends among adults over the age of 18 years old up till 2018 has been on declining trends, yet the current e-cigarette uses percentage trend since 2011 to 2019 has been on an incline trend, especially among whites men and women [3].
It is obvious that the primary cause of global mortality and victimizing over 120,000 people annually in the U.S. by itself is the lung cancer, some studies have shown some relations between the lung cancer and vitamin D [4,5,6]; but no further investigation on the possible effect of cotinine on vitamin D or conversely. In a study it showed that the vitamin D deficiency was common in the US population, especially among minority groups blacks and Hispanics knowing that the vitamin D deficiency was linked to some of the important risk factors of leading causes of death in the nation [7].

Previous epidemiology studies showed that the self-reported smoking habits and status has been widely used to assess detrimental health effects of smoking and to orient counselling and other preventive interventions. However, it can be unreliable if the subjects are under pressure because of social or medical disapproval. Furthermore, the quantity of smoke products inhaled and absorbed varies by the manner of smoking. Because of these difficulties, increased emphasis has been placed on measuring exposure through the use of biological markers to provide more accurate estimates of smoking status and of the dose received. Therefore, serum cotinine may be a better method of quantifying risks from cigarette use in epidemiological studies [8,9].

Therefore, the purpose of this chapter is to utilize serum cotinine and to investigate further a potential correlation between this biomarker to vitamin D status among adult women in the nation.

National Health and Nutrition Examination Surveys “NHANES”

National Health and Nutrition Examination Survey, or NHANES is a program of the National Center for Health Statistics (NCHS), which is part of the CDC supervision in the nation since 1960s. The program has utilized the assessment of health and
nutritional condition of noninstitutionalized citizens in the population. It represents a sample of 5,000 individuals living in different counties across the nation. It is unique since the combination of interviews and physical examinations, including but not limited to demographic, dietary and laboratory tests is the foundation of the datasets [10,11].

**NHANES Sample Design and Data Collection**

The process of data collection began with series of questionnaires conducted at the participants home, followed by physical examination at the Mobile Examination Center (MEC), where a series of physical measurements, blood and urine specimens were collected for laboratory testing. NHANES has used a probability sampling design in a multi-stage and complex format to select a sample representative of the civilian noninstitutionalized citizens population of the U.S., whom the subject represents around 50,000 other U.S. citizens. The first stage was the selection of the primary sampling unit, or individual counties. The national counties were all divided into 15 categories based on their representations, and one county was selected from each category, where they formed 15 counties in the NHANES surveys for each year. The second stage was the selection of segments within the counties. Within each county, smaller groups were formed with many households in each group, then 20 to 24 of these small groups were chosen. On the third stage dwelling units, or households were selected. All the residents within the selected small groups were identified, with a sample selection of approximately 30 households in each group. The fourth, the selection of individuals within a household by a computer program selecting some, all, or none of the household members randomly (Figure 1).
Due to some public health interests, NHANES was designed to represent large statistics of certain subgroups, and in some cases oversampling (Blacks and Mexican-Americans) was done to surge the reliability and accuracy of the health status indicators for these population subgroups [11]. Utilizing these datasets enabled this investigation to study the effect of cotinine on 25-OH-D$_3$ trends for statistical analysis.

Figure 1: Welcome NHANES Participants. How was I selected?

Materials and Methods

The continuous NHANES surveys from 2001 to 2006 were used, in which data were collected in two-year cycles (NHANES 2001-2002, NHANES 2003-2004, and NHANES 2005-2006). The continuity of the datasets has allowed statistical estimates and flexibility for larger groups and specific ethnicity groups in the form of questionnaires and exam components annually. Each cycle has data sets of participants which includes demographics data (Gender, Age, Race/Ethnicity), laboratory data (Vitamin D, Cotinine) and few other data such as dietary and physical examination. All these 6 years were merged together to construct a single file for the
analysis. Each participant is assigned a unique identifier known as sequence number (SEQN), which helped to make sure that all the observation and results are matched for each participant correctly. After all datasets were merged, the datasets were reviewed for those participants that had either missing data (such as “refused to answer”, or their response was “don’t know”), since the missing data may have misrepresented the analysis results. “For NHANES 2001-2002, there were 13,156 participants selected for the sample, 11,039 of them were interviewed (83.9%), and 10,477 (79.6%) were examined in the MEC unit. For NHANES 2003-2004, there were 12,761 participants selected, 10,122 of them were interviewed (79.3%) and 9,643 (75.6%) were examined in the MEC. For the NHANES 2005-2006 10,348 individuals of all ages were participated in the sample collection, but no further details were available on the interview and MEC”.

Response variable (Vitamin D (nmol/L)) and explanatory variable (Cotinine (ng/mL)) were measured from 2001 to 2006 where the three data sets were combined. Age, race/ethnicity and gender were used as additional explanatory variables (Table 1).

Table 1: Variables of interest for the Nutrition Epidemiology investigation.

<table>
<thead>
<tr>
<th>Years</th>
<th>Vitamin D (nmol/L)</th>
<th>Cotinine (ng/mL)</th>
<th>Gender (M/F)</th>
<th>Age (Years)</th>
<th>Ethnicity (White, Black, Hispanic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001-2002</td>
<td>LBDVIDMS</td>
<td>LB2COT</td>
<td>RIAGENDR</td>
<td>RIDAGEYR</td>
<td>RIDRETH1</td>
</tr>
<tr>
<td>2003-2004</td>
<td>LBDVIDMS</td>
<td>LBXCOT</td>
<td>RIAGENDR</td>
<td>RIDAGEYR</td>
<td>RIDRETH2</td>
</tr>
<tr>
<td>2005-2006</td>
<td>LBDVIDMS</td>
<td>LBXCOT</td>
<td>RIAGENDR</td>
<td>RIDAGEYR</td>
<td>RIDRETH3</td>
</tr>
</tbody>
</table>

“The purpose of the NHANES datasets was to be used to evaluate risks for deficiencies and toxicities of specific nutrients in the population, to provide accurate data, and to
estimate the contribution of diet, supplements, and other factors to serum levels of nutrients. The data was used for research to further define nutrient requirements and optimal levels for disease prevention and health promotion.”

**NHANES 2001-2006 Laboratory Data**

**Vitamin D (nmol/L)** - Vitamin D samples were collected from all the participants from the age of 1-year and older at MECs. The Diasorin assay was used for the extraction and measurement of the vitamin. First, the 25-OH-D₃ metabolite was extracted from serum with acetonitrile, then it was assayed using RIA antibody method. The samples, antibody and tracer were all incubated at 20-25°C for 90-minutes. The samples after being processed, were stored under appropriate frozen temperature at -30°C, and were transferred to the Division of Laboratory Sciences, National Center for Environmental health, Centers for Disease Control and Prevention located in Atlanta, Georgia for further analysis. The quality assurance (QA) and quality control (QC) throughout the process is assured by the 1988 Clinical Laboratory Improvement Act mandates, as well as all recommended QC procedures by manufacturers were followed. All the data were reviewed, and incomplete or improbable values were sent to the performing laboratory for re-confirmation.

**Cotinine (ng/mL)** - Cotinine is the major metabolite of nicotine and because of its more stable half-life it is preferred metabolite for smoking assessment compared to the nicotine’s shorter half-life. It is used to measure the frequency and extent of tobacco use (an active smoker) and exposure (as passive or second-hand smoker). The samples for the analysis of cotinine were collected from participants 3 years of age or older, who did not meet any exclusion criteria. The serum cotinine was measured by ID
HPLC-APCI MS/MS technique (isotope dilution-high performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry), and its concentrations are derived from the ratio of native to labeled cotinine in the sample by comparisons to a standard curve. For the years 2003-2004 and 2005-2006 the serum cotinine detection limit was 0.015 ng/mL, and the below the limit of detection value was 0.011 ng/mL, whereas in 2001-2002 there were two detection limits and below the limit of detection values. One of the detection limits was 0.05 ng/mL and the below the limit of detection value was 0.035 ng/mL. The other detection limit was 0.015 ng/mL and the below the limit of detection value was 0.011 ng/mL. There were no changes to the equipment or lab site for all these years. The quality assurance (QA) and quality control (QC) throughout the process is assured by the 1988 Clinical Laboratory Improvement Act mandates, as well as all recommended QC procedures by manufacturers were followed.

**NHANES 2001-2006 Demographic Data**

The demographics information was collected during the in-home interviews, and prior to the health examination, and CAPI (Computer Assisted Personal Interviewing) methodology software was used, with pre-programmed data edit and consistency checks alerting the interviewer for unusual or possible false values recorded. The demographics provides interview questions with the information such as gender, age, race/ethnicity, education, marital status, country of birth, pregnancy status, household size, total family and household income, and ration of income to poverty. After the data collection completion, later the collected data was reviewed by the field office reviewers for accuracy and completeness. For the nature of this investigation age (19-49 years of
age), gender (female) and race/ethnicity (Non-Hispanic White, Non-Hispanic Black, Hispanic American and Other ethnicities are the three variables used (Figure 2). The two groups of Mexican Americans and Other Hispanics were combined to represent the Hispanic population in throughout the nation. All the required guidelines for the data and variables are provided in (Appendix B).

**Figure 2:** Participants Selection Criteria for the investigation.

**Statistical Analysis**

Data were analyzed using Stata 14 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). Demographic characteristics and blood serum levels of participants were described using average values and standard deviations for continuous variables and proportions for categorical variables. Pearson's correlation coefficients and Ps were reported to examine the bivariate relationship between cotinine and vitamin D for both the total sample and subsamples stratified by race/ethnicity (White, Black, and Hispanic).
Results

The analysis of the outcome indicated that there is a correlation between cotinine and vitamin D amongst the women with different ethnicities. There is a statistically significant and negative relationship between cotinine and vitamin D for the total participants \((n=4,272)\) with the \(P<0.01\), White \((P<0.0001)\), Black \((P<0.01)\) with higher correlation compared to Hispanic \((P<0.96)\) and Other ethnicity \((P<0.51)\) (Table 2), (Figures 3-7).

Table 2: Correlation between Cotinine and Vitamin D among Women.

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>Correlation Coefficient (Rho)</th>
<th>p-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>4,272</td>
<td>-0.047</td>
<td>&lt;.01</td>
<td>**</td>
</tr>
<tr>
<td>White</td>
<td>1,877</td>
<td>-0.119</td>
<td>&lt;.0001</td>
<td>***</td>
</tr>
<tr>
<td>Black</td>
<td>956</td>
<td>-0.123</td>
<td>&lt;.01</td>
<td>**</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1,241</td>
<td>-0.001</td>
<td>0.967</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>198</td>
<td>0.046</td>
<td>0.519</td>
<td></td>
</tr>
</tbody>
</table>

Note: *\(p<.05\), **\(p<.01\), ***\(p<.001\)

Figure 3: Cotinine vs. Vitamin D Correlation in Black Women.
Figure 4: Cotinine vs. Vitamin D Correlation in Hispanic Women.

Figure 5: Cotinine vs. Vitamin D Correlation in Other Women.
Figure 6: Cotinine vs. Vitamin D Correlation in All Women.

Figure 7: Cotinine vs. Vitamin D Correlation in White Women.
Discussion

The evidence from previous NHANES III data from 1988-1994 among adults over the age of 35 years-old study revealed that gender, ethnicity, taking vitamin D supplements, physical activity, alcohol consumption, geographical region, body mass index and other factors were all associated with different levels of vitamin D among the participants in this study [12]. The prevalence of hypovitaminosis D from previous NHANES reports did not account for assay changes and drifts over time. Thus, published NHANES reports on vitamin D status for the U.S. population were likely either over- or underestimated. The comparison between NHANES 1988-1994 (n=18,641) with 2001-2006 (n=23,424) revealed that the recent decline in vitamin D status in the nation is more likely due to increased prevalence of lifestyle changes such as limited presence of vitamin D in foods, decreased exposure of UV-B radiation from sunlight, obesity and other potential lifestyle changes [13]. The outcome from previous NHANES III also showed that there was a strong relationship between serum concentration of vitamin D and pulmonary function after the data was adjusted for age, gender, ethnicity, and smoking [14]. Most of the cohort studies conducted in the U.S. have shown that vitamin D deficiency is more common in the black ethnicity compared to Hispanics and whites since the skin pigmentation, melanin is the essential factor, and more prevalence in women [15]. In the investigation let by Benowitz et al., it showed that the Non-Hispanic black ethnicity had a slower cotinine metabolism compared to its Non-Hispanic white counterparts for both the nicotine and cotinine oxidative metabolism [16]. In this investigation based on the NHANES statistical analysis it showed that the increased of cotinine in serum also played a factor in decreased serum 25-OH-D$_3$ levels amongst the
women with different ethnicity as well. The analyses demonstrated that among all three smoking categories, black female active smokers have lower vitamin D (13.37 ng/ml), than Hispanic (19.21 ng/ml) or white (24.92 ng/ml) females. It was demonstrated that the active smoker black females have the highest percentage of vitamin D deficiency and inadequacy in the population compared to other ethnic females. As shown in Table 2, the level of cotinine, overall, is significantly and negatively associated with the level of vitamin D ($\rho=-.047$, $P<.01$). When stratified by race/ethnicity, this association remains statistically significant for White females ($\rho=-.119$, $P<.0001$) and Black females ($\rho=-.123$, $P<.01$), but not for Hispanic females ($\rho=-.001$, $P=.967$). A European cohort study performed by Baltar et al., showed that not only tobacco smoking has a direct impact on cotinine level, but also other non-smokers who are exposed to tobacco smoking (also known as passive smoker or second-hand smoker), have increased level of cotinine level as well [17].

In another study led by Brot and his team, they also showed that the calcium and vitamin D metabolism were affected by smoking significantly [18]. One study such as the one by Afzal et al., showed that the higher risk of tobacco-related cancers were associated with lower plasma vitamin D levels, but not with risk of other cancers [19], and other studies such as the one done by Cheng et al., showed that vitamin D intake was associated with a lower lung cancer risk in non-smoking and postmenopausal women [20].

There are many epidemiological studies that showed the effect of tobacco smoking on increasing cotinine levels, and the effects of smoking on bone density and bone fractures; Including many studies that showed the effect of low vitamin D on
COPD and Lung Cancer, and the advantage of taking vitamin D in lowering the risk of the cancer. But, yet not so many investigations have been completed to demonstrate if there is a correlation between cotinine and vitamin D or vice versa.

**Conclusion**

The blood serum cotinine concentrations can also affect vitamin D concentrations in addition to other previously investigated and known factors such as gender, ethnicity, dietary supplement intake and sun exposure. Further research is necessary to better understand how cotinine may affect the vitamin D status among women with different ethnic backgrounds and smoking habits.
References


CHAPTER 3
NUTRITION EPIDEMIOLOGY Part - II
Effect of Serum Cotinine on Serum 25-hydroxyvitamin D$_2$, 25-hydroxyvitamin D$_3$, Total Vitamin D (25-OH-D$_2$ and D$_3$) and COPD Symptoms Using NHANES 2007 – 2014 Datasets Among Women of Different Ethnic Backgrounds
Introduction

The tobacco epidemic is amongst the worst public health threats the world has ever known. The 23rd, 31st and 33rd World Health Assemblies of World Health Organization (WHO) focused on the effect of tobacco smoking as a growing problem of public health in most of the industrialized as well as the developing countries. Among the risk groups affected by the negative health impacts of smoking are pregnant women, lactating mothers, children, and youth. Over time, increasing evidence has shown that smoking is a primary cause of many health concerns, such as chronic obstructive pulmonary disease (COPD) including chronic bronchitis and emphysema, lung cancer, and several other serious health problems [1,2,3].

Nicotine in tobacco, is a major component in commercial products and is extremely addictive. Nicotine is recognized as a general risk factor in many devastating health conditions causing death to millions annually worldwide. Tobacco smoking also has harmful effects on non-smokers who are exposed to tobacco smoke involuntarily through Second-Hand Smoke (SHS) exposure. It has also been implicated in adverse health outcomes to those non-smokers causing 1.2 million deaths annually. The National Health Interview Survey 1996-2010 and the Medical Expenditure Panel Survey 1998-2011 data show considerably higher cost of health care associated with obesity and smoking among non-Hispanic white women compared to men, ethnic minorities, and younger counterparts associated with smoking, and it was non-discriminatory across gender, ethnicity and age [4,5,6]. After nicotine enters the body, it transforms into a major by-product metabolite called Cotinine. Measuring cotinine concentration is the most reliable way of determining the level of exposure to nicotine in both
Environmental Tobacco Smoke (ETS) exposed smokers and SHS-exposed non-smokers. It is also the preferred method of measuring nicotine exposure because of its longer half-life, specifically in blood serum (~16 hours) compared to urine, saliva, and hair [7,8].

Of the one billion smokers of the world, approximately 200 million of the smokers are women, as they are more targeted by the tobacco industry, as they tend to smoke “light” cigarettes more than men. In some countries more investigation is needed to better understand the potential trend toward the increasing use of tobacco among women [9].

The National Center for Health Statistics (NCHS) is part of the Centers of Disease Control and Prevention (CDC) organization. It is the principal health statistics agency in the country, which runs the National Health and Nutrition Examination Surveys (NHANES) program. It is a program that evaluates and reviews the health and nutritional status of populations in the United States. The NHANES survey is unique because it combines attributes such as interviews and physical examinations. NHANES is a major program with the responsibility for producing vital and health statistics for the nation. The previous data from NHANES showed that women had higher nicotine metabolite ratios in serum compared to their male counterparts among both smokers and non-smokers in the order of Non-Hispanic white more than Non-Hispanic black women, and had lower nicotine metabolites in urine. These data can be further used to study differences in how nicotine is metabolized by women who are smokers and non-smokers [10].
The 2010 “World No Tobacco Day” sponsored by WHO emphasized the marketing of tobacco in relation to gender. The objective of this event was to further discuss the harmful effects of tobacco marketing toward women [11]. Smokers and second-hand smokers are more susceptible to COPD incidents than non-smokers, and the number of women smokers developing COPD is rapidly increasing. Therefore, women who smoke are at enhanced risk of emerging potentially fatal COPD. In industrialized countries, the prevalence of COPD is now almost as high in women as it is in men [12]. It is a serious public health dilemma that makes breathing difficult for 16 million Americans and applies to a disease that causes airflow blockage and breathing-related illness, with millions more yet undiagnosed and untreated. In 2008, chronic lower respiratory diseases such as COPD became one of the third major cause of mortality [13,14]. Because smoking is the main cause and the leading risk factor for COPD, in 2000 to 2004 it impacted to about 80% of COPD deaths, however much of this disease is potentially preventable by quitting to smoke. But the CDC reports from 2017 showed that the occurrence of COPD was greater among women and older adults than men [15,16]. Symptoms of COPD include frequent coughing, wheezing, whistling, excess phlegm or mucus, shortness of breath and trouble taking a deep breath [17].

According to one study that analyzed the National Health Interview Survey (NHIS) data during 1999 to 2011, the prevalence of COPD among non-Hispanic whites contrasted with non-Hispanic blacks and Hispanics was generally higher. And in women the annual age-adjusted prevalence was higher than in men, especially among younger women [18]. A few other studies showed that a high proportion of smoking-related pulmonary patients with COPD falsely declared themselves to be non-smokers, and
those who claimed to be non-smokers continued to smoke. The accurate classification of smoking status is vital to the treatment of lung diseases [19,20]. However, not only cigarette smoking but SHS exposure could also affect the progression of the disease as well. Yet, the importance of the impact of exposure on COPD health outcomes remain unclear [21].

It is likely that nutritional status of COPD patients impacts the outcome of the disease. One element of the nutritional status is the amount of saturated fat intake; however, the correlation between lung function and fat intake remains to be determined unclear. As vitamin D is a known fat soluble substrate, the results from a NHANES 2007-2012 study previously demonstrated benefits of increased demand for macronutrient substrates with potential anti-inflammatory activity. Overall intakes of fat in COPD patients could theoretically benefit the individuals. It was observed that an increase in lung function was associated with saturated fatty acids (SFA) intake in individuals with COPD. However, specific associated mechanisms are yet not yet identified [22]. Previously it was shown that vitamin D intake was associated with lower lung cancer risk in non-smoking, post-menopausal women [23]. It also has been observed that low levels of vitamin D and its deficiency are common among COPD patients, and women who currently smoke have lower dietary intakes of vitamin D than non-smokers [24,25].

Vitamin D can be either acquired from dietary sources or produced in the body. Dietary vitamin D is available in two forms, vitamin D₂ (ergocalciferol) from botanical sources and vitamin D₃ (cholecalciferol) from animal sources, both of which are collectively labeled as vitamin D. However, foods containing natural vitamin D and
dietary sources generally account for a small amount of the total vitamin in the body. The primary source of vitamin D in the form of vitamin D₃ can be produced from its non-enzymatic dermal synthesis from 7-dehydrocholesterol in the skin by exposure to ultraviolet (UV) light, mainly from light in UV-B range (280-315 nm) [26,27]. All the modern-day medical innovations notwithstanding, the deficiency of vitamin D continues to be a global pandemic affecting citizens in both developed and developing countries. The deficiency of the vitamin not only causes bone disorders and fractures in adults, but it also has been associated with other diseases such as cancers, autoimmune diseases, hypertension, and infectious disease [28,29].

Brot et al., (1999) performed a cross-sectional study in Denmark on 45 to 58-year-old women (n=510) with different smoking lifestyles that showed the effect of smoking on serum vitamin D. The smokers had significantly decreased levels of the serum vitamin D metabolite, 25(OH)D₃, suggesting that smoking holds a considerable influence on the vitamin metabolism [30]. Cutillas-Marco et al., (2012), in a cross-sectional investigation, revealed, that smoking was associated with the risk of vitamin D deficiency among southern European Caucasian men and women participants (n=177). The mechanism that explains the effects of cigarette smoking on vitamin D metabolism remains unclear, but it eventually exaggerates bone loss in smokers [31]. The result of NHANES-III from 1988 to 1994 data analyses conducted by Black et al., (2005) regarding the association of vitamin D levels and pulmonary function in people over the age of 20-years old (n=14,091), showed there was a strong relationship between serum levels of vitamin D and the volume of air one can exhale (pulmonary function) [32]. There have been other studies that showed the beneficial effects of vitamin D on
respiratory health. One study that analyzed NHANES 2001-2006 data showed a significant association of lower vitamin D levels with respiratory symptoms and with COPD [33]. In Belgium, studies done by Janssens et al., (2009) (n=414) and Mekove et al., (2016) (n=152) revealed that vitamin D deficiency occurs frequently in COPD patients and correlated with the disease severity as well. Patients admitted to hospital with COPD for exacerbation are a risk group for vitamin D deficiency and insufficiency with stronger prevalence in women than in men [34, 35]. Many epidemiological and experimental investigations have shown possible effects of smoking on vitamin D; however, none of these studies used the serum levels of cotinine as a marker for smoking. Because the concept that vitamin D is associated with chronic disease not involving calcium metabolism is comparatively new, and less is understood about the potential mechanisms relating the vitamin to the disease.

According to Mercy Medical Center report in 2018, approximately 42% of the US residents are vitamin D deficient, with some people such as pre-menopausal women, those with poor nutrition habits, people at the age of 65 and older, non-Hispanic whites who avoid even minimal sun exposure, and those who take prescription medication long term for heartburn, acid reflux, and constipation having even higher levels of deficiency [36]. A NHANES study in 2015 indicated that there was a potential correlation between the levels of cotinine and vitamin D among women with different ethnic backgrounds. There was a statistically significant and negative relationship between these metabolites. The correlation is higher for non-Hispanic white and non-Hispanic black women with a higher correlation compared to Hispanic and other ethnicities. The study demonstrated that in women smokers, non-Hispanic black women had lower vitamin D
(13.3 ng/mL) levels, than Hispanic (19.2 ng/mL) or white (24.9 ng/mL) women and the low vitamin D correlated to higher levels of cotinine. Therefore, increased blood serum levels of cotinine, or a higher rate of smoking, may have contributed to decreased vitamin D levels in addition to other known factors such as diet, amount of sun exposure, gender, and ethnicity [37].

The aim of this investigation is to continue the analysis of the effect of smoking on vitamin D status and COPD symptoms by using serum cotinine as the biomarker of tobacco use. In addition to the strong evidence presented in previous epidemiological studies, this investigation will propose potential answers to these questions: Question #1 – Does an increase in cotinine levels predict a decrease the vitamin D levels in blood serum among women? Questions #2 – Does cotinine have a negative association with both vitamin D₂ and vitamin D₃ equally in women? Question #3 – How are Cotinine and Vitamin D levels influencing symptoms of COPD? for the correlation between smoking and vitamin D.

Materials & Methods

The continuous NHANES surveys from 2007 to 2014 collected data in two-year cycles (NHANES 2007-2008, NHANES 2009-2010, NHANES 2011-2012, and NHANES 2013-2014). The continuity allocates annual statistical estimates for broad groups and specific ethnicity groups as well as flexibility in the content of the questionnaires and exam components. Each cycle has data sets from participants, which includes demographics data (Gender, Age, Ethnicity), laboratory data (vitamin D “vitamin D₂, D₃, total vitamin D” and cotinine) and other data such as diet behavior/nutrition, and
respiratory health questionnaire data (Table 1). In the ethnicity category there were both Mexican-Americans and other Hispanics, these two were combined to represent the Hispanics category. The entire NHANES dataset is publicly available and no individuals can be linked to any records.

All the eight years of continuous datasets were merged to create a single file for the statistical analysis purposes. Each participant is assigned a unique identifier known as Sequence Number (SEQN), which assures that all the observations and results are properly linked for each participant. After merging, the data were reviewed for those participants that had missing data (such as refused to answer, or the response was “don’t know”), since the missing data may distort the analysis results. The final sample size for this investigation is \( n=5729 \) women (Non-Hispanic White \( n=2274 \), Non-Hispanic Black \( n=1175 \), Hispanic \( n=1585 \), and other races, including multi-racial \( n=695 \)) (Table 3). Men were not included in this investigation so the outcomes would be comparable to the pattern in our previous epidemiology study.

Table 1: NHANES Datasets and Variables of interest.

<table>
<thead>
<tr>
<th>Years</th>
<th>Laboratory Data</th>
<th>Demographics Data</th>
<th>Questionnaire Data</th>
<th>Dietary Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007-2014</td>
<td>25 OH ( \text{D}_2 ) (nmol/L)</td>
<td>Gender (F)</td>
<td>Respiratory Health</td>
<td>Dietary Supplement Use 30-Day - Total Dietary Supplement (( \text{D}_2 + \text{D}_3 )) – “microgram µg”</td>
</tr>
<tr>
<td></td>
<td>25 OH ( \text{D}_3 ) (nmol/L)</td>
<td>Age (Years of Age)</td>
<td>(# wheezing or whistling attacks; Bring up phlegm most days; Coughing most days;)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total Vitamin D ( (\text{D}_2 + \text{D}_3) ) (nmol/L)</td>
<td>Ethnicity (White, Black, Hispanic)</td>
<td>Diet Behavior &amp; Nutrition (How healthy is the diet?)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cotinine (ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RDQ080: #wheezing or whistling attacks past year
RDQ050: Bring up phlegm most days (3 months period)
RDQ031: Coughing most days (over 3 months period)

The values of vitamin D\textsubscript{2} and D\textsubscript{3} were converted from nmol/L to ng/mL following the recommended conversion equation by CDC below for parallel comparison with cotinine units in ng/mL (Table 2).

**Table 2:** Conversion of Vitamin D values from nmol/L to ng/mL.

| Conversion (D3 “25OHD3”): | 1 nmol/L = 0.40066 ng/mL |
| Conversion (D2 “25OHD2”): | 1 nmol/L = 0.41266 ng/mL |

**Table 3:** Number and Percentage of Participants by the Ethnicity 2007-2014.

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Number of Participants (n)</th>
<th>Total Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Hispanic White</td>
<td>2274</td>
<td>39.69</td>
</tr>
<tr>
<td>Non-Hispanic Black</td>
<td>1175</td>
<td>20.51</td>
</tr>
<tr>
<td>Hispanics</td>
<td>1585</td>
<td>27.67</td>
</tr>
<tr>
<td>Others</td>
<td>695</td>
<td>12.13</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td><strong>5729</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

The participants were further sub categorized based on the total blood serum levels of their vitamin D and cotinine. Based on the cotinine levels they were categorized into three groups of Non-smokers/Minor SHS ([Cotinine] <1 ng/mL), Light-smokers/Some SHS (1 ng/mL<[Cotinine]<70 ng/mL) and Heavy-smokers/Major SHS ([Cotinine]>70 ng/mL) according to the Salimetrics® (Table 4 & Figure 1).
Table 4: Number and Percentage of Participants by the Cotinine (Smoking) Status.

<table>
<thead>
<tr>
<th>Smoking &amp; Exposure Status</th>
<th>Number of Participants (n)</th>
<th>Total Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers/Minor SHS – [Cotinine] &lt;1 ng/mL</td>
<td>3774</td>
<td>71.36</td>
</tr>
<tr>
<td>Light-smokers/Some SHS - 1&lt; [Cotinine] &lt;70 ng/mL</td>
<td>468</td>
<td>8.85</td>
</tr>
<tr>
<td>Heavy-smokers/Major SHS - [Cotinine] &gt;70 ng/mL</td>
<td>1047</td>
<td>19.8</td>
</tr>
<tr>
<td>Total:</td>
<td>5289</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 1: Blood serum cotinine levels in population subgroups.

Also, based upon the vitamin D status, they were categorized into the three groups of deficient, inadequate, and sufficient based on the National Institute of Health (NIH) Office of Dietary Supplements (Table 5 & Figure 2).
Table 5: Number and Percentage of Participants by the Vitamin D Status.

<table>
<thead>
<tr>
<th>Vitamin D Status</th>
<th>Number of Participants (n)</th>
<th>Total Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficient (&lt;12 ng/mL)</td>
<td>555</td>
<td>10.85</td>
</tr>
<tr>
<td>Inadequate (12&lt;Vit.D]&lt;20 ng/mL)</td>
<td>1393</td>
<td>27.23</td>
</tr>
<tr>
<td>Sufficient (20&lt;Vit.D]&lt;50 ng/mL) (Over Upper-Limit)</td>
<td>3041</td>
<td>59.45</td>
</tr>
<tr>
<td></td>
<td>(126)</td>
<td>(2.46)</td>
</tr>
<tr>
<td>Total:</td>
<td>5115</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 2: Total blood serum 25-OH-Vitamin D levels in population subgroups.

Analysis of Covariance (ANCOVA) model was employed to include the continuous variable in addition to the variables of interest, both the dependent and independent variables, as means for control.
Scheffe’s method\textsuperscript{iv} was used to test all possible contrasts at the same time. It applies to the set of all possible contrasts among the factor level means, not just the pairwise differences considered by Tukey’s method\textsuperscript{v}, which it applies simultaneously to the set of all pairwise comparisons. Binomial regression linear model\textsuperscript{vi} was used to predict the odds of seeing an event, given a vector of regression variables. The Bonferroni\textsuperscript{vii} test is a type of multiple comparison method used in statistical analysis. It is a test method used to reduce the instance of a false positive. The family-wise error rate\textsuperscript{viii} was also used to determine the probability of making at least one false conclusion.

Relative Risk (RR)\textsuperscript{ix} is often used when the study involves comparing the likelihood, or chance, of an event occurring between two groups. Relative Risk is considered a descriptive statistic, not an inferential statistic as it does not determine statistical significance. Relative Risk utilizes the probability of an event occurring in one group compared to the probability of an event occurring in the other group. It requires the examination of two dichotomous variables, where one variable measures the event (occurred vs. not occurred) and the other variable measures the groups (group 1 vs. group 2).
Results

Question #1 – Does an increase in cotinine levels predict a decrease in the total vitamin D levels in blood serum among women?

An ANCOVA model was constructed modeling total serum vitamin D level against cotinine level. The model included adjustment for the subject age, ethnicity, as well as a measure of diet behavior and nutrition (NHANES variable DBQ700 “How healthy is the diet?”). Scheffe’s method was employed to control the family-wise error rate for comparisons among subgroups in the model. In the total 5,279 women ages 22-49 in the 2007-2014 NHANES cohort 5,115 subjects had the required values of cotinine and vitamin D measurements available and were included in the model. It was found that among the non-Hispanic white heavy-smoker group, the higher level of cotinine was associated with a decrease in vitamin D levels of -1.03 ng/mL (95% CI (-1.87 ng/mL, -0.18 ng/mL)), $P < 0.0001$) relative to the non-Hispanic white non-smoker group versus the Hispanics $P=0.56$ and non-Hispanic black $P=0.12$ heavy-smoker and non-smoker groups. (This value is the mean difference in vitamin D levels [ng/mL] between the heavy-smoker ([Cotinine] > 70 ng/mL) non-Hispanic white group and the non-smoker ([Cotinine] < 1 ng/mL) non-Hispanic white group. It is a comparison between two groups: a factor level coefficient in the ANCOVA model. It does not imply that there is a -1.03 ng/mL mean difference in vitamin D levels per 1 ng/mL increase in cotinine. Hence, we believe that the units are correctly assigned as currently written). In summary, per the outcome, the data show that the elevated levels of cotinine are associated with decreased total vitamin D ($D_2 + D_3$) levels (Table 6 & Figure 3).
Table 6: Results Summary of the Laboratory Data Variables.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of subjects (n)</th>
<th>Mean Vitamin D$_2$ [ng/mL]</th>
<th>Mean Vitamin D$_3$ [ng/mL]</th>
<th>Mean Total Vitamin D [ng/mL]</th>
<th>Cotinine [ng/mL]</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic White</td>
<td>2274</td>
<td>1.25</td>
<td>31.30</td>
<td>31.58</td>
<td>66.12</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Non-Hispanic Black</td>
<td>1175</td>
<td>1.69</td>
<td>17.92</td>
<td>19.05</td>
<td>53.43</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1585</td>
<td>1.17</td>
<td>22.11</td>
<td>22.60</td>
<td>16.16</td>
<td>0.0361</td>
</tr>
<tr>
<td>Smoking Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smoker/Minor SHS</td>
<td>3774</td>
<td>1.35</td>
<td>26.17</td>
<td>26.72</td>
<td>0.07</td>
<td>(Reference)</td>
</tr>
<tr>
<td>Light smoker/Minor SHS</td>
<td>468</td>
<td>1.24</td>
<td>22.97</td>
<td>23.51</td>
<td>21.90</td>
<td>0.4586</td>
</tr>
<tr>
<td>Heavy smoker/Major SHS</td>
<td>1047</td>
<td>1.28</td>
<td>25.33</td>
<td>25.84</td>
<td>249.16</td>
<td>0.0374</td>
</tr>
</tbody>
</table>

*p-values reported for ANCOVA model of Vitamin D3.

Figure 3: Effect of cotinine concentration on total vitamin D$_2$+D$_3$ concentration.
Question #2 – Does cotinine have a negative association with both vitamin D\textsubscript{2} and vitamin D\textsubscript{3} equally in women?

No association between cotinine status and vitamin D\textsubscript{3} levels were identified for the Hispanic ($P=0.43$), non-Hispanic black ($P=0.12$) or other race groups ($P=0.56$). No association between cotinine status and vitamin D\textsubscript{2} levels were identified in the cohort. A plot of vitamin D\textsubscript{3} levels by cotinine status is shown in the figure below (Figure 4-6). Note that non-significant trends were observed for all categories except for the non-Hispanic white ethnic group. Non-smoker/Minor SHS: mean $[D_3] = 21.7$ ng/mL; Standard Error = 1.59 ng/mL; Heavy-smoker/Major SHS: mean $[D_3] = 21.4$ ng/mL; Standard Error = 1.64 ng/mL

The $[D_3]$ difference between heavy-smoker/Major SHS and non-smoker/Minor SHS (main effect of Cotinine level) was non-significant ($P=0.256$). It is the interaction between cotinine level and ethnicity that was significant ($P=0.004$) and is the notable finding in this investigation.

It is not surprising that the main effect of cotinine level on vitamin D\textsubscript{3} concentration was not significant. The significant interaction effect in the presence of a non-significant main effect indicates the presence of a crossover effect by ethnicity. That is, the observed difference in average vitamin D levels across smoking status for some ethnicities are opposite that of some other group. This can be visualized in the figure below:
Figure 4: Vitamin D₃-Cotinine correlation in different ethnicities.

Figure 5: Effect of cotinine concentration on vitamin D₂ concentration.
Figure 6: Effect of cotinine concentration on total vitamin D₃ concentration.

Note that among the non-Hispanic white population, a decline in vitamin D₃ levels was observed for heavy-smokers relative to non-smokers; whereas a (non-significant) increase was observed for the “Other Races” and “Hispanic category”. This crossover effect washes out the presence of an overall mean difference in the main effect of smoking.
Question #3 – How are Cotinine and Vitamin D levels influencing symptoms of COPD?

A negative binomial generalized linear model was constructed regressing the self-reported number of wheezing and whistling attacks that participants experienced against vitamin D$_3$ levels. The model included adjustment for subject age, ethnicity, cotinine level, diet behavior and nutrition. Bonferroni’s method was employed to control the family-wise error rate for comparisons among subgroups in the model. The hypothesis of interest was that there is a significant association between COPD symptoms and vitamin D levels, after adjusting for other factors that might alter COPD symptoms. The cotinine level is in the model as a proxy variable for smoking status. This allows to make the assertion that there is an association between vitamin D levels and COPD symptoms independent of smoking status. The need for adjustment using cotinine is represented by the Directed A-cyclic Graph (DAG) below (Figure 7):

![Directed A-cyclic Graph](image)

**Figure 7:** Directed A-cyclic Graph (DAG).
The green arrow is the hypothesis of interest. There is an established relationship between smoking and cotinine concentration, and the ANCOVA model suggests a relationship between smoking and vitamin D. If some measure of smoking status (i.e. cotinine concentration) were not included in the model, it would be unclear whether the change in COPD symptoms were associated with vitamin D levels or due to a latent smoking variable which was also altering vitamin D status. With cotinine concentration in the model it can be said that, even with taking smoking status into account, vitamin D appears to present a relationship with COPD symptoms. Among the total group of the participants 546 individuals had measurements available and were included in the model. Vitamin D₃ showed a protective effect against wheezing and whistling; a 10 ng/mL decrease in vitamin D₃ was associated with an increased risk of wheezing and whistling attack (relative risk (RR) 1.33 (95% CI (1.03,1.07), \( P=0.02 \)). No effect of vitamin D₂ status on RR of wheezing and whistling attacks were identified. A plot of expected wheezing and whistling attacks by vitamin D₃ level is shown in the figure below (Figure 8).
Figure 8: Number of wheezing & whistling attacks versus serum vitamin D₃ levels.

Given the SAS application limitation in calculating the CI, instead Relative Risk (or rate ratio “IRR”) was used to measure the strength of the association between vitamin D₃ and number of wheezing and whistling attacks in the past year.

\[
\text{IRR}(D₃) = 0.97197 ≥ (1/0.97197)^{10} = 1.328\quad \text{IRR for 10 unit decrease in vitamin D₂}
\]

CI: lower bound = \((1/\exp(-0.0034))^{10} = 1.034\), upper bound = \((1/\exp(-0.0535))^{10} = 1.707\)

Per the outcome, there appears to be association between vitamin D₃ levels and number of wheezing and whistling attacks, which means the participants with lower vitamin D₃ levels experienced higher numbers of wheezing and whistling attacks. There was observational evidence that vitamin D₃ was associated with an increase in number of wheezing and whistling attacks after adjusting for smoking status, age, diet, and ethnicity. Although there was no evidence of association between vitamin D₃ (either
lower or higher levels) with bringing up phlegm most days and coughing most days, there was marginal evidence ($P < 0.1$) of an association between vitamin D$_2$ with bringing up phlegm and coughing. If more datapoints were available, we may have observed a stronger association; but there was no evidence of association between vitamin D$_2$ with number of wheezing and whistling attacks. A negative binomial model to account for overdispersion of the data and considered some other secondary effect combinations, which improved the fit. It still appears that there is an effect of ethnicity on the protective effect of vitamin D$_3$ against whistling and wheezing fits; although after controlling for multiple testing it is not possible to identify exactly the source of this relationship.

As for the heavy-smoker/Major SHS vs non-smoker/Minor SHS vitamin D$_3$ levels, the mean is indeed lower in the Major SHS group (mean [D$_3$] = 23.8 ng/mL) vs Minor SHS (mean [D$_3$] = 23.96 ng/mL). The difference in the means appears to be result of more non-Hispanic white participants in the heavy-smoker/Major SHS vs non-smoker/Minor SHS. Hence the higher levels of vitamin D$_3$ observed on average suggest that there are other factors that influence D$_3$ (ethnicity, age, diet). It is important to account for the differences in these variables among the groups before considering the vitamin D$_3$ levels. Therefore, we analyzed the effect of smoking groups on vitamin D$_3$ using a linear model. The heavy-smoker/Major SHS group has a less healthy diet (15.10% in “Excellent” diet category) vs non-smoker/Minor SHS (78.06% in “Excellent” diet category), (Table 7). Data also show, most critically, that the heavy-smoker/Major SHS group is (59.98% non-Hispanic white) vs. non-smoker/Minor SHS group (35.06% non-Hispanic white), (Table 8).
Table 7: How healthy is the diet among non-smokers, light-smokers, and heavy-smokers.

<table>
<thead>
<tr>
<th>SMOKING &amp; EXPOSURE STATUS</th>
<th>DBQ700 (HOW HEALTHY IS THE DIET)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Excellent</td>
</tr>
<tr>
<td>NON-SMOKERS/MINOR SHS</td>
<td>Excellent</td>
</tr>
<tr>
<td></td>
<td>268 (7.36%)</td>
</tr>
<tr>
<td>LIGHT-SMOKERS/SOME SHS</td>
<td>Excellent</td>
</tr>
<tr>
<td></td>
<td>30 (5.00%)</td>
</tr>
<tr>
<td>HEAVY-SMOKERS/MAJOR SHS</td>
<td>Excellent</td>
</tr>
<tr>
<td></td>
<td>53 (5.06%)</td>
</tr>
</tbody>
</table>

Table 8: Ethnicity Distribution Among the Smoking Groups.

<table>
<thead>
<tr>
<th>Smoking &amp; Exposure Status</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-Hispanic White</td>
</tr>
<tr>
<td>Non-smokers/Minor SHS</td>
<td>Hispanics (33.41%)</td>
</tr>
<tr>
<td>Light-smokers/Some SHS</td>
<td>Hispanics (23.93%)</td>
</tr>
<tr>
<td>Heavy-smokers/Major SHS</td>
<td>Hispanics (10.89%)</td>
</tr>
</tbody>
</table>
The conclusion is that the mean [vitamin D₃] among heavy smokers is significantly higher than among non-smokers for the Non-Hispanic White cohort. The difference in the number of people in each ethnic group does impact the statistical analysis but not in a way that we have identified would bias the effect estimates. The manner in which the unequal sample sizes between ethnic groups impacts the analysis is with respect to power: the power to detect a significant difference in mean [vitamin D₃] among heavy smokers vs. non-smokers is reduced as the sample size decreases. Hence, we would expect that the power to detect a difference would be lowest for the Other Ethnicities group and highest for the Non-Hispanic White group. It is possible that, given a larger sample of non-Hispanic White subjects, other interesting relationships may have been identified.

For analysis of a particular ethnic group, the measurements and number of people in other ethnic groups do not impact the statistical estimates used for the difference test. Therefore, it does not appear bias should be present due to imbalanced ethnic groups for these effect estimates. As we know that ethnicity has a considerable influence on total vitamin D₃ levels, the ethnicity imbalance is the major contributor (Table 9).

Table 9: Mean and Standard Error Vitamin D₃ Comparison based on the Ethnicity.

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Vitamin D₃ Mean (ng/mL)</th>
<th>STANDARD ERROR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hispanic</td>
<td>21.17</td>
<td>1.645</td>
</tr>
<tr>
<td>Non-Hispanic White</td>
<td>29.62</td>
<td>1.613</td>
</tr>
<tr>
<td>Non-Hispanic Black</td>
<td>16.42</td>
<td>1.628</td>
</tr>
<tr>
<td>Other Ethnicities</td>
<td>21.72</td>
<td>1.724</td>
</tr>
</tbody>
</table>
All differences, excluding the difference between Hispanic and Other races, were significant after applying a Scheffe multiple testing adjustment ($P < 0.0001$ for all comparisons). Significant differences are visualized in the figure below (Figure 9).

![Figure 9](image)

**Figure 9:** Means covered by the same bar are not significantly different. i.e. the only non-significant difference is between “Hispanic” and “Other races”. Ethnic groups that share a line are not significantly different (only Hispanic and Other races are not different from each other). Values are reported in [vitamin D$_3$] ng/mL.

**Discussion**

Vitamin D can be obtained from different sources in two forms, vitamin D$_2$ (ergocalciferol) and vitamin D$_3$ (cholecalciferol). So, we have a combination of both available to us as part of our lifestyle, either from sunlight exposure, diet and dietary supplement, and fortified foods [38]. Vitamin D in the form of vitamin D$_3$ can be made from 7-dehydrocholesterol in the skin by exposure to ultraviolet light, mainly from light in UVB spectrum. Biancuzzo et al., (2013) showed that vitamin D$_2$ and D$_3$ both were
effective in raising and maintaining serum concentration of 25-OH-D. Vitamin D₂ is metabolized in a fashion similar to that of vitamin D₃ to both its 25-hydroxy and 1,25-dihydroxy metabolites [39]. One study showed the supplementation of vitamin D₂ produces appreciable amounts of serum 25-OH-D₂, with a lesser affinity for Vitamin D Binding Protein (DBP) and results in a shorter circulating half-life than that of 25-OH-D₃ [40]. Previously, it was initially thought that both forms of the vitamin follow the same metabolic pathway. However, minor differences in the chemical structure (chemistry of side chains) between the two forms of vitamin D result in differences in the site of hydroxylation, which ultimately leads to the production of active metabolites [41].

Bouillon et al., (2016) showed that there are many similarities between the transport and actions of both forms, but the true equivalency between the two is still questionable and more studies are required, before it can be said that vitamin D₂ is as a good an analog as vitamin D₃, rather than being truly bioequivalent [42]. Structurally, vitamin D₂ differs from vitamin D₃ in that its side chain has a C24 methyl group and a C22-C23 double bond that are responsible for the differences in oxidative processes occurring on the side chain relative to those observed for vitamin D₃ [43]. Therefore, as the data showed here, the reason that cotinine affects vitamin D₃ more than vitamin D₂ may primarily be due to the minor chemical structure between the two forms. Another reason that it was observed cotinine has more effect on decreasing vitamin D₃ level compared to vitamin D₂, may also be due to the explanation by a previous study which showed that vitamin D₃ is more effective than vitamin D₂ at raising total 25-OH-D levels and most assays of serum vitamin D do not distinguish between the two vitamers [44].
The pigmentation (melanin levels) found in the skin has been established as a physiologic mechanism by which non-Hispanic black and Hispanics are predisposed to vitamin D deficiency. More melanin absorbs and scatters ultraviolet (UV) rays from sunlight, which results in the less efficient conversion of 7-dehydrocholesterol to pre-vitamin D₃. In general women are lighter in skin pigmentation than their male counterparts, hence by natural selection it was suggested an attribute favored possibly due to greater vitamin D requirements during pregnancy and lactation [45,46,47]. Therefore, skin pigmentation is one of many determinants of vitamin D production, given significant competition between melanin pigments and vitamin D precursors in absorbing UVB radiation. Throughout human evolution our skin colors have evolved as an adaptation to surrounding climate, with persons of the darkest pigmentation having an origin near the equator and lighter-skinned populations arising in regions closer to the poles [48].

In a study done on Hispanic women (n=345) in low-income immigrant neighborhoods in New York City using data collected from Women’s Health Across the Nation FFQ, two dietary routines were identified, a healthy dietary routine high in vegetables, legumes, potatoes, fish and other seafood; and an energy dense dietary routine high in red meat, poultry, pizza, French fries and high-energy drinks. Among the two diets, the healthy dietary routine was positively associated with neighborhoods linguistic isolation, and negatively associated with neighborhood poverty. These results indicated that among Hispanic immigrants living in immigrant communities is associated with healthy dietary routines [49]. As the results indicated in our study, the heavy-
smokers/ Major SHS group declared less healthy diet compared to the non-smoking women.

Cigarette-smoking behavior differs substantially by race and gender in the United States among Non-Hispanic blacks and whites as demonstrated by the data analyses from National Youth Tobacco Survey 2004-2013, National Survey on Drug Use and Health 2002-2013, National Health Interview Survey 2004-2013 and NHANES 2001-2012 [50]. As previously reported in one study, higher levels of cotinine in blood per cigarette smoked by blacks compared with whites was explained by both slower clearance of cotinine and higher intake of nicotine per cigarette in blacks. Greater nicotine uptake (30% greater nicotine uptake in blacks than whites), and higher cotinine half-life in blacks than in whites, and therefore greater tobacco effects per cigarette could, in part, explain some of the ethnic differences in smoking-related disease risks [51].

In COPD patients, nutritional status is a well-recognized predictive indicator and poor nutrition was characterized by loss of fat-free body mass or muscle wasting. Many of the COPD patients are in a state of hyper metabolism, which expends more calories per kilogram, potentially caused by the increased work of breathing [52,53,54]. Previous study showed an association between an increased risk of vitamin D deficiency and COPD, and the characteristics of the disease were significantly related to 25(OH)D levels [55]. Prior investigations have shown that vitamin D has potentially positive effects on respiratory health. One such study that analyzed data from NHANES 2001-2006 showed that lower levels of vitamin D were significantly associated with respiratory symptoms and with COPD [56]. Another study that analyzed NHANES 2001-2010 data
also showed an association between vitamin D insufficiency and wheezing in adults after stratifying the analysis by ethnicity and current smoking; vitamin D insufficiency was associated with current wheeze in non-Hispanic whites and blacks women [57]. Women, since they are more likely to be significantly burdened by the symptoms of COPD than men, may benefit from more intensive and earlier management of COPD symptoms specifically wheezing, and this appears most pronounced among younger women [58]. Other studies also showed that COPD susceptibility was not associated with low vitamin D serum levels, but the COPD severity was inversely associated with vitamin D deficiency, as well as increased risk of COPD. The severity was in reverse association with the vitamin deficiency but not with COPD exacerbation [59,60]. It was suggested by one study to routinely monitor vitamin D levels in COPD patients and to consider vitamin D supplementation to those with vitamin D deficiency [61].

The data from these analyses showed that an increase in cotinine concentration is associated with a decrease in total vitamin D among women. There is evidence of an association between cotinine and vitamin D₃, but no evidence was observed as an association with vitamin D₂. Therefore, elevated cotinine levels are associated with a decrease in total vitamin D levels. There is evidence of an association between vitamin D₃ and RDQ080 (lower vitamin D₃ means higher numbers of coughing and wheezing episodes) after adjusting for smoking status, age, diet, and ethnicity. There is no evidence of an association between vitamin D₂ and RDQ080. We observed marginal evidence (P<0.1) of an association between vitamin D₂ and RDQ050 and RDQ031.
Conclusion

In addition to cotinine, other factors may affect vitamin D concentrations such as gender, ethnicity, dietary supplement intake and sun exposure. These data from NHANES 2007-2014 confirmed the previous conclusion from NHANES 2001-2006 investigation that smoking affects vitamin D levels among women. Further research is necessary to better understand whether vitamin D exerts beneficial effects on respiratory health in women since they are more likely to be significantly burdened by the symptoms of COPD.
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CHAPTER 4
In Vitro Investigation of Effect of Blood Serum Cotinine on Blood Serum Vitamin D Concentration in Russian Women.
Introduction

Based on the analysis of epidemiological data in the U.S. population provided by CDC, the metabolite of nicotine, cotinine, concentration in blood serum may have an effect on vitamin D blood serum concentration amongst non-Hispanic White, non-Hispanic Black and Hispanic women. That study was the first investigation that showed a correlation between cotinine and 25-OH-D₃ in human serum. The purpose of this chapter is to continue the investigation further. Therefore, this in vitro study was undertaken to demonstrate if this correlation is valid when these variables are measured for the purpose of comparison. Thus, we have obtained samples from a few Russian women who had donated blood to a tissue bank to further demonstrate if an increase in serum cotinine was associated with the decrease of the vitamin D serum level, utilizing the ELISA immunoassay technique.

After smoking, the blood concentration of nicotine rises quickly, and reaches its highest peak at the completion of smoking. Cotinine concentration in the serum is the predominant metabolite used as the biomarker of nicotine because of its longer half-life (~16 hours), compared to nicotine (~2 hours). The relationship between smoking and serum cotinine concentration was shown in another study where the active tobacco smoking increased the level of cotinine, and it was shown that passive smokers (second hand smokers) also had higher levels of cotinine in their serum [1]. Cotinine is an important metabolite in mammalian species, out of the six primary metabolites of nicotine, with a much slower metabolism than nicotine. Blood nicotine concentration is lower (between 10-37 ng ml⁻¹) compared to its metabolite, cotinine at 250-300 ng ml⁻¹. The relationship between the two metabolites was expressed based on steady state
exposure conditions “$D_{nic} = CL_{COT} \times C_{COT} \div f$”, where $D_{nic}$ is the intake (dose) of nicotine, $CL_{COT}$ is the clearance of cotinine, $C_{COT}$ is the steady state blood concentration of cotinine and $f$ is the fraction of nicotine converted to cotinine [2]. Cotinine is the product of C-oxidation of nicotine, and it is further metabolized via 3’-hydroxylation to form trans-3’-hydroxycotinine, and other metabolites such as 5’-hydroxycotinine, norcotinine, and cotinine N-oxide [3,4].

Figure 1: Metabolites of Cotinine.

The metabolic pathway of vitamin D is initiated in skin, and continues in liver, where the pre-vitamin D molecule is hydroxylated to form 25-hydroxyvitamin D$_3$. It is the major circulating form for the vitamin in the serum. Due to its stability this metabolite is
used to assess the clinical levels of the vitamin status, and the low circulating levels are associated with the vitamin deficiency [5,6].

![Metabolic Pathway of Vitamin D](image)

**Figure 2:** 25-hydroxyvitamin D₃ Metabolic Pathway.

This chapter is intended to investigate further the correlation between cotinine and 25-OH-D₃ concentrations in Russian Caucasian women’s serum samples.

**Materials and Methods**

(All the individual data, SOPs, Patient Consent Form and Ethics Committee Approval are available in Appendix-B).

The design of this chapter was to determine the correlation between 25-OH-D₃ and cotinine among smokers versus non-smoker Caucasian women’s serum samples, using ELISA (Enzyme-linked immunosorbent assay) kits for cotinine and vitamin D (25(OH)D₃).
Hypothesis: As the cotinine concentration increases in Caucasian Russian women who are heavy and light smokers, the vitamin D concentration decreases compared to the non-smoking women.

Materials:

Blood Serum Samples: The population of this study was a sample size of n=24 Russian women, with a smoking history of Non-smokers (n=10), Light/Passive smokers (n=4) and Heavy-smokers (n=10). All the subjects were classified as healthy donors with no medical history, between the ages of 27 to 47 years old, and the BMI range of 17.6 to 28.3 (Table 1). The serum specimens were procured in Moscow (Russia), and were purchased from ProteoGenex Inc, Culver City, CA 90230.

Table 1: Blood Serum Donors. Russian women with different smoking history.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Date of collection</th>
<th>Sex</th>
<th>Age</th>
<th>Specimen type</th>
<th>Volume</th>
<th>Ethnicity</th>
<th>Height, cm</th>
<th>Weight, kg</th>
<th>BMI</th>
<th>Patient diagnosis</th>
<th>Smoking history</th>
<th>Medical history</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Smokers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D4129S</td>
<td>08/02/2012</td>
<td>F</td>
<td>28</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>175</td>
<td>70</td>
<td>22.9</td>
<td>healthy donor</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>D6977S</td>
<td>10/02/2014</td>
<td>F</td>
<td>45</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>170</td>
<td>80</td>
<td>27.7</td>
<td>healthy donor</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>D5606S</td>
<td>11/14/2013</td>
<td>F</td>
<td>30</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>158</td>
<td>64</td>
<td>17.6</td>
<td>healthy donor</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>D6708S</td>
<td>08/02/2014</td>
<td>F</td>
<td>47</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>160</td>
<td>70</td>
<td>27.2</td>
<td>healthy donor</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>D4074S</td>
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<td>F</td>
<td>43</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>165</td>
<td>75</td>
<td>27.5</td>
<td>healthy donor</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>D4292S</td>
<td>02/23/2012</td>
<td>F</td>
<td>30</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>170</td>
<td>70</td>
<td>24.4</td>
<td>healthy donor</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>D4546S</td>
<td>01/14/2013</td>
<td>F</td>
<td>35</td>
<td>serum</td>
<td>2 x 1mL</td>
<td>Caucasian</td>
<td>158</td>
<td>67</td>
<td>26.8</td>
<td>healthy donor</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>D5789S</td>
<td>03/13/2014</td>
<td>F</td>
<td>28</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>152</td>
<td>48</td>
<td>20.8</td>
<td>healthy donor</td>
<td>2 years 0.5 pack/day</td>
<td>no</td>
</tr>
<tr>
<td>D6677S</td>
<td>08/07/2014</td>
<td>F</td>
<td>45</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>160</td>
<td>70</td>
<td>27.3</td>
<td>healthy donor</td>
<td>11 years 0.5 pack/day</td>
<td>no</td>
</tr>
<tr>
<td>D6810S</td>
<td>09/23/2014</td>
<td>F</td>
<td>47</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>158</td>
<td>67</td>
<td>26.8</td>
<td>healthy donor</td>
<td>13 years 0.5 pack/day</td>
<td>no</td>
</tr>
<tr>
<td>D7457S</td>
<td>03/16/2015</td>
<td>F</td>
<td>38</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>166</td>
<td>78</td>
<td>28.3</td>
<td>healthy donor</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Light Smokers</td>
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<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>152</td>
<td>48</td>
<td>20.8</td>
<td>healthy donor</td>
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<td>no</td>
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<td>Caucasian</td>
<td>160</td>
<td>70</td>
<td>27.3</td>
<td>healthy donor</td>
<td>11 years 0.5 pack/day</td>
<td>no</td>
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<td>D6810S</td>
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<td>47</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>158</td>
<td>67</td>
<td>26.8</td>
<td>healthy donor</td>
<td>13 years 0.5 pack/day</td>
<td>no</td>
</tr>
<tr>
<td>D7457S</td>
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<td>F</td>
<td>38</td>
<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>166</td>
<td>78</td>
<td>28.3</td>
<td>healthy donor</td>
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<td>Heavy Smokers</td>
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<td>F</td>
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<td>serum</td>
<td>3 x 1mL</td>
<td>Caucasian</td>
<td>163</td>
<td>72</td>
<td>27.1</td>
<td>healthy donor</td>
<td>6 years 1 pack/day</td>
<td>no</td>
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<tr>
<td>D7301S</td>
<td>12/24/2014</td>
<td>F</td>
<td>40</td>
<td>serum</td>
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<td>Caucasian</td>
<td>165</td>
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<td>27.5</td>
<td>healthy donor</td>
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<tr>
<td>D7313S</td>
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<td>serum</td>
<td>2 x 1mL</td>
<td>Caucasian</td>
<td>166</td>
<td>78</td>
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<td>38</td>
<td>serum</td>
<td>2 x 1mL</td>
<td>Caucasian</td>
<td>171</td>
<td>67</td>
<td>22.9</td>
<td>healthy donor</td>
<td>8 years 1 pack/day</td>
<td>no</td>
</tr>
<tr>
<td>D5508S</td>
<td>11/05/2013</td>
<td>F</td>
<td>31</td>
<td>serum</td>
<td>2 x 1mL</td>
<td>Caucasian</td>
<td>166</td>
<td>70</td>
<td>25.4</td>
<td>healthy donor</td>
<td>5 years 1 pack/day</td>
<td>no</td>
</tr>
<tr>
<td>D6533S</td>
<td>06/09/2014</td>
<td>F</td>
<td>45</td>
<td>serum</td>
<td>2 x 1mL</td>
<td>Caucasian</td>
<td>158</td>
<td>70</td>
<td>28</td>
<td>healthy donor</td>
<td>15 years 1 pack/day</td>
<td>no</td>
</tr>
<tr>
<td>D6716S</td>
<td>08/22/2014</td>
<td>F</td>
<td>45</td>
<td>serum</td>
<td>2 x 1mL</td>
<td>Caucasian</td>
<td>168</td>
<td>72</td>
<td>25.5</td>
<td>healthy donor</td>
<td>11 years 1 pack/day</td>
<td>no</td>
</tr>
<tr>
<td>D7380S</td>
<td>02/12/2015</td>
<td>F</td>
<td>45</td>
<td>serum</td>
<td>2 x 1mL</td>
<td>Caucasian</td>
<td>167</td>
<td>72</td>
<td>25.6</td>
<td>healthy donor</td>
<td>15 years 1 pack/day</td>
<td>no</td>
</tr>
<tr>
<td>D7422S</td>
<td>03/27/2015</td>
<td>F</td>
<td>40</td>
<td>serum</td>
<td>2 x 1mL</td>
<td>Caucasian</td>
<td>168</td>
<td>72</td>
<td>25.5</td>
<td>healthy donor</td>
<td>10 years 1 pack/day</td>
<td>no</td>
</tr>
</tbody>
</table>
Human Serum Cotinine ELISA Kit (96-Strip-wells): Two customized quantitative Sandwich ELISA kits (Item#MBS019457) with sensitivity of 1.0 ng/mL, and detection range of 6.25 ng/mL to 200 ng/mL were purchased from MyBioSource, Inc. San Diego, CA 92115.

Human Serum 25 Hydroxy Vitamin D3 ELISA Kit (96-Strip-Wells): Two quantitative Sandwich ELISA kits (Item#MBS042776) were purchased from MyBioSource, Inc. San Diego, CA 92115.

Method:

**Blood Serum Samples:** Undiluted serum or standards were added to each well in the ELISA kits, except the blank wells. The procedures for both assays were the same.

**Human Serum ELISA Kit (96-Strip-wells):**

**Assay Procedure** - The following steps were followed to prepare the process:

All reagents and samples were brought to room temperature (~20°C) for 30 minutes before starting the assay procedure. Standards and samples were applied to the plate in duplicate wells of a 96-well strip palate that had an immobilized antibody to cotinine or 25-OH-cholecalciferol in 50 µl aliquots.

We then added 100 µl of HRP-conjugate reagent to each well, except the blank well, covered the strips with an adhesive strip and incubated for 60 minutes at 37°C. The microtiter plate was then washed 4 times manually. The wash solution contained PBS (phosphate buffered saline) with the pH of 7.4-7.6, and Tween 20. For manual washing we removed the incubation mixture by emptying contents of the plate into a sink and, using a squirt bottle, filled each well completely with Wash Solution (1X), then emptied the contents of the plate into waste. This procedure was repeated for a total of
four times. After the final wash, the plate was blot dried by hitting the plate onto paper
towels until no moisture appeared.

We then added Chromogen Solution A (50 µL) and Chromogen Solution B (50 µL) to each well successively, gently mixed and kept the plate protected from light to
incubate for 15 minutes at 37°C. We then added 50 µL of Stop Solution to each well.
The color in the wells changed from blue to yellow because of the low pH. The plates
were read at the optical density (O.D.) of 450 nm with a plate reader within 15 minutes.
Results & Statistical Analysis

After running the ELISA kits on both Vitamin D and Cotinine for all the sera, the values shown below (Table 2) were determined at the end based on the O.D. 450 nm readings.

Table 2: Vitamin D (ng/mL) and Cotinine (ng/mL) values of participants.

<table>
<thead>
<tr>
<th>Smoking Habit</th>
<th>25(OH)D3 (ng/mL)</th>
<th>Cotinine (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy Smokers</td>
<td>30.73</td>
<td>87.64</td>
</tr>
<tr>
<td>Heavy Smokers</td>
<td>42.63</td>
<td>130.39</td>
</tr>
<tr>
<td>Heavy Smokers</td>
<td>15.36</td>
<td>137.72</td>
</tr>
<tr>
<td>Heavy Smokers</td>
<td>63.18</td>
<td>164.49</td>
</tr>
<tr>
<td>Heavy Smokers</td>
<td>20.61</td>
<td>30.92</td>
</tr>
<tr>
<td>Heavy Smokers</td>
<td>33.41</td>
<td>87.25</td>
</tr>
<tr>
<td>Heavy Smokers</td>
<td>9.05</td>
<td>214.01</td>
</tr>
<tr>
<td>Heavy Smokers</td>
<td>42.87</td>
<td>124.10</td>
</tr>
<tr>
<td>Heavy Smokers</td>
<td>21.85</td>
<td>167.74</td>
</tr>
<tr>
<td>Heavy Smokers</td>
<td>12.28</td>
<td>138.55</td>
</tr>
<tr>
<td>Light Smokers</td>
<td>41.89</td>
<td>125.67</td>
</tr>
<tr>
<td>Light Smokers</td>
<td>26.27</td>
<td>107.07</td>
</tr>
<tr>
<td>Light Smokers</td>
<td>10.58</td>
<td>165.79</td>
</tr>
<tr>
<td>Light Smokers</td>
<td>39.91</td>
<td>21.15</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>25.41</td>
<td>133.29</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>42.51</td>
<td>32.76</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>38.34</td>
<td>154.61</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>26.64</td>
<td>158.68</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>11.63</td>
<td>37.21</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>89.87</td>
<td>38.38</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>40.40</td>
<td>33.00</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>22.32</td>
<td>25.43</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>39.25</td>
<td>109.64</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>59.48</td>
<td>51.78</td>
</tr>
</tbody>
</table>

Also, the mean values and standard deviation (σ) for both cotinine and vitamin D of all the subjects were calculated, (Table 3), and were graphed (Figure 2). The comparison of the vitamin D means indicates that as the mean values of cotinine increased, the heavy-smokers and light/passive-smokers (both combined to represent Smokers
category) had a lower mean of vitamin D compared to the non-smoking women (29.22 ng/mL vs. 39.59 ng/mL). The vitamin D means were also compared to the National Institute of Health (NIH) Serum 25-OH-D3 Concentrations and Health, and since the mean vitamin D is greater than 20 ng/mL, on average the vitamin D levels in the subjects are considered adequate for bone and overall health in healthy individuals. But the vitamin D levels may not be considered adequate for preventions of development of diseases such as cancer and other chronic diseases as indicated in some studies [7,8,9].

Table 3: Mean values and Standard Deviation of both Cotinine and Vitamin D in ng/mL for Non-smokers, Light-Smokers and Heavy-Smokers.

<table>
<thead>
<tr>
<th>Mean vs. Mean</th>
<th>Mean Vit-D (ng/mL)</th>
<th>Mean Cot (ng/mL)</th>
<th>S.D. Vit-D</th>
<th>S.D. Cot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Smokers</td>
<td>39.59</td>
<td>77.48</td>
<td>22.07</td>
<td>54.96</td>
</tr>
<tr>
<td>Light Smokers</td>
<td>29.66</td>
<td>104.92</td>
<td>14.49</td>
<td>60.99</td>
</tr>
<tr>
<td>Heavy Smokers</td>
<td>29.22</td>
<td>124.22</td>
<td>15.65</td>
<td>52.60</td>
</tr>
</tbody>
</table>

The correlation coefficient indicated a negative relationship ($r = -0.31$, $P=0.15$) between the two metabolites for all the 24 subjects (Table 4, Figure 4).

Table 4: Pearson Correlation Statistics for Vitamin D and Cotinine in the $n=24$.

<table>
<thead>
<tr>
<th>Variable</th>
<th>With Variable</th>
<th>$n$</th>
<th>Sample Correlation</th>
<th>Fisher’s z</th>
<th>Bias Adjustment</th>
<th>Correlation Estimate</th>
<th>95% Confidence Limits</th>
<th>H0: Rho=Rho0</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-OH-D3</td>
<td>Cotinine</td>
<td>24</td>
<td>-0.305</td>
<td>-0.315</td>
<td>-0.006</td>
<td>-0.299</td>
<td>-0.626</td>
<td>0.118</td>
<td>0</td>
</tr>
</tbody>
</table>
A weak negative correlation exists between serum cotinine and vitamin D. This indicates that smoking may exert a negative influence on 25-OH-D$_3$ metabolite, yet this effect did not reach statistical significance, possibly due to small sample size, and the presence of other uncontrolled variations, such as age, diet, ethnicity and other factors.

**Discussion**

Previous studies have examined several factors affecting the vitamin D deficiency. On one of the studies previously identified that smokers have lower levels of 25-OH-D$_3$ and its active form 1,25-(OH)$_2$D$_3$ [8]. Also, other studies in contrast it was shown that again smoking was associated with poorer vitamin D status, along with excessive body weight in current women smokers compared to never smokers [10,11]. Another study showed that vitamin D insufficiency was common in childbearing-age women, and low circulating level of 25-OH-D$_3$ during late pregnancy was associated
with reduced bone mineral content during the childhood years, which, as many previous studies showed, will develop osteoporosis in adulthood [12,13].

In another study conducted by Benowitz et al., it was observed that in women the half-life of cotinine, independent from their ethnicity, was significantly lower compared to men [14]. This indicated that gender is a factor in the speed of metabolism of cotinine. In this investigation, we show that as the serum cotinine increased, the serum concentration of 25-OH-D₃ decreased as the smoking habit changed amongst the non-smokers, light-smokers, and heavy-smoker Russian women. Although it was shown that as the serum concentration of cotinine increases, the serum concentration of the 25-OH-D₃ decreases, however the increase of cotinine did not contribute to a major reduction in the level of 25-OH-D₃ amongst all three groups.

The relationship between just the heavy-smokers and non-smoking women was also measured, and there was no significant correlation between the two variables’ concentration in serum. Based on National Institute of Health – Office of Dietary Supplements classification, amongst the 24 samples, only 3 (1- heavy smokers, 1- light smoker, and 1- non-smoker) had low 25-OH-D₃, less than 12 ng/mL, that is considered deficient and associated with osteomalacia in adults. Amongst the 24 samples, only 2 (2- heavy smokers, 0- light smoker, and 0- non-smoker) had low 25-OH-D₃ less than 20 ng/mL (but higher than 12 ng/mL) that is considered inadequate for bone and overall health in healthy individuals in adults. For others, the 25-OH-D₃ was above or equal to 20 ng/mL which is generally considered adequate for bone and overall health in healthy individuals. Therefore, either other mechanisms mediate the influence of smoking
(cotinine) on serum vitamin D (25-OH-D₃), or the subjects in this study were misclassified according to their self-reported smoking habits.

Conclusion

A weak negative correlation ($r=-0.305$, $P=0.1472$, $n=24$) that was not statistically significant was observed between serum cotinine concentration and serum vitamin D concentration amongst all three categories of smoking. A 95% confidence interval for this population correlation coefficient correspond to $[-0.626, 0.118]$. The sample size for this investigation was small, therefore a larger sample size would be beneficial for better understanding of this correlation. Further studies are needed with greater control of nuisance variables to rule out whether serum cotinine concentration and serum vitamin D concentration are uncorrelated.
References


CHAPTER 5

Enzymes Interaction of Cotinine CYP2A6 and 25-OH-D₃ CYP2R1 and CYP3A4 In Human Hepatic Microsomes Cells and Its Gene Expression
Introduction

In chapter 3 it was suggested that the elevated levels of cotinine in serum may affect the concentration of vitamin D (25-OH-D₃) in the serum. Although it has been demonstrated by other researchers that age, gender, and diet effect on the levels of 25-OH-D₃ in the serum, cotinine may be another potential factor to be considered. The purpose of this chapter is to further investigate the potential of an enzyme-enzyme interaction between cotinine and vitamin D metabolism influenced by cytochrome P450s.

The Cytochrome P450 (or CYP450) gene family is a group of enzymes implanted primarily in the lipid bilayer of the liver cells and other cells throughout the body. The enzymes are involved in the synthesis and metabolism of various biochemicals within cells, such as metabolism of many drugs, steroids and carcinogens. Each gene is named with CYP to indicate its part of the CYP450 gene family, followed by a number associated with specific group within the family, and a letter representing the gene’s subfamily, and another number assigned to the specific gene within the subfamily [1,2].

25-OH-D₃ CYP2R1 Metabolism

The first step of vitamin D activation process occurs in the liver, where the hydroxylation happens on carbon-25, and forms the 25-OH-D₃ [3]. The formation of the vitamin in liver is mainly because of the hydroxylase enzymes activated in this process, including the CYP2R1, CYP27A1, CYP3A4, CYP2D11, CYP2D25 and perhaps others, but the CYP2R1 is the most important enzyme in the first process of activation (Figure 1).
Figure 1: Activation of Vitamin D in liver at the presence of CYP2R1 enzyme (25-hydroxylase) to form 25(OH)D₃.

CYP2R1 has shown high specificity for the C-25 position on vitamin D, but not other sterol substrates [4,5]. The enzyme is the key for the hydroxylation, and it could contribute to 25-OH-D₃ sufficiency status [6]. The discovery of CYP2R1 in 2003 ended the three decades of search for the hydroxylase responsible in vitamin D formation. It is a 501 amino-acid, liver microsomal mRNA, which hydroxylates both vitamin D₃ and vitamin D₂, and it is present mainly in the liver and testis. (The enzyme's mRNA is abundant in both tissues, and not so abundant in others). An inherited mutation (the change in leucine residue at position 99 to a proline, which eliminates the hydroxylase enzyme activity of the CYP) in the CYP2R1 gene also has been identified in people with low 25-OH-D₃ concentrations and rickets. The expression of CYP2R1 with CYP27B1 prompted to activation of vitamin D enzyme, 1α-hydroxylase, whereas the co-expression with 24 hydroxylase CYP24A1 caused inactivation, which confirmed that the
biochemical properties of CYP2R1 was more reliable as being the microsomal hydroxylase [7,8].

25-OH-D3 CYP2R1 Gene Expression

The CYP2R1 is a member of family number 2, in the R subfamily and the first member, located on the 11th chromosome (Figure 2). It has a quaternary homodimer structure, and its proteins are monooxygenase, which is a catalyzer to reactions in drug metabolism, synthesis of cholesterol, steroids and other lipids. It is the microsomal hydroxylase that converts vitamin D into the active ligands for the VDR. Previously it was demonstrated that the gene mutation was associated with 25-hydroxyvitamin D deficiency [9].

Figure 2: CYP2R1 Gene in Genomic Location on Chromosome-11.

Cotinine CYP2A6 Metabolism

Most of the nicotine, 70-80%, is metabolized into cotinine by the CYP2A6 enzyme. Cotinine is the major metabolite of nicotine via an oxidation process. With a half-life of ~16 hours (clearance average of 45 mL/min), it is a stable biomarker of its parent metabolite, so therefore, it is a valuable index in smokers. Cotinine ‘s further metabolism leads to trans-3’-hydroxycotinine formation, which is the major urinary metabolite of nicotine in tobacco users. It was demonstrated that CYP2A6 was responsible for the conversion of cotinine to trans-3’-hydroxycotinine catalyzation, which is a NADPH (Nicotinamide adenine dinucleotide phosphate) dependent reaction, with a
high activity for metabolizing nicotine and cotinine [10,11]. The metabolism of nicotine into cotinine occurs in two steps. During the first step, the oxidation of nicotine occurs in the presence of CYP2A6, where nicotine $\Delta^{1',5'}$-imminium ion is formed [12]. During the second step, the imminium ion at the presence of cytosolic aldehyde oxidase, is converted to cotinine (Figure 3) [13]. Approximately 4% of the total CYP450 enzyme protein is represented by CYP2A6 (with 494 amino acids) of adult liver microsomes [14]. In vivo investigation supported that CYP2A6 played a significant role in nicotine metabolism in the liver, and the nicotine’s urinary metabolites are derived from cotinine [15].

Figure 3: Metabolism of Nicotine to Cotinine at the presence of CYP2A6.

Cotinine CYP2A6 Gene Expression

The CYP2A6 is a member of family 2, in the A Subfamily A, and the 6th member, located on the 19th chromosome (Figure 4), with a quaternary structure (no recent data available). Its proteins are monooxygenase with catalyzer properties involved in reactions such as drug metabolism, synthesis of cholesterol, steroids and other lipids. It is in the endoplasmic reticulum and is known for its nicotine metabolization function.
Individuals with certain gene mutations were said to have a weak nicotine metabolizing efficiency [16].

![CYP2A6 Gene in Genomic Location on Chromosome-19.](image)

**Figure 4:** CYP2A6 Gene in Genomic Location on Chromosome-19.

**Materials and Methods**

**Metabolic stability in microsomes**

Pooled human microsomes (Sigma, St Louise, MO) were suspended in 66.7 mM potassium phosphate buffer (pH=7.4) with NADPH and treated with 30 ng/mL vitamin D with and without 300 ng/L cotinine. Tubes were incubated at 37 °C for 0, 5, 15, 30, 60, and 120 min in a water bath, and the reaction was quenched by adding 150 uL methanol. Disappearance of vitamin D was measured by a vitamin D ELISA kit (Abcam, Cambridge, MA).

**Cell culture**

The human cell line HepG2 (HB-8065) was obtained from American Type Culture Collection (ATCC) (Manassas, VA). Cells were routinely passaged every 3-4 days and maintained in high glucose DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA), and 1% penicillin-streptomycin (Fisher Scientific, Pittsburg, PA) at 37 °C and 5% CO₂. Cells were sub-cultured into 24-well plates and, once confluent, exposed to 30 ng/mL vitamin D with and without 300 ng/mL cotinine. Cell media was sampled at 0, 5, 15, 30, 60, and 120 min post exposure.
and disappearance of vitamin D was measured by the vitamin D ELISA kit (Abcam, Cambridge, MA).

**RNA extraction and qPCR**

The total RNA was isolated from cells using TRIzol reagent (Life Technologies, Carlsbad, CA). RNA was quantified using Synergy H1/Take 3 plate setup (BioTek). The cDNAs were synthesized using 2 µg of RNA for each sample using high-capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA) on an ABI GeneAMP 9700 (Life Technologies, Carlsbad, CA).

The resulting cDNA was amplified in duplicate by real-time quantitative PCR (qPCR) using SYBR green PCR Master Mix (Life Technologies, Carlsbad, CA) that is designed for quantitative real-time PCR using a set of two PCR primers that flank the target region. Primers were as follows: β-actin (housekeeping gene), forward primer: 5’-AGA GCT ACG AGC TGC CTG AC -3’, reverse primer: 5’- AGC ACT GTG TTG GCG TAC AG -3’; CYP3A4, forward primer: 5’- CCA AGC TAT GCT CTT CAC CG -3’, reverse primer: 5’- TCA GGC TCC ACT TAC GGT GC -3’; CYP2R1, forward primer: 5’- CAG CCT CAT CCG AGC TTC -3’, reverse primer: 5’- CCA CAG TTA ATA TGC CTC CA -3’. qPCR amplifications were performed on an ABI 7500 Fast real time PCR (Life Technologies) using 1 cycle at 50 °C for 2 min and 1 cycle of 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The dissociation curve was completed with 1 cycle of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. mRNA expression was analyzed using the ΔΔCT method and normalized with respect to the expression of the β-actin using ABI 7500 Fast System SDS Software v1.3.0 (Life Technologies).
Amplification of specific transcripts was further confirmed by obtaining melting curve profiles. All results were expressed as fold change from vehicle controls.

**Results**

Data (from rows 3-9 of table, Appendix-C) are grouped by microsomes 1&2, other microsomes, and hepatic cells. There is very little difference within these groups but statistically significant among the three groups. Below are the individual column regressions. In the graph below, X is the log of dose. The microsome 1 and 2 group has a strongly significantly nonzero slope. The other microsomes have a smaller but still significant slope but the HepG2 group shows no significant dose response. The data (all available at Appendix-C) from the cell culture investigation of microsome and HepG2 liver cells showed that cotinine appears to increase the metabolism of vitamin D. Cotinine activated the CYP enzyme and increased the metabolism of vitamin D in the liver, which resulted in the disappearance of the vitamin from blood (Figure 5).
Figure 5: Effect of Cotinine on Microsomal and Hepatocyte Vitamin D Metabolism.

There is a linear and negative relationship between the log of the concentration of vitamin D (X axis) and the average concentration of cotinine (Y axis). In other words, a lower level of vitamin D (X axis) is associated with a higher level of cotinine (Y axis). The data from gene expression experiments showed that at the presence of cotinine, mRNA expression of the CYP2R1 and CYP3A4 (metabolizing 25-OH-D₃ to 4β,25 (OH)₂D₃) enzymes increased. Such as at the presence of cotinine, within two hours there was a three folds increase of the both enzymes expression (Figure 6 & Figure 7). Consequently, the presence of cotinine upregulated both enzymes so less 25-OH-D₃ will be present in serum to go through the second vitamin D hydroxylation (1α,25 hydroxylation) in kidney to convert the 25-OH-D₃ to the active form 1α,25(OH)₂D₃ and complete the full vitamin D metabolism.
Figure 6: mRNA gene expression of CYP2R1 at the presence and absence of Cotinine.

Figure 7: mRNA gene expression of CYP3A4 at the presence and absence of Cotinine.
Discussion

Among the vitamin D hydroxylation enzymes in liver (CYP2R1, CYP3A4, CYP27A1 and CYP2J3), the CYP2R1 is the most likely candidate for the microsomal vitamin D 25-hydroxylase metabolic pathway. As demonstrated by Cheng et al., the CYP2R1, unlike other 25-hydroxylases, would hydroxylate both vitamin D₂ and vitamin D₃ equally well at physiologically relevant substrate concentrations. One other study demonstrated that vitamin D upregulated transcription of several hydroxylases such as CYP3A through the VDR pathway in hepatic cells (17). The gene expression experiments in this chapter demonstrated that at the absence of cotinine, the mRNA expression of these two genes are below two-fold, but with the presence of cotinine the mRNA expression increased to three-fold within the same time between 60 to 120 minutes in both scenarios. Therefore, the presence of cotinine in microsomes may have increased the activities of CYP2R1 and CYP3A further that may have decreased the bioavailability of 25-OH-D₃ in serum to further continue the metabolic pathway to the second hydroxylation (1α,25(OH)₂D₃ hydroxylase) step, where the 25-OH-D₃ is transformed into the active metabolite of calcitriol 1α,25(OH)₂D₃. As mentioned earlier the previous data suggested that CYP2R1 is a strong candidate for the microsomal 25-OH-D₃ hydroxylation by Cheng et al., 2003. On the other hand, in another study it was shown that the human hepatic microsomal CYP3A4 had the highest activity for 25-hydroxylase in both vitamin D₂ and vitamin D₃ as well [18]. In another study it was also shown that CYP3A4 is a 24-hydroxylase for vitamin D₂ and D₃ as well in the hepatic microsomes [19]. Therefore, induction of CYP3A4 gene expression by cotinine may enhance 25-OH-D₃ catabolism leading to vitamin D inactivation, and hence modulate
vitamin D₃ effects in body. CYP3A4 is considered to be the most important of the family of drug-metabolizing cytochrome P450 enzymes, contributing importantly to the clearance of perhaps half of therapeutic agents that undergo metabolic biotransformation [20].
Conclusion

Cotinine may induce CYP3A4 expression in the liver cells and microsomes and accelerate vitamin D catabolism and may contribute to vitamin D deficiency, although further investigation is required to demonstrate the mechanistic link between the effect of cotinine on the CYP2R1 and CYP3A4 to further clarify the impact of smoking on vitamin D status.
References


CHAPTER 6

Conclusions and Future Investigation Interests
In this dissertation, we explored how tobacco smoking may have affected vitamin D levels among women who are smokers, non-smokers and heavy-smokers, including those who come in contact with tobacco smoke passively. In order to study this further the nicotine metabolite, cotinine, was used as the biomarker to indicate smoking, and to determine its potential effect on vitamin D metabolism.

The first chapter provided a history of tobacco products use and smoking, a review of lung cancer statistics in the United States and the world, a history of vitamin D discovery and its deficiency, both nicotine and vitamin D metabolisms, and a summary of studies that have been conducted to investigate the correlation between smoking and vitamin D.

Based on the literature review pertaining to the effect of smoking on vitamin D levels, and the potential effect of vitamin D as a therapeutic agent on lung cancer and other respiratory diseases this research was further carried on into epidemiology and in-vitro studies.

The second chapter used the National Health and Nutrition Examination Surveys from 2001-2006, which are publicly available health data provided by Centers for Disease Control and Prevention (CDC). This chapter concluded that in addition to other previously investigated and known factors such as gender, ethnicity, dietary supplement intake and sun exposure the blood serum cotinine concentrations were negatively correlated with vitamin D$_3$ concentrations among different ethnic groups of women with different tobacco smoking lifestyles.

The third chapter used the National Health and Nutrition Examination Surveys from 2007-2014, which are publicly available health data provided by Centers for
Disease Control and Prevention (CDC). This chapter confirmed the findings from chapter two, and concluded that the blood serum cotinine concentrations continued to affect vitamin D₃ and total vitamin D (D₂ and D₃ combined) concentrations among different ethnic groups of women with different tobacco smoking lifestyles, but not as much effect was seen for decreasing vitamin D₂ concentration. This difference may be further explained due to the chemical structure difference between the two forms of the vitamin. Also, this survey was utilized to investigate the potential effect of vitamin D levels on COPD symptoms. The conclusion was that there is evidence of an association between vitamin D₃ and higher numbers of wheezing and whistling attacks after the data were adjusted for smoking status, age, diet, and ethnicity, and there is no evidence of an association between vitamin D₂ and numbers of wheezing and whistling attacks.

Chapter four further explored the outcomes of the previous two chapters’ epidemiological investigation in laboratory settings. In this chapter the non-Hispanic white women (n=24) blood serum samples were received from an outside provider. ELISA technique was used to investigate whether different serum levels of cotinine may have an effect on vitamin D₃ serum levels. A weak negative correlation (r=-0.305, P=0.14) was observed between serum cotinine concentration and serum vitamin D concentration amongst all three categories of smoking, but it was not statistically significant. The sample size for this investigation was small, thus so a larger sample size from carefully controlled subjects would be beneficial to better document this correlation.

In chapter five the effect of cotinine metabolism was investigated in liver cells, where the primary vitamin D metabolism occurs. It was observed that the presence of
serum cotinine up-regulates the two primary enzymes CYP2R1 and CYP3A4 that play major roles in conversion of pre-vitamin D to the major circulating form of the vitamin 25-OH-D$_3$. But CYP3A4 is also the enzyme that plays an important role of catabolizing the 25-OH-D$_3$ into an inactivated form. This chapter concluded that cotinine may induce CYP3A4 expression in the liver cell microsomes and accelerate vitamin D catabolism and may contribute to vitamin D deficiency.

Therefore, my dissertation highlighted the potential effects of smoking on vitamin D status in the United States by a combination of nutrition epidemiology and in-vitro investigations.

In conclusion, it is recommended to further continue this study to better understand how cotinine may affect the vitamin D status among women with different ethnic backgrounds and smoking habits. Also, more research is needed to better understand whether vitamin D exerts beneficial efforts on respiratory health in women, since they are more likely to be significantly burdened by the symptoms of COPD. Continued in-vitro investigations with greater control of nuisance variables may rule out whether serum cotinine concentration and serum vitamin D concentration are uncorrelated, and demonstrate the mechanistic link between the effect of cotinine on the CYP2R1 and CYP3A4 in kidney cells, where the 25-OH-D$_3$ is converted to its biologically form of 1,25 (OH)$_2$D$_3$. Continued research will further clarify the impact of smoking on vitamin D status.
**Future Interests:**

Interaction between vitamin D and the immune system has been recognized for many years [1]. Previously, it was hypothesized that vitamin D supplementation may prevent acute respiratory infections based on the fact that these infections are much more common during the winter, which coincides with lower vitamin D in the population [2]. One study showed in general that vitamin D supplementation provided protection against acute respiratory tract infection, especially in those who have deficient levels of vitamin D [3]. As the results of the current Coronavirus Disease 2019 (COVID-19) pandemic, a recent study has shown that the countries in the Northern Hemisphere have experienced relatively higher Covid-19 mortality rates with an estimated 4.4% rise in mortality for each 1 degree latitude north of 28 degrees North after adjustment for age of population. This suggests a role for UVB radiation for vitamin D synthesis. Factors such as age, ethnicity, obesity, diabetes, and hypertension are among those linked with severe Covid-19 diagnosis, and these factors are also associated with deficiency of vitamin D or its response [4]. Therefore, future laboratory experiments along with randomized and placebo-control clinical trials will be beneficial to assess if vitamin D may have any potential therapeutic role in combatting against Covid-19.
References:


It’s not that I’m so smart, it’s just that I stay with problems longer.

- Albert Einstein
Classification for Kingdom Plantae Down to Genus Nicotiana L. Retrieved from https://plants.usda.gov/java/ClassificationServlet?source=display&classid=NICOT


National Health and Nutrition Examination Survey
https://www.cdc.gov/nchs/nhanes/participant/participant-selected.htm

NHANES 2001-2002

NHANES 2003-2004

NHANES 2005-2006

How to Merge NHANES Data in SAS -
https://www.cdc.gov/nchs/tutorials/NHANES/Preparing/AppendMerge/Task2.htm

Key Concepts About Missing Data in NHANES -
https://www.cdc.gov/nchs/tutorials/NHANES/Preparing/CleanRecode/Info1.htm


Getting Started with NHANES 2005-2006 Data Analysis

Key Concepts About Missing Data in NHANES -
https://www.cdc.gov/nchs/tutorials/NHANES/Preparing/CleanRecode/Info1.htm


Vitamin D Fact Sheet for Health Professional https://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional/

NIH Office of Dietary Supplements – Vitamin D Fact Sheet for Health Professionals https://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional/
. gen lifestyle1=0
. replace lifestyle1=2 if lifestyle=="Heavy Smokers"
   (10 real changes made)
. replace lifestyle1=1 if lifestyle=="Light Smokers"
   (4 real changes made)
. replace lifestyle1=0 if lifestyle=="Non-Smokers"
   (0 real changes made)
. lab var lifestyle1 "smoking status"
. lab define lifestyle1 0"0 Non-Smokers" 1"1 Light Smokers" 2"2 Heavy Smokers"
. lab value lifestyle1 lifestyle1
. tab1 lifestyle lifestyle1, m

-> tabulation of lifestyle

<table>
<thead>
<tr>
<th>Life-Style</th>
<th>Freq.</th>
<th>Percent</th>
<th>Cum.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy Smokers</td>
<td>10</td>
<td>41.67</td>
<td>41.67</td>
</tr>
<tr>
<td>Light Smokers</td>
<td>4</td>
<td>16.67</td>
<td>58.33</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>10</td>
<td>41.67</td>
<td>100.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>24</strong></td>
<td><strong>100.00</strong></td>
<td></td>
</tr>
</tbody>
</table>

-> tabulation of lifestyle1

<table>
<thead>
<tr>
<th>smoking status</th>
<th>Freq.</th>
<th>Percent</th>
<th>Cum.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Non-Smokers</td>
<td>10</td>
<td>41.67</td>
<td>41.67</td>
</tr>
<tr>
<td>1 Light Smokers</td>
<td>4</td>
<td>16.67</td>
<td>58.33</td>
</tr>
<tr>
<td>2 Heavy Smokers</td>
<td>10</td>
<td>41.67</td>
<td>100.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>24</strong></td>
<td><strong>100.00</strong></td>
<td></td>
</tr>
</tbody>
</table>
. gen smoking=(lifestyle1==1 | lifestyle1==2)

. lab define smoking 0"0 Non-Smoker" 1"1 Heavy/Light Smoker"

. lab value smoking smoking

. tab1 smoking lifestyle1, m

-> tabulation of smoking (create a binary variable for smoking status)

```
              smoking |     Freq.  Percent  Cum.
---------------------+------------------------
          0 Non-Smoker |         10   41.67  41.67
         1 Heavy/Light Smoker |         14   58.33  100.00
---------------------+------------------------
            Total |         24  100.00
```

-> tabulation of lifestyle1

```
            smoking status |     Freq.  Percent  Cum.
---------------------------+------------------------
          0 Non-Smokers |         10   41.67  41.67
          1 Light Smokers |          4   16.67  58.33
         2 Heavy Smokers |         10   41.67  100.00
---------------------------+------------------------
           Total |         24  100.00
```

. save "C:\Users\Joe\Desktop\Kiano\smoking women bmi\smoking.dta", replace

file C:\Users\Joe\Desktop\Kiano\smoking women bmi\smoking.dta saved

. /* 1. overall relationship between Cotinine & Vit D (ALL subjects) */
. ** visual inspection: negative relationship **
. twoway fpfitci cotinine vit_d || scatter cotinine vit_d /* curvilinear relationship */
If we allow for non-linear relationship: For the total sample (N = 24; including Non-smokers, light smokers, and heavy smokers) There is a negative relationship between Vit D (X-axis) and Cotinine level (Y-axis), especially when the Vit D level is less than 20 ng/ml.

```
if we only allow for linear relationship: For the total sample, there is a negative relationship between Vit D (X-axis) and Cotinine level (Y-axis),
```

```
** correlation: negative but not significant (small sample size) **
```

```
.pwcorr cotinine vit_d bmi, sig /* total sample: not significant */
```

```
<table>
<thead>
<tr>
<th>cotinine</th>
<th>vit_d</th>
<th>bmi</th>
</tr>
</thead>
<tbody>
<tr>
<td>cotinine</td>
<td>1.0000</td>
<td></td>
</tr>
<tr>
<td>vit_d</td>
<td>-0.3051</td>
<td>1.0000</td>
</tr>
</tbody>
</table>
```
|                | Coef.   Std. Err. | t    | P>|t| | [95% Conf. interval] |
|----------------|------------------|------|------|-------------------------|
| cotinine       | -.1010869        | .0672764 | -1.50 | 0.147   | .2406095, .0384358 |
| _cons          | 44.03706         | 7.891452 | 5.58  | 0.000   | 27.67119, 60.40293 |

Source | SS   | df  | MS   | Number of obs = 24  
---------|------|-----|------|---------------------|
F(1, 22) = 2.26
Model | 761.433577 | 1    | 761.433577 | Prob > F = 0.1472 |
Residual | 7419.7668 | 22   | 337.262127 | R-squared = 0.0931 |
Adj R-squared = 0.0518
Total | 8181.20038 | 23   | 355.704364 | Root MSE = 18.365 |

Source | SS   | df  | MS   | Number of obs = 24  
---------|------|-----|------|---------------------|
F(2, 21) = 1.34
Model | 928.269788 | 2    | 464.134894 | Prob > F = 0.2824 |
Residual | 7252.93059 | 21   | 345.377647 | R-squared = 0.1135 |
Adj R-squared = 0.0290
Total | 8181.20038 | 23   | 355.704364 | Root MSE = 18.584 |

Source | SS   | df  | MS   | Number of obs = 24  
---------|------|-----|------|---------------------|
F(3, 20) = 0.92
Model | 994.702451 | 3    | 331.567484 | Prob > F = 0.4478 |

**regression analysis**

```
. reg vit_d cotinine /* not significant */
. reg vit_d cotinine lifestyle1 /* not significant */
. reg vit_d cotinine lifestyle1 bmi /* not significant */
```
Residual |  7186.49793        20  359.324896   R-squared =  0.1216
-----------+------------------------------------------------------------------
        Adj R-squared =  -0.0102
Total |  8181.20038        23  355.704364   Root MSE   =  18.956

| vit_d | Coef. Std. Err.    t  P>|t| [95% Conf. interval] |
|--------------------------------|
| cotinine | -0.087624  .0787102 -1.11 0.279 .2518106 .0765626 |
| lifestyle1 | -3.047029  4.671172 -0.65 0.522 -12.79092 6.696864 |
| bmi | .5837767  1.357687  0.43 0.672 -2.248308 3.415862 |
| _cons | 31.17787  33.1414  0.94 0.358 -37.95387  00.3096 |

/* 2. 2-group comparison: Heavy Smokers vs. Non-Smokers */
** visual inspection (similar results as the total sample) **
. twoway fpfitci cotinine vit_d || scatter cotinine vit_d if lifestyle1~=1

If we allow for non-linear relationship: For heavy smokers and non-smokers, there is a negative relationship between Vit D (X-axis) and Cotinine level (Y-axis), especially when the Vit D level is less than 20 ng/ml.

. twoway lfitci cotinine vit_d || scatter cotinine vit_d if lifestyle1~=1
If we only allow for linear relationship: For heavy smokers and non-smokers, there is a negative relationship between Vit D (X-axis) and Cotinine level (Y-axis).

Model summary

Likelihood:
\[
\text{vit}_d \sim \text{normal}(\text{xb}_\text{vit}_d,\{\text{var}\})
\]

Priors:
\[
\{\text{vit}_d: \_\text{cons} \text{ cotinine} \text{ coti\_life} \text{ lifestyle1} \text{ bmi}\} \sim 1 \text{ (flat)} \quad (1)
\{\text{var}\} \sim \text{jeffreys}
\]

(1) Parameters are elements of the linear form \(\text{xb}_\text{vit}_d\).

Bayesian normal regression

\[
\begin{align*}
\text{MCMC iterations} & = 12,500 \\
\text{Random-walk Metropolis-Hastings} & \quad \text{Burn-in} = 2,500 \\
\text{Metropolis-Hastings} & \quad \text{MCMC sample size} = 10,000 \\
\text{Hastings sampling} & \quad \text{Number of obs} = 14 \\
\text{Burn-in} & \quad \text{Acceptance rate} = 0.2743 \\
\text{MCMC sample size} & \quad \text{Efficiency: min} = 0.001259 \\
\text{Number of obs} & \quad \text{avg} = 0.02832 \\
\text{Acceptance rate} & \quad \text{Log marginal likelihood} = -55.013512 \\
\text{Efficiency: min} & \quad \text{max} = 0.06291
\end{align*}
\]

\[
\begin{array}{lcccc}
\text{Equal-tailed} & \text{Mean} & \text{Std. Dev.} & \text{MCSE} & \text{Median [95% Cred.Interval]} \\
\hline
\text{vit}_d & \cdot1036871 & .1528962 & .006901 & -.1083223 \text{-.3899873} .2247368 \\
\text{cotinine} & \cdot.0720683 & .2620055 & .01137 & -.0640334 \text{-.6044262} .4344972 \\
\text{coti\_life} & .4999386 & 29.04824 & 1.15815 & .5108911 \text{-.56.26412} 58.62897 \\
\text{lifestyle1} & .2844986 & 1.941399 & .451635 & .45579 \text{-.3.613757} 3.629169 \\
\text{bmi} & \end{array}
\]
Note: There is a high autocorrelation after 500 lags.

3. Correlation Matrix: no significant (linear) relationship between Vit D and cotinine:
First row: correlation coefficient
Second row: P

```
. pwcorr vit_d logvitd cotinine logcotinine, sig

          vit_d  logvitd  cotinine  logcotinine

   vit_d  1.0000
   logvitd  0.9392  1.0000  0.0000
   cotinine -0.3051 -0.3429  1.0000  0.1472  0.1010
   logcotinine -0.2459 -0.2595  0.9706  1.0000  0.2467  0.2208  0.0000
```
August 2nd, 2016

To whom it may concern:

Please accept this letter as confirmation of the following:

• All samples are collected by ProteoGenex, Inc. legally and ethically, under Ethics Committee (EC) approval, and processed according to strict Standard Operating Procedures (SOPs).
• All samples are obtained with signed informed consent, with an appropriate IRB/EC approval and in accordance with current Federal Regulations in addition to ICH, HIPAA and GCP guidelines pertaining to protection of human subjects.
• The samples are collected with understanding and consent that the samples will be used for research by commercial or academic institutions.
• The samples are collected from donors screened and found negative for HIV, HBV, HCV, and syphilis.
• ProteoGenex follows stringent procedures to ensure donors’ privacy and confidentiality protection. All personally identifying information is redacted from all pathology reports and summarizing clinical spreadsheets. All specimens are de-identified with a Sample ID, so that the donor’s identity is kept anonymous.

Applicable documents are attached.

Sincerely,

[Signature]

Marina Prilutskaya, PhD
Head of Operations
ProteoGenex SOP for Collection of Serum

STANDARD OPERATING PROCEDURE

<table>
<thead>
<tr>
<th>TITLE</th>
<th>Collection of Human Body Fluid Samples: Serum</th>
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<tr>
<td>Issue Date</td>
<td>Expiry Date</td>
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<tr>
<td>01/2005</td>
<td>03/01/2018</td>
</tr>
</tbody>
</table>

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Document invalid if past expiry date

1. PURPOSE
   1.1. To establish standardized collection and handling of serum that will be used in pre-clinical studies.

2. SCOPE
   2.1. This procedure applies to all blood samples collected by ProteoGenex.

3. PROCEDURE
   3.1. General Information
       3.1.1. Handle all samples with Universal Precaution.
       3.1.2. When filling the cryotubes, always leave some headroom to allow expansion during freezing.
       3.1.3. Samples should be shipped on dry ice.
   3.2. Specimen labeling and patient data collection
       3.2.1. Label all sample tubes with a collection site name and patient ID number
       3.2.2. Documentation including patient data and specific information about the collection process must be collected along with the samples.
       Patient and sample data requirements are specified by clients.
   3.3. Isolation of Serum
       3.3.1. In all cases, blood samples should be obtained at the time point specified by clients.
ProteoGenex SOP for Collection of Serum

3.3.2. Process blood sample as soon as possible. Freezing of serum aliquots must occur no longer than 6 hours after blood collection.

3.3.3. Use 2 Vacutainer SST tube to collect approximately 15 milliliters (total) of blood for serum separation.

3.3.4. Invert the vacutainer tube five times, and then allow blood to clot at room temperature for a minimum of 30 minutes and a maximum of 2 hours.

3.3.5. Centrifuge blood in vacutainer tube for 10 minutes at 1300xG-1500xG at room temperature.

3.3.6. Carefully transfer serum (leaving a residual amount of it to avoid contamination with pelleted cells) into labeled vials (2 milliliter cryotubes).

3.3.7. Store serum samples at -80°C.

Approval Signatures

Marna Pributskaia, PhD
Head of Operations
1. PURPOSE AND OBJECTIVE:

The objective of this SOP is to describe activities and procedures for obtaining and documenting Informed Consent in research involving human subjects.

2. SCOPE:

To ensure that written Informed Consent is obtained from each patient in accordance with Regulatory requirements, GCP/ICH.

3. RESPONSIBILITY:

The Principal Investigator (PI) Study Coordinator (SC) and/or other designated site staff will obtain written Informed Consent from all patients/donors where specimens for research are obtained.

4. POLICY AND PROCEDURES:

4.1. For studies in which the Ethical Committee requires informed consent in written form, the principal investigator or appropriate research personnel will fully inform the subject or the subject’s legally authorized representative of all pertinent Ethical Committee approved research. The process includes:
   a. Giving the subject adequate information concerning the research in language that is as non-technical as possible (eighth grade language or lower if more appropriate for subject population);
   b. Providing ample time and opportunity for the subject or the subject’s legally authorized representative to inquire about the details of the research project and to decide whether or not to participate in the research, as well as to consider other available options, if any;

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c. Responding to subject’s or the subject’s legally authorized representative’s questions to his/her/their satisfaction;
d. Ensuring to the degree possible that the subject has comprehended the information provided about the research;
e. Obtaining the subject’s or the subject’s legally authorized representative’s voluntary consent;
f. Documenting that the process has occurred,
g. The investigator and all research personnel are responsible for continuing the informed consent process throughout the subject’s participation in the study.

4.2. Based upon the requirements of the Code of Federal Regulations, Proteogenex has determined the minimally required elements of an informed consent document. Required elements include:
a. Study Purpose
b. Number of People Taking Part in the Study
c. Procedures Involved in the Study
d. Risks of Taking Part in the Study
e. Benefits of Taking Part in the Study
f. Alternatives to Taking Part in the Study
g. Confidentiality
h. Costs/Compensation
i. Contacts for Questions or Problems
j. Voluntary Nature of the Study
k. Subject’s Consent
l. Signatures and Date

4.3. When written consent is required, the PI or appropriate research personnel will file the original signed consent form with the subject’s research records. A copy of the consent form will be provided to the subject or the subject’s legally authorized representative at the time of consent and a copy will also be placed in the subject’s medical record, if appropriate.

4.4. In research involving patients, informed consent will be obtained for each research subject prior to altering a subject’s care for the purpose of research. Informed consent must be obtained prior to performing any non-routine procedure (e.g., eligibility testing) if being done exclusively for the purpose of screening for, or participating in, the research project. This could be done using an abbreviated screening informed consent document and/or the regular consent form. The consent must be obtained according to Ethical Committee, sponsor, and GCP requirements, if applicable.

4.5. Written consent should be signed and personally dated by the subject or subject’s legally authorized representative, and by the person who conducted the informed consent discussion.
4.6. The person who conducts the informed consent process (explains the protocol, reviews the consent, answers potential subject’s questions) must sign the consent form as the consenter. This signature confirms that the consent process was properly conducted, not that the subject signed the consent.

Approval Signatures

Masha Prilutskaya, PhD
Head of Operations
RUSSIAN ONCOLOGICAL RESEARCH CENTER n.a. N.N. BLOKHIN
ETHICS COMMITTEE REVIEW FORM

Protocol/Study No | PG-ONC 2003/1
Title: COLLECTION OF TISSUE, BLOOD AND BONE MARROW SAMPLES FOR
DIAGNOSTIC AND PHARMACEUTICAL RESEARCH

developed by ProteoGenex Inc., USA
Following documents have been submitted to the Ethics Committee on date 24.12.2015

☑ Protocol version _____________ dated _____________
☑ Patient Information/Consent Form version _____________ dated _____________
☐ Investigator's Brochure version _____________ dated _____________
☐ Amendment(s) number _____________ dated _____________
☐ Insurance number _____________ dated _____________
☐ Regulatory approval protocol _____________ dated _____________
☑ CV of Investigator(s) Name: N.A. Dyakova dated _____________
☐ CV of Investigator(s) Name: _____________ dated _____________
☐ Other (specify) Annual Report

☑ The Continuation of Study was: Approved
☐ Conditionally approved (see attached letter)
☐ Deferred pending further information (see attached letter)
☐ Rejected (see attached letter)

☑ Is review of study status required Yes ☐ No
☐ If Yes, ☐ Annually ☐ Periodically (when)

The investigator(s) is/are EC member(s) and therefore did not vote

Date: 11 January 2016
Chairman of EC D.Z. Kupchan Signature

The EC is organized and operates in compliance with the ICH GCP requirements and Russian Law
ProteoGenex SOP
Quality Assurance/Quality Control (QA/QC) Program

STANDARD OPERATING PROCEDURE

| TITLE: Quality Assurance/Quality Control Program |
|-------------------------------|---------------------------------|
| Issue Date | Expiry Date | SOP No. | Version | Copy | Pages |
| 01/2005 | 03/01/2018 | 023 | No.2 | 1 | 2 |

Document control Signature

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1. PURPOSE
   1.1. To establish standardized Quality Assurance/Quality Control process

2. SCOPE
   2.1. This program applies to all QA/QC procedures implemented by ProteoGenex

3. QUALITY ASSURANCE/QUALITY CONTROL (QA/QC) PROGRAM

The objective of ProteoGenex, Inc. Quality Assurance/Quality Control (QA/QC) program is to ensure that all specimens collected and processed, and all data collected are valid, of known precision and accuracy, properly documented and stored. QA program is designed to provide a description of the overall procedures used to collect, process, and store specimens and document clinical information. The QA program also ensures that collection, preservation, labeling, and storage procedures are established so as to maintain sample integrity and identification.

The following is a summary of the key elements of ProteoGenex QA program:

1. Competence and expertise of the technical staff
2. Strict adherence to principles of good laboratory practice (GLP).
3. Consistent use of Standard Operating Procedures (SOPs).
4. Development and adherence to carefully designed protocols
5. The use of mechanically stable, reliable, and well-maintained analytical instruments
6. Appropriate calibrations and standards.
7. All equipment is maintained properly according to SOPs
8. Adequate supervision of all technical operations by management and senior personnel.
9. Documentation and Sample Tracking Procedures That Ensure Traceability
10. Protocols for the Training and Implementation of all QA/QC Related Activities
11. Maintains QA/QC training record for personal
12. Documentation System That Details the Requirements for all Tests and Services Including all Record Keeping and Validation Procedures
13. Data management system include computerized inventory tracking system
14. Auditing Program That Monitors Compliance with all the Applicable Regulations and Guidelines

Approval Signatures

Marina Prihatskaya, PhD
Head of Operations

[Signature]

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ProteoGenex SOP
Quality Assurance Overview

STANDARD OPERATING PROCEDURE

<table>
<thead>
<tr>
<th>TITLE: Quality Assurance Overview</th>
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</thead>
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</tr>
<tr>
<td>01/2015</td>
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</tbody>
</table>

Document control Signature ______________________

Copy control: This SOP is only in force if signed here and under approval signatures (document end).

1. PURPOSE
   1.1. To provide guidance for QA Overview

2. SCOPE
   2.1. This SOP lists the specific steps required for the QA process

3. PROTEOGENEX QA OVERVIEW
   3.1. All human biological specimens are handled using sterile or aseptic techniques. Samples that are intended to be frozen are snap-frozen directly in liquid nitrogen within 30 minutes or less after surgical excision.
   3.2. A representative piece of tissue is cut off and used to make a representative paraffin embedded tissue block and H&E slide.
   3.3. The remaining sample is cut into 0.5 cm fragments. Each fragment is snap frozen individually in liquid nitrogen.
   3.4. Samples must be pre-frozen in liquid nitrogen before being placed in the tubes to assure they do not stick to the tubes. Tubes must be pre-labeled.
   3.5. Tissue samples will be stored in liquid nitrogen.
   3.6. Informed consent must be obtained from each donor. Patient identification information must be de-identified.
   3.7. Samples should be handled as if potentially infectious. All reasonable effort should be made to warn clients if any known infectious agent is contained in the samples.
   3.8. Prior to shipment ProteoGenex will contact clients and will provide the date of delivery.
   3.9. At a time of shipment ProteoGenex will contact clients and will provide tracking # for the shipment. All fresh/frozen samples should be shipped on dry ice 10 lbs per 2ft³ container.
   3.10. The following items are required for the shipment
       1) List of samples included in shipment
2) The samples
3) H&E slides (if requested)
4) Complete clinical, pathological (ProteoGenex) and biological data need to be send in electronic version

3.11. Pathology QC:
1) Be sure that H&E slides were prepared from each fresh frozen tissue, and from each FFPE tissue block, collected for ProteoGenex.
2) Be sure each H&E slide was reviewed by pathologist to confirm tissue identity and normal histology and to ensure that representative cellular components are present.
3) Be sure H&E slide compared with confirmed diagnosis in the pathological report.
4) Tissue samples with >50% of tumor cells will be acceptable for shipment to the clients (unless other requested)
5) Pathologist passes or fails sample

3.12. RNA QC:
1) Be sure that extraction of total RNA was performed on all fresh frozen samples collected for ProteoGenex.
   A. Small pieces of fresh frozen samples are cut on dry ice for RNA testing
   B. RNA is extracted using TriZol reagent
   C. RNA is analyzed using a Agilent Bioanalyser 2100
2) Be sure that the RNA ratio of 28S to 18S peaks is equal or greater then 1.7

3.13. Clinical data QC:
1) Be sure that all data are reviewed to ensure that it accurate and complete
2) Data is determined to be compatible with pathology information.

Approval Signatures

Marina Prihtskaya, PhD
Head of Operations
RESEARCH SUBJECT INFORMATION AND CONSENT FORM

1. Purpose of Study: You are being asked to participate in this biomedical research study because your surgical tissue sample and or blood, and specimens from other people with similar conditions, have the potential to provide valuable information to researchers that may help them find new ways to detect and treat diseases such as yours in the future. The tissue that you donate must be obtained from your surgical treatment and would normally be discarded. Tissue samples from this study will only be used after a pathological diagnosis has been established and the use of these specimens will not alter or compromise your treatment in any way.

2. Research: If you take part in this study, you are being asked to donate tissue and or blood that are removed to treat your disease/condition. No additional treatment or tests other than those that are normally prescribed for your medical care will be performed. You may be asked to have a blood sample collected. The amount of blood will not exceed 40ml. The tissue and or blood will be used specifically for research to examine the expression of molecules related to various diseases including cancer, heart disease, diabetes, inflammatory, and other conditions. These studies will examine how much of these molecules are present in diseased and normal cells in order to identify genes important in the development of disease. In addition, some of the tissue and or blood may be stored for an indefinite period of time in a tissue repository for future biomedical research purposes. The tissue and or blood may be used for research and shared with collaborators at academic or commercial institutions. The research that may be performed includes the examination of RNA, DNA, or proteins in cells. You will not be given the results of any research performed on the tissue with all information identifying you being removed. The researchers who use the tissue and or blood may need to know some things about your health before and after surgery (for example: your age, sex, ethnic group, smoking habits, clinical diagnosis, medical treatment for your condition, family medical history, etc., if available. Medical information about you may be given to researchers along with your tissue and or blood, but your name and any other identifying information will be removed. The researchers will not know who you are.

3. Benefits: There will be no direct benefit to you, however, information from this study may benefit other patients with similar medical problems in the future. The research may lead to commercial products that are sold in the future. Your sample is only one of many and your sample will not be linked to you. Therefore, use of your tissue sample or blood and information does not create any right, title, or interest in the specimens or to any derivative materials or products that may be developed as a result of the research.

4. Risks: This research will have no risks to you. It will have no affect on your care.

5. Voluntary Participation/Withdrawal: Taking part in this study is voluntary. You may choose not to take part, or if you decide to take part, you can later change your mind and
withdraw from the study. Your decision will not change the present or future health care or other services that you receive. The study doctor, or the sponsor, may stop your participation in this study without your consent. Whether or not you choose to take part in the study, you will receive the standard treatment for patients with your condition.

6. Costs: There will be no additional costs for this procedure.

7. Compensation: You will not be paid for participating in this study.

8. Confidentiality: All information collected about you during the course of this study will be kept confidential to the extent permitted by law. Only a code number will identify you in the research records. Information, which identifies you personally, will not be released without your written permission. Your health records may be reviewed by the study sponsor, its agents, the Human Investigation Committee, approved persons from other organizations taking part in this study, and appropriate federal agencies. Information from this study may be published, but your identity will be kept confidential in any publications.

9. Questions: This study has been explained to you and all of your questions have been answered. If you have any questions in the future or in the case of a research related injury or illness, you may contact the PI or one of his/her associates at the following telephone number . If you have any questions about your rights as a research subject, the Chair of the Human Investigation Committee can be contacted at .

10. Consent to Participate in a Research Trial: To voluntarily agree to take part in this study, you must sign on the line below. If you choose to take part in this study, you may withdraw at any time. You are not giving up any of your legal rights by signing this form. Your signature below indicates that you have read, or have been read this entire consent form, including the risks and benefits, and have had all your questions answered. You will be given a copy of this consent form.

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<tr>
<th>Signature of Investigator/Designee Obtaining Consent</th>
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**Patient Information Leaflet and Informed Consent**

By signing below you give consent to ___________________ to make tissue and/or blood that is removed as a result of the procedure available for biomedical research. The research is to help find out more about what causes certain human diseases and how to better diagnose, treat, or even cure them. The tissue will be used specifically for research to look at the expression of molecules that may be related to various diseases, such as cancer, heart disease, diabetes, inflammatory and other conditions, etc.

In addition, some of the tissue may be stored for an indefinite period of time in a tissue repository for future biomedical research purposes. The tissue may be used for research and shared with collaborators at academic or commercial institutions. The research that is done may include the examination of DNA, RNA or proteins in cells. You will not be given the results of any research performed on the tissue, because all information identifying you as the donor will be removed. The research will not benefit you directly, but may benefit someone like you in the future. The researchers who use the tissue may need to know some things about your health before and after surgery (for example: your age, sex, ethnic group, smoking history, your diagnosis, medical treatment for your condition, family medical history as it pertains to your condition, etc.). Medical information about you may be given to researchers along with the tissue, but your name and any other identifying information will be removed. The researchers will not know who you are and your identity will be kept confidential. The research may lead to commercial products that are sold in the future. Use of your tissue sample and information does not create any right, title, or interest in the tissue or to any derivative materials or products that may be developed as a result of the research. Your participation is voluntary. You are free to decline or withdraw from participation without your care being affected. There will be no additional costs for this procedure and you will not be paid for participating.

If you have questions, you may call ________________________________

☐ No, I do not give permission.

☐ Yes, I give permission but only for my condition, which is: ________________________________

☐ Yes, I give permission for the use of my tissue in all studies

_Was copy of this consent given to patient?_  ☐ YES  ☐ No

______________________________ (Patient’s Name)

______________________________  ________________________

Patient’s Signature  Date
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C  0.841 0.811 0.226 0.249 0.912 0.898 0.647 0.527 0.313 0.369 1.396 1.289  
D  0.800 0.906 0.242 0.275 0.489 0.407 2.013 1.797 0.936 0.797 0.477 0.495  
E  0.624 0.739 0.627 0.795 1.014 1.183 0.993 0.865 0.210 0.206 0.904 0.919  
F  0.529 0.579 0.747 0.925 0.310 0.280 0.568 0.637 0.709 0.973 1.310 1.213  
G  0.275 0.291 0.434 0.510 0.281 0.268 0.396 0.394 0.056 0.059 0.050 0.054  
H  0.054 0.211 0.281 0.902 1.679 2.628 0.050 0.064 0.060 0.065 0.096  


**Coding & Data**  

**First Analysis (SAS-Code-1.docx)**  

<table>
<thead>
<tr>
<th>Class</th>
<th>Levels</th>
<th>Values</th>
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<tbody>
<tr>
<td>type</td>
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</tr>
<tr>
<td>gp</td>
<td>3</td>
<td>Hep Mic Mic12</td>
</tr>
</tbody>
</table>

Model $Y = function \ of \ (gp, \ type, \ ID(gp), \ ID(type))$  
ID is a continuous variable, given by log(dose)  
$> \ log(1010)$  
[1] 6.917706  
$> \ log(279)$  
[1] 5.631212  
$> \ log(71.6)$  
[1] 4.271095  
$> \ log(24.4)$  
[1] 3.194583  
$> \ log(4.8)$  
[1] 1.568616  

Model has log(dose) as covariate and after rerunning this model, the
There are significant differences between 3 GP levels (Mic12, Mic, Hep; p= 0.0071); there are not significant differences between types levels (microsome 1-6, HepG2_07-10, p= 0.2624) at each GP level; ID Slope for each GP level is significantly different from 0 (p<.0001), at least one of GP levels has an ID slope different from 0; there are not significant difference between ID slope by TYPE within each level of GP (p=0.3382). This may lead to fit a single regression line for each GP level (it means there is no significant within-group difference; therefore, we can divide the sample into 3 general GP groups [Mic, Mic12, Hep] rather than 10 groups and check the “common” slope in each GP group).

Coefficients Parameter estimates of model

There is a significant and negative relationship between Vit D and Cotinine in Hep (tray#7-10; p=.0448) & Mic 12 (tray #1-2; p<.0001). But there is no significant relationship for Mic (tray #3-6).
Equation is given by
Prediction line $Y_{\hat{}} = \text{Intercept} + \text{effect\_gp} + \text{effect\_type(gp)} + \text{slope\_id\_within(gp)} =+ \text{slope(gp and Type)},$
For Mic12, microsome1,
$Y_{\hat{}} = 0.89999997530 +0.0000+ 0.1410890675 - 0.1053610215*ID -0.0241518837*ID$

Lines are represented in graph below
Fitting a common slope for each TYPE within each GP level

Fitting a model by each tray (total = 10 types):

X axis = id = natural log (Vit D)
Y axis = Cotinine level

The graph shows that:
(1) There is a negative relationship between X and Y in Mic12 (symbol as x; steeper slopes) and Hep (symbol as +; slopes for Hep are less steep than those for Mic12).
(2) There is no relationship between X and Y in Mic (tray#3-6, symbol as o; cancel each other out).
All types within a common GP have the same slope (common slope), but different intercept (so it allows variation of the average cotinine level when Vit D is zero for each tray [type]), graph below:
GP shows distinct behavior with Mc12 showing a steeper negative slope (-.1174369634 ± 0.00781259), and HEP the smallest negative slope (-.0084376768 ± 0.00820975). MICrosome 4 (NOT important) from GS=MIC shows better response to dose, with an intercept equal to 0.9420442867 + 0.0109376224 + 0.0418000000) followed by Microsome 3.

LSMEANS for GS at average value of LD are presented. Pairwise mean comparison was carried on using the Least significant different (LSD) test, at a significance level $\alpha = 0.05$.

Results show that at the average level for log(dose) these three means are significantly different, with Mic (A) showing the highest Y predicted mean value (0.887) and Mic12 (C; 0.486) the lowest Y predicted mean. (The average value of log(Vit D) is expected to be highest in Mic and lowest in Mic 12)

HEP shows lower predicted mean than MIC.

### T Comparison Lines for Least Squares Means of gp

<table>
<thead>
<tr>
<th>LS-means with the same letter are not significantly different.</th>
<th>y LSMEAN</th>
<th>gp</th>
<th>LSMEAN Number</th>
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<td>0.8873790</td>
<td>Mic</td>
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<td>B</td>
<td>0.7806258</td>
<td>Hep</td>
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<tr>
<td>C</td>
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</table>

At log(dose) = 2.08, HEP shows lower predicted mean than MIC.

### T Comparison Lines for Least Squares Means of gp

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<tr>
<th>LS-means with the same letter are not significantly different.</th>
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<th>LSMEAN Number</th>
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Comparison Lines for Least Squares Means of gp

LS-means with the same letter are not significantly different.

<table>
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<th>y LSMEAN</th>
<th>gp</th>
<th>LSMEAN Number</th>
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<tr>
<td>B</td>
<td>0.78062545 Hep</td>
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<tr>
<td>C</td>
<td>0.48623425 Mic12</td>
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</table>

At log(dose) = 4.124

At log(dose) = 6.9177, HEP shows lower predicted mean than MIC, but not significant difference

Greater difference is observed at log(dose) = 2.08 between HEP and MIC, lower difference at log(dose) = 6.09. (So the differences in cotinine level between 3 GP groups become smaller when Vitamin D level becomes larger; people with a higher Vitamin level tend to have similar pattern of smoking?)
Second model has factors,

<table>
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<th>Class</th>
<th>Levels</th>
<th>Values</th>
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<td>HepG2_07 HepG2_08 HepG2_09 HepG2_10 microsome1 microsome2 microsome3 microsome4 microsome5 microsome6</td>
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And Type III SS

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<td>0.06304202</td>
<td>13.96</td>
<td>&lt;.0001</td>
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</table>

Results in type (pvalue=0.0377), ID (pvalue<.0001) and ID(Type) (pvalue<.0001) are significant at significance level alpha = 0.05

Response Y is a function of Type, ID and ID(Type). Since ID is continues the model represent a set of regression lines, one for each of the types with intercept given by estimated Intercept plus Type effect and slope for ID given by the common ID plus the change in slope associated with each type, these lines would be represented by a plot with X-axis= ID and Y-axis=Y, the dependent variable in the model.

Lines are presented in page 9 of pdf.

Page 11-12 shows the results from pairwise comparison of TYPE lsmeans calculated at average value of ID, these differences show how the regression groups around their intercept. Check regression lines plots

Third model looks at factors Type and GP
There are significant differences between regression lines intercepts due to GP, and ID slope within each level of GP are jointly significant different from 0, at least one of these three slopes is not zero. (NOT important)

Table of Parameter estimates, show size of the effect of each term in the model:

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<th>Estimate</th>
<th>Standard Error</th>
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<td>ID(gp) Hep</td>
<td>-0.0084</td>
<td>0.0083</td>
<td>-1.02</td>
<td>0.3142</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID(gp) Mic</td>
<td>-0.0198</td>
<td>0.0084</td>
<td>-2.46</td>
<td>0.0207</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID(gp) Mic12</td>
<td>-1.1744</td>
<td>0.0278</td>
<td>-44.88</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Intercept for gp=Hep=0.8154 (p<.0001)
; for gp=Mic=0.969 (p<.0001)
for gp=Mic12=0.971 (p<.0001)
ID Slope for Hep = -0.00844 (p=0.3142, non significant)
ID Slope for Mic = -0.01987 (p=0.0207)
ID Slope for mic12 = -0.11744 (p<.0001)

Graphs below are representation of these lines.
Here are your data (rows 3-9 of your table) grouped by microsomes 1&2, other microsomes, Hep. There is very little difference within these groups but statistically significant differences between the three groups. Below are the individual column regressions. In both graphs, X is the log of dose. The microsome 1 and 2 group has a strongly significantly nonzero slope. The other microsomes have a smaller but still significant slope but the HepG2 group shows no significant dose response. Below are lines for each of your columns. (same findings as previous pages)
(shows the detail: allow different intercept for each tray [type] but the same slope for each GP [Mic, Mic 12, Hep])

Procedure Details
Plate Type 96 WELL PLATE
Eject plate on completion
Read Absorbance Endpoint
Random
Wavelengths: 405
Read Speed: Normal, Delay: 100 msec, Measurements/Data Point: 8

Layout

<table>
<thead>
<tr>
<th>NBS</th>
<th>BO</th>
<th>1010 ng/ml</th>
<th>279 ng/ml</th>
<th>71.6 ng/ml</th>
<th>24.4 ng/ml</th>
<th>4.8 ng/ml</th>
<th>0.5 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: SPL1</td>
<td>SPL1</td>
<td>SPL9</td>
<td>SPL16</td>
<td>SPL23</td>
<td>SPL30</td>
<td>SPL30</td>
<td>SPL30</td>
</tr>
<tr>
<td>B: SPL2</td>
<td>SPL2</td>
<td>SPL10</td>
<td>SPL17</td>
<td>SPL24</td>
<td>SPL31</td>
<td>SPL31</td>
<td>SPL31</td>
</tr>
<tr>
<td>C: SPL3</td>
<td>SPL3</td>
<td>SPL11</td>
<td>SPL18</td>
<td>SPL25</td>
<td>SPL32</td>
<td>SPL32</td>
<td>SPL32</td>
</tr>
<tr>
<td>D: SPL4</td>
<td>SPL4</td>
<td>SPL12</td>
<td>SPL19</td>
<td>SPL26</td>
<td>SPL33</td>
<td>SPL33</td>
<td>SPL33</td>
</tr>
<tr>
<td>E: SPL5</td>
<td>SPL5</td>
<td>SPL13</td>
<td>SPL20</td>
<td>SPL27</td>
<td>SPL34</td>
<td>SPL34</td>
<td>SPL34</td>
</tr>
<tr>
<td>F: SPL6</td>
<td>SPL6</td>
<td>SPL14</td>
<td>SPL21</td>
<td>SPL28</td>
<td>SPL35</td>
<td>SPL35</td>
<td>SPL35</td>
</tr>
<tr>
<td>G: SPL7</td>
<td>SPL7</td>
<td>SPL15</td>
<td>SPL22</td>
<td>SPL29</td>
<td>SPL36</td>
<td>SPL36</td>
<td>SPL36</td>
</tr>
<tr>
<td>H: SPL8</td>
<td>SPL8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*** SAS variables (SAS-Code-1.docx) ***  
Y = lowercase(***type) = microsome1-6, HepG2_07-10
**Results**

### Actual Temperature: 27.5

<table>
<thead>
<tr>
<th></th>
<th>VdD ng/ml</th>
<th>Cotinine</th>
<th>mean (Tray 3, 4)</th>
<th>mean (Tray 5, 6)</th>
<th>mean (Tray 7, 8)</th>
<th>120</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.468124137</td>
<td>0.789</td>
<td>0.5635</td>
<td>0.568</td>
<td>0.6155</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>4.8</td>
<td>1.387688626</td>
<td>0.6265</td>
<td>15</td>
<td>15</td>
<td>15.6350</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>24.4</td>
<td>1.65470202</td>
<td>0.424</td>
<td>30</td>
<td>30</td>
<td>0.478</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>279</td>
<td>2.44604205</td>
<td>0.3825</td>
<td>60</td>
<td>60</td>
<td>0.891</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>1010</td>
<td>3.00432174</td>
<td>0.138</td>
<td>120</td>
<td>120</td>
<td>0.903</td>
<td>120</td>
<td>120</td>
</tr>
</tbody>
</table>

- *dose = Vit D* (category)
- *id = natural log(*dose)=log(Vit D)*

<table>
<thead>
<tr>
<th></th>
<th>Absorbance data, microsomes</th>
<th>VitD log data, microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>VdD 30 ng/ml</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Cotinine 300 ng/ml</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Time, min</td>
<td>Time, min</td>
<td>Time, min</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5635</td>
<td>0.568</td>
</tr>
<tr>
<td>4.8</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>24.4</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>279</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>1010</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

**Calculations of VitD concentrations in ng/ml according to the equation**

\[ x = y - 0.9705/0.2704 \]

### Absorbance at 405 nm

There is a linear and negative relationship between the log of the concentration of VitD(X axis) and the average concentration of cotinine (tray 1 & tray 2; Y axis). In other words, a lower level of VitD(X axis) is associated with a higher level of cotinine (Y axis).

These are predicted values produced by the regression equation (NOT very useful). They use data from tray 1 and tray 2 to create a regression equation, and then predict the log of the concentration of VitD in microsomes (tray 3-6)

**Calculations of VitD concentrations in ng/ml according to the equation**

\[ x=10^y \]

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iii https://www.statisticssolutions.com/general-uses-of-analysis-of-covariance-ancova/


vii https://www.investopedia.com/terms/b/bonferroni-test.asp

viii https://www.statisticshowto.com/familywise-error-rate/

ix https://www.statisticssolutions.com/relative-risk/