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Tesis doctoral

Evaluation of biomarkers for studying new challenges in tobacco control.

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Evaluation of biomarkers for studying new challenges in tobacco control

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«Non omnia possumus omnes».

—Virgil

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Resumen

El tabaquismo es reconocido a nivel global como un factor de riesgo significativo para la salud pública y una de las principales causas de muertes prevenibles. España ha implementado políticas de control del tabaco con el objetivo de proteger la salud pública de los daños del tabaco. La Ley 28/2005, en vigor desde el 1 de enero de 2006, prohibió fumar en lugares públicos cerrados. Posteriormente, la Ley 42/2010, implementada el 2 de enero de 2011, amplió la prohibición de fumar a todos los espacios interiores públicos y ciertas áreas al aire libre, brindando una mayor protección. A pesar de estos avances, el tabaquismo sigue siendo un grave problema de salud pública y se están considerando medidas adicionales, como el empaquetado neutro y la expansión de la prohibición de fumar.

La evaluación de la efectividad de las políticas de control del tabaco se realiza mediante diversas medidas, incluyendo la prevalencia del consumo de tabaco, las tasas de enfermedades y mortalidad relacionadas, los costos económicos y los cuestionarios. Los biomarcadores proporcionan una medida directa de la exposición al tabaco y son fundamentales en esta evaluación. En España, las leyes de espacios libres de humo han demostrado reducir

la exposición al humo de segunda mano y disminuir los niveles de cotinina en la población no fumadora. Estos hallazgos destacan la importancia de incorporar biomarcadores en la evaluación del impacto de las políticas de control del tabaco, ya que proporcionan datos objetivos sobre los niveles de exposición y la efectividad de las intervenciones.

El objetivo de este estudio es explorar nuevos biomarcadores para abordar los desafíos emergentes en el consumo de tabaco. Se investigará cómo las legislaciones han influido en los niveles de biomarcadores relacionados con el tabaco en individuos expuestos al humo de segunda mano y se evaluará la efectividad de las leyes en la reducción del consumo de tabaco. Además, se investigó si la tasa de metabolismo de la nicotina y la relación entre sus metabolitos pueden ser biomarcadores confiables de la dependencia de la nicotina. Comparando estos biomarcadores en fumadores, usuarios de cigarrillos electrónicos y no fumadores después de la implementación de las leyes de espacios libres de humo, se obtuvo información sobre su impacto en diferentes productos de tabaco. También se evaluó la aplicabilidad del Test de Fagerström para la Dependencia de Cigarrillos en la medición de las concentraciones de nicotina y cotinina tanto en cigarrillos tradicionales como electrónicos, validando su aspecto bioquímico.

Los resultados de esta investigación se presentan en cuatro artículos científicos, dos de los cuales han sido publicados en revistas indexadas, uno está en proceso de revisión y otro está siendo discutido con los coautores. Estos resultados muestran cambios notables en los biomarcadores relacionados con el tabaco en fumadores y no fumadores después de la implementación de las leyes. La exposición al tabaco ha disminuido en los no fumadores, mientras que en los fumadores se han observado niveles más altos de cotinina salival y nitrosaminas específicas del tabaco en la saliva. Además, se ha encontrado que la tasa de metabolismo de la nicotina y la relación de sus metabolitos son biomarcadores confiables de la dependencia de la nicotina. Después de la implementación de las leyes de espacios libres de humo, los fumadores y los usuarios de cigarrillos electrónicos presentan tasas de metabolismo de la nicotina más altas en comparación con los no fumadores.

En conclusión, la implementación de las leyes de control del tabaco en España ha tenido un impacto significativo en los biomarcadores relacionados con el tabaco. Tras las medidas legislativas se ha reducido la concentración de biomarcadores en la población no fumadora. Asimismo, la tasa de metabolismo de la nicotina y la relación de sus metabolitos han demostrado ser indicadores fiables de la dependencia de la nicotina en diferentes productos

de tabaco. Estos hallazgos resaltan la importancia de seguir desarrollando estrategias basadas en evidencia y políticas de control del tabaco para proteger la salud pública.

Abstract

Smoking is globally recognized as a significant risk factor for public health and one of the leading causes of preventable deaths. Spain has implemented tobacco control policies with the aim of protecting public health from the harms of tobacco. The Law 28/2005, in effect since January 1, 2006, banned smoking in enclosed public places. Subsequently, the Law 42/2010, implemented on January 2, 2011, extended the smoking ban to all indoor public spaces and certain outdoor areas, providing greater protection. Despite these advancements, smoking remains a serious public health problem, and additional measures such as plain packaging and expanding the smoking ban are being considered.

The evaluation of tobacco control policies is conducted through various measures, including tobacco consumption prevalence, rates of related diseases and mortality, economic costs, and questionnaires. Biomarkers provide a direct measure of tobacco exposure and are crucial in this evaluation. In Spain, smoke-free laws have been shown to reduce exposure to secondhand smoke and decrease cotinine levels in the non-smoking population. These findings highlight the importance of incorporating biomarkers in the assessment of the impact of tobacco

control policies, as they provide objective data on exposure levels and intervention effectiveness.

The objective of this study is to explore new biomarkers to address emerging challenges in tobacco consumption. It will investigate how the legislation has influenced levels of tobacco-related biomarkers in individuals exposed to secondhand smoke and evaluate the effectiveness of the laws in reducing tobacco consumption. Additionally, it investigated whether the nicotine metabolism rate and the ratio of its metabolites can serve as reliable biomarkers of nicotine dependence. By comparing these biomarkers in smokers, e-cigarette users, and non-smokers after the implementation of smoke-free laws, information was obtained regarding their impact on different tobacco products. The applicability of the Fagerström Test for Cigarette Dependence in measuring nicotine and cotinine concentrations in both traditional and electronic cigarettes was also evaluated, validating its biochemical aspect.

The results of this research are presented in four scientific articles, two of which have been published in indexed journals, one is in the process of peer review, and another is being discussed with co-authors. These results demonstrate notable changes in tobacco-related biomarkers in smokers and non-smokers after the imple-

mentation of the laws. Tobacco exposure has decreased in non-smokers, while higher levels of salivary cotinine and specific tobacco-specific nitrosamines have been observed in smokers. Additionally, the nicotine metabolism rate and the ratio of its metabolites have been found to be reliable biomarkers of nicotine dependence. After the implementation of smoke-free laws, smokers and e-cigarette users exhibit higher nicotine metabolism rates compared to non-smokers.

In conclusion, the implementation of tobacco control laws in Spain has had a significant impact on tobacco-related biomarkers. Concentrations of biomarkers have decreased in the non-smoking population, while the nicotine metabolism rate and the ratio of its metabolites have proven to be reliable indicators of nicotine dependence in different tobacco products. These findings underscore the importance of continuing to develop evidence-based strategies and tobacco control policies to protect public health.

Contents

Part 1

Introduction

Chapter 1.1 Implications of Tobacco Use

Smoking is widely regarded as a significant public health risk factor and a leading cause of preventable deaths worldwide. This is true not only for individuals who smoke but also for those exposed to smoking[1, 2]. The World Health Organization (WHO) has reported that smoking was responsible for eight million deaths in 2020, with approximately one million of these deaths resulting from non-smokers being exposed to Second-hand smoke (SHS)[2].

Tobacco is known to be linked to a variety of negative health outcomes and premature death, including:

- Cancer. While the development of cancer can have various mechanisms, one common aspect is the harmful impact of cigarettes, which contains over 7000 chemicals[3]. Many of them are carcinogens, having the potential to damage DNA and thus leading to mutations in cells and initiating the process of carcinogenesis. In addition, these carcinogens have the potential to undermine the body's immune system, hindering its ability to eliminate cancer cells, which leads to uncontrolled growth of these kind of cells. Smoking is responsible for the majority of cancer-related deaths,

with approximately 30% of all cancer cases being linked to smoking[1]. It is estimated that nearly 90% of lung cancer deaths are caused by smoking. However, there are other cancers in which evidence is sufficient to infer a causal relationship to smoking, including cancers of the bladder, breast, cervical, colorectal, endometrial, esophageal, kidney, larynx, liver, oral cavity and pharynx, pancreas, and stomach. Approximately 30% of cancer deaths around the world can be attributed to smoking; thus making smoking the leading known cause of cancer[1].

- Cardiovascular Disease. Smoking is a major risk factor for the development of heart disease and stroke, which are the leading causes of death in the developed world[2]. Blood clots can pose a significant health risk when they form within the body[3]. Smoking induces chemical changes in the blood that increase its viscosity, thereby leading to the formation of potentially fatal clots. Such clots can obstruct blood flow to vital organs such as the heart or brain. Furthermore, inhalation of tobacco smoke can induce alterations in blood composition, including elevated triglyceride levels and decreased levels of high-density lipoprotein cholesterol. Tobacco smoke constituents can also impede the repair of arterial lining injuries, which can increase the probability of clot formation within a damaged artery.

All of this increases the risk of arteriosclerosis, the build-up of plaque in the arteries that restricts blood flow, leading to heart attacks and strokes. It also increases the risk of developing peripheral artery disease, a condition in which plaque builds up in the arteries that supply blood to the legs and arms, causing pain and numbness[3]. There is enough evidence to infer a causal relationship between tobacco smoke and abdominal aortic aneurysm, atherosclerosis/peripheral vascular disease, cerebrovascular disease, and coronary heart disease[1].

- Respiratory Disease. The well-established role of tobacco as a major cause of infectious respiratory diseases is widely known[1–3]. Tobacco smoke contains irritants that can cause inflammation in the airways, making it harder to breathe and triggering various diseases. One of these diseases is chronic obstructive pulmonary disease[1], which is a progressive condition that causes irreversible damage to the lungs, leading to airflow blockage and breathing-related problems. Smoking can also trigger or exacerbate other respiratory diseases, such as asthma, chronic obstructive bronchopulmonary disease, chronic respiratory symptoms, influenza, infections, pneumonia, acute respiratory illness, tuberculosis, and reduced or impaired lung function and growth levels[1, 2]. Several studies have been conducted on the

relationship between tobacco and COVID-19 during the pandemic. The current evidence indicates that smokers are more likely to experience severe outcomes from the disease. However, there are conflicting findings regarding smoking's role in COVID-19. Some studies suggest that smoking may be a protective factor against COVID-19, while others suggest it may be a risk factor. Therefore, its exact role in the infection process remains unclear[2].

- Other Health Risks. Smoking has a negative impact on dental disease, diabetes mellitus, diminished health status, eye diseases, hip fractures, liver cirrhosis, low bone density, and peptic ulcer, among others[1].

It is worth highlighting that SHS exposure is a significant public health issue, with devastating consequences for the health and well-being of those who are exposed to it[1–3]. One key finding is that the number of deaths caused by coronary heart disease due to SHS exposure greatly exceeds the number of SHS-related deaths from lung cancer. Additionally, the projected rise in the likelihood of having a stroke due to exposure to SHS is approximately 20-30%.[1, 2]. This underscores the importance of addressing the problem of SHS exposure in order to reduce the incidence of heart disease, which is one of the leading causes of death worldwide. Fur-

thermore, it can also trigger other diseases such as lung cancer and cerebrovascular disease[1]. In addition to the risk of heart disease, exposure to SHS has been shown to have negative health effects on several specific subpopulations. Children, for example, are particularly vulnerable to the effects of SHS exposure. Children who are exposed to SHS are at increased risk of sudden infant death syndrome, reduced infant birth weight, respiratory infections, and asthma attacks[1, 2]. This is a major concern, given that children are often unable to control their own exposure to SHS. Furthermore, adults with certain underlying health conditions may be at increased risk of negative health outcomes due to SHS exposure. For instance, individuals with asthma or heart disease may experience exacerbation of their symptoms when exposed to SHS. This can lead to increased hospitalizations and other health complications[1, 2], making it all the more crucial to address the problem of SHS exposure in order to protect the health and well-being of vulnerable populations.

In addition to the adverse health effects of tobacco, there are significant economic costs associated with smoking. According to the WHO[2], the cost of healthcare related to tobacco consumption amounts to €1.3 trillion globally each year. In Europe, this cost is estimated to range between €10 and €400 per inhabitant per year,

which includes the expenses associated with treating tobacco-related diseases[4]. Moreover, tobacco use has a notable environmental impact as well[5]. The production and disposal of tobacco products cause soil erosion, deforestation, diminishment of biodiversity, emission of greenhouse gases, water pollution, pesticides and fertilizers used. Tobacco products, including cigarette butts, also generate a significant amount of waste that takes years to decompose and contributes to environmental contamination.

Chapter 1.2 The Tobacco Epidemic

Despite the adverse impact on the well-being of the user and the surroundings, tobacco consumption remained prevalent worldwide in 2021, with 17.5% of the population (almost one billion of the global population) aged 15 years and above using it, including 847 million men and 153 million women. It is noteworthy that over 80% of the one billion tobacco users worldwide are residing in low- and middle-income nations[2]. In 2022, approximately 22% of the Spanish population aged 15 years and over reported smoking, with 20% of them being daily smokers. The remaining 2% reported being light smokers. The non-smoking community accounted for the majority, comprising 56% of the population. Meanwhile, former smokers represented 22% of the population. There were over 7.9 million daily smokers in Spain as of 2022, which amounts to 16% of the total population. Of this group, approximately 3.3 million were women (6.9% of the total population) and 4.5 million were men (9.5% of the total population)[6, 7]. This widespread use of tobacco products, including cigarettes, cigars, and smokeless tobacco, and the associated negative health, economic, and environmental consequences is referred to as the tobacco epidemic[1].

The tobacco epidemic generally follows a similar diffusion model, as identified from an epidemiological perspective[8]. Four stages of the tobacco epidemic have been identified. In the first stage, the prevalence of tobacco use is less than 15% in men, and women barely use tobacco. The annual per capita consumption (per adult) is not more than 500 cigarettes, and there is little impact on mortality. This stage may last for two decades. In the second stage, which usually lasts between two to three decades, the prevalence of tobacco use in men reaches its maximum value, ranging between 50% and 80%. Women start using tobacco in this phase, and consumption rapidly increases. The average consumption is estimated to be between 1000 and 3000 cigarettes per year, mostly among men (2000-4000 cigarettes per year). At the end of this stage, around 10% of deaths in men are related to tobacco use. In the third stage, the prevalence of tobacco use in men begins to decline, reaching around 40% by the end of this stage. Tobacco use among women stabilizes, and the prevalence is not expected to reach that of men. The highest cigarette consumption is observed during this stage among both men and women (3000-4000 cigarettes per year). During this stage, mortality associated with tobacco use reaches up to 25-30% in men and 5% in women. This stage can last up to three decades. In the fourth stage, the

prevalence of tobacco use in women (around 30%) approaches that of men (around 35%), which has been and continues to decline in both sexes. The highest mortality rates from tobacco-related causes are observed during these years, 30-35% in men and 20-25% in women. The shift between the different stages is determined by three factors: the prevalence of tobacco use (percentage of daily smokers), the amount of consumption (amount smoked in a period), and the mortality attributable to tobacco use[8]. Currently, in Spain, there is a downward trend in the prevalence of tobacco use in the general population, which began decades earlier in men than in women[6–8]. Therefore, Spain is in the fourth stage of the tobacco epidemic.

1.2.1 Early efforts to combat Tobacco Epidemic

In 2003, the WHO developed the Framework Convention on Tobacco Contro (FCTC) as a response to the tobacco epidemic[9]. This treaty is the first to have been negotiated under the auspices of the WHO and was adopted by the World Health Assembly (WHA), the decision-making body of the WHO, on May 21, 2003. It became effective on February 27, 2005, and has gained rapid and widespread acceptance, making it one of the most widely ratified treaties in the history of the United Nations. To date, the treaty has been ratified by 180

countries worldwide, including 50 member states of the WHO European region[9].

The FCTC is significant because it recognizes that tobacco use is a global public health problem and that it is the leading cause of preventable death worldwide[1, 2, 9]. It is based on the principle that everyone has the right to the highest attainable standard of health, and it offers a comprehensive approach to reducing tobacco consumption and exposure to tobacco smoke. By reaffirming this fundamental right and providing guidelines for evidence-based policies, the FCTC has become an essential tool in the global fight against the tobacco epidemic. Its provisions include measures to protect people from exposure to tobacco smoke, regulate tobacco product marketing and sales, support tobacco cessation programs, prevent tobacco industry interference in public health policies and promote international cooperation in tobacco control efforts[9].

The FCTC has been a valuable tool in the battle against tobacco use, but it faced significant challenges[2]. One of the said challenges was the persistent opposition from the tobacco industry, which has employed various tactics to undermine tobacco control efforts. For instance, the tobacco industry has lobbied against measures to increase taxes on tobacco products, claimed that the FCTC would

have detrimental economic consequences, portrayed tobacco as a problem affecting only high-income countries, challenged the WHO's mandate to develop a tobacco control treaty, argued that tobacco control policy conflicts with principles of good governance and national sovereignty, advocated for corporate social responsibility as an alternative to tobacco control policy, claimed that tobacco is not harmful to health or its effects are negligible, opposed graphic warning labels on tobacco packaging, and attempted to discredit scientific evidence linking tobacco use to health problems, among other strategies[2].

To address these challenges, the MPOWER package was developed by the WHO to provide a comprehensive approach to reducing tobacco use[10]. The MPOWER package was adopted by the WHA, in May 2008. This package is designed to support the implementation of effective interventions to reduce tobacco demand at the country level, in accordance with the guidelines provided in the FCTC. MPOWER focuses on six proven tobacco control measures: Monitoring tobacco use and prevention policies, implementing policies to Protect people from tobacco smoke creating smoke-free public spaces, Offer help to quit tobacco use, Warn people about the dangers of tobacco, Enforce bans on tobacco advertising, promotion, and sponsorship, and Raise taxes on tobacco

products. These measures have been shown to be effective in reducing tobacco use and improving public health outcomes, but their implementation has faced resistance from the tobacco industry and some governments[2].

Recognizing the urgency of curbing the tobacco epidemic, the World Bank published a significant communication in 1999 titled: "Curbing the epidemic: Governments and the economics of tobacco control", which urged nations to intervene in the economics of tobacco control by reviewing a series of measures that could effectively reduce tobacco-related deaths and illnesses[11]. These interventions were deemed cost-effective and included raising taxes on tobacco products, restricting smoking in public places and workplaces, banning tobacco advertising and promotion, providing better consumer information, mandating direct warning labels on tobacco product packaging, and supporting smokers who want to quit by increasing access to nicotine replacement therapy and other cessation treatments. The Tobacco Control Scale was later developed based on these interventions to assess the implementation of tobacco control policies at the national level in the European Union using a new assessment method[12]. The scale serves as a useful tool for tracking progress in tobacco control and evaluating the effectiveness of policies in reducing tobacco use and its associated harms.

As a result of the global efforts to combat the tobacco epidemic, several countries, including Spain, have already adopted measures to reduce tobacco use and exposure to SHS.

1.2.2 Implementing Tobacco Control Policies

Currently, at least one MPOWER measure is implemented in 75% of all countries, which have a combined population of over 5.3 billion people. In half of these countries, two or more MPOWER measures have been adopted[2]. For example, in Europe, there have been several achievements in tobacco control policies. The European Union (EU) has implemented the Tobacco Products Directive, which includes measures such as the requirement for larger health warnings on cigarette packs and a ban on flavored cigarettes[13]. The EU also established the European Network for Smoking and Tobacco Prevention to promote tobacco control policies and coordinate efforts among member states[14].

Spain has also made significant progress in tobacco control policies, and two laws have been implemented to protect public health from the harms of tobacco. The first law, known as Law 28/2005, came into effect on January 1st, 2006 which prohibits smoking in all enclosed public places, including bars and restaurants[15]. The law was well accepted by the population, and sup-

port increased especially among smokers[16]. However, the social perception of compliance was lower in bars, restaurants, and leisure spaces compared to other areas of exposure. The law did not result in changes in tobacco consumption, which has been declining in Spain for some years. Nevertheless, the law significantly reduced exposure to SHS in the workplace, particularly in the hospitality industry. Furthermore, the law had a positive impact on health, specifically in reducing respiratory symptoms in hospitality workers, and data suggests a reduction in cases of myocardial infarction in the general population. The law did not have a negative economic impact on the hospitality industry in terms of employment and sales volume. However, there were geographical imbalances in the inspection and enforcement of compliance with the law among different autonomous communities. The most unfavorable situation caused by this legislation was the high degree of exposure to SHS suffered by hospitality workers. Despite this limitation, the law marked a significant step forward in tobacco control in Spain[16].

Realizing the need for more comprehensive tobacco control measures, Spain introduced the second law, Law 42/2010, on January 2nd, 2011[17]. This law extended the prohibition of smoking in all public indoor areas and some outdoor areas, providing greater protection for the

public from the harms of tobacco. The law also included stricter measures to ensure compliance with the smoke-free policies and hefty fines for violators. The evaluation of both tobacco control laws in Spain revealed that while significant progress has been made, there are still challenges ahead[18]. To further denormalize tobacco use, pending actions include implementing plain packaging and developing preventive campaigns. Regulatory policies for tobacco taxes should also be reviewed to equalize the price of different products. Future interventions should consider creating new smoke-free spaces, particularly in areas where minors and vulnerable groups may be exposed, such as homes and private vehicles. Regulations for smoke-free spaces should also apply equally to e-cigarettes. Other pending actions include expanding and systematizing cessation support, assessing the need for specific interventions for vulnerable groups, and promoting training for healthcare professionals in effective smoking cessation interventions[18]. These two laws demonstrate Spain's commitment to tobacco control and its efforts to protect public health.

1.2.3 Current Challenges in Tobacco Epidemic

While tobacco control policies and interventions have been successful in reducing smoking prevalence in many countries (including Spain), there are still many chal-

lenges in tobacco control.

One of the primary challenges in tobacco control is the growing prevalence of alternative nicotine products, including Electronic Nicotine Delivery Systems (ENDSs) such as Electronic cigarettes (e-cigs) and Heat-Not-Burn Products (HNBs). These products, along with Electronic Non-Nicotine Delivery Systems (ENNDSs), have gained popularity in recent years[2]. The key difference is that ENNDSs do not contain nicotine, whereas ENDSs do. Both types of devices are electronic devices that deliver an aerosolized solution to the user, which is typically inhaled. However, the absence of nicotine in ENNDSs makes them less addictive compared to ENDSs. Both have been marketed as a safer alternative to traditional tobacco products and have attracted many young people to start vaping. However, research has shown that neither of them is safe and can be harmful to health. The long-term health effects of ENDSs and ENNDSs are still unknown, and more research is needed to understand their potential risks. On the other hand, HNB products are products that, like traditional cigarettes, heat tobacco to release nicotine[2].

Another challenge is the marketing and advertising of tobacco products, especially to young people[2]. Tobacco companies have long used advertising and promotional

strategies to attract new users, particularly among youth populations. The use of social media and other online platforms has made it easier for tobacco companies to reach and engage young people. Additionally, the use of flavored tobacco products, which are more attractive to young people, has increased in recent years, further increasing the likelihood of youth smoking initiation[2].

In 2020 alone, the sale of these types of products increased by 40% in Spain, largely supported by the intensive advertising campaigns of the tobacco industry[19]. Despite the general trend among the population to think that these products are less harmful than conventional ones[20], there is no evidence to support this idea[19]. In addition, the aerosol expelled by ENDSs and ENNDSs emits toxic substances for humans. For these reasons, the official position of the Spanish Ministry of Health is to consider them as a danger to the consumer's health[20]. Therefore, these products must adhere to the action lines approved by the Public Health Commission in May 2019. This measure legally equates these new products and traditional cigarettes, to some extent, with the regulation of new products being much more lax than that of traditional cigarettes[20].

Furthermore, tobacco control policies encounter significant opposition from the tobacco industry, which pos-

sesses considerable financial resources to influence and hinder tobacco control initiatives[2]. Tobacco companies often challenge tobacco control policies in court, delaying the implementation of regulations or watering down their effectiveness.

Comprehensive tobacco control policies, including increasing taxes, implementing smoke-free laws, and regulating tobacco advertising, have proven to be highly effective in reducing tobacco use and associated health risks[2]. Many countries are implementing these policies to also tackle the use of ENDSs and ENNDSs. Consequently, smoking prevalence and tobacco-related deaths have significantly decreased. Despite the existence of tobacco control policies, however, there is still a need to strengthen them. Countries are adopting similar regulatory measures for ENDSs due to the effectiveness of tobacco control policies in reducing tobacco use. Many countries have implemented legislative measures such as bans on sales and advertisements, health warnings on packaging, and restrictions on use in public places to regulate ENDS. According to the WHO, 111 countries have implemented some form of regulation on ENDS, with 32 of these countries completely banning the sale of ENDSs (covering 2.4 billion people) and the remaining 79 countries adopting one or more legislative measures to regulate ENDSs (covering 3.2 billion people). It is worth

noting that only 30 countries have completely banned the use of ENDSs in all indoor public places, workplaces, and public transport. The use of ENDS in public places where smoking is prohibited may re-normalize smoking in public. Only eight countries mandate the inclusion of large graphic health warnings on ENDSs packaging, and 22 countries have completely banned the advertising, promotion, and sponsorship of ENDSs devices, e-liquids, or both. There is a growing trend of monitoring ENDSs use among children, adolescents, and adults through nationally representative surveys, with 87 countries now collecting data on prevalence[2].

The launch of new tobacco-related products is an example of a new challenge in tobacco control, but there are still old challenges that have been persisting for years, such as exposure to SHS, which contributes to the worsening of non-smokers' health[2]. Research on exposure to SHS has laid the foundation for studying another new challenge in tobacco control, the persistence of certain toxic substances on surfaces (known as thirdhand smoke). Currently, little is known about this type of exposure in new products and its effects on health, although there are indications that it is associated with the onset of various diseases, especially in the pediatric population. Despite all these efforts, we are still far from victory, as more than 1 billion people around the world still

smoke[2].

Chapter 1.3 Monitoring the Tobacco Epidemic

To evaluate the effectiveness of tobacco control policies, various metrics can be used, such as tobacco use prevalence, tobacco-related illness and mortality rates, economic costs of tobacco use, and questionnaires[2]. While biomarkers provide a direct measure of exposure to tobacco, the other measures can be obtained through indirect means or secondary sources. For instance, in Spain, the implementation of smoke-free laws led to a 20% reduction in self-reported exposure to SHS and an 87.6% reduction in cotinine concentration in the non-smoking population, which were determined through surveys and biomarker analysis, respectively[21].

1.3.1 Use of Questionnaires

Questionnaires have been used extensively in tobacco control as a reliable tool for data collection, allowing researchers and policymakers to gather information on the prevalence[2], patterns, attitudes, and perceptions of tobacco use. These surveys provide valuable data that can be used to inform about the development, implementation, and evaluation of tobacco control policies and programs. Furthermore, questionnaires can be tailored to specific populations and subgroups, allowing

researchers to identify trends in tobacco use and attitudes towards tobacco control measures across different demographics[2].

Another commonly used questionnaire in tobacco control is the Fagerström Test for Cigarette Dependence (FTCD)[22]. The FTCD is a widely accepted instrument for measuring nicotine dependence and is often used in clinical settings to assess a smoker's level of dependence on tobacco. The FTCD is a useful tool for identifying individuals with high levels of cigarette dependence, which may require more intensive treatment and support in quitting smoking. The FTCD was originally developed to measure nicotine dependence[23, 24]. Since then it was better proposed as a measure of dependence on traditional cigarettes[22]. The test has been validated and it is a widely used tool among researchers in spite of its poor psychometric properties[24–27] and its limited potential as a predictor of nicotine and cotinine concentration. Its widespread use in research and clinical settings makes it a valuable asset in tobacco control efforts. However, with the introduction of e-cigs in recent years, there has been a debate about whether these tests can be applied to e-cigs smokers as well as traditional cigarette smokers[27].

In addition to questionnaires, the strength of a national

tobacco surveillance system is assessed by the frequency and periodicity of nationally representative youth and adult surveys in countries[2]. A strong national tobacco surveillance system is essential for the effective evaluation of tobacco control policies, as it provides accurate and up-to-date information on key indicators such as tobacco use prevalence, exposure to SHS, and the economic costs of tobacco use. The MPOWER framework recognizes the importance of a strong national tobacco surveillance system, and provides a comprehensive approach to tobacco control that encompasses various measures for policy development, implementation, and evaluation[2].

Countries are grouped in the top Monitoring category in the MPOWER framework when all criteria listed below are met for both youth and adult surveys: whether a survey was carried out recently, whether the survey was representative of the country's population, whether a similar survey was repeated within 5 years (periodic), and whether the youth and adult populations were surveyed through school-based and household population-based surveys respectively[2]. Surveys were considered recent if conducted in the past 5 years and representative only if a scientific random sampling method was used to ensure nationally representative results. Subnational surveys or national surveys of specific population groups provide insufficient information to enable tobacco control

action for the total population[2].

Spain has implemented several anti-smoking measures over the years, and questionnaires have been a crucial tool in evaluating the effectiveness of these policies[2]. The WHO evaluated the available documents and generated a summary of the measures and indicators that demonstrate the achievements of each country in implementing the MPOWER measures. The results of the evaluation in Spain indicated that compliance with bans on advertising, sponsorship, and adherence to smoke-free laws was high, with approximately 80% of establishments adhering to the laws. These findings demonstrate the effectiveness of questionnaires in monitoring the implementation of anti-smoking policies[2].

Another illustrative instance of the application of questionnaires in evaluating the effectiveness of anti-smoking measures in Spain is the National Health Survey conducted in 2017[7] and the European Health Survey in Spain conducted in 2020[28].

The National Health Survey has been conducted several times in Spain since its inception in 1987; The most recent survey was conducted in 2017[7]. It is dedicated to providing statistical information on the population's health and its determinants, the magnitude and distribution of disease and disability, and access to and use of

health services. The survey is aimed at the entire population residing in primary family households throughout the national territory. The survey has a whole section dedicated to investigating tobacco smoke exposure in both adults and minors. Its primary focus is on the number of passive smokers and the duration of time they spend in environments filled with tobacco smoke, including their own homes, means of transportation, enclosed public places, and indoor areas of their workplace. Moreover, the survey provides comprehensive information on the length of time individuals typically spend in different environments laden with tobacco smoke, including their own homes, means of transportation, and enclosed public places[7].

In addition to the National Health Survey, the European Health Survey in Spain also provides information on the health status of the Spanish population, in order to plan and evaluate healthcare actions[28]. Its primary goal is to obtain data on health status, healthcare utilization, and health determinants in a harmonized and comparable way at the European level. The target population includes individuals aged 15 years and over who are habitual residents in their main family households throughout the national territory. It has a section devoted to measuring exposure to tobacco smoke in enclosed places[28].

The completion of the two surveys mentioned above has allowed for a comprehensive characterization of the smoking problem in Spain[7, 28]; the percentage of daily smokers among women and men was recorded to be 16.4% and 23.3%, respectively. Notably, the highest proportion of daily smokers among men was observed in the age group of 25-34, whereas for women, it was in the 45-54 age group. It was also evident that the population of daily smokers is predominantly concentrated in the age range of 25-64, with the rates being approximately 30% in men and 20% in women. However, the percentage of daily smokers tends to decrease after the age of 65, albeit more prominently in women than in men. Additionally, occasional smoking rates were reported by 2.1% of women and 2.6% of men, with the highest percentage of occasional smokers among men being in the 25-34 age group (3.9%) and among women being in the 15-24 age group (3.9%). Moreover, 16.7% of women and 27.6% of men reported being former smokers, with the highest proportion of former smokers among women being in the 55-64 age group (27.5%) and among men being in the 75-84 age group (51.8%). The majority of women (64.8%) and men (46.4%) have never smoked, with the highest percentages for women in the 85 and over age group (95.6%) and for men in the 15-24 age group (73.9%)[7, 28].

Concerning exposure to tobacco smoke, the surveys results revealed that 87.5% of women and 85.3% of men have either never been exposed or have been exposed to tobacco smoke in enclosed spaces almost never[7, 28]. The population aged 65 and over reported the least exposure, with 95.1% of women and 92.9% of men indicating no exposure. However, among the 15-24 age group, a considerable proportion of men (10.2%) and women (9.4%) were exposed to tobacco smoke in enclosed spaces every day. With regards to alcohol consumption, 74.6% of men aged 15 and over reported consuming alcohol in the past 12 months, compared to 56.8% of women. Notably, the highest percentage of alcohol consumers by age group was observed among those aged 25-34 (80.6% for men and 64.7% for women). The survey also found that men consume alcohol more frequently than women, with 19.7% of men reporting daily consumption compared to 5.9% of women. Finally, 25.4% of men and 43.2% of women reported never consuming alcohol. In addition to national surveys, questionnaires have been used to evaluate anti-smoking measures in specific populations in Spain. For example, a study conducted among pregnant women aimed to evaluate the effectiveness of a smoking cessation program. The study used questionnaires to assess smoking behavior before and after the program. The results showed that the program was effective in

reducing smoking among pregnant women, with 25% of participants quitting smoking during the program. This study demonstrates the utility of questionnaires in evaluating the effectiveness of anti-smoking measures in specific populations, such as pregnant women[7, 28].

In conclusion, questionnaires are a useful tool for evaluating anti-smoking measures, but their use should be balanced with an awareness of their limitations[2]. To minimize the potential for response bias, survey designers should use carefully phrased questions and ensure the anonymity and confidentiality of the responses. Additionally, surveys should be designed with a mix of closed-ended and open-ended questions to allow for more nuanced responses[2].

1.3.2 Biomarkers of Tobacco

In order to conduct a thorough and rigorous evaluation of anti-smoking measures, it is common practice to incorporate multiple data sources, including survey results, biomarkers, and observational studies[29, 30]. Biomarkers, in particular, provide a valuable tool for assessing tobacco use and exposure. These measurable indicators of biological processes, diseases, or exposures can be used for diagnostic, predictive, and monitoring purposes[31]. By examining biomarkers of tobacco use and exposure, researchers are able to gain insight into the

underlying mechanisms of tobacco-induced diseases and a more objective measure of tobacco use and exposure than self-reported measures. In addition, biomarkers are able to detect exposure to tobacco products that may not be captured by self-reporting, such as SHS even in the case of exposure to smokeless tobacco products. Furthermore, biomarkers can provide information on the intensity and duration of tobacco exposure, which can help researchers better understand the relationship between tobacco use and disease[29–31].

Among the various biomarkers used to assess tobacco exposure and use, tobacco-specific biomarkers are unique to tobacco smoke and can be used to assess exposure to tobacco smoke. In contrast, tobacco-related biomarkers are biomarkers of the biological effects of tobacco smoke exposure[32].

1.3.3 Tobacco-Specific Biomarkers

1.3.3.1 Nicotine and Its Metabolites

Nicotine is a well-known biomarker that is commonly used in clinical and research settings to assess tobacco use and exposure[29]; It is a naturally occurring chemical compound found in tobacco leaves that is responsible for the addictive properties of tobacco products such as cigarettes, oral snuff, pipe tobacco, cigars, chewing to-

bacco, and ENDSs. When tobacco products are smoked or chewed, nicotine is absorbed into the bloodstream and distributed throughout the body, where it is metabolized into various byproducts. Because nicotine is a specific and sensitive marker of tobacco use and exposure, it is commonly used as a biomarker in clinical and research settings[29].

Once inhaled, nicotine is absorbed into the body and primarily metabolized in the liver by the Enzyme cytochrome P450 2A6 (CYP2A6), which converts nicotine to cotinine, a major metabolite of nicotine[29]. Cotinine is then further metabolized to trans - 3' - hydroxycotinine (3-HC), which is excreted in the urine. Because cotinine and 3-HC have longer half-lives than nicotine (nicotine half-life averages 2h in plasma while cotinine and 3-HC average 16h and 7h, respectively) and can be measured in human fluids such as saliva, plasma, blood, or urine for up to ten days after nicotine metabolism, they are often preferred biomarkers for assessing tobacco exposure over nicotine itself. Although approximately 80% of nicotine is metabolized to cotinine, the measurement of the same biomarker differs depending on the biological matrix and the point of the pharmacokinetic pathway. In smokers, blood or plasma nicotine concentrations during afternoon sampling are typically in the range of 10 to 50 ng/ml. Furthermore, at the 6-hour

mark following oral nicotine dosing, the correlation coefficients between plasma 3-HC ratios and oral nicotine and cotinine clearances were found to be 0.78 and 0.63, respectively.

There are several methods for detecting nicotine and its metabolites in biological samples, including blood, urine, and saliva. The most commonly used method is Chromatography-mass spectrometry (GC-MS)[29], which is a highly sensitive and specific technique for detecting trace amounts of nicotine and its metabolites[29].

1.3.3.2 Tobacco-specific nitrosamines

Tobacco-Specific Nitrosamines (TSNAs) are a group of potent carcinogenic compounds found in tobacco products[33]. The formation of TSNAs occurs during the curing and processing of tobacco leaves. During the curing process, tobacco leaves are dried and fermented, which results in the breakdown of the tobacco proteins and the release of free amino acids. Nitrogen-containing compounds, such as nitrates and nitrites, are also present in tobacco leaves. These compounds can react with the free amino acids and nicotine present in the tobacco leaves to form TSNAs. The formation of TSNAs is enhanced by high temperatures and high humidity levels, which are commonly used during tobacco processing. The most common TSNAs found in tobacco products

include N-Nitrosonornicotines (NNNs), 4 - (methylnitrosamino) - 1 - (3 - pyridyl) - 1 - butanones (NNKs), and 4 - (methylnitrosamino) - 1 - (3 - pyridyl) - 1 - butanols (NNALs)[33, 34].

TSNAs are highly toxic and can cause various types of cancer[35]. Studies have shown that NNNs and NNKs are carcinogenic and mutagenic, which means that they can cause mutations in DNA, leading to the development of cancer cells[34, 35]. TSNAs can be detected with Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and has also been used as a biomarker for the effectiveness of smoking cessation interventions, as its levels decrease after quitting smoking[34, 35]. The half-lives of TSNAs vary depending on the specific compound. For example, NNAL has a half-life of around 26h[35]. Therefore, reducing exposure to TSNAs through smoking cessation is crucial in preventing the harmful effects of these toxic compounds on human health.

In addition, the Rate of Nicotine Metabolism (RNM), obtained by dividing the concentration of cotinine by that of nicotine, has been shown to be associated with the number of cigarettes smoked per day and the degree of nicotine dependence[36]. Higher RNM values indicate faster nicotine metabolism, which in turn is associated with increased tolerance to nicotine and a greater risk

of addiction[36]. Therefore, RNM can serve as a useful biomarker for predicting the risk of nicotine dependence and related health effects. However, since the half-life of nicotine is very short, this ratio is highly dependent on the time elapsed since the last cigarette was smoked and the number of cigarettes smoked, resulting in RNM being highly variable throughout the day. To overcome this issue, the use of Nicotine Metabolite Ratio (NMR), that is the ratio of 3-HC to cotinine, is encouraged. The NMR is a reliable indicator of nicotine metabolism, as it remains consistent across various biological fluids[37, 38]. For example, if one method identifies a person as a slow metabolizer, they are highly likely to be categorized as such in other fluids, although the cut-off points may vary. This trait allows the NMR to capture both inter-individual and environmental differences. In addition, the NMR is more stable than the RNM, exhibiting minimal variation throughout the day, lower sensitivity to the time elapsed since the last cigarette in smokers, and sustained stability over a year's time. Even individuals who have recently quit smoking show stable NMR values. NMR values in saliva and urine can serve as proxies for plasma NMR, although the former tend to be more variable[37, 38]. Both of them are related to the activity of the CYP2A6[37], further emphasizing its reliability as biomarkers for nicotine metabolism.

1.3.4 Tobacco-Related Biomarkers

Another group of biomarkers that has gained interest in recent years are Volatile Organic Compounds (VOCs)[32]. These are small molecules released by the incomplete combustion of tobacco and other substances present in cigarette smoke. They can be detected in exhaled breath, saliva, and urine samples, making them easy to collect and non-invasive. VOCs have been shown to be reliable biomarkers for assessing exposure to tobacco smoke, and some have been linked to specific health effects, such as lung cancer and cardiovascular disease. For instance, acrolein, a VOCs found in cigarette smoke, is a known respiratory irritant and has been shown to be associated with irritations, damage to tissues, the formation of DNA adducts, mutagenicity, and potent carcinogenic effects. Similarly, benzene, another VOCs found in cigarette smoke, has been reported to have strong carcinogenic effects[32].

However, the detection and quantification of VOCs can be challenging, as they are present in low concentrations and can be influenced by factors such as diet and environmental exposure[32]. Nonetheless, advances in analytical techniques have led to the development of sensitive and specific methods for detecting VOCs (like GC-MS), which have greatly improved our ability to

monitor tobacco smoke exposure and associated health risks[39].

Apart from biomarkers related to tobacco smoke exposure, there are also biomarkers related to the biological effects of smoking. For example, DNA adducts are formed when TSNAs in tobacco smoke bind to DNA molecules, leading to mutations that can contribute to cancer development. These adducts can be measured in various biological samples, including blood and urine, and have been shown to be associated with the risk of lung cancer and other smoking-related diseases[32].

1.3.5 Clinical applications of Biomarkers

Biomarkers can be used to assess tobacco use and exposure in individuals, as well as in populations[31]. For example, in population-based studies, nicotine biomarkers can be used to estimate the prevalence of tobacco use and exposure, as well as to identify subgroups that are at higher risk for tobacco-related diseases.

Several studies have utilized nicotine biomarkers to assess tobacco use and exposure in various populations. For instance, a study conducted in Spain analyzed urinary cotinine levels in a sample of non-smoking pregnant women to assess their exposure to SHS[40]. The study found that urinary cotinine levels were positively associ-

ated with exposure to SHS, and exposure to SHS during pregnancy, in turn, resulted in adverse fetal outcomes[41, 42].

Cotinine levels, among other biomarkers, can be a valuable means of monitoring the efficacy of smoking cessation interventions, including behavioral counseling and the use of nicotine replacement therapy (NRT), as indicated by the WHO[2]. A decrease in nicotine or cotinine levels in biological samples can indicate successful smoking cessation[43]. Moreover, studies have shown that the implementation of smoking cessation interventions during pregnancy can be effective in reducing the number of women who continue to smoke in late pregnancy, as well as lowering the incidence of low birthweight and preterm birth. It is therefore crucial for these interventions to be widely available in all healthcare settings in order to support people in quitting smoking and to reduce social inequalities associated with tobacco addiction[42, 43].

Biomarkers can be used to diagnose and monitor tobacco-related diseases, such as lung cancer, chronic obstructive pulmonary disease, and cardiovascular disease[29]. Nicotine and its metabolites have been shown to be associated with an increased risk of these diseases, and their detection in biological samples can aid in early diagnosis and treatment[29].

The effectiveness of tobacco control policies, such as smoke-free laws and tobacco taxes, can be evaluated through the use of biomarkers[44]. By assessing changes in nicotine biomarker levels in populations before and after the implementation of tobacco control policies, researchers can determine the impact of these policies on reducing tobacco use and exposure[44].

1.3.6 Constraints of Biomarkers

While biomarkers are useful tools for assessing tobacco use and exposure, there are several limitations to their use. Firstly, biomarkers can give false-positive or false-negative results in certain situations. For example, some medications or dietary constituents can interfere with the metabolism of nicotine and its metabolites, leading to inaccurate results[29]. Nicotine biomarkers and TSNAs have a relatively short detection window, which means they are only useful for assessing recent tobacco use or exposure[29, 35]. Secondly, biological variables such as, age, sex, Body Mass Index (BMI), and genetics can all affect the metabolism of nicotine and other biomarkers. For example, studies have shown that sex hormones play a role in the metabolism of cotinine, with women having a faster nicotine metabolism than men. However, the literature is unclear about the effect of age and BMI on biomarker levels[29].

Moreover, the same biomarker measured on different biological matrices may result in different quantities[29, 32, 34]. For instance, cotinine can be measured in blood, urine, saliva, and hair, and the concentration of cotinine can vary depending on the biological matrix used. This variation is due to the differences in absorption, distribution, metabolism, and elimination of the biomarker in different biological matrices[29, 32, 34]. Therefore, it is crucial to carefully select the appropriate biological matrix for the measurement of the specific biomarker of interest, taking into consideration the study objectives and the characteristics of the biomarker in question. Overall, biomarker measurement requires careful consideration and standardization of procedures to ensure the accuracy and comparability of results.

To sum up, biomarkers are powerful tools for assessing tobacco exposure, tobacco related health effects, and smoking behavior. While some biomarkers, such as nicotine, have undergone extensive research and validation, other biomarkers, such as ratios, VOCs and DNA adducts, require further validation. Despite the limitations of biomarkers, such as their susceptibility to interference by certain medications or dietary constituents and their variability across different biological matrices, nicotine biomarkers remain a valuable tool for assessing tobacco use and exposure and improving pub-

lic health. Nonetheless, further research and validation of biomarkers are necessary to enhance their accuracy and reliability. We should note that biomarkers cannot replace self-reported measures of smoking behavior, but they can be used in combination with them to provide a more comprehensive assessment of tobacco use and exposure. By integrating survey results, biomarker data, and observational studies, researchers can offer a more complete evaluation of anti-smoking measures. This approach can address the limitations of self-reported measures, such as underreporting or social desirability bias, and provide a more precise evaluation of tobacco use and exposure. Only by combining biomarker data with other sources of information, such as questionnaires, researchers can generate a more complete picture of the state of the tobacco epidemic at both the individual and population levels.

Part 2

Hypothesis and Objectives

Chapter 2.1 Hypothesis

- The Spanish comprehensive smoke-free legislations in Spain (Law 28/2005 and Law 42/2010) have led to a decrease in tobacco-related biomarkers among smokers.
- The Spanish comprehensive smoke-free legislations in Spain (Law 28/2005 and Law 42/2010) have also led to a decrease in tobacco-related biomarkers among non-smokers.
- The Rate of Nicotine Metabolism and the Nicotine Metabolite Ratio can be used as biomarkers of nicotine dependence.
- Smokers and electronic cigarettes with nicotine users after the smoke-free legislations in Spain (Law 28/2005 and Law 42/2010) have higher Nicotine Metabolite Ratio and Rate of nicotine metabolism than non-smokers.
- The Fagerström Test for Cigarette Dependence is associated with electronic cigarette use and therefore can be used as a reliable predictor of nicotine and cotinine concentration in electronic cigarettes, similar to its predictive ability in traditional cigarettes.

Chapter 2.2 Objectives

- To investigate the effect of the Spanish comprehensive smoke-free legislations (Law 28/2005 and Law 42/2010) on the levels of tobacco-related biomarkers among smokers.
- To examine the impact of the Spanish comprehensive smoke-free legislations (Law 28/2005 and Law 42/2010) on the levels of tobacco-related biomarkers among non-smokers.
- To determine whether the Nicotine Metabolite Ratio and the Rate of nicotine metabolism can serve as reliable biomarkers of nicotine dependence.
- To compare the Nicotine Metabolite Ratio and the Rate of Nicotine Metabolism in smokers and electronic cigarettes with nicotine users after the implementation of the Spanish smoke-free legislations (Law 28/2005 and Law 42/2010) with non-smokers.
- To assess if the predictive property of the Fagerström Test for Cigarette Dependence to measure nicotine and cotinine concentration in traditional cigarettes is also applicable to electronic cigarettes, thus validating the biochemical aspects of the test.

Part 3

Methodology

In order to achieve the objectives of this doctoral thesis, two epidemiological studies were carried out. These studies collected information on smoking-related characteristics, sociodemographic characteristics, anthropometric characteristics, and biomarkers information. For a more comprehensive understanding of the methodology used, please refer to the papers of the doctoral thesis under Research objectives and results. Nonetheless, a brief overview of the methodologies is presented below.

Chapter 3.1 dCOT3 Study

The first project, titled "Impact of Spanish legislation for tobacco control on tobacco consumption and passive exposure to tobacco in the adult population: a cohort study with biomarkers" (dCOT3), is a longitudinal study of a representative sample of the non-institutionalized adult general population in the city of Barcelona, aged 16 years or older. The baseline sample of 1,245 individuals was determined during the years 2004-2005, and a follow-up was conducted during 2013-2014 with 736 participants. From the baseline population, 235 individuals were excluded: 101 died, 49 no longer resided in Barcelona, and 85 did not sign the informed consent or were minors ($<$ 18 years old) whose legal guardians did not accept the informed consent. During the follow-up, 72.9% of the sample agreed to participate, 18.5% refused to participate, and 7.2% had moved out of Barcelona. All individuals who had participated in the study in 2004-2005 and continued to reside in the city of Barcelona were included if they agreed to participate in the follow-up and were interviewed at their homes after being contacted by letter and phone appointment. Prior to obtaining informed consent, selected subjects were personally interviewed. In cases where they could not answer for themselves due to inca-

pacity or disability or did not understand the language (Spanish or Catalan), a close person who lived with or spent at least 8 hours a day with the index subject answered the questionnaire. The same Computer-Assisted Personal Interview (CAPI) questionnaire on smoking-related characteristics, sociodemographic characteristics, anthropometric characteristics, and biomarker information used in the baseline research in 2004-2005 (PI020981) was used to enable comparisons, implemented using a personal computer. After completing the questionnaire, saliva and urine samples were collected. Information on the methodology can be found on this website: https://www.icoprevencio.cat/uct/es/portfolio/dcot3/.

Chapter 3.2 e-cig Study

The second project (PI15/00291), which is titled "Pattern of use, acceptability, and risk perception of electronic cigarettes: prospective cohort study with biomarkers," has a representative sample of adult e-cig users from the population of Barcelona. The study included adults aged 18 years or older who were residents of Barcelona and e-cig users at the time of recruitment. These individuals agreed to be interviewed at their homes during the week following recruitment to answer a CAPI questionnaire about e-cig use, after which a saliva sample was collected.

Chapter 3.3 Biomarkers Determination

To determine the concentration of biomarkers in saliva samples, a standardized protocol was utilized[34]. Participants were instructed to rinse their mouths and stimulate saliva production by sucking on a lemon candy (Smint®), and then provide 9 mL of saliva by spitting directly into a test tube with the help of a funnel. Each individual sample was divided into 3 mL aliquots and stored at -20°C until analysis. The frozen samples were sent to the Group of Integrative Pharmacology and Systems Neuroscience of the Municipal Institute for Medical Research (IMIM-Hospital del Mar) in Barcelona, where biomarkers were determined using alkaline single liquid-liquid extraction with dichloromethane/isopropanol followed by LC-MS/MS. The limit of quantification for nicotine, cotinine, and 3-HC was 0.5 ng/mL, 0.1 ng/mL, and 0.04 ng/mL, respectively. Values below the limit of quantification were halved to avoid overestimation or underestimation bias.

Part 4

Research objectives and results

The doctoral thesis comprises four scientific articles, two of which have been published in journals included in the Journal Citation Reports (SCI/SCCI) or SCOPUS.; one is currently under peer review in a journal indexed in both systems; the other is being discussed with coauthors. The correspondence with the journal that accepted the paper included in this thesis can be found in Annex II to Annex III. During my training for this doctoral thesis, I also participated in other article related to biomarkers, which is published in a journal indexed in Web of Science (see Annex IV). The articles included in this thesis are listed below.

1. Article I: Pérez-Martín H, Lidón-Moyano C, González-Marrón A, Fu M, Pérez-Ortuño R, Ballbè M, Martín-Sánchez JC, Pascual JA, Fernández E, Martínez-Sánchez JM. Changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers before and after Spanish smoke-free legislation. Cancer Epidemiology 80, 102226. ISSN: 1877-7821. https://doi.org/10.1016/j.canep.2022.102226. Cancer Epidemiology is included in the CiteScore of Scopus with a CiteScore in 2022 of 5.0 (position 45/115 in the category Epidemiology).

   - Objective: To evaluate variations in salivary cotinine cut-offs to discriminate smokers and non-smokers

before and after the implementation of smoke-free legislation (Law 28/2005 and Law 42/2010) in a sample of the adult population of Barcelona, Spain.

- Results: The mean salivary cotinine concentration was significantly lower post-2010 law (-85.8%, $p < 0.001$). The ROC curves determined that the optimal cotinine cut-off points for discriminating non-smokers and smokers were 10.8 ng/mL (pre-2005 law) and 5.6 ng/mL (post-2010 law), with a post-2010 law sensitivity of 92.6%, specificity of 98.4%, and an area under the curve of 97.0%. The post-2010 law cotinine cut-off points were 5.6 ng/mL for males and 1.9 ng/mL for females.

2. Article II: Pérez-Martín H, Lidón-Moyano C, González-Marrón A, Fu M, Pérez-Ortuño R, Ballbè M, Martín-Sánchez JC, Pascual JA, Fernández E, Martínez-Sánchez JM. Changes in concentrations of Tobacco Specific Nitrosamines in saliva in the general population of Barcelona before and after implementation of tobacco control legislation. This Manuscript is in peer review in Journal Citation Report of ISI-Web of Science.

   - Objective: To evaluate the changes TSNAs levels in the saliva of smokers and non-smokers before and after the implementation of the Spanish smoke-free legislation.

- Results: Salivary concentration of TSNAs in Smokers at baseline decreased by -90.5% [- 95.0; -82.2], -48.7% [-60.1; -34.0], and -86.2% [-90.1; -80.9] for NNN, NNK and NNAL, respectively. Continuing smokers increased its concentration of NNN by 149.8% [36.8; 356.1], while no significant change in the TSNAs concentration of Continuing non-smokers was observed.

3. Article III: Pérez-Martín H, Lidón-Moyano C, González-Marrón A, Fu M, Pérez-Ortuño R, Ballbè M, Martín-Sánchez JC, Pascual JA, Fernández E, Martínez-Sánchez JM. Variation in Nicotine Metabolization According to Biological Factors and Type of Nicotine Consumer. Healthcare 11 (MDPI, 2023), 179. ISBN: 2227-9032. https://www.mdpi.com/2227-9032/11/2/179. Healthcare is included in the Journal Citation Report of ISI-Web of Science with and impact factor in 2021 of 3.160 (position 35/88 in the category HEALTH POLICY & SERVICES).

   - Objective: To describe the NMR among tobacco smokers and e-cigs users and nonusers in Barcelona, Spain.
   - Results: Exclusive users of e-cig without nicotine have the lowest rate of nicotine metabolism (Geometric mean: 0.08, p-values < 0.001) while cigarette smokers have the highest (Geometric mean: 2.08,

p-values < 0.001). Nonusers have a lower nicotine metabolic rate than cigarette smokers (Geometric means: 0.23 vs. 0.18, p-value < 0.05). Younger individuals (18–44 years) have a higher rate of nicotine metabolism than older individuals (45–64 years and 65–89) (Geometric means: 0.53 vs. 0.42 and 0.31, respectively, p-values < 0.01) and individuals with lower body mass index (21–25 kg/m2) have a higher rate of nicotine metabolism than the rest (26–30 kg/m2 and 31–60 kg/m2) (Geometric means: 0.52 vs. 0.35 and 0.36, respectively-values < 0.01).

4. Article IV: Pérez-Martín H, Lidón-Moyano C, Martínez-Sánchez JM. Validity of adaptation of the Fagerström Test for Cigarette Dependence in Electronic Cigarette users: A Bayesian approach with biomarkers. This Manuscript is in peer review in Journal Citation Report of ISI-Web of Science.

   - Objective: To determine if the predictive property of the Fagerström Test for Cigarette Dependence (FTCD) to assess nicotine and cotinine concentration in traditional cigarettes is applicable to e-cigarettes.
   - Results: There is strong evidence suggesting that nicotine levels are inversely related to the time to the first cigarette in the morning and the number of cigarettes per day in Exclusive cigarette smokers (Bayes Factors of 11.940 and 4.955). We also found

evidence for this with cotinine (Bayes Factors of 65.328 and 5.427). For Exclusive e-cig users (with nicotine), we just found a moderate association between nicotine and time to first cigarette (Bayes Factors of 4.954).

Part 5

Scientific articles of the doctoral thesis

Chapter 5.1 Article I

Changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers before and after Spanish smoke-free legislation

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Changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers before and after Spanish smoke-free legislation

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ABSTRACT

Introduction: High levels of cotinine in non-smokers indicate passive exposure to tobacco smoke. This study aims to evaluate variations in salivary cotinine cut-offs to discriminate smokers and non-smokers before and after the implementation of smoke-free legislation (Law 28/2005 and Law 42/2010) in a sample of the adult population of Barcelona, Spain.

Methods: This longitudinal study analyzes salivary cotinine samples and self-reported information from a representative sample (n = 676) of the adult population from Barcelona before and after the approval of smoke-free legislation. We calculated the receiver operating characteristic (ROC) curves, to obtain optimal cotinine cut-off points to discriminate between smokers and non-smokers overall, by sex and age, and their corresponding sensitivity, specificity, and area under the curve. We used linear mixed-effects models, with individuals as random effects, to model the percentage change of cotinine concentration before and after the implementation of both laws.

Results: The mean salivary cotinine concentration was significantly lower post-2010 law (−85.8%, $p < 0.001$). The ROC curves determined that the optimal cotinine cut-off points for discriminating non-smokers and smokers were 10.8 ng/mL (pre-2005 law) and 5.6 ng/mL (post-2010 law), with a post-2010 law sensitivity of 92.6%, specificity of 98.4%, and an area under the curve of 97.0%. The post-2010 law cotinine cut-off points were 5.6 ng/mL for males and 1.9 ng/mL for females.

Conclusion: The implementation of Spanish smoke-free legislation was effective in reducing secondhand smoke exposure and, therefore, also in reducing the cut-off point for salivary cotinine concentration. This value should be used to better assess tobacco smoke exposure in this population.

1. Introduction

Smoking is considered a major risk factor for the health of both smokers and people exposed to smoke [1,2]. According to the World Health Organization (WHO), eight million people died from tobacco use in 2020 and around one million of those deaths were of non-smokers

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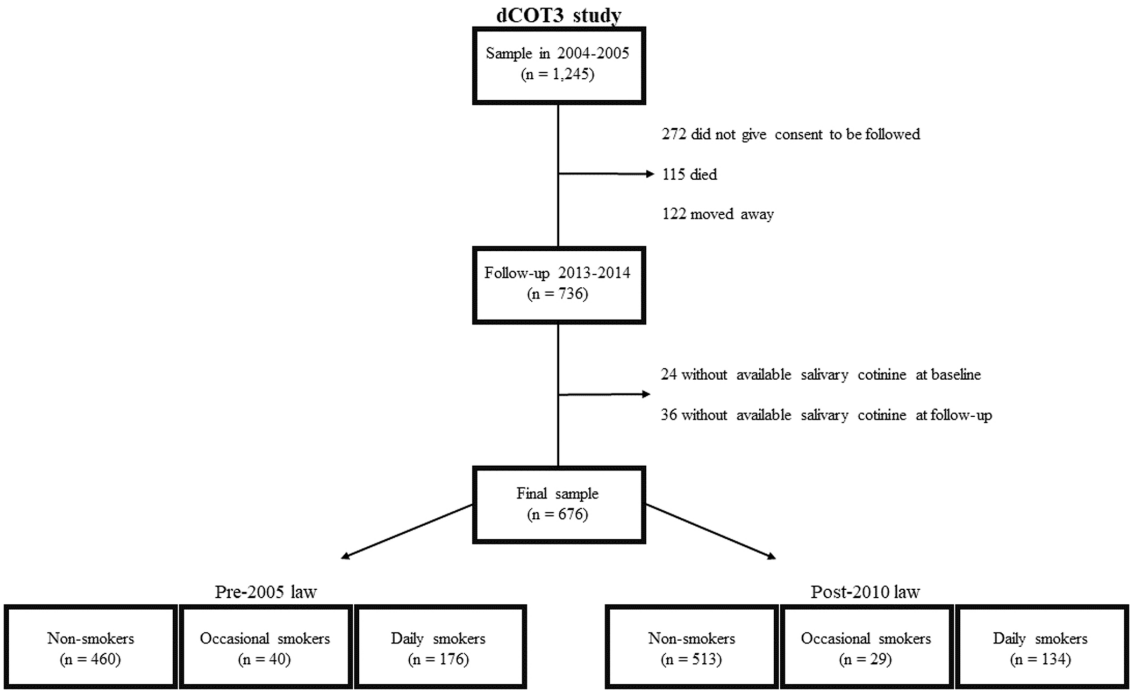

Fig. 1. Flow chart of Samples for dCOT3 Pre-2005 law and Post-2010 law.

exposed to second-hand smoke (SHS) [3]. It is estimated that around 15% of the global population were smokers in 2018 [4]. To attenuate the tobacco epidemic, the WHO Framework Convention on Tobacco Control (WHO FCTC) reaffirmed the necessity of a consistent tobacco control legislation aiming at reducing demand, in addition to tax increase and regulation of tobacco producers and sellers, which several parties have already adopted [5]. In Spain, two laws came into effect. The first one (Law 28/2005) came into effect on the 1st of January 2006 [6] and supposed a great improvement in public health since this law decreased the prevalence of smokers in the young population, as well as the acute myocardial infarction morbidity and the prevalence of SHS exposure in non-smokers [7,8], but it still allowed smoking in some hospitality sectors. Hence, this law did not fully protect the population against passive smoking 5]. The second one (Law 42/2010) came into effect on the 2nd of January 2011, extending the prohibition of smoking in all public indoor areas without exceptions and in some outdoor areas [9]. With both laws in place, secondhand smoke exposure has since been significantly reduced [10].

Passive exposure and tobacco consumption can be estimated with the information obtained from various standardized questionnaires, a particularly useful tool when monitoring the evolution of the tobacco epidemic in the population[5,11]. However, self-reported tobacco consumption is not always a reliable source of information and subjects may underreport it due to the increasing population awareness of tobacco's negative health consequences [11]. To avoid this issue, biomarkers of tobacco exposure (e.g., nicotine, cotinine, trans-3-hydroxy-cotinine) have been broadly used as an objective measure to differentiate between smokers and non-smokers [11–13]. Nicotine is an addictive substance used as a biomarker that is present in a variety of tobacco products. Once a person is exposed to tobacco smoke, nicotine is mostly metabolized into cotinine within a few hours [14]. Cotinine has a much longer in vivo half-life (16–20 h) than nicotine (2 h) and can be measured in a variety of human fluids (e.g., saliva, plasma, blood, and urine) [15,16], turning cotinine into a tobacco consumption biomarker widely studied. As cotinine concentration provides an objective measure of passive smoking in non-smokers and of tobacco use in smokers, cotinine cut-offs are a great resource for differentiating smoking status [11–13]. Based on our study conducted in 2009, the optimal cut-off point to discriminate smoking status in the adult population from Barcelona, Spain, was found to be 9.2 ng/mL [11]. However, this study was realized between the implementation of both Spanish smoke-free legislations. Since passive exposure to tobacco smoke has decreased from 2009, we believe that this cut-off point needs to be reassessed. Our research group evaluated the impact of smoke-free legislation using cotinine from a general population cohort to validate the results [5,10], but we did not evaluate if cotinine cut-offs changed after the implementation of both laws. Thus, this study aims to assess the changes in the salivary cotinine cut-offs in an adult sample of cigarette smokers and non-smokers before and after the implementation of the Spanish smoke-free legislation.

2. Methods

We used data from a follow-up study "Determinants of Cotinine project-phase 3 (dCOT3 study)". This cohort study included data of the adult (>16 years) population in Barcelona (Catalonia, Spain). The baseline was carried out during the years 2004–2005 (n = 1245) and one follow-up was realized during 2013–2014 (n = 736). Saliva samples were collected by trained staff in both interviews employing the same protocol that prevents contamination from recent smoking, and analyzed in the same lab using same validated procedures, which can be found elsewhere [11,17]. After rinsing their mouths and sucking a lemon candy (Smint ®) to stimulate saliva production, participants provided 9 mL of saliva by spitting it into a funnel placed in a test tube. Each sample was separated into 3 mL aliquots, and frozen to − 20ºC for storage. The frozen samples were sent to the Municipal Institute for

Table 1
Optimal cut-off points of salivary cotinine concentration Post-2010 law (2013–2014), sensitivity, specificity and area under the curve for different comparisons, overall and according to sex and age.

		Post-2010 law				
	n (%)	% smokers	CP-Post (ng/mL)	Se (%)	Sp (%)	% AUC (95% CI)
Smokers (daily and occasional) vs. non-smokers						
Overall	676	24.1	5.6	92.6	98.4	97.0 (95.0; 99.0)
Sex						
Male	310 (45.9)	27.4	5.6	95.3	97.9	98.0 (96.0; 100.0)
Female	366 (54.1)	21.3	1.9	93.6	96.9	96.0 (92.0; 99.0)
Age (years old)						
17–44	311 (46.0)	32.8	1.3	97.1	94.3	98.0 (96.0; 100.0)
> 44	365 (54.0)	16.7	5.6	91.8	99.3	96.0 (92.0; 99.0)
Daily smokers vs. Non-smokers						
Overall	647	20.7	18.0	99.3	98.6	99.0 (98.0; 100.0)
Sex						
Male	295 (45.6)	18.2	18.0	99.9	97.8	100.0 (99.0; 100.0)
Female	352 (54.4)	23.7	26.0	98.4	99.3	99.0 (96.0; 1.0)
Age (years old)						
17–44	294 (45.4)	28.9	18.0	99.9	97.6	100.0 (99.0; 100.0)
> 44	353 (54.6)	13.9	26.0	98.0	99.3	98.0 (94.0; 100.0)
Occasional smokers vs. Non-smokers						
Overall	542	5.4	0.9	79.3	94.0	88.0 (79.0; 96.0)
Sex						
Male	240 (44.3)	6.3	0.9	86.7	93.3	92.0 (82.0; 99.0)
Female	302 (55.7)	4.6	1.9	71.4	96.9	84.0 (67.0; 98.0)
Age (years old)						
17–44	226 (41.7)	7.5	1.3	82.4	94.3	89.0 (77.0; 98.0)
> 44	316 (58.3)	3.8	0.9	75.0	94.8	87.0 (69.0; 99.0)

Medical Research (IMIM-Hospital del Mar) in Barcelona. Cotinine was measured by alkaline single liquid-liquid extraction with dichloromethane/isopropanol [18]. This method has a quantification limit of 0.10 ng/mL and a detection limit of 0.03 ng/mL.

We used the self-reported information on the smoking status and salivary cotinine concentration from the baseline and the follow-up [5,11]. Subjects that did not have available salivary cotinine at baseline (n = 24) or the follow-up (n = 36) were excluded. The final sample included 676 individuals (Fig. 1). The variable smoking status was self-reported with five possible options: 1) smoker of at least one cigarette a day; 2) occasional smoker (they smoke, but not every day); 3) former daily smoker (at least one year without smoking), but used to smoke at least one cigarette a day; 4) former non-daily smoker (at least one year without smoking), but used to smoke occasionally; 5) never smoker. For the purpose of our analysis, former smokers (categories 3 and 4) and never smokers were all recategorized as non-smokers.

We used receiver operating characteristic (ROC) curves to obtain the areas under the curve (AUCs) and the optimal cotinine cut-off values to discriminate between smokers and non-smokers, with an approach to maximize the sum of sensitivity and specificity. In total, six ROC curves were obtained according to the smoking status, three corresponding to the data before the first law and three using the data after the law (pre-2005 law and post-2010 law, respectively). These ROC curves were calculated for: 1) smokers (daily and occasional) versus non-smokers; 2) daily smokers versus non-smokers, and 3) occasional smokers versus non-smokers.

To assess significant changes in cotinine geometric means, we used linear mixed-effects models, with individuals as random effects, adjusted to model the percentage change (and 95% confidence intervals) of salivary cotinine concentrations (after log 10 transformation) for the baseline and follow-up. Each analysis was stratified by sex and age. Age was categorized into two levels (17–44 years old and >44 years old) to ensure an equitable distribution of the sample and the quality of the ROC curves. Data were analyzed using R-4.0.4.

The data generated in this study are not publicly available as information could compromise participants consent but are available upon reasonable request from the corresponding authors.

3. Results

The baseline sample for this study consisted of 460 (68.1%) non-smokers, 176 (26.0%) daily smokers, and 40 (5.9%) occasional smokers. In the follow-up after the implementation of the latest Spanish smoke-free legislation these numbers changed to: 513 (75.9%) non-smokers, 134 (19.8%) daily smokers, and 29 (4.3%) occasional smokers. The count and percentage at baseline of males in the sample was 310 (46%), and there were 365 (54%) people over 44 years old.

Table 1 shows the optimal post-2010 law cut-off points, sensitivity, specificity, and the area under the ROC curve, overall and stratified according to sex and age. The optimal cut-off point of salivary cotinine concentration that discriminates between smokers (daily and occasional) and non-smokers was 5.6 ng/mL, with a sensitivity of 92.6% and a specificity of 98.4% (AUC = 97.0%). The cut-off point was higher in males than in females (5.6 vs 1.9 ng/mL, with sensitivities and specificities higher than 93.0%). According to groups of age, the optimal cut-off point was higher in individuals older than 44 years than in the group of 17 – 44 years (5.6 vs 1.3 ng/mL, with sensitivities and specificities higher than 90.0%).

n: sample size; CP-Post: cut-off point post- 2010 law; Se: sensitivity; Sp: specificity; AUC: areas under the curves; CI: confidence interval.

The optimal cut-off point that discriminates between daily smokers and non-smokers was 18.0 ng/mL, with a sensitivity of 99.3% and a specificity of 98.6% (AUC = 99.0%). The cut-off point was lower in males than in females (18.0 vs 26.0 ng/mL with sensitivities and specificities higher than 97.0%). According to groups of age, the optimal cut-off point was higher in older individuals (18.0 vs 26.0 ng/mL with sensitivities and specificities higher than 97.0%).

The optimal cut-off point that discriminates between occasional smokers and non-smokers was 0.9 ng/mL, with a sensitivity of 79.3% and a specificity of 94.0% (AUC = 88.0%). The cut-off point was lower in males than in females (0.9 vs 1.9 ng/mL with sensitivities and specificities higher than 71.0%). According to groups of age, the optimal cut-off point was higher in individuals from 17 to 44 years than in those older than 44 years (1.3 vs 0.9 ng/mL with sensitivities and specificities higher than 75%). We found a leftward shift in the salivary cotinine concentration cut-off point after the implementation of the two laws (Fig. 2).

Significant differences in the cut-off points were found before and after the implementation of both Spanish laws. In all cases, the adjusted mean percentage change decreased by more than 80.0% (Table 2).

4. Discussion

We found a significant decrease in the salivary cotinine

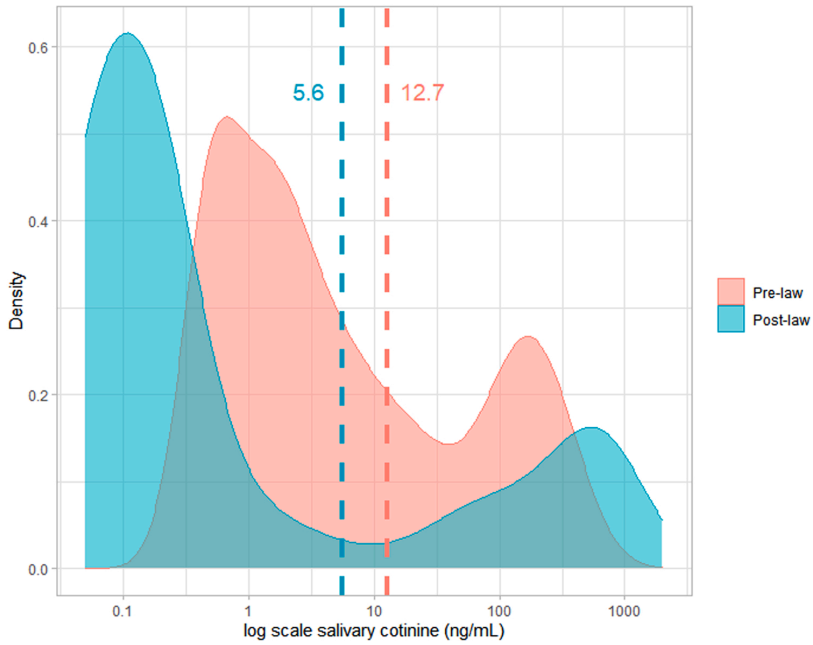

Fig. 2. Density plot of log cotinine in saliva (ng/mL) in a representative sample from the general population of Barcelona, Spain, before and after the implementation of Spanish smoke-free legislation, with the corresponding cut-off points to distinguish smokers from non-smokers.

concentration cut-off point to discriminate between smokers and non-smokers in Barcelona after the implementation of the Law 42/2010. The results were similar for the stratified models by sex and age; in all cases, the decrease was higher than 80% compared to 2005 values. There were also significant reductions in salivary cotinine concentration cut-off points when we compared non-smokers with daily smokers (86.8%) and non-smokers with occasional smokers (93.8%).

Similar studies indicate that the cut-off point for distinguishing between smokers and non-smokers have decreased in the last decades, coinciding with the implementation of tobacco control legislation [12, 19–24]. We had previously estimated the salivary cotinine cut-off in 2005 to be 9.2 ng/mL, much lower than the ones estimated in the general population of other countries [11,12,19–24]; however, this cut-off point was calculated before the implementation of the new smoke-free legislation. In this study, we show that the general cotinine cut-off point in the population of Barcelona has decreased from 9.2 to 5.6 ng/mL after the implementation of the new legislation. The aforementioned decrease in the cotinine cut-off point is reflected in Fig. 2, which shows a reduction in SHS exposure in nonsmokers after the update of the law (previously described by Fernández et al. [25] and Lidón-Moyano et al. [5]). This is sufficient proof of the effectiveness of this type of measures to reduce passive exposure to tobacco smoke. In addition, an increase in the mean salivary cotinine concentration of smokers is also observable, as described in a previous study by Lidón-Moyano et al. [26].

Despite this, the salivary cotinine cut-off point calculated post-2010 for adult non-smokers in Barcelona is still higher than other populations in advanced stages of the tobacco epidemic. In the U.S., previous studies of the adult population reported cut-off points around 3–4 ng/mL [12, 13,25]. Although the cut-off point has lowered in Barcelona, passive exposure is still prevalent and tobacco control measures must continue to be implemented in order to diminish the tobacco epidemic in our population.

Optimal cut-off points varied between different types of smokers and non-smokers, being the cut-off point between non-smokers and daily smokers higher than that between non-smokers and occasional smokers. Furthermore, males have a higher cut-off point than females when comparing non-smokers and smokers, which is consistent with previously reported results with these same data before the law 42/2010 was approved [11]. However, in contrast with these results, females have higher cut-off points than males when comparing non-smokers versus daily smokers and versus occasional smokers. In addition to this, in the particular case of daily smokers versus non-smokers, the cut-off calculated for females is higher after the implementation of the legislation. This may be a direct consequence of increased smoking prevalence and cigarette consumption among women between 40 and 64 years in the last 20 years as a result of their latest incorporation to the tobacco epidemic, resulting in an increase of their cotinine levels [27–29]. When comparing smokers (daily and occasional) vs. non-smokers, salivary cotinine cut-offs overall and stratified by sex in our sample have similar values to the ones reported in the U.S.A. [13,27]. Other aspect that could affect the reduction of the cut-off is the intensity (number of cigarettes per day) and duration of smoking (time of smoking). In this sense, we have self-reported information about the number of cigarettes smoked during the last 24 and 48 h just before interview and saliva collection. We observed a statistically significant reduction in the number of cigarettes smoked in the last 24 and 48 h before and after the coming into effect of Spanish smoking legislations (data not shown).

The overall optimal cut-off point post-2010 has sensitivity, specificity, and AUC values all greater than 90%, much higher values than the ones observed in the pre-2005 cut-off points —which are between 70% and 80%, but do not reach 90% (data not shown)—. It should be noted that salivary cotinine cut-offs for occasional smokers have lower sensitivity and specificity than for daily smokers. As salivary cotinine has a half-life of approx. 17 h, the differences between the cut-offs observed suggest that cotinine may not be a good biomarker for occasional smokers as other biomarkers of long-term exposure (up to weeks later) to tobacco smoke, like tobacco-specific nitrosamines [30–32]. Further

Table 2
Comparison of salivary cotinine concentration cut-off points before the implementation of Law 28/2005 and after the implementation of Law 42/2010 in Spain and change percentage of cotinine geometric mean (with 95% confidence intervals) between different smoking status groups, overall and according to sex and age.

	n	CP – Pre (ng/mL)	CP – Post (ng/mL)	% change (95% CI)	p-value
Smokers (daily and occasional) vs. Non-smokers					
Overall	676	10.8	5.6	-85.8 (−88.3; −82.8)	< 0.001
Sex					
Male	310	25.1	5.6	-85.8 (−89.6; −80.7)	< 0.001
Female	366	10.8	1.9	-85.8 (−88.9; −81.9)	< 0.001
Age (years old)					
17–44	311	12.4	1.3	-81.2 (−86.2; −74.3)	< 0.001
> 44	365	10.8	5.6	-88.8 (−91.2; −85.8)	< 0.001
Daily smokers vs. Non-smokers					
Overall	647	22.6	18.0	-86.8 (−89.2; −84.0)	< 0.001
Sex					
Male	295	25.1	18.0	-87.0 (−90.5; −82.2)	< 0.001
Female	352	10.8	26.0	-86.7 (−89.6; −83.0)	< 0.001
Age (years old)					
17–44	294	22.6	18.0	-83.1 (−87.7; −76.7)	< 0.001
> 44	353	25.1	26.0	-89.3 (−91.6; −86.4)	< 0.001
Occasional smokers vs. Non-smokers					
Overall	542	2.1	0.9	-93.8 (−94.8; −92.6)	< 0.001
Sex					
Male	240	2.7	0.9	-94.7 (−96.0; −92.9)	< 0.001
Female	302	1.8	1.9	-93.0 (−94.3; −91.3)	< 0.001
Age (years old)					
17–44	226	3.5	1.3	-94.4 (−95.8; −92.6)	< 0.001
> 44	316	2.1	0.9	-93.3 (−94.6; −91.7)	< 0.001

CP-Pre: Cut-off point pre-2005 law; CP-Post: Cut-off point post- 2010 law; 95% CI: 95% confidence interval; % change (95% CI): Change percentage of cotinine geometric mean.

studies should assess other biomarkers potentially more suitable for occasional smokers.

One limitation of our study is that we analyzed self-reported data. This kind of data may be subdued to information bias affecting the information on smoking status, and therefore, cotinine cut-off points. However, the participants agreed to provide a saliva sample for cotinine analysis, and thus this bias is almost negligible. Also, our results tally with the cut-off points reported in other studies after the implementation of tobacco control legislation [12,27]. In addition, the prevalence of users of other tobacco products and electronic cigarettes was very low in our sample (1.18%) so we could not control for type of product. However, due to the small sample the impact expected is limited. Another limitation of the study is that working with cohort data overestimates the elderly representation. Accordingly, the sample was weighted to minimize limitations and the baseline sample was representative of the city of Barcelona. A full description of the methodology can be found elsewhere [26,30,33,34]. On the other hand, a strength of our study is to obtain updated cut-off values to distinguish between smokers and non-smokers, with different cut-off values by sex and age groups, being able to describe changes over time. Another strong point of this study is that it is the first study that includes data of cotinine in saliva (a matrix widely used to determine cotinine) in the general population which was collected before and after the implementation of Spanish smoke-free legislation.

There was a large reduction in salivary cotinine cut-off points to distinguish between smokers and non-smokers, non-smokers and daily smokers, and non-smokers and occasional smokers after the implementation of Spanish smoke-free legislation. The updated cut-off point to discriminate between smokers and non-smokers is around 5.6 ng/mL in the adult population, but it differs according to sex and age. When possible, more specific cut-off points according to sex and age should be used.

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Author's contribution

HPM analyzed the data and drafted the first manuscript with the supervision of CLM, AGM and JMMS. MF, MB, EF and JMMS contributed to the design and coordination of the study. RPO and JAP analyzed saliva samples. All authors contributed substantially to the interpretation of the data and the successive versions of the manuscript. All authors contributed to the manuscript and approved its final version. JMMS is the principal investigator of the project.

Conflict of interest

All authors declare that they have no conflicts of interest.

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Chapter 5.2 Article II

Changes in concentrations of Tobacco Specific Nitrosamines in saliva in the general population of Barcelona before and after implementation of tobacco control legislation

Changes in concentrations of Tobacco Specific Nitrosamines in saliva in the general population of Barcelona before and after implementation of tobacco control legislation

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Abstract

Introduction: The objective of this study was to evaluate the changes in tobacco-specific nitrosamines (TSNAs) levels in the saliva of smokers and non-smokers before and after the implementation of the Spanish smoke-free legislation.

Methods: We conducted a longitudinal study to assess self-reported data and salivary concentrations of TSNAs, including NNN, NNK, and NNAL, in a sample of 272 adults from the general population of Barcelona. The participants were surveyed in 2004-2005 and followed up in 2013-2014, before and after the implementation of the smoke-free legislation in 2006, which was extended in 2011. The geometric mean (GM) and its 95% confidence interval of TSNAs concentrations were calculated from saliva samples collected at baseline and follow-up. Data was analyzed overall and stratified by Sociodemographic characteristics, Smoking-related characteristics, and Anthropometric characteristics. We used linear mixed-effects models to examine the adjusted percentage change in TSNAs concentrations before and after legislation.

Results: Salivary concentration of TSNAs in Smokers at baseline decreased by -90.5% [-95.0; -82.2], -48.7% [-60.1; -34.0], and -86.2% [-90.1; -80.9] for NNN, NNK and NNAL, respectively. Continuing smokers increased its concentration of NNN by 149.8% [36.8; 356.1], while no significant change in the TSNAs concentration of Continuing non-smokers was observed.

Conclusions: Salivary TSNA concentrations decreased in those who quit smoking at follow-up. However, the concentration of salivary NNN increased in people who continued to smoke at follow-up. Assessing TSNAs can be useful in evaluating the impact of tobacco legislation. Our findings support the implementation of tighter smoking regulations to improve the overall health of the population.

Keywords: cigarette; tobacco; tobacco-specific nitrosamines; saliva; smoking.

Implications

To protect the population from the harmful effects of tobacco, tobacco control laws have been established. Therefore, it is crucial to investigate the changes in all tobacco-related substances following the implementation of these laws. Analyzing tobacco-specific nitrosamines (a class of carcinogens found in tobacco) can help assess the effectiveness of tobacco control legislation.

Introduction

For many decades, the harmful effects of tobacco have been the subject of extensive research, focusing on both smokers and individuals exposed to secondhand smoke (SHS) [1,2]. All the available evidence has raised awareness among the scientific community and health authorities, prompting the World Health Organization (WHO) to call on parties to develop or improve existing tobacco control legislation through mechanisms like the WHO Framework Convention on Tobacco Control (FCTC). The goal is to reduce the prevalence of tobacco use among the general population, particularly by reducing exposure to SHS among non-smokers [2,3].

As a result, Spain introduced tobacco control legislation on January 1st, 2006 (Law 28/2005) [4], which resulted in a substantial improvement in public health. The new law led to a reduction in SHS exposure and its associated diseases, such as myocardial infarction, which continued to decline [5,6]. However, this legislation did not fully protect the population from passive smoking, as smoking was still permitted in some public facilities [7]. Consequently, the Spanish government enacted a second tobacco control legislation on January 2nd, 2011 (Law 42/2010) [8], which extended smoking restrictions to all public indoor areas, including bars and restaurants, while still allowing smoking in most outdoor areas. With the implementation of both laws, exposure to SHS has been significantly reduced, as evidenced by studies that used various biomarkers of tobacco use and exposure [5,9,10].

To evaluate the effects of tobacco control policies, studies in various countries have used nicotine and its major metabolite, cotinine as biomarkers of tobacco consumption and SHS exposure [11]. Cigarette smoke contains over sixty strong human carcinogens, including tobacco-specific nitrosamines (TSNAs) that are responsible for a significant portion of

tobacco carcinogenesis [12]. Relevant TSNAs include N'-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and its main metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which are mainly formed from tobacco alkaloids during tobacco curing [12]. NNN can also be endogenously formed from nicotine and nornicotine in the oral cavity and stomach due to the acidic environment and local microbiota metabolism [12]. High concentrations of NNN in oral fluid have been proposed as a proxy for cancer risk [13]. Therefore, studying these biomarkers to assess potential health effects due to exposure to SHS may be relevant, particularly in assessing the impact of tobacco control legislation. These biomarkers may demonstrate specific health benefits from implementing restrictions on smoking in public places to protect the health of non-smokers and encouraging smokers to quit or reduce their smoking.

The objective of this study is to evaluate the changes in salivary concentrations of TSNAs, including NNN, NNK, and NNAL before and after the implementation of Spanish tobacco control legislation (laws 28/2005 and 42/2010) in a sample of active smokers and Continuing non-smokers residing in Barcelona, Spain.

Methods

Study design and sampling procedure

We analyzed data collected in the dCOT3 study cohort, which was conducted in Barcelona, Spain. The baseline analysis was conducted in 2004-2005, and involved a representative sample of the adult population aged 16 years and above. The details of the baseline analysis are described elsewhere [14,15]. The baseline study consisted of 1245 individuals. We followed up with 1010 participants from the initial study of 1245 individuals, as 101 had passed away,

49 had migrated outside of the Barcelona province, and 85 either declined to be followed or were under 18 years old in 2004-2005. The follow-up survey was conducted between May 2013 and February 2014, after the implementation of both Spanish smoking legislations. Of the eligible sample, 72.9% agreed to participate and completed the questionnaire, 18.5% refused to participate, 7.2% had relocated elsewhere, and 1.3% had passed away.

Saliva samples were collected and analyzed for the determination of cotinine as a biomarker of tobacco exposure [13]. To stimulate saliva production, participants rinsed their mouths and sucked a lemon candy (Smint ®), and then they spitted 9 mL of saliva into a tube using a funnel. Each sample was separated into 3 mL aliquots, and frozen for storage at -20 ºC. The frozen samples were later sent to IMIM-Hospital del Mar Medical Research Institute in Barcelona for analysis. Additionally, participants were given a self-reported questionnaire to provide information on smoking characteristics, exposure to SHS, and sociodemographic variables.

The follow-up included the same questionnaire and cotinine and TSNAs analysis in saliva samples. The TSNAs analyses were done on frozen aliquots from saliva samples collected at baseline and follow-up from both smokers and non-smokers. While there were no statistically significant differences in age, sex, level of education, and smoking status between the followed-up sample and the participants who were lost, the final sample was slightly overrepresented by older individuals compared to the general population of Barcelona.

Out of the overall sample of the dCOT3 study, only 282 subjects had TSNAs concentrations available at baseline and follow-up, and 10 Continuing non-smokers were excluded due to their cotinine values not being consistent with active smoking (> 10 ng/ml of cotinine per cigarette smoked daily) [16]. The final sample available for analysis consisted of the remaining

272 subjects. The complete methodology for the baseline and follow-up can be found elsewhere [13,17,18].

Variables

TSNAs concentrations

The concentrations of TSNAs (NNN, NNK, and NNAL) were measured using liquid chromatography coupled with tandem mass spectrometry with multiple reaction monitoring (LC/MS/MS) [13]. The method has a limit of quantification of 1.0, 2.0, and 0.5 pg/mL for NNN, NNK, and NNAL, respectively. To avoid underestimation or overestimation bias, a value of half the quantification level was assigned for TSNAs concentrations below the limit of quantification. This approach was necessary because 19.44% of NNN, 21.40% of NNK, and 24.01% of NNAL concentrations were below the limit of quantification.

Co-variables

1) *Sociodemographic characteristics*

Participants' date of birth was reported, and their age at the time of the baseline sampling was calculated. Age was then categorized into groups based on the tertiles of the unrefined sample in the dCOT3 study cohort at baseline, with the groups being ((18,44], (44,64], and > 64 years old). Participants also reported their biological sex at baseline.

2) *Smoking-related characteristics*

Cotinine concentration at baseline was determined using the same analytical procedure used for TSNAs, with a limit of quantification of 100 pg/mL. The participants were divided into three categories depending on the cotinine levels in the unprocessed sample, using tertiles:

the first group comprised individuals with levels at or below 10 ng/mL ([0,10]), the second group included those with levels greater than 10 ng/mL but less than or equal to 50 ng/mL ((10,50]), and the third group consisted of participants with levels above 50 ng/mL (>50).

Smoking status was assessed at both baseline and follow-up using five categories: 1) Daily smoker, 2) Occasional smoker (defined as those who did not smoke every day), 3) Former daily smoker, 4) Former occasional smoker (defined as those who quit smoking more than 6 months before the survey time), and 5) Never smoker. Based on the possible combinations of these categories at baseline and follow-up, a variable called Smoking Status was created and categorized as follows: 1) Continuing non-smokers (non-smokers at both baseline and follow-up), 2) Smokers at baseline (Smokers at baseline who had quit at follow-up), 3) Smokers at follow-up (former or never Smokers at baseline, who had started smoking at follow-up), and 4) Continuing smokers (smokers at both baseline and follow-up).

The information of the number of years smoking at follow-up was self-reported by all Continuing smokers (Smokers at baseline and follow-up) and categorized according to the quartiles present in the sample as follows: 1) Less than 25 years (or ≤ 24), 2) Between 25 and 37 years (or (24,37]), 3) Between 38 and 49 years (or (37,49]), and 4) More than 49 years (or > 49).

The information about the number of years without smoking at follow-up for every smoker at baseline was collected. This was done by subtracting the age at follow-up from the age they quit smoking, which was obtained from the response given to the question "At what age did you quit smoking?" Based on the quartiles present in the unprocessed sample of the variable years without smoking Smokers at baseline were divided into four groups. These groups were defined as follows: the first group comprised individuals who had smoked for

12 years or less (≤ 12), the second group included those who had smoked between 12 and 19 years ((12,19]), the third group consisted of individuals who had smoked between 19 and 30 years ((19,31]), and the fourth group included smokers who had smoked for more than 31 years (>31).

The Fagerström Test for Cigarette Dependence (FTCD) was used to assess the level of dependence on cigarettes among participants at both baseline and follow-up [19]. The FTCD score ranges from 0 to 10 and reflects the individual's physical dependence on cigarettes, with higher scores indicating greater dependence. The FTCD score can be divided into five levels of dependence, including Very Low Dependence (0-2), Low Dependence (3-4), Medium Dependence (5), High Dependence (6-7), and Very High Dependence (8-10) [20]. However, due to the small sample size, we grouped participants into three categories based on their FTCD score: Low Dependence (0-4), Medium Dependence (5), and High Dependence (6-10).

Additionally, the variable pack-years was computed by multiplying the self-reported number of cigarettes smoked daily by the number of self-reported years smoking cigarettes at baseline. This variable was categorized into four categories: 1) 0 pack-years, 2) between 0 and 5 pack-years, 3) between 6 and 10 pack-years, and 4) more than 10 pack years.

3) Anthropometric characteristics

Participants in the study self-reported their height and weight at baseline, which were then used to calculate their body mass index (BMI). BMI was calculated (kg/m^2) and grouped into four categories (in accordance to WHO guidelines [21], although the underweight range

was extended to increase the sample size): ((10, 20] kg/m², (20,25] kg/m², (25,30] kg/m², and > 30kg/m²).

Statistical analysis

To analyze the data, we calculated the geometric mean (GM) and its 95% confidence interval (95% CI) of TSNAs concentrations globally and stratified by sex, age, BMI, smoking status, and years since quitting smoking. For former smokers at follow-up, the analysis was limited to those who had quit smoking, due to the asymmetry in their distribution. In the case of non-smokers at both baseline and follow-up, the analysis was stratified only by sex, age, and BMI.

The Wilcoxon signed-rank test for paired data was performed to compare the GMs of NNN, NNK, and NNAL at baseline and follow-up. The adjusted percentage change (and 95% CI) of TSNAs concentrations between the baseline and follow-up was obtained after log 10 transformation using linear mixed-effects models, with individuals as random effects, adjusted for sex, age, and BMI (when a variable was used for stratification, it was not included in the adjusted model). The level of significance was set at $\alpha = 0.05$. The data were analyzed using R-4.0.4.

Results

The entire study sample comprised of 272 participants, with 44.9% (n = 122) being men. The age distribution was as follows: 28.8% (n = 78) of participants were under the age of 45 years, while 35.3% (n = 96) were between the ages of 45 and 64 years at baseline. In terms of smoking status, 55.9% (n = 152) were Continuing non-smokers, 2.57% (n = 7) were Smokers at follow-up, 17.7% (n = 48) were Smokers at baseline, and 23.9% (n = 65) were Continuing smokers.

The overall percentage change of NNN was 1.5% [95%CI 1.2; 1.8] and 1.3% [1.1; 1.6] pg/mL at baseline and follow-up, respectively (GMs comparation p-value = 0.038). The corresponding concentrations for NNK were 1.4% [1.3; 1.5] and 1.3% [1.2; 1.4] pg/mL (GMs comparation p-value = 0.012), while for NNAL, they were 0.5% [0.5; 0.6] and 0.4% [0.4; 0.4] pg/mL (GMs comparation p-value < 0.001). The adjusted percentage change in concentration also showed a decreasing trend for NNN (-14.3% [-32.1; 8.3]), NNK (-9.6% [-17.3; -1.2]), and NNAL (-28.7% [-37.3; -18.9]). After stratifying for sex, age, and BMI, the reduction was only significant for NNK and NNAL as shown in Supplementary table 1. None of the TSNAs values changed significantly in Continuing non-smokers. The adjusted percentage changes for NNN, NNK, and NNAL in for the former ones were 1.3% [-8.4; 12.1], -3.1% [-10; 4.3], and -3.4% [-6.8; 0.1], respectively.

The GMs of NNAL showed a significant decrease in both men and women, and a greater percentage change was observed in men (-38.4% [-50.9; -22.6]) compared to women -19.8 [-30.5; -7.6]. Age was also found to be a significant factor, with significant differences in NNAL concentrations observed in participants between the ages of (44,64] years and those older than 64 years, showing adjusted percentage changes of -33.0% and -32.5%, respectively. Participants over the age of 64 also showed a significant decrease in NNK concentration (adjusted percentage change of -16.8%). Significant differences in NNN concentration were observed in individuals within the (10,20] kg/m^2 BMI category, with an adjusted percentage change of -60.9%. Similarly, significant differences were observed in NNAL concentration in individuals within the (25,30] kg/m^2 BMI category, showing an adjusted percentage change of -36.7%. Smokers at baseline showed significant decreases in all TSNAs values, with adjusted percentage changes of -90.5%, -48.7%, and -86.2% for

NNN, NNK, and NNAL, respectively. Continuing smokers, on the other hand, showed a significant increase in NNN concentration, with an adjusted percentage change of 149.8%.

All the analyses of Smokers at baseline data (Supplementary Table 2) with a sample size of 8 or more in each category showed significant differences in the specific TSNAs before and after the implementation of tobacco control legislation, except for NNK in individuals aged 18-44 years and in those who smoked for 24-37 years. Furthermore, all adjusted percentage changes in each TSNA indicated a general reduction of TSNAs by at least 28%.

Continuing smokers' data (Supplementary Table 3) showed an increase in the GM. The highest change was observed in NNN with an adjusted percentage change of 149.8% [36.8; 356.1], followed by NNK (15.9% [-9.0; 47.6]) and NNAL (11.6% [-15.6; 48.0]). However, these overall increases were not significant for NNK (p-value = 0.375) and NNAL (p-value = 0.564). This pattern was also observed after adjusting the models by tobacco consumption (data not shown).

Regarding Continuing non-smokers data (Supplementary table 4), the overall results show a non-significant reduction in the concentration of NNN (1.5% [1.2; 1.8] to 1.3% [1.1; 1.6]) after the legislation. The adjusted percentage change in NNN concentration was 1.3% [-8.4; 12.0]. Similarly, there was a non-significant reduction in the concentration of NNK (1.4% [1.3; 1.5] to 1.3% [1.2; 1.4]) after the legislation. The adjusted percentage change in NNK concentration was -3.1% [-9.9; 4.3], which was not statistically significant. The overall results also show a significant reduction (p-value < 0.001) in the concentration of NNAL (0.5% [0.5; 0.6] to 0.4 [0.3; 0.4]), with an adjusted percentage change of -3.4% [-6.7; 0.1].

When stratified by sex, a significant reduction in NNAL levels was observed for both men and women (p-values of 0.007 and 0.016, respectively), with an adjusted percentage change of -5.3% [-2.9; 3.3] and -5.3% [-10.1; -0.3], respectively. The GMs went from 0.5 [0.5; 0.6] to 0.4 [0.3; 0.4].

Stratifying by age, no significant change was observed for NNN in any age group. However, for NNK, a statistically significant decrease was observed in the groups > 64 years old (p-value = 0.031), the percentage of change was -3.5% [-14.8; 9.4]. For NNAL, a statistically significant decrease was observed in the groups (44,64] years old and > 64 years old (p-values of 0.037 and 0.001, respectively), with adjusted percentage changes of -0.5% [-1.4; 0.5] and -6.7% [-13.3; 0.4].

When stratified by BMI categories, a statistically significant decrease in NNN was observed in participants with a BMI between (10,20] kg/m^2 (p-value = 0.023), with an adjusted percentage change of -17.4% [-40.8; 15.3] and GMs values of 2.3 [1.0; 5.2] and 0.9 [0.6; 1.4] for baseline and follow-up, respectively. No significant changes were observed for NNK in any BMI categories. Significant decreases were found on people between (20,25] kg/m^2 and (25,30] kg/m^2 (p-values of 0.089 and 0.014, respectively), with adjusted percentage changes of -0.9% [-5.2; 3.7] and -6.7% [-13.3; 0.4], respectively. Their respective GMs values went from 0.5 [0.4; 0.6] to 0.4 [0.4; 0.5] and from 0.6 [0.5; 0.8] to 0.4%[0.3; 0.4].

It is worth noting that in previous studies conducted with this cohort, we observed a 28.7% increase in the cotinine salivary GM [22]. Additionally, we reported a significant decrease in smoking prevalence (34.5% at baseline vs. 26.1% at follow-up), the average number of cigarettes per day (15 at baseline vs. 10 at follow-up), and a significant increase in the use of hand-rolled tobacco (3.1% at baseline vs. 30.9% at follow-up) [23].

Discussion

Upon analyzing changes in TSNAs concentrations in saliva samples of 272 individuals from the general adult population of Barcelona before and after the implementation of both Spanish tobacco control laws, an overall decrease of 90.5% for NNN, 48.7% for NNK and 86.2% for NNAL was observed in Smokers at baseline (n = 48). This decrease was particularly notorious among men, older individuals and those with a higher value of Pack years at baseline. On the other hand, we also observed an overall increase of 149.8% in the NNN of Continuing smokers (n = 65). The increase was particularly notorious among women and individuals between 45 and 64 years of age.

The need to study these biomarkers and evaluating their changes before and after the implementation of tobacco control laws is crucial, especially given that TSNAs are markers that serve as proxies for health risks, yet have been little studied in the general population. This study is the first to describe the variation of TSNAs before and after the implementation of tobacco control legislation in Spain. Our results are consistent with other studies using salivary cotinine, a well-known biomarker of tobacco consumption and exposure [24]. In a previous study with data from the same cohort, we detected a significant increase in salivary cotinine concentration among Continuing smokers after the implementation of tobacco control legislation, indicating compensatory smoking behavior due to restrictions in public places [19,22]. Besides, we also observed significant decreases in the smoking prevalence, the average number of cigarettes per day, and the percentage of conventional tobacco consumption [24], thus indicating some sort of compensatory smoking behavior as a consequence of restrictions to smoking in public places. Similar reductions in cotinine

concentration were observed in studies using different cohorts of Continuing non-smokers in Ireland [25], Scotland [26], and the US [27] reporting a similar salivary cotinine decrease in Continuing non-smokers after the implementation of tobacco-related legislation as in our study.

The results of our study on salivary TSNAs need to be interpreted with caution, and further research is needed. However, the similarities we observed between salivary cotinine and TSNAs in Smokers at baseline, Continuing non-smokers, and Continuing smokers are significant enough to allow us to extrapolate the patterns observed with cotinine to TSNAs. TSNAs are directly related to DNA damage and are considered a better biomarker of tobacco-related health risks[28]. Therefore, using TSNAs can help assess the impact of tobacco control legislation and extensions by studying changes in their concentrations.

By and large, using saliva samples is considered preferable to using urine samples in to estimate tobacco smoking due to the ease of sample collection and the less invasive nature of the procedure for the subjects involved [13,29]. Furthermore, saliva samples provide a more comprehensive profile of TSNAs, particularly for NNN. However, most studies on TSNAs to date have relied on urine samples [30], in which NNAL, the main metabolic product of NNK, has been found to be present in a higher concentration [31,32]. In contrast, our research group was the first to test TSNAs in oral fluid, which offers a much simpler and faster method of *in situ* sample collection for individuals exposed to tobacco smoke [13]. Our previous research revealed that, in non-smokers, salivary NNK levels were almost triple those of NNN and NNAL (33.0, 1.6, and 1.2 pg/mL, respectively), whereas in smokers, NNN levels were almost four times higher than those of NNK and NNAL (17.0, 4.0, and 1.7, respectively). Furthermore, TSNAs have been shown to be a consistent oral fluid biomarker of both active

and passive tobacco exposure [29], making them suitable biomarkers for epidemiological studies assessing cancer risk. Nevertheless, information on salivary TSNAs remains scarce, and more research is needed to fully understand their potential use as a biomarker.

One possible explanation for the significant increase in salivary NNN levels among Continuing smokers is that some smokers may have switched to cigarette brands with higher levels of TSNAs [33]. This could occur due to differences to differences in cultivation and curing practices used by different cigarette brands. Such a switch could result in increased exposure to TSNAs and other harmful chemicals, including NNN. Moreover, other unmeasured factors may be contributing to the observed increase in NNN levels among Continuing smokers. Changes in the composition or processing of tobacco products over time could also contribute to the observed increase in NNN levels. For example, changes in the curing process of tobacco leaves or the addition of new flavorings or additives could affect the levels of TSNAs in cigarette smoke [33]. Additionally, some smokers may have increased their smoking or puff frequency over time [34,35], which could lead to increased exposure to different tobacco biomarkers, including NNN.

It is also possible that the significant increase in salivary NNN observed in Continuing smokers may be due to their high dependence on tobacco, which is consistent with the results observed in the FTCD at follow-up. TSNAs have the potential to be used as tobacco biomarkers [13] , and they act as a proxy for tobacco dependence [18]. The controversial hardening hypothesis suggests that the smoking population consists of groups of smokers based on their tobacco dependence, with highly dependent smokers finding it harder to quit smoking than those who are less dependent [36]. However, this hypothesis is population-based [37,38], and we cannot determine what happens to smokers who are not part of our sample or

new smokers who are not in our sample. Therefore, further studies are needed to confirm whether hardening is occurring in the population from which our sample is taken [36]. Furthermore, Supplementary table 1 and Figures 1 and 2 shows that lower concentrations of NNN are less common in the follow-up compared to those observed at baseline, whereas higher concentrations of NNN are more common after the follow-up.

Lastly, it may be possible that the observed increase in NNN levels among Continuing smokers is simply a chance finding, and not indicative of a true trend. In any case, further research would be needed to explore these possibilities and determine the underlying causes of this phenomenon.

In terms of limitations, there are several factors that should be considered. Firstly, our salivary-based TSNA analysis has not been validated against the more commonly used urinary TSNA analysis. Although we have previously discussed this in another study [13], we recognize that this is an area that requires further investigation. Secondly, the sample representativeness is a concern due to the dropout rates and attrition, which have affected the sample characteristics by aging it. We attempted to address this limitation by using statistical methods to adjust for potential confounding factors. Thirdly, we attempted to control for the timing of sample collection in every type of smokers by asking the Time since Last Cigarette, but we could not control for non-smokers thus our results may be biased. Lastly, our study may have been underpowered to detect small changes in TSNA levels, although the power analysis revealed a 40% power with our sample size. However, we aimed to maximize the internal validity of our analysis rather than the external validity.

In addition to these limitations, there are a few more worth noting. One is the lack of stratification by SHS among smokers and Continuing non-smokers, which may limit the

control of potential tobacco exposure. Another is that although there were no statistically significant differences in age, sex, level of education, and smoking status between the followed-up sample and the participants lost, the final sample was slightly skewed towards older individuals compared to the population of Barcelona. Another potential limitation is information bias, which may result from the use of self-reported questionnaires and may affect the accuracy of the information on smoking status. However, the questionnaires were administered by trained interviewers at both baseline and follow-up, which may increase the internal validity of the results. Moreover, the study utilized salivary cotinine, a validated biomarker of smoking status, to identify cotinine values inconsistent with active smoking. Another potential limitation is the low prevalence of smokers at follow-up due to the progressive decrease in smoking prevalence in developed countries, which may compromise the significance of the analysis. However, the high participation rate in the DCOT study follow-up may help mitigate this limitation [39–41].

Conclusions

The study found that there was a decrease in the concentration of salivary TSNAs among Smokers at baseline after the implementation of smoking legislation in Spain. However, an increase in TSNAs concentration was observed among Continuing smokers. The use of TSNAs has been found to be useful in assessing the impact of tobacco laws. Therefore, the results indicate that stricter smoking regulations could improve the overall health of the population.

Data Availability Statement

The data underlying this article will be shared on reasonable request to the corresponding author.

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Author's contribution

HPM analyzed the data and drafted the first manuscript with the supervision of CLM, AGM and JMMS. MF, MB, EF and JMMS contributed to the design and coordination of the study. RPO and JAP analyzed saliva samples. All authors contributed substantially to the interpretation of the data and the successive versions of the manuscript. All authors

contributed to the manuscript and approved its final version. JMMS is the principal investigator of the project.

Conflict of interest

All authors declare that they have no conflicts of interest.

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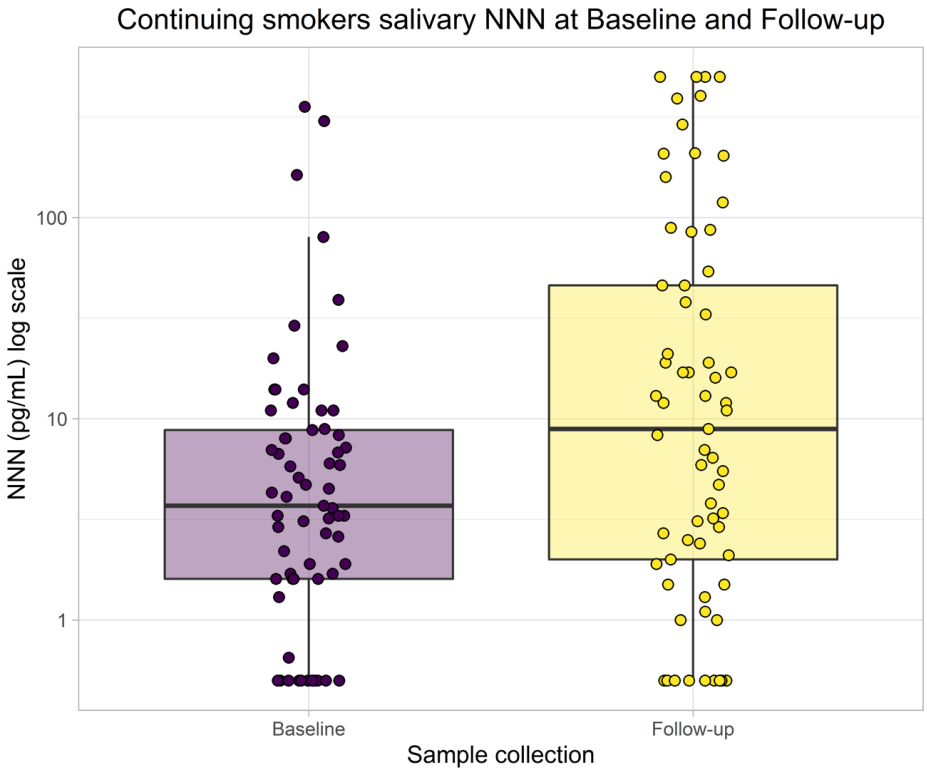

Figure 1: Boxplots representing the salivary concentration of NNN (in logarithmic scale) at baseline and follow-up of Continuing smokers.

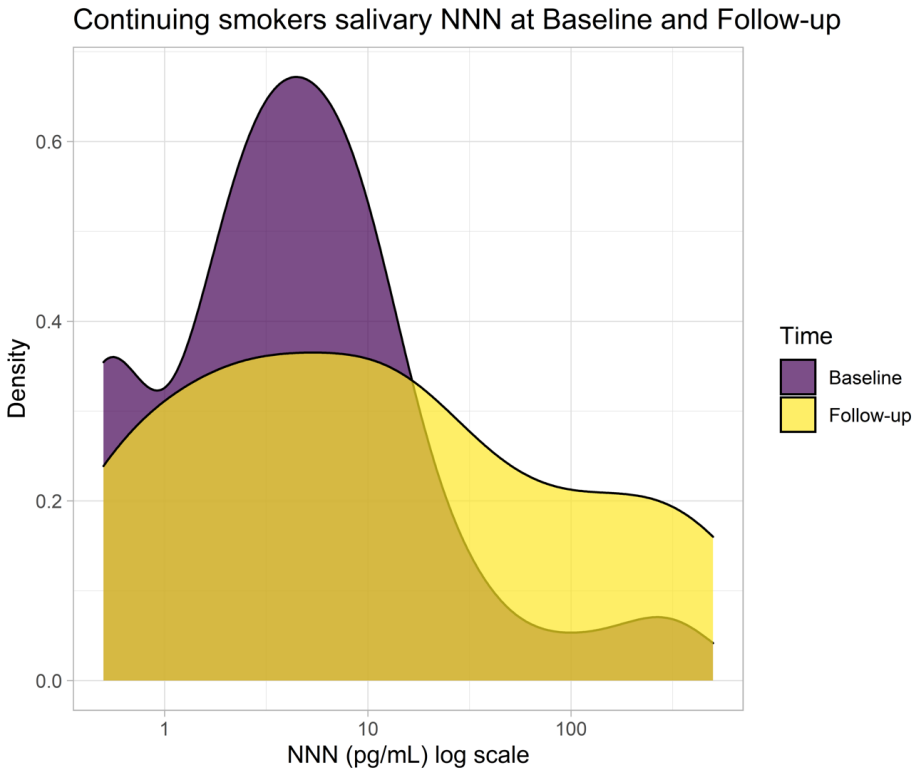

Figure 2: Density plot representing the salivary concentration of NNN (in logarithmic scale) of Continuing smokers depending on when the sample was collected.

Supplementary table 1: Geometric mean (GM) with its 95% confidence interval (CI) and adjusted percentage change (% change) and 95% confidence interval of NNN, NNK, and NNAL concentrations of all smokers at the baseline independently of tobacco consumption at the follow-up (all sample) according to sociodemographic and smoking characteristics at baseline.

		NNN (pg/mL)			
		GM [95 % CI]			
	n	Baseline	Follow-up	p-value*	% change** [95% CI]
Overall	272	1.5 [1.2; 1.8]	1.3 [1.1; 1.6]	0.038	-14.3 [-32.1; 8.3]
Sex					
Men	122	2.3 [1.6; 3.3]	1.57 [1.1; 2.2]	0.318	-32.5 [-55.4; 2.1]
Women	150	1.1 [0.9; 1.3]	1.11 [0.9; 1.4]	0.837	4.3 [-19.3; 34.8]
Age (Years)					
(18,44]	78	1.7 [1.2; 2.4]	1.5 [1.0; 2.3]	0.500	-11.6 [-44.1; 39.8]
(44,64]	96	1.5 [1.1; 2.00]	1.6 [1.1; 2.4]	0.280	9.9 [-25.5; 62.0]
> 64	98	1.4 [1.0; 2.0]	0.9 [0.7; 1.2]	0.171	-34.3 [-55.3; -3.5]
BMI (kg/m²)					
(10,20]	16	2.3 [1.0; 5.3]	0.9 [0.6; 1.4]	0.023	-60.9 [-81.2; -18.6]
(20,25]	128	1.4 [1.1; 1.9]	1.5 [1.1; 2.1]	0.699	3.7 [-26.9; 47.2]
(25,30]	95	1.6 [1.1; 2.3]	1.1 [0.8; 1.5]	0.391	-29.2 [-50.9; 2.1]
> 30	31	1.5 [0.8; 2.6]	1.5 [0.8; 3.1]	0.687	4.8 [-55.2; 145.2]
Smoking Status					
Continuing non-smokers	152	0.6 [0.6; 0.7]	0.6 [0.6; 0.7]	0.672	1.3 [-8.4; 12.1]
Smokers at follow-up	7	0.6 [0.4; 0.8]	3.3 [0.4; 25.1]	0.178	472.8 [-25.4; 4299.0]
Smokers at baseline	48	7.5 [4.0; 14.3]	0.7 [0.6; 0.9]	<0.001	-90.5 [-95.0; -82.2]
Continuing smokers	65	4.2 [2.9; 6.1]	10.4 [6.1; 17.7]	0.002	149.8 [35.7; 359.6]
Years without smoking (follow-up)					
≤ 12	20	0.6 [0.5; 0.7]	1.2 [0.6; 2.5]	0.114	100.5 [-45.9; 151.3]
(12,19]	18	0.6 [0.5; 0.7]	0.7 [0.5; 1.1]	0.353	26.5 [-88.7; -16.6]
(19,31]	18	0.6 [0.5; 0.8]	0.6 [0.5; 0.7]	0.590	-14.0 [-56.6; 19.4]
> 31	16	0.8 [0.5; 1.1]	0.8 [0.5; 1.3]	0.498	10.0 [-81.9; 82.1]
		NNK (pg/mL)			
Overall	272	1.4 [1.3; 1.5]	1.3 [1.2; 1.4]	0.012	-9.6 [-17.3; -1.2]
Sex					
Men	122	1.6 [1.4; 1.9]	1.4 [1.2; 1.6]	0.124	-14.7 [-27.7; 0.7]
Women	150	1.3 [1.2; 1.4]	1.2 [1.1; 1.3]	0.200	-5.3 [-13.7; 3.9]
Age (Years)					
(18,44]	78	1.3 [1.2; 1.5]	1.3 [1.1; 1.5]	0.705	-2.9 [-18; 15.1]
(44,64]	96	1.4 [1.3; 1.6]	1.3 [1.2; 1.5]	0.596	-7.2 [-18.7; 6.1]
> 64	98	1.5 [1.3; 1.7]	1.2 [1.1; 1.4]	0.031	-16.8 [-29.5; -1.8]
BMI (kg/m²)					
(10,20]	16	1.2 [1.0; 1.5]	1.1 [0.9; 1.4]	0.584	-6.6 [-32.5; 67.6]

(20,25]	128	1.4 [1.3; 1.5]	1.3 [1.2; 1.5]	0.261	-5.7 [22.5; 73.6]
(25,30]	95	1.5 [1.3; 1.7]	1.3 [1.1; 1.4]	0.116	-15.1 [24.6; 99.4]
> 30	31	1.5 [1.2; 1.8]	1.3 [1.1; 1.6]	0.572	-9.8 [-49.1; 117.9]
Smoking Status					
Continuing non-smokers	152	1.1 [1.1; 1.2]	1.1 [1.0; 1.2]	0.419	-3.1 [-10; 4.3]
Smokers at follow-up	7	1.0 [1.0; 1.0]	1.1 [0.9; 1.4]	1.000	10.4 [-9.4; 34.6]
Smokers at baseline	48	2.2 [1.7; 2.8]	1.1 [1.0; 1.2]	<0.001	-48.7 [-60.4; -33.5]
Continuing smokers	65	1.8 [1.5; 2.2]	2.1 [1.7; 2.6]	0.117	15.9 [-9.3; 48.0]
Years without smoking (follow-up)					
≤ 12	20	1.0 [1.0; 1.1]	1.2 [0.96; 1.37]	0.181	9.9 [-11.9; 41.5]
(12,19]	18	1.2 [1.0; 1.3]	1.1 [0.9; 1.2]	0.588	-8.1 [-28.8; 59.7]
(19,31]	18	1.1 [1.0; 1.3]	1.2 [1.0; 1.4]	0.752	4.4 [-25.9; 56.1]
> 31	16	1.0 [1.0; 1.2]	1.1 [1.0; 1.3]	0.371	6.6 [-31.7; 30.6]
		NNAL (pg/mL)			
Overall	272	0.5 [0.5; 0.6]	0.4 [0.4; 0.4]	<0.001	-28.7 [-37.3; -18.9]
Sex					
Men	122	0.7 [0.6; 0.9]	0.5 [0.4; 0.6]	0.007	-38.4 [-50.9; -22.6]
Women	150	0.4 [0.4; 0.5]	0.3 [0.3; 0.4]	0.016	-19.8 [-30.5; -7.6]
Age (Years)					
(18,44]	78	0.5 [0.4; 0.7]	0.5 [0.4; 0.6]	0.534	-17.6 [-35.4; 5.0]
(44,64]	96	0.7 [0.5; 0.8]	0.4 [0.4; 0.5]	0.037	-33.0 [-46.8; -15.6]
> 64	98	0.5 [0.4; 0.6]	0.3 [0.3; 0.4]	<0.001	-32.5 [-45.0; -17.1]
BMI (kg/m^2)					
(10,20]	16	0.7 [0.4; 1.2]	0.4 [0.3; 0.5]	0.193	-43.6 [-77.2; 67.8]
(20,25]	128	0.5 [0.4; 0.6]	0.4 [0.3; 0.5]	0.089	-17.8 [-53.3; -9.9]
(25,30]	95	0.6 [0.5; 0.8]	0.4 [0.3; 0.5]	0.014	-36.7 [-48.0; 15.4]
> 30	31	0.5 [0.3; 0.8]	0.3 [0.3; 0.4]	0.155	-34.8 [-90.8; 4.5]
Smoking Status					
Continuing non-smokers	152	0.3 [0.3; 0.3]	0.3 [0.3; 0.3]	0.102	-3.4 [-6.8; 0.1]
Smokers at follow-up	7	0.3 [0.2; 0.5]	0.4 [0.2; 0.8]	0.371	45.1 [-28.6; 194.8]
Smokers at baseline	48	1.9 [1.4; 2.7]	0.3 [0.2; 0.3]	<0.001	-86.2 [-90.1; -80.8]
Continuing smokers	65	1.3 [1.0; 1.7]	1.4 [1.1; 1.9]	0.211	11.6 [-16.0; 48.4]
Years without smoking (follow-up)					
≤ 12	20	0.3 [0.2; 0.3]	0.3 [0.2; 0.4]	0.181	13.6 [-8.1; 40.6)
(12,19]	18	0.3 [0.3; 0.3]	0.3 [0.3; 0.3]	1.000	-1.6 (-4.9; 1.8)
(19,31]	18	0.3 [0.2; 0.3]	0.3 [0.3; 0.3]	1.000	-1.9 (-5.9; 2.2)
> 31	16	0.3 [0.2; 0.3]	0.3 [0.3; 0.3]	1.000	-4.2 (-11.4; 3.5)

* p-values were obtained from paired Wilcoxon tests comparing salivary nitrosamine (baseline vs follow-up). ** adjusted percentage changes were calculated using linear mixed-effects models, with individuals as random effects, adjusted for sex, age, and BMI (for the analyses in which the variable was used to stratify, it was not used to adjust for) to model the adjusted percentage change of salivary TSNAs concentrations (after log 10 transformation) between the baseline and follow-up.

Supplementary table 2: Geometric mean (GM) with its 95% confidence interval (CI) and percentage change (% change) and 95% confidence interval of NNN, NNK, and NNAL concentrations of Smokers at baseline (smokers at baseline who had quit at follow-up) according to sociodemographic and smoking characteristics at baseline.

		NNN (pg/mL)			
	n	GM [95 % CI]		p-value*	% change** [95% CI]
		Baseline	Follow-up		
Overall	48	7.5 [4.0; 14.3]	0.7 [0.6; 0.9]	<0.001	-90.5 [-95.0; -82.2]
Sex					
Men	27	12.8 [5.2; 31.4]	0.7 [0.5; 0.8]	<0.001	-94.9 [-97.8; -88.5]
Women	21	3.8 [1.58; 9.25]	0.8 [0.6; 1.2]	0.001	-78.9 [-90.2; -54.8]
Age (Years)					
(18,44]	16	4.5 [1.5; 13.8]	0.6 [0.5; 0.7]	0.003	-87.3 [-95.3; -65.1]
(44,64]	21	5.3 [2.2; 12.9]	0.9 [0.6; 1.3]	<0.001	-83.7 [-92.5; -64.6]
> 64	11	30.5 [6.0; 155.8]	0.8 [0.4; 1.0]	<0.001	-97.8 [-99.5; -90.9]
BMI (kg/m²)					
(10,20]	4	5.1 [0.2; 162.8]	0.7 [0.2; 2.2]	0.181	N.C.***
(20,25]	21	5.9 [2.4; 14.5]	0.6 [0.5; 0.7]	<0.001	-89.8 [-95.3; -77.8]
(25,30]	19	7.6 [2.3; 25.0]	0.8 [0.6; 1.2]	<0.001	-89.1 [-96.4; -67.1]
> 30	4	38.5 [1.5; 992.6]	0.8 [0.2; 4.4]	0.125	N.C.***
Pack years					
0	21	2.9 [1.23; 6.84]	0.6 [0.5; 0.8]	0.001	-78.6 [-89.8; -55.2]
(0,5]	16	7.4 [2.8; 19.4]	0.8 [0.5; 1.2]	0.001	-89.8 [-95.4; -77.1]
(5,10]	7	45.5 [4.9; 426.1]	0.6 [0.4; 1.0]	0.016	-98.6 [-99.6; -94.5]
> 10	4	51.8 [1.3; 2009.4]	1.4 [0.2; 8.9]	0.125	-97.3 [-99.5; -84.8]
Fagerström test for independence score					
Low (0-4)	24	8.2 [2.8; 23.6]	0.8 [0.6; 1.1]	<0.001	-90.4 [-96.4; -74.2]
Medium (5-6)	8	9.8 [3.0; 32.4]	0.6 [0.4; 0.7]	0.008	-94.3 [-97.5; -86.8]
High (7-10)	3	6.1 [1.7; 22.0]	0.5 [0.5; 0.5]	0.250	N.C.***
Cotinine level					
[0,10]	3	0.7 [0.1; 3.9]	0.5 [0.5; 0.5]	1.000	N.C.***
(10,50]	4	12.4 [0.1; 2277.2]	0.5 [0.5; 0.5]	0.181	-32.1 [-59.6; 14.1]
> 50	41	8.5 [4.4; 16.5]	0.8 [0.6; 1.0]	<0.001	-96.0 [-99.6; -62.6]
		NNK (pg/mL)			
Overall	48	2.2 [1.7; 2.8]	1.1 [1.0; 1.2]	<0.001	-48.7 [-60.1; -34.0]
Sex					
Men	27	2.6 [1.8; 3.8]	1.1 [1.0; 1.4]	0.002	-56.1 [-50.6; 0.0]

Women	21	1.7 [1.3; 2.3]	1.1 [1.0; 1.2]	0.003	-37.3 [-20.4; 0.0]
Age (Years)					
(18,44]	16	1.7 [1.3; 2.3]	1.1 [0.9; 1.5]	0.124	-33.4 [-53.8; -4.0]
(44,64]	21	2.0 [1.5; 2.8]	1.1 [1.0; 1.3]	0.002	-45.6 [-58.6; -28.5]
> 64	11	3.5 [1.5; 8.2]	1.1 [0.9; 1.4]	0.013	-68.6 [-85; -34.3]
BMI (kg/m²)					
(10,20]	4	1.7 [0.6; 4.8]	1.0 [1.0; 1.0]	0.371	N.C.***
(20,25]	21	1.8 [1.3; 2.4]	1.1 [0.9; 1.4]	0.024	-36.7 [-54.6; -70.9]
(25,30]	19	2.4 [1.5; 4.0]	1.1 [1.0; 1.3]	0.004	-54.2 [-11.7; -28.0]
> 30	4	4.6 [2.3; 9.1]	1.2 [0.7; 2.2]	0.125	N.C.***
Pack years					
0	21	1.7 [1.2; 2.3]	1.1 [1.0; 1.2]	0.014	-35.7 [-52.4; -13.2]
(0,5]	16	2.1 [1.6; 2.8]	1.1 [0.9; 1.5]	0.037	-45.7 [-61.4; -23.7]
(5,10]	7	2.4 [1.2; 4.7]	1.0 [1.0; 1.0]	0.059	-57.5 [-71.3; -37.0]
> 10	4	8.8 [1.0; 77.9]	1.5 [0.7; 3.5]	0.125	-82.5 [-94.0; -48.8]
Fagerström test for independence score					
Low (0-4)	24	2.1 [1.5; 2.8]	1.1 [1.0; 1.2]	<0.001	-46.6 [-58.5; -31.3]
Medium (5-6)	8	2.2 [1.3; 3.7]	1.0 [1.0; 1.0]	0.036	-54.4 [-67.4; -36.2]
High (7-10)	3	2.5 [0.3; 19.4]	1.0 [1.0; 1.0]	0.371	N.C.***
Cotinine level					
[0,10]	3	1.4 [0.3; 5.0]	1.0 [1.0; 1.0]	1.000	N.C.***
(10,50]	4	2.3 [0.4; 13.3]	1.0 [1.0; 1.0]	0.371	-28.2 [-53.9; 11.9]
> 50	41	2.2 [1.7; 2.9]	1.1 [1.0; 1.3]	<0.001	-56.9 [-79.5; -9.2]
		NNAL (pg/mL)			
Overall	48	1.9 [1.4; 2.7]	0.3 [0.2; 0.3]	<0.001	-86.2 [-90.1; -80.9]
Sex					
Men	27	2.6 [1.7; 4.0]	0.3 [0.2; 0.3]	<0.001	-89.2 [-92.9; -88.1]
Women	21	1.4 [0.8; 2.3]	0.3 [0.2; 0.3]	<0.001	-81.1 [-83.6; -69.7]
Age (Years)					
(18,44]	16	1.1 [0.6; 2.1]	0.2 [0.2; 0.2]	0.003	-77.0 [-86.1; -61.8]
(44,64]	21	2.2 [1.4; 3.6]	0.3 [0.2; 0.4]	<0.001	-87.3 [-91.8; -80.4]
> 64	11	3.3 [1.5; 7.2]	0.3 [0.3; 0.3]	<0.001	-92.4 [-96.1; -84.9]
BMI (kg/m²)					
(10,20]	4	1.4 [0.1; 14.0]	0.3 [0.3; 0.3]	0.181	N.C.***
(20,25]	21	1.4 [0.8; 2.4]	0.3 [0.2; 0.4]	<0.001	-79.0 [-87.3; -65.1]
(25,30]	19	2.4 [1.5; 3.8]	0.3 [0.2; 0.3]	<0.001	-89.1 [-92.9; -83.5]
> 30	4	6.4 [2.6; 16.0]	0.3 [0.3; 0.3]	0.125	N.C.***

Pack years					
0	21	1.3 [0.8; 2.1]	0.3 [0.2; 0.3]	<0.001	-79.4 [-86.3; -69.0]
(0,5]	16	1.8 [1.2; 3.1]	0.3 [0.2; 0.4]	0.001	-83.8 [-89.6; -74.2]
(5,10]	7	3.7 [1.5; 9.0]	0.3 [0.3; 0.3]	0.016	-93.2 [-96.1; -88.0]
> 10	4	8.5 [1.4; 53.5]	0.3 [0.3; 0.3]	0.125	-97.1 [-98.7; -93.6]
Fagerström test for independence score					
Low (0-4)	24	1.9 [1.3; 2.9]	0.3 [0.3; 0.3]	<0.001	-86.6 [-90.6; -80.8]
Medium (5-6)	8	2.1 [1.0; 4.6]	0.3 [0.2; 0.4]	0.008	-87.1 [-91.5; -80.6]
High (7-10)	3	5.2 [2.1; 13.1]	0.3 [0.3; 0.3]	0.250	N.C.***
Cotinine level					
[0,10]	3	0.4 [0.1; 2.9]	0.3 [0.3; 0.3]	1.000	N.C.***
(10,50]	4	1.1 [0.1; 15.2]	0.3 [0.3; 0.3]	0.371	-37 [-66.1; 17]
> 50	41	2.3 [1.7; 3.2]	0.3 [0.2; 0.3]	<0.001	-76.3 [-92.4; -25.9]

* p-values were obtained from paired Wilcoxon tests comparing salivary nitrosamine (baseline vs follow-up). ** percentage changes were calculated using linear mixed-effects models, with individuals as random effects, adjusted for sex, age, and BMI (for the analyses in which the variable was used to stratify, it was not used to adjust for) to model the percentage change of salivary TSNAs concentrations (after log 10 transformation) between the baseline and follow-up. *** N.C.: Not computable value.

Supplementary table 3: Geometric mean (GM) with its 95% confidence interval (CI) and percentage change (% change) and 95% confidence interval of NNN, NNK, and NNAL concentrations of Continuing smokers (smokers at baseline and follow-up) according to sociodemographic and smoking characteristics at baseline.

		NNN (pg/mL)			
		GM [95 % CI]		p-value*	% change** [95% CI]
	n	Baseline	Follow-up		
Overall	65	4.2 [2.9; 6.1]	10.4 [6.1; 17.7]	0.020	149.8 [36.8; 356.1]
Sex					
Men	37	5.2 [2.9; 9.1]	9.1 [4.4; 18.6]	0.086	71.8 [-22.8; 282.6]
Women	28	3.2 [2.0; 5.1]	12.4 [5.4; 28.8]	0.007	311.3 [81.5; 831.7]
Age (Years)					
(18,44]	29	3.6 [2.0; 6.2]	5.7 [2.7; 12.0]	0.545	55.4 [-29.1; 240.9]
(44,64]	26	3.4 [2.1; 5.7]	18.0 [7.6; 42.7]	<0.001	458.5 [129.9; 1257.1]
> 64	10	11.1 [2.5; 49.1]	14.0 [2.3; 84.9]	0.492	26.0 [-82.9; 830.3]
BMI (kg/m²)					
(10,20]	7	3.9 [1.4; 10.]	1.6 [0.7; 3.8]	0.150	-58.7 [-81.7; -6.6]
(20,25]	33	4.5 [2.6; 7.8]	14.9 [7.0; 31.7]	0.013	232.3 [41.2; 682.3]
(25,30]	18	5.7 [2.4; 13.0]	8.9 [3.2; 25.2]	0.130	58.7 [-44.8; 356.5]
> 30	5	2.4 [0.6; 9.6]	57.2 [4.6; 710.7]	0.062	N.C.***
Pack years					
0	30	4.2 [2.6; 7.0]	13.2 [5.6; 30.7]	0.008	217.2 [35.6; 642.1]
(0,5]	26	3.4 [1.7; 6.6]	7.3 [3.3; 16.2]	0.148	115.2 [-13.7; 436.7]
(5,10]	4	3.5 [0.4; 32.8]	2.4 [0.3; 19.2]	0.625	-31.6 [-74.9; 86.8]
> 10	5	13.5 [1.2; 159.1]	54.1 [5.2; 567.6]	0.438	299.9 [-44.9; 2801.8]
Fagerström test for independence score					
Low (0-4)	35	3.3 [2.2; 5.0]	8.7 [4.5; 16.7]	0.022	159.1 [28.8; 421.2]
Medium (5-6)	11	8.5 [2.8; 25.8]	39.7 [8.4; 188.2]	0.067	365.8 [18.9; 1724.9]
High (7-10)	5	6.1 [2.3; 16.7]	15.8 [1.0; 240.9]	0.312	156.7 [-28.3; 819.3]
Years smoking (follow-up)					
≤ 24	24	3.5 [1.8; 6.7]	6.3 [2.6; 14.6]	0.721	N.C.***
(24,37]	19	3.9 [1.8; 8.2]	11.8 [4.3; 31.9]	0.055	202.2 [-0.3; 816.2]
(37,49]	16	4.4 [2.3; 8.5]	13.4 [4.2; 43.0]	0.004	226.7 [25.8; 748.6]

	> 49	6	9.3 [0.9; 95.6]	29.5 [1.8; 487.7]	0.438	N.C.***
Cotinine level						
	[0,10]	3	0.6 [0.4; 0.8]	0.9 [0.4; 11.2]	1.000	N.C.***
	(10,50]	10	1.4 [0.7; 2.8]	3.4 [1.0; 11.4]	0.322	202.2 [-0.3; 816.2]
	> 50	52	5.8 [3.9; 8.7]	14.8 [8.2; 26.8]	0.004	226.7 [25.8; 748.6]
			NNK (pg/mL)			
Overall		65	1.8 [1.5; 2.2]	2.1 [1.69; 2.59]	0.375	15.9 [-9.0; 47.6]
Sex						
	Men	37	2.0 [1.6; 2.7]	2.2 [1.6; 3.0]	0.572	30.7 [-0.2; 71.3]
	Women	28	1.5 [1.2; 1.9]	2.0 [1.5; 2.7]	0.065	32.1 [-0.2; 74.7]
Age (Years)						
	(18,44]	29	1.4 [1.2; 1.7]	1.7 [1.2; 2.4]	0.224	21.3 [-14.8; 72.8]
	(44,64]	26	1.9 [1.4; 2.4]	2.4 [1.7; 3.4]	0.055	29.5 [-6.1; 78.3]
	> 64	10	3.6 [1.8; 7.4]	2.8 [1.6; 4.9]	0.514	-23.0 [-64.6; 67.3]
BMI (kg/m²)						
	(10,20]	7	1.1 [0.8; 1.5]	1.3 [0.9; 2.1]	0.371	17.0 [-8.8; 50.0]
	(20,25]	33	1.9 [1.5; 2.5]	2.2 [1.5; 3.1]	0.581	12.1 [-23.6; 64.2]
	(25,30]	18	2.1 [1.4; 3.2]	2.1 [1.4; 3.2]	0.666	1.9 [-30.8; 50.1]
	> 30	5	1.5 [0.7; 3.1]	3.2 [1.4; 7.3]	0.223	N.C.***
Pack years						
	0	30	1.9 [1.4; 2.4]	2.1 [1.5; 3.0]	0.527	14.2 [-23.8; 71.1]
	(0,5]	26	1.6 [1.2; 2.1]	1.9 [1.4; 2.6]	0.187	22.9 [-13.5; 74.4]
	(5,10]	4	2.2 [0.4; 11.4]	1.6 [0.3; 7.9]	0.371	-24.8 [-53.3; 21.2]
	> 10	5	2.7 [1.3; 5.6]	3.4 [1.2; 9.8]	0.201	28.1 [-15.7; 94.7]
Fagerström test for independence score						
	Low (0-4)	35	1.5 [1.2; 1.9]	1.8 [1.3; 2.5]	0.206	19.6 [-14.3; 66.9]
	Medium (5-6)	11	2.1 [1.4; 3.2]	3.9 [2.4; 6.6]	0.019	85.7 [23.9; 178.4]
	High (7-10)	5	2.9 [1.1; 7.7]	3.1 [1.2; 8.3]	0.812	9.5 [-32.9; 78.7]
Years smoking (follow-up)						
	≤ 24	24	1.3 [1.1; 1.6]	1.6 [1.1; 2.0]	0.455	N.C.***
	(24,37]	19	1.8 [1.3; 2.4]	2.1 [1.4; 3.2]	0.196	20.3 [-22.5; 85.2]
	(37,49]	16	2.3 [1.5; 3.6]	2.6 [1.7; 4.0]	0.327	15.2 [-31.5; 93.5]
	> 49	6	3.6 [1.0; 10.6]	3.0 [1.5; 6.1]	0.787	N.C.***
Cotinine level						
	[0,10]	3	1.1 [0.7; 1.7]	1.0 [1.0; 1.0]	1.000	N.C.***
	(10,50]	10	1.6 [0.8; 3.0]	1.6 [1.0; 2.6]	0.855	20.3 [-22.5; 85.2]
	> 50	52	1.9 [1.5; 2.3]	2.3 [1.8; 3.0]	0.098	15.2 [-31.5; 93.5]
			NNAL (pg/mL)			
Overall		65	1.3 [1.0; 1.7]	1.4 [1.1; 1.9]	0.564	11.6 [-15.6; 48.0]
Sex						

Men	37	1.5 [1.0; 2.2]	1.6 [1.1; 2.4]	0.297	5.2 [-28.8; 56.4]
Women	28	1.0 [0.7; 1.6]	1.2 [0.9; 1.8]	0.449	21.4 [-19.4; 83.0]
Age (Years)					
(18,44]	29	0.8 [0.6; 1.2]	1.1 [0.8; 1.6]	0.081	30.4 [-13.5; 96.6]
(44,64]	26	1.5 [1.0; 2.1]	1.7 [1.1; 2.8]	0.185	18.4 [-19.0; 73.9]
> 64	10	3.3 [1.3; 8.8]	2.0 [0.8; 4.9]	0.232	-38.9 [-76.8; 61.4]
BMI (kg/m²)					
(10,20]	7	0.8 [0.3; 2.0]	0.6 [0.3; 1.1]	0.834	0.0 [-67.8; 65.4]
(20,25]	33	1.3 [0.9; 2.0]	1.7 [1.2; 2.3]	0.371	0.0 [-9.8; 72.2]
(25,30]	18	1.6 [0.9; 3.1]	1.7 [0.9; 3.4]	0.298	0.0 [-40.7; 92.9]
> 30	5	1.4 [0.7; 2.7]	1.5 [0.3; 6.4]	0.855	N.C.***
Pack years					
0	30	1.2 [0.8; 1.7]	1.5 [1.0; 2.2]	0.046	19.4 [-11.2; 61.1]
(0,5]	26	1.2 [0.7; 2.1]	1.3 [0.8; 2.2]	0.861	10.3 [-38.0; 96.2]
(5,10]	4	1.3 [0.1; 15.2]	0.8 [0.2; 2.8]	0.423	-40.1 [-77.5; 59.9]
> 10	5	2.3 [0.9; 6.0]	2.9 [0.8; 10.2]	1.000	28.9 [-27.5; 129.1]
Fagerström test for independence score					
Low (0-4)	35	1.1 [0.8; 1.4]	1.1 [0.8; 1.6]	0.092	9.0 [-22.9; 55.0]
Medium (5-6)	11	1.3 [0.6; 2.7]	2.5 [1.4; 4.6]	0.206	90.0 [13.5; 218.1]
High (7-10)	5	3.4 [1.2; 9.5]	2.5 [0.7; 9.1]	1.000	-25.3 [-68.3; 75.9]
Years smoking (follow-up)					
≤ 24	24	0.8 [0.5; 1.2]	1.1 [0.73; 1.63]	0.125	N.C.***
(24,37]	19	1.2 [0.8; 2.0]	1.4 [0.7; 2.6]	0.132	10.5 [-45.7; 124.8]
(37,49]	16	2.1 [1.2; 3.5]	1.7 [1.0; 2.9]	0.551	11.9 [-18.3; 53.4]
> 49	6	2.6 [0.4; 15.7]	3.2 [1.0; 10.19]	0.844	0.0 [0.0; 0.0]
Cotinine level					
[0,10]	3	0.3 [0.2; 0.4]	0.4 [0.0; 4.0]	1.000	N.C.***
(10,50]	10	0.9 [0.3; 2.7]	1.0 [0.4; 2.7]	0.624	N.C.***
> 50	52	1.5 [1.1; 2.0]	1.7 [1.2; 2.2]	0.259	-3.8 [-53.8; 100.0]

* p-values were obtained from paired Wilcoxon tests comparing salivary nitrosamine (baseline vs follow-up). ** percentage changes were calculated using linear mixed-effects models, with individuals as random effects, adjusted for sex, age, and BMI (for the analyses in which the variable was used to stratify, it was not used to adjust for) to model the percentage change of salivary TSNAs concentrations (after log 10 transformation) between the baseline and follow-up. *** N.C.: Not computable value.

Supplementary table 4: Geometric mean (GM) with its 95% confidence interval (CI) and percentage change (% change) and 95% confidence interval of NNN, NNK, and NNAL concentrations of Continuing non-smokers at baseline and follow-up according to sociodemographic and smoking characteristics at baseline.

		NNN (pg/mL)			
		GM [95 % CI]			
	n	Baseline	Follow-up	p-value*	% change** [95% CI]
Overall	272	1.5 [1.2; 1.8]	1.3 [1.1; 1.6]	0.553	1.3[-8.4; 12.0]
Sex					
Men	122	2.3 [1.6; 3.2]	1.6 [1.1; 2.2]	0.318	10.2[-7.6; 31.5]
Women	149	1.1 [0.9; 1.3]	1.1 [0.9; 1.4]	0.837	-3.4[-14.5; 9.1]
Age (Years)					
(18,44]	78	1.7 [1.2; 2.4]	1.5 [1.0; 2.3]	0.500	6.4[-6.9; 21.7]
(44,64]	96	1.5 [1.1; 2.0]	1.6 [1.1; 2.4]	0.280	2.4[10.7; 15.9]
> 64	97	1.4 [1.0; 2.0]	0.9 [0.7; 1.2]	0.171	-1.3[-17.7; 18.3]
BMI (kg/m²)					
(10,20]	16	2.3 [1.0; 5.2]	0.9 [0.6; 1.4]	0.023	-17.4[-40.8; 15.3]
(20,25]	128	1.4 [1.1; 1.9]	1.5 [1.1; 2]	0.699	5.6[-7.9; 21.0]
(25,30]	94	1.6 [1.1; 2.3]	1.1 [0.8; 1.5]	0.391	-2.6[-15.6; 12.3]
> 30	31	1.4 [0.8; 2.6]	1.5 [0.7; 3.1]	0.687	3.9[43.8; 55.2]
		NNK (pg/mL)			
Overall	272	1.4 [1.3; 1.5]	1.3 [1.2; 1.4]	0.056	-3.1[-9.9; 4.3]
Sex					
Men	122	1.6 [1.4; 1.9]	1.4 [1.2; 1.6]	0.124	-5.8[-9.0; 14.1]
Women	150	1.3 [1.2; 1.4]	1.2 [1.1; 1.3]	0.200	-5.8[-14.3; 3.5]
Age (Years)					
(18,44]	78	1.3 [1.2; 1.5]	1.3 [1.1; 1.5]	0.705	-3.9[-16; 10]
(44,64]	96	1.4 [1.3; 1.6]	1.3 [1.2; 1.5]	0.596	-2.0[-10.9; 7.8]
> 64	98	1.5 [1.3; 1.7]	1.2 [1.1; 1.4]	0.031	-3.5[-14.8; 9.4]
BMI (kg/m²)					
(10,20]	16	1.2 [1.0; 1.5]	1.1 [0.9; 1.3]	0.584	N.C.***
(20,25]	128	1.4 [1.2; 1.5]	1.3 [1.2; 1.5]	0.261	-2.1[-11.3; 7.9]
(25,30]	95	1.5 [1.3; 1.7]	1.3 [1.1; 1.4]	0.116	-3.1[-16.2; 12]
> 30	31	1.4 [1.2; 1.8]	1.3 [1.1; 1.6]	0.572	-6.6[-20.8; 10.1]
		NNAL (pg/mL)			
Overall	272	0.5 [0.5; 0.6]	0.4 [0.3; 0.4]	<0 .001	-3.4[-6.7; 0.1]
Sex					
Men	122	0.7 [0.6; 0.9]	0.5 [0.4; 0.6]	0.007	-5.3[-2.9; 3.3]
Women	150	0.4 [0.4; 0.5]	0.3 [0.3; 0.4]	0.016	-5.3[-10.1; -0.3]
Age (Years)					
(18,44]	78	0.5 [0.4; 0.7]	0.5 [0.4; 0.6]	0.534	0.7[-3.1; 4.7]
(44,64]	96	0.6 [0.5; 0.8]	0.4 [0.4; 0.5]	0.037	-0.5[-1.4; 0.5]
> 64	98	0.5 [0.4; 0.6]	0.3 [0.3; 0.4]	0.001	-6.7[-12.9; -0.1]
BMI (kg/m²)					
(10,20]	16	0.6 [0.4; 1.2]	0.4 [0.3; 0.5]	0.193	N.C.***
(20,25]	128	0.5 [0.4; 0.6]	0.4 [0.4; 0.5]	0.089	-0.9[-5.2; 3.7]
(25,30]	95	0.6 [0.5; 0.8]	0.4 [0.3; 0.4]	0.014	-6.7[-13.3; 0.4]
> 30	31	0.5 [0.3; 0.8]	0.3 [0.2; 0.4]	0.155	-3.1[-8.8; 3]

* p-values were obtained from paired Wilcoxon tests comparing salivary nitrosamine (baseline vs follow-up). ** percentage changes were calculated using linear mixed-effects models, with individuals as random effects, adjusted for sex, age, and BMI (for the analyses in which the variable was used to stratify, it was not used to adjust for) to

model the percentage change of salivary TSNAs concentrations (after log 10 transformation) between the baseline and follow-up. *** N.C.: Not computable value.

Chapter 5.3 Article III

Variation in Nicotine Metabolization According to Biological Factors and Type of Nicotine Consumer

healthcare

MDPI

Article

Variation in Nicotine Metabolization According to Biological Factors and Type of Nicotine Consumer

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Abstract: This study aims to describe the nicotine metabolite ratio among tobacco smokers and electronic cigarette (e-cigarette) users and nonusers. We analyzed pooled data from a longitudinal and a cross-sectional study of the adult population from the city of Barcelona. The final sample included information on 166 smokers, 164 e-cigarettes users with nicotine, 41 e-cigarette users without nicotine, 95 dual users (users of both products), and 508 nonusers. We used log-linear models to control for the potential confounding effect of the daily number of cigarettes smoked. Salivary nicotine metabolic rate assessment included the rate of nicotine metabolism (cotinine/nicotine) and the nicotine metabolite ratio (trans-3′-hydroxycotinine/cotinine). Exclusive users of e-cigarette without nicotine have the lowest rate of nicotine metabolism (Geometric mean: 0.08, p-values < 0.001) while cigarette smokers have the highest (Geometric mean: 2.08, p-values < 0.001). Nonusers have lower nicotine metabolic rate than cigarette smokers (Geometric means: 0.23 vs. 0.18, p-value < 0.05). Younger individuals (18–44 years) have a higher rate of nicotine metabolism than older individuals (45–64 years and 65–89) (Geometric means: 0.53 vs. 0.42 and 0.31, respectively, p-values < 0.01) and individuals with lower body mass index (21–25 kg/m^2) have a higher rate of nicotine metabolism than the rest (26–30 kg/m^2 and 31–60 kg/m^2) (Geometric means: 0.52 vs. 0.35 and 0.36, respectively-values < 0.01). Nicotine metabolic rates are useful biomarkers when reporting smoking status and biological differences between individuals.

Keywords: biomarker; cotinine; electronic cigarette; nicotine; nicotine metabolism; smoking

1. Introduction

Nicotine is a natural compound found in tobacco leaves. Several tobacco products such as cigarettes, oral snuff, pipe tobacco, cigars and chewing tobacco have approximately 2% of nicotine per unit [1]. The most frequent nicotine concentration in the liquid of electronic cigarettes (e-cigarettes) ranges from 0 to 36 mg/mL in high nicotine vaping products [1]. The harmful health effects of tobacco consumption are well known, as it has

been associated with more than 25 types of cancer and cardiovascular diseases, among other conditions [2–4].

High-dose nicotine absorption in the body happens mainly through smoking or exposure to tobacco smoke. Once tobacco smoke is inhaled, nicotine is absorbed into the organism. After this, the clearance of the compound results in its conversion into better assimilable metabolites. Nicotine is almost entirely degraded to cotinine and cotinine is mostly degraded to trans-3′-hydroxycotinine; both transformations are catalyzed by the enzyme CYP2A6 [5–7]. Both compounds have been well studied due to their relative in vivo long half-life (around 16–20 h and 6 h, respectively) [5,8]. Cotinine and trans-3′-hydroxycotinine can be measured in human fluids such as saliva, plasma, blood or urine during ten days after nicotine metabolization [5,9]. Around 80% of nicotine is metabolized to cotinine, turning cotinine into a well-fitted biomarker of tobacco consumption and exposure [5,9]. However, different biological matrixes represent measurements of the same biomarker taken at different points in the pharmacokinetic pathway, as seen with the higher concentration of cotinine in urine in comparison with that in saliva or plasma [10,11]. In addition, the metabolization of nicotine can be affected by various factors. Some of the most common in the literature are gender, age and BMI. In the case of sex, it has been found that sex hormones play a role in the metabolism of cotinine and that women have a faster nicotine metabolism than men. In the case of age and BMI, the bibliography is not so clear in this regard. This is because there are countless factors that can influence the liver (and consequently the CYP2A6 enzyme), sometimes even in contradictory ways [12–14].

To study nicotine addiction and smoking behavior, ratios are a great alternative to work with [15]. One of the most intuitive ratios is the rate of nicotine metabolism (RNM), obtained by dividing the concentration of cotinine by that of nicotine (cotinine/nicotine) [16,17]. Even so, as the half-life of nicotine is so short (2 h) [15], these ratios are heavily reliant on the time since the last cigarette was smoked and the number of cigarettes smoked, so the metabolic ratio of nicotine is highly variable during the day [16,18]. To overcome the problem, the use of the nicotine metabolite ratio (NMR), that is, the ratio of trans-3′-hydroxycotinine to cotinine, is further encouraged [17]. The NMR is consistent in different biological fluids (e.g., a person who is determined to be a slow metabolizer by one method is highly likely to be below the cut point for slow metabolism in other fluids, but the cut points may be different between biological matrixes) and captures both inter-individual and environmental differences. Besides, it is more stable than the RNM, meaning that it has minimal variation during the day, lower dependence on time since the last cigarette in smokers and it is stable over a year time (the NMR continues to give very similar values even a year after a reduction in tobacco consumption has begun. This quality also applies to people who have recently quit smoking) [17,19]. The NMR values in saliva and urine can be used as proxies of the NMR in plasma, although values in urine are more variable [5]. Moreover, the NMR is consistently associated with the activity of the enzyme CYP2A6 [5–7].

The NMR has been validated as a reliable biomarker of nicotine metabolism and cigarette dependence, especially in saliva and urine, whose collection uses non-invasive techniques [17,20]. On the other hand, while nicotine metabolites have been extensively studied in smokers, information about nicotine metabolites in users of e-cigarettes is scarce [21]. Some studies analyzing tobacco-specific biomarkers among users of e-cigarettes showed that despite its commercialization as a smoking cessation aid, nicotine and cotinine concentrations may be higher in e-cigarette' users, making e-cigarettes even more addictive than traditional cigarettes [22,23]. Therefore, to examine the variability in nicotine metabolism, the objective of this study is to investigate potential variations in the RNM and NMR in tobacco smokers and users of e-cigarettes according to different biologic factors and smoking status.

2. Materials and Methods

This is a pooled analysis carried out with data retrieved from two different studies. The first one was a longitudinal study on tobacco smoking patterns "Determinants of

Cotinine project-phase 3 (dCOT3 study)." This was a cohort study of a sample of adults (≥16 years at baseline) from the general population of Barcelona (Catalonia, Spain). The baseline was carried out during the years 2004–2005 (n = 1245) and one follow-up was carried out during 2013–2014 (n = 736). The study included self-reported information about smoking patterns and tobacco exposure, and a saliva sample was collected at the follow up for the determination of various biomarkers of tobacco exposure. After cleansing the follow-up [24], removing subjects that did not have available saliva samples (n = 44), subjects without available nicotine, cotinine or trans-3′-hydroxycotinine information (n = 11), and seven nonusers whose cotinine concentrations were incompatible with being a nonuser (>10 ng/mL) [25], this sample retained data of 674 individuals. The second study was a cross-sectional study (n = 302) conducted in 2017–2018 containing data on adult (≥18 years old) users of e-cigarettes living in Barcelona. As an alternative to a probabilistic sampling technique, the consumer panels technique [26] was used in order to enroll users of e-cigarettes. Although this technique renders the sample unrepresentative of the general population, it minimizes the limitations of the reduced sample size, given the low prevalence of use in this population. Individuals who declared to be current users of e-cigarettes were asked to take part in the study. A questionnaire on e-cigarette use patterns was used and a saliva sample was also collected to determine nicotine, cotinine and trans-3′-hydroxycotinine. Two individuals whose information was missing and could not be categorized were excluded, rendering a second sample of 300 e-cigarette users. Thus, the final merged sample retained data of 974 individuals (Figure 1). The design and methodology of both studies are detailed elsewhere [25,26]. Both studies received approval by the ethics committee of the Bellvitge University Hospital (PR118/11 y PR133/15, respectively) and all participants signed informed consent.

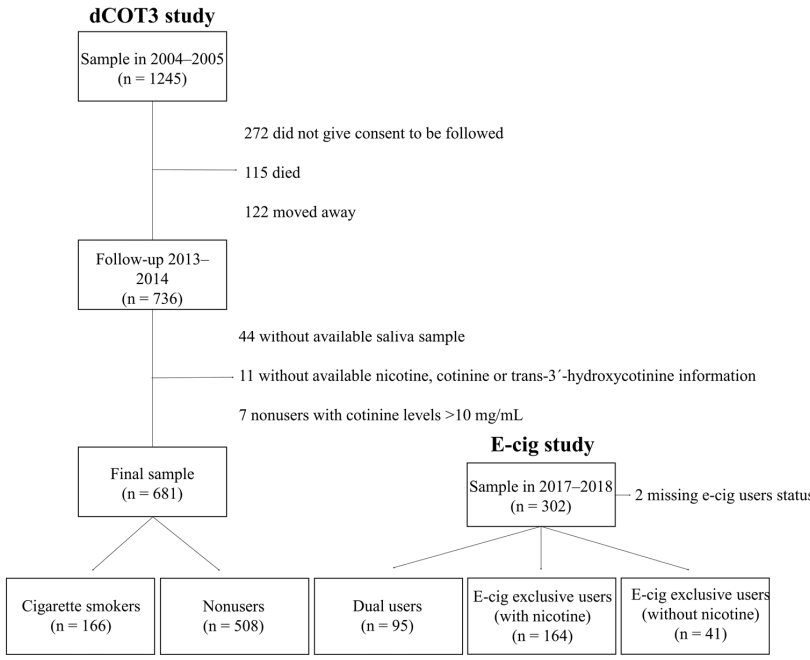

Figure 1. Flow chart of the selection of individuals from the dCOT3 and E-cig studies (n = 974).

2.1. Determination of Biomarkers in Saliva and Computation of the Rate of Nicotine Metabo-Lism and Nicotine Metabolite Ratio

In order to determine nicotine, cotinine and 3′-hydroxycotinine concentrations we analyzed salivary samples employing a common protocol [9,27]. After rinsing their mouths

and sucking a lemon candy (Smint®) to stimulate saliva production, participants provided 9 mL of saliva by directly spitting in a test tube with the help of a funnel. Each individual sample was separated into 3 mL aliquots and stored at −20 °C. The frozen samples were sent to the Group of Integrative Pharmacology and Systems Neuroscience of the Municipal Institute for Medical Research (IMIM-Hospital del Mar) in Barcelona. All biomarkers were determined by alkaline single liquid-liquid extraction with dichloromethane/isopropanol followed by liquid chromatography-tandem mass spectrometry; this methodology is described elsewhere [28]. The limit of quantification of this method was 0.5 ng/mL for nicotine, 0.1 ng/mL for cotinine and 0.04 ng/mL for trans-3′-hydroxycotinine. Values under the limit of quantification were halved to avoid overestimation or underestimation bias. Then, the rate of nicotine metabolism, or RNM (cotinine/nicotine) and the nicotine metabolite ratio, or NMR (trans-3′-hydroxycotinine/cotinine) were calculated.

2.2. Smoking Status and Use of E-Cigarettes

According to self-reported information, we classified the participants into the five following groups: (a) dual users (participants who were both current cigarette smokers and users of e-cigarettes), (b) cigarette smokers, (c) e-cigarette exclusive users with nicotine, (d) e-cigarette exclusive users without nicotine, and (e) nonusers. The inclusion of users of e-cigarettes without nicotine and nonusers of any products is due to the fact that they can have low levels of nicotine metabolites, and can generally be attributable to passive exposure to nicotine [29]. Inclusion of non-users is justified since exposure to tobacco smoke was not controlled for.

Nonusers were individuals who declared to have never smoked or to have formerly smoked/used tobacco/nicotine products or e-cigarettes. Subjects were considered current cigarette smokers if they declared smoking cigarettes daily or occasionally (people who smoked regularly within a week but did not smoke every day of the week) at the moment of the survey. Any person who used e-cigarettes and did not smoke for at least six months was considered an exclusive e-cigarette user. If exclusive users of e-cigarettes declared that the e-liquid contained nicotine, regardless of the concentration, the users were categorized as "e-cigarette exclusive users with nicotine". Otherwise, they were categorized into "e-cigarette exclusive users without nicotine". Individuals who reported smoking cigarettes and using e-cigarettes were considered dual users.

2.3. Biological Variables

Our study also included information on self-reported biological variables, namely sex, age and body mass index (BMI). We categorized the individuals' age into 3 groups (according to the sample tertiles): (a) between 18 and 44 years old, (b) between 45 and 64 years old and (c) between 65 and 89 years old. Similarly, we also categorized the individuals' BMI in a total of 4 groups (in accordance to WHO guidelines [30], although the underweight range was extended to increase the sample size): (a) between 10 and 20 kg/m^2, (b) between 21 and 25 kg/m^2, (c) between 26 and 30 kg/m^2 and (d) between 31 and 60 kg/m^2.

2.4. Statistical Analysis

Because of the skewness in nicotine metabolites values [25], we calculated the geometric mean (GM) and geometric standard deviation (GSD) of RNM and NMR. Analyses were stratified by the five groups of smoking status and use of e-cigarette and biological variables. We compared both RNM and NMR across all groups using the Mann Whitney test for independent samples and Kruskal-Wallis H test, both with Bonferroni correction (multiplying the p-values by the number of comparisons) according to smoking status and use of e-cigarette, sex, age and BMI. Given the potential confounding effect of the daily number of cigarettes smoked and the variable for the e-cigarette, we realized two (adjusted and unadjusted) log-linear models for each one of the ratios according to the smoking and e-cigarette categories. We performed a sensitivity analysis to find differences

between the complete sample and those participants with a cotinine level greater than the limit of quantification. To ease the interpretation of our results, we decided to follow Siegel et al. [20] methodology and classify participants based on overall RNM and NMR quartiles, labeling those in the first quartile as "slow metabolizers," those in the second quartile as "moderate metabolizers," and those in the third and fourth quartiles as "fast metabolizers." The level of significance was set at $\alpha = 0.05$. Data were analyzed using R (R Foundation for Statistical Computing, Vienna, Austria) version 4.0.4.

3. Results

The GM of RNM and NMR overall and stratified by smoking and e-cigarette use, sex, age and BMI are shown in Table 1. E-cigarette exclusive users without nicotine showed the lowest RNM value (GM: 0.08, *p*-values < 0.001). Cigarette smokers showed the highest ratio of them all (GM:2.08, *p*-values < 0.001). Nonusers have significantly higher values (GM:0.27, *p*-values < 0.001) than e-cigarette exclusive users without nicotine, but lower than dual users and e-cigarette exclusive users with nicotine. There was no significant difference between e-cigarette exclusive users with nicotine and dual users (GM:0.49 vs. GM:0.48). Regarding NMR, significant differences were found between cigarette smokers and nonusers (GM:0.27 vs. GM:0.23, *p*-values < 0.05).

Table 1. Geometric mean (GM) and Geometric Standard Deviation (GSD) of Rate of Nicotine Metabolism (RNM) and Nicotine Metabolite Ratio (NMR) in saliva samples according to smoking status and use of e-cigarette, sex, age and body mass index (BMI).

	***n* (%)**	**RNM GM (GSD)**	**NMR GM (GSD)**
Overall	974	0.43 (4.27)	0.22 (2.09)
Smoking and e-cigarette status [a]			
Nonusers of any product [a5]	508 (52.16)	0.27 (2.30) [(a1, a2, a3, a4) ***]	0.23 (1.99) [a2 *]
E-cigarette exclusive users without nicotine [a4]	41 (4.21)	0.08 (8.12) [(a1, a2, a3, a5) ***]	0.23 (1.80)
E-cigarette exclusive users with nicotine [a3]	164 (16.84)	0.49 (3.45) [(a2, a4, a5) ***]	0.22 (1.90)
Dual users [a1]	95 (9.75)	0.48 (4.70) [(a2, a4, a5) ***]	0.24 (1.80)
Cigarette smokers [a2]	166 (17.04)	2.08 (4.90) [(a1, a3, a4, a5) ***]	0.18 (2.61) [a5 *]
Sex [b]			
Female [b1]	442 (45.38)	0.43 (3.98)	0.24 (2.11) [b2 ***]
Male [b2]	532 (54.62)	0.43 (4.52)	0.21 (2.06) [b1 ***]
Age (years) [c]			
18–44 [c1]	371 (38.09)	0.53 (4.97) [c3 ***]	0.21 (1.96) [c3 ***]
45–64 [c2]	363 (37.27)	0.42 (4.17) [c3 **]	0.22 (2.13)
65–89 [c3]	240 (24.64)	0.31 (3.18) [c1 ***, c2 **]	0.25 (2.20) [c1 ***]
BMI (kg/m^2) [d]			
10–20 [d1]	64 (6.57)	0.54 (4.93)	0.26 (2.07)
21–25 [d2]	378 (38.81)	0.52 (4.56) [d3 ***, d4 **]	0.22 (2.12)
26–30 [d3]	336 (34.50)	0.35 (3.93) [d2 ***]	0.22 (2.09)
31–60 [d4]	186 (49.21)	0.36 (3.86) [d2 **]	0.23 (2.01)

Letters as superscripts ([a], [b], [c], and [d]) indicate the qualitative variable (Smoking and e-cigarette status, Sex, Age (years), and BMI (kg/m^2) respectively). Numbers as superscripts indicate the level of the variable whose letter precedes them. The superscripts for statistical significance (* significant at $p < 0.050$; ** significant at $p < 0.010$; *** significant at $p < 0.001$) report on the significant *p*-values (after adjusting by Bonferroni correction) when comparing the categories within each variable to which that row corresponds with the rest of the categories within that same variable, always within the same category.

Younger individuals showed higher RNM than older individuals (0.53 vs. 0.31). Similarly, individuals with BMI 21–25 have higher RNM than those in the higher categories (0.52 vs. 0.35–0.36). On the other hand, NMR was higher in females (0.24 vs. 0.21; *p*-value <0.001) and lower in younger individuals (0.21 vs. 0.25; *p*-value < 0.001). No differences in NMR were found between individuals according to BMI. The comparison of the unadjusted

and the adjusted log-linear models for both the RNM and the NMR (Table 2) did not show much difference from each other, indicating that there is no significant confusion effect in any of the ratios. For the RNM, all the categories of smoking status and use of e-cigarette were significant (<0.01) in both models, while for the NMR only the category cigarette smokers (<0.01) was significant. The quartile division of the RNM classifies those individuals whose RNM is lower than 0.20 as slow metabolizers (first quartile), between 0.2 and 0.36 (second quartile) as moderate metabolizers and those with a value higher than 0.36 as fast metabolizers. In the case particular case of the NMR, the first quartile was 0.14 and the second 0.23.

Table 2. Log-linear models for Smoking and e-cigarette status (nonusers as reference) for Rate of Nicotine Metabolism (RNM) and Nicotine Metabolite Ratio (NMR). The first one (Unadjusted Model) and the second one with its presence (Adjusted Model).

Title 1	**RNM**			**NMR**		
Log-linear Models	**exp(Estimate) [c]**	**CI [d]**	***p*-Value**	**exp(Estimate) [c]**	**CI [d]**	***p*-Value**
Unadjusted model [a]						
Intercept	0.27	0.24; 0.30	<0.001	0.23	0.22; 0.25	<0.001
E-cigarette exclusive users without nicotine	0.08	0.05; 0.14	<0.001	0.23	0.17; 0.32	0.96
E-cigarette exclusive users with nicotine	0.49	0.36; 0.68	<0.001	0.22	0.18; 0.27	0.37
Dual users	0.48	0.33; 0.70	<0.001	0.24	0.19; 0.30	0.71
Cigarette smokers only	2.08	1.51; 2.85	<0.001	0.18	0.15; 0.22	<0.001
Adjusted model [b]	0.27	0.24; 0.30	<0.001	0.23	0.22; 0.25	<0.001
Users of e-cigarettes without nicotine	0.09	0.05; 0.14	<0.001	0.23	0.17; 0.32	0.97
Users of e-cigarettes with nicotine	0.65	0.45; 0.95	<0.001	0.21	0.16; 0.26	0.18
Dual	0.60	0.4; 0.89	<0.001	0.23	0.18; 0.29	0.91
Cigarette smokers	2.43	1.74; 3.39	<0.001	0.18	0.14; 0.22	<0.001
Daily number of cigarettes smoked	0.26	0.23; 0.29	<0.001	0.23	0.22; 0.25	0.29

[a] Log-linear model computed without including the daily number of cigarettes as covariable. [b] Log-linear model adjusted for the daily number of cigarettes and its equivalent for e-cigarette. [c] Exponential of the sum of the estimates (but for the daily number of cigarettes). [d] Confidence Interval.

4. Discussion

By describing the relationship between NMR and biological differences, previous studies have categorized individuals according to the rate of metabolism with which they metabolize nicotine and its derivatives [17,20]. The quartile division method of NMR for the classification of slow (<0.14), moderate (0.14–0.23) and fast metabolizers (>0.23) in saliva was in line with previous studies, on which the first and second quartile for NMR dividing between slow and moderate metabolizers were found to be 0.17 and 0.29 [31], and 0.18 and 0.3 [32], respectively. In contrast with slower metabolizers, faster metabolizers of nicotine are thought to clear nicotine much faster, develop more symptoms of nicotine dependence, and increase their nicotine dose in order to minimize withdrawal symptoms [20]. To the best of our knowledge, this is the only study comparing salivary nicotine rate of metabolism in Spain by tobacco consumption including e-cigarettes in the general population and also the first time that a division point in the RNM has been described with the intention of dividing a sample into different types of metabolizers. Regarding biological factors, previous studies showed that NMR is higher in females [33,34] and that females are faster metabolizers than males [7,13,33], while age [34] and body mass index (BMI) have been negatively associated with NMR [31]. In the particular case of the NMR, faster nicotine metabolism among nonusers as compared to cigarette smokers has been reported [33].

4.1. Nicotine Metabolite Ratio & Rate of Nicotine Metabolism According to Smoking Status and Use of E-Cigarette

Regarding the NMR and according to our results, smokers, users of e-cigarettes (with and without nicotine) and nonusers in our sample are moderate metabolizers, while dual users are fast metabolizers, having a higher NMR, which is the opposite of expectations, since the NMR should be lower in consumers of tobacco products [35]. Our findings when comparing the adjusted and unadjusted linear models indicate no confounding effect attributable to the number of cigarettes per day. Consistent with the findings on NMR, it is possible that the discrepancies observed in dual users and users of e-cigarettes may be attributable to variations in tobacco consumption topography [32,36]. In this sense, future studies must investigate the relationship between nicotine metabolization ratios with the consumption topography of different tobacco products.

On the other hand, regarding the RNM, results show that e-cigarette (without nicotine) users are slow metabolizers, nonusers are moderate metabolizers, and smokers, e-cigarette (with nicotine) users and dual users are fast metabolizers. Given the higher nicotine dependence of fast metabolizers [20], our results support the previously described hypothesis of faster metabolizers taking a higher nicotine dose in order to alleviate withdrawal symptoms. Due to the low nicotine concentration in nonusers and users of e-cigarettes without nicotine, perhaps there are differences in the nicotine metabolization between passive smokers depending on the level of exposure. However, the GM of RNM for users of e-cigarettes without nicotine is extremely low. Results for this unstable rate may not be representative of the whole population.

In line with a previous study [7], NMR showed utility as a biomarker of nicotine metabolism in users of e-cigarettes. In this sense, it is important to continue studying the similitudes in the nicotine metabolism between users of e-cigarettes and cigarette smokers through NMR and RNM in the future.

4.2. Nicotine Metabolite Ratio & RNM According to Biological Factors

We found significant differences in nicotine metabolism according to sex in NMR (p-values < 0.001); the quartile division in our sample suggests that female are fast metabolizers and male are moderate metabolizers. However, no differences were found when comparing the GM of RNM between males and females. Another study suggests that females are more likely to have faster nicotine metabolism and have higher dependence on nicotine products, possibly due to the effect of sex hormones on CYP2A3 [12,13]. Nevertheless, the GM of NMR for female (0.24) and male (0.21) obtained in our study is lower than the one reported in that study (0.43 and 0.35, respectively) [13]. Discrepancies between both studies may be due to the differences between the two populations, as their sample was composed of failed quitters and ours of cigarette smokers, users of e-cigarettes, and nonusers, and, as previously reported, when the NMR is higher, nicotine metabolization is faster, and faster metabolizers may have a much harder time quitting smoking [37].

When comparing the GM of RNM by age, a negative association was clearly observed, being older individuals (65–89 years) faster nicotine metabolizers than younger ones (<64 years). NMR showed a positive association with age, but just between participants <44 years (moderate metabolizers) and participants 65–89 years (fast metabolizers) individuals. A previous study reported a nicotine clearance similar to the one we observed in RNM [38]. However, that study counted with limited subjects (n = 40) and was conducted introducing nicotine via intravenous infusion and measured in plasma and urine, so differences in nicotine metabolization between different biological matrixes are to be expected. As previous studies reported similar trends concerning age and rates of nicotine metabolism, our results suggest that accumulated exposure to nicotine and natural metabolic changes associated with age may enhance its metabolization rate [12,13]. In this case, the differences observed are negatively associated with age, being the value for older individuals (65–89 years) practically the same as nonusers.

Our results also shows that there is a negative association between the RNM and the BMI, while no association was found between the NMR and the BMI, although the quartile analysis categorized overweight and underweight individuals as fast metabolizers (>0.23), while the rest were categorized as moderate metabolizers. Previous studies with a similar sample size reported BMI being negatively associated with NMR [14,31,39]. However, understanding the effect and metabolic rates of novel forms of nicotine intake in smoking status, use of e-cigarette and biological factors needs further investigation.

Discrepancies between both ratios could be attributed to NMR with independence of the number of cigarettes smoked [17]. Based on this, we should use the most stable ratio (NMR) as a good unbiased indicator of nicotine metabolization. However, if reported together, they could be used to determine the differences and have a rough estimation of smoking quantities.

4.3. Limitations

Our study has some limitations that should be mentioned. Although the questionnaire has been self-declared and could represent a reporting bias, there is sufficient evidence that self-reported data on smoking performs well when working with trained interviewers. In addition, as one portion of the pooled data was taken from a longitudinal study (this sample is aged) and the other portion used a non-probabilistic sampling technique, our sample is not representative of the general population. We did not analyze nicotine concentration in the e-cigarettes to compare with the self-reported data. Neither did we control for passive exposure. Furthermore, classifying individuals in the sample by quartiles has the downside that the selection of the sample influences that classification. Also, there is female underrepresentation in our sample, and as females are faster metabolizers than males, our results may present underestimation bias. Lastly, there are some significant concerns about the use of metabolite ratios that must be mentioned. One of them is that the NMR may not be reliable when calculated using values that are below the limit of quantification, not only because small measurement errors can lead to large differences in ratios but also because such low values indicate that cotinine and trans-3′-hydroxycotinine are not at steady state in the body. When not at steady state, the concentrations of trans-3′-hydroxycotinine are not solely formation-dependent and therefore the assumptions underlying the use of the NMR as a measure of nicotine metabolism are not met [40]. However, we performed a sensitivity analysis between the complete sample size and those participants with a cotinine level greater than the limit of quantification and could not find significant differences between groups. Other potential issue related to the use of metabolite ratios is that the RNM is highly dependent on time since last use which is why it is not commonly used as a measure of nicotine metabolism rate. Regrettably, time since last use is not found among the data obtained through the questionnaires passed.

5. Conclusions

We did not find significant differences in the NMR between dual users, e-cig (with nicotine) users, and cigarette smokers or between nonusers and e-cigarette (without nicotine) users; however, we successfully identified different types of metabolizers according to smoking status and use of e-cigarettes. Both in RNM and NMR, dual users were fast metabolizers. At the level of absorption and metabolic rates, the use of e-cigarettes with nicotine could be analogous to the use of conventional tobacco products. These findings warrant further investigation given the potential of the NMR to inform about the biotransformation of nicotine and, consequently, about smoking status and biological differences in nicotine metabolic rate between individuals.

Author Contributions: H.P.-M. analyzed the data and drafted the first manuscript with the supervision of C.L.-M., A.G.-M. and J.M.M.-S., M.F., M.B., E.F. and J.M.M.-S. contributed to the design and coordination of the study. R.P.-O. and J.A.P. analyzed saliva samples. J.M.M.-S. is the principal investigator of the project. All authors contributed substantially to the interpretation of the data and

the successive versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Bellvitge University Hospital (protocol codes PR118/11 and PR133/15, date of approvals 2 May 2012 and 21 May 2015, respectively).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

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Chapter 5.4 Article IV

Validity of adaptation of the Fagerström Test for Cigarette Dependence in Electronic Cigarette users: A Bayesian approach with biomarkers

Validity of adaptation of Fagerström Test for Cigarette Dependence in Electronic Cigarette users: A Bayesian approach with biomarkers

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ABSTRACT

Background: Validation of biochemical dependence using e-cigarette adapted questionnaires is still controversial.

Objective: The aim of this study is to determine if the predictive property of the Fagerström Test for Cigarette Dependence (FTCD) to assess nicotine and cotinine concentration in traditional cigarettes is applicable to e-cigarettes, thus validating the biochemical aspects of the FTCD

Methods: The final sample included information on 128 and Exclusive cigarette smokers and 164 as Exclusive e-cig users (with nicotine). We analyzed saliva samples to assess nicotine and cotinine concentration, and passed a questionnaire reporting information on sex, age, BMI, and the FTCD to both type of smokers. Then we used Bayesian hierarchical modelling to calculate the geometric means of each group with 95% credible intervals , both adjusted and unadjusted. We also compared the nicotine and cotinine concentration between the three levels of the categorized total score of the FTCD.

Results: There strong evidence suggesting that nicotine levels are inversely related to the time to the first cigarette in the morning and the number of cigarettes per day in Exclusive cigarette smokers (Bayes Factors of 11.940 and 4.955). We also found evidence for this with cotinine (Bayes Factors of 65.328 and 5.427). For Exclusive e-cig users (with nicotine), we just found a moderate association between nicotine and time to first cigarette (Bayes Factors of 4.954).

Conclusions: While some of the items in the FTCD related with nicotine and cotinine levels for Exclusive cigarette smokers, this is not the case for Exclusive e-cig users (with nicotine).

Keywords:

INTRODUCTION

Cigarette smoking has long been associated with health risks due to its addictive nature and potential carcinogenic effects on humans (Organization y Cancer, 2004; Pan et al., 2019; Services, 2014). As such, researchers have developed various tests over time that are designed to measure dependence to traditional cigarettes (Etter et al., 2003; Fagerström, 1978). One such test is the Fagerström Test for Cigarette Dependence (FTCD), which was originally developed to measure nicotine dependence (Fagerström, 1978; Heatherton et al., 1991). Since then it was better proposed as a measure of dependence to traditional cigarettes (Fagerström, 2012). The test has been validated and it is a widely used tool among researchers in spite of its poor psychometric properties (Becoña y Vázquez, 1998; Etter, 2005; Heatherton et al., 1991; Sharma et al., 2021) and its limited potential as a predictor of nicotine and cotinine concentration (Etter et al., 1999).

However, with the introduction of e-cigarettes over recent years there has been debate about whether or not these tests can be applied to e-cigarette smokers as well as traditional ones (Rest et al., 2021; Sharma et al., 2021). To date, most studies centered on adapting existing questionnaires for tobacco dependence, mostly focusing on the psychometric qualities and not in the biochemical dependence (Foulds et al., 2015; Sharma et al., 2021). Furthermore, studies linking biomarkers such as nicotine or cotinine to e-cigarettes are of particular interest because these are substances commonly used to monitor the smoking epidemic in traditional cigarettes (Lidón-Moyano et al., 2017) and it has been shown that e-cigarettes can have cotinine levels equal to or higher than traditional cigarettes —or at least people can metabolise more nicotine from them— (Diamantopoulou et al., 2019).

Hence, the aim of this study is to determine if the predictive property of the FTCD to assess nicotine and cotinine concentration in traditional cigarettes is also applicable to e-cigarettes, thus validating the biochemical aspects of the FTND.

MATERIAL AND METHODS

Study design and sampling procedure

We combined the results of two different studies to perform a pooled analysis. One of them was a cohort study on tobacco smoking patterns "Determinants of Cotinine project-phase 3 (dCOT3 study)" which recruited 1,245 individuals at baseline (2004-2005) and maintained information on 736 at the follow-up (2013-2014) from a representative sample of the adult (≥16 years) population of Barcelona (Catalonia, Spain) (Lidón-Moyano et al., 2018). All participants answered a self-reported questionnaire to obtain information about tobacco consumption and sociodemographic variables. In addition, a sample of saliva (6 ml) was collected for the determination of nicotine and cotinine. The other was a cross-sectional study (2017-2018) which used the consumer panels technique to enroll 302 adult (≥18 years) users of e-cigarettes from the same city (Bunch et al., 2018). A questionnaire on e-cig use patterns was used and a saliva sample was also collected. There were 44 individuals whose saliva sample were not available, 11 individuals without available nicotine or cotinine information, and two e-cig users whose information were missing. Of all of the remaining individuals (n = 981), 171 (128 complete answers to the FTCD) declared themselves as exclusive cigarette smokers and 164 as exclusive e-cig users (with nicotine). Thus, the final merged sample retained data of 292 individuals. The design and methodology of both studies are detailed elsewhere (Bunch et al., 2018; Lidón-Moyano et al., 2018). The research and ethics committee of the Bellvitge University Hospital provided ethical approval for the studies protocol (PR118/11 and PR133/15, respectively), including the informed consent form. Both studies meet the code of the Declaration of Helsinki.

Study variables

Salivary nicotine and cotinine

We collected saliva samples (6 mL) and analyzed them employing a common protocol in order to determinate nicotine and cotinine (Pérez-Ortuño et al., 2015), both biomarkers of tobacco exposure (Benowitz, 1996; Benowitz et al., 2009). Frozen samples were analyzed employing LC/MS/MS after a single alkaline liquid-liquid extraction with dichloromethane/isopropanol (Pérez-Ortuño et al., 2015) by the Group of Integrative Pharmacology and Neuroscience of the Municipal Institute for Medical Research (IMIM-Hospital del Mar) in Barcelona. The limit of quantification of this method was 0.5 ng/mL for nicotine and 0.1 ng/mL for cotinine. Values under the limit of quantification were halved to avoid overestimation or underestimation bias.

Socio-demographic variables

Our study also included information on self-reported biological variables, namely gender, age, and body mass index (BMI). We divided the age of individuals into three groups (according to the quartiles of the sample) (a) 18 to 44 years, (b) 45 to 64 years, and (c) 65 to 89 years. Similarly, we divided individuals' BMI into a total of four groups (according to

WHO guidelines, although the range of underweight was expanded to increase the sample size): (a) between 10 and 20 kg/m^2, (b) between 21 and 25 kg/m^2, (c) between 26 and 30 kg/m^2 and (d) between 31 and 60 kg/m^2.

Smoking status and use of e-cigarettes

According to self-reported information, we classified the participants into the two following groups: a) dual users (participants who were both current cigarette smokers and users of e-cigarettes), b) exclusive cigarette smokers, c) e-cigarette exclusive users with nicotine, d) e-cigarette exclusive users without nicotine, and e) nonusers. Subjects were considered exclusive cigarette smokers if they declared smoking cigarettes daily or occasionally at the moment of the survey. If exclusive users of e-cigarettes declared that the e-liquid contained nicotine, regardless the concentration, the users were categorized as exclusive e-cig users (with nicotine). Dual users, e-cigarette exclusive users without nicotine, and nonusers were discarded from the study as the FTCD its designed for nicotine dependence.

Fagerström Test for Cigarette Dependence (FTCD)

FTCD was passed in the self-reported questionnaire and its six items were evaluated, yielding to a total score ranging from 0 to 10 (Fagerström, 1978, 2012; Heatherton et al., 1991). Two of said are multiple-choice items, each one with four possible options that scored from 0 to 3. The rest are binary items that scored from 0 to 1. The list of items with scores was as follows (as translated from Spanish):

1. How soon after you wake up do you smoke your first cigarette?
    a. Within 5 minutes (3)
    b. 6 to 30 minutes (2)
    c. 31 to 60 minutes (1)
    d. After 60 minutes (0)
2. Do you find it difficult to refrain from smoking in places where it is forbidden?
    a. Yes (1)
    b. No (0)
3. Which cigarette would you hate most to give up?
    a. The first one in the morning (1)
    b. Any other (0)
4. How many cigarettes per day do you smoke?
    a. 31 or more (3)
    b. 21 to 30 (2)
    c. 11 to 20 (1)
    d. 10 or less (0)
5. Do you smoke more frequently during the first hours after waking than during the rest of the day?
    a. Yes (1)
    b. No (0)
6. Do you smoke when you are so ill that you are in bed most of the day?

a. Yes (1)
b. No (0)

In the case of exclusive cigarette smokers, the fourth item was not passed as a categorical question, but numerical and later categorize as presented in the FTCD. On the other hand, the number of cigarettes smoked per day for exclusive e-cigarette users (with nicotine) was estimated based on its nicotine concentration. In addition to all of that, we categorized the total score in a new variable with three levels based on cigarette dependence (Fagerström, 2012; Fagerstrom et al., 1990): 1) Low dependence: A total score of 3 or less, 2) Medium dependence: between 4 and 6, 3) High dependence: More than 6.

Statistical analysis

We calculated Exclusive cigarette smokers and Exclusive e-cigarette users (with nicotine) absolutes and relatives' frequencies, both overall and for each level of every item in the FTCD. Besides, as salivary nicotine and cotinine distributions have been reported as skewed (Lidón-Moyano et al., 2017), we used Bayesian hierarchical modelling to calculate their geometric means (GM) with 95% credible intervals (95% CI), both unadjusted and adjusted by Sex, Age and BMI. In addition, we calculated the probabilities that the nicotine and cotinine concentration was 10%, 20%, or 30% higher than the reference level at each level for each question of every item in the FTCD. Furthermore, we calculate the Bayes factor (or BF by its acronym) of each model as additional thresholds to decide when to reject a null hypothesis. Finally, we compared the nicotine and cotinine concentration between the three levels of the categorized total score of the FTCD of Exclusive cigarette smokers and Exclusive e-cig users (with nicotine) through Bayesian ANOVAs, in which we also computed the BF. Data were analyzed using R-4.2.4. and WinBUGS-1.4.3.

RESULTS

A total of 471 adults constituted the final sample. Of these, 34.03% were females who, aging 19-44 years (55.479%), 45-64 years (47.603%), and >= 65 years (11.644%), 11.644% were underweight adults (BMI = 10-20 kg/m^2), 56.507% were within normal weight (BMI = 21-25 kg/m^2), 35.274% were overweight (BMI <= 26-30 kg/m^2), and, 13.356% were obese (BMI > 31 kg/m^2).

There was strong evidence (BF = 11.940 (Andraszewicz et al., 2015)) that for Exclusive cigarette smokers, having higher levels of Nicotine was inversely associated with the time it takes a person to smoke the first cigarette of the day (also "Time to the first cigarette of the day or TTF) (Table 1). There was also moderate evidence (BF = 4.955) that for Exclusive cigarette smokers, having higher levels of Nicotine was directly associated with the mean number of cigarettes smoked per day (CPD) (Table 1). This was the same for Exclusive e-cig users (with nicotine), as there was moderate evidence (BF = 4.954) that having higher levels of Nicotine was inversely associated with TTF, however, in the particular case of Exclusive e-cig users (with nicotine) there was anecdotal evidence (BF = 0.382) that the level

of Nicotine is not associated with the mean number of CPD. There was evidence that there was no association (BFs < 0.800) between the rest of the FTCD items and nicotine concentration, both in the case of Exclusive cigarette smokers and Exclusive e-cig users (with nicotine).

The above results were also observed for Cotinine (Table 2), existing very strong evidence (BF = 65.328) that there is an inverse association between Exclusive cigarette smokers having a higher concentration of Cotinine and TTF; As well as there was moderate evidence (BF = 5.427) that for Exclusive cigarette smokers, having higher levels of Cotinine was directly associated with the mean number of CPD, while there was moderate evidence in Exclusive e-cig users (with nicotine) pointing out that such relationship did not exist (BF = 0.254). There was no evidence (BF = 1.098) that an association exists between Exclusive e-cig users (with nicotine) having a higher concentration of Cotinine and TTF. When adjusting by Sex, Age, and BMI the GMs for Table 1 and 2 remained similar (Appendix 1).

Probabilities of Exclusive cigarette smokers and Exclusive e-cig users (with nicotine) who smoke within the first 30 minutes in the morning have at least 30% more Nicotine or Cotinine than those who smoke it after 60 minutes are 70% or more (Tables 1-2). Likewise, Probabilities of Exclusive cigarette smokers who smoke at least 11 cigarettes per day (on average) have at least 30% more Nicotine or Cotinine than those who smoke 10 or less also are 70% or more. There is anecdotal evidence supporting the hypothesis that there is no difference in nicotine for both types of smokers (BFs = 1.544 and 1.794, respectively) when comparing the categorized FTCD total score (Figure 1). In addition, our results show that there is strong evidence that an association exits between cotinine and the categorized FTCD total score for Exclusive cigarette smokers (BF = 20.097), but there is very strong evidence supporting that an association does not exist between cotinine and the categorized FTCD total score for Exclusive e-cig users (with nicotine) (BF = 0.386).

Table 1: Bayesian hierarchical model results for nicotine concentration, both overall and for each level of each item of the Fagerström test for nicotine dependence in Exclusive cigarette smokers and Exclusive e-cig users (with nicotine). n: absolute frequency, %: relative frequency as a percentage, GM: Geometric Mean, 95% CI: 95% credibility intervals, BF: Bayes Factor.

	Nicotine						
	Exclusive cigarette smokers						
	n (%)	**GM [95% CI]**	**exp(beta) [95% CI]**	**P (beta > 1.1)**	**P (beta > 1.2)**	**P (beta > 1.3)**	**BF**
Overall	128	83.548 [67.885; 101.552]	-	-	-	-	
How soon after you wake up do you smoke your first cigarette?							11.940
After 60 minutes	42 (32.812)	73.454 [52.124; 102.452]	-	-	-	-	
31 to 60 minutes	23 (17.969)	87.677 [33.625; 207.315]	1.194 [0.645; 2.02]	0.564	0.439	0.332	
6 to 30 minutes	47 (36.719)	134.796 [58.324; 291.634]	1.835 [1.120; 2.847]	0.979	0.952	0.912	
Within 5 minutes	16 (12.500)	214.771 [73.468; 529.456]	2.924 [1.409; 5.168]	0.997	0.994	0.987	
Do you find it difficult to refrain from smoking in places where it is forbidden?							0.498
No	108 (84.375)	99.487 [80.034; 123.600]	-	-	-	-	
Yes	20 (15.625)	144.925 [64.142; 300.472]	1.457 [0.801;2.431]	0.796	0.704	0.601	
Which cigarette would you hate most to give up?							0.727
Any other	71 (57.724)	94.491 [71.389; 122.200]	-	-	-	-	
The first one in the morning	52 (42.276)	125.438 [60.921; 243.731]	1.328 [0.853; 1.995]	0.788	0.646	0.495	
How many cigarettes per day do you smoke?							4.955
10 or less	38 (31.933)	68.576 [47.483; 94.605]	-	-	-	-	
11 to 20	47 (39.496)	120.391 [50.782; 257.002]	1.756 [1.069; 2.717]	0.967	0.923	0.864	
21 to 30	25 (21.008)	146.162 [56.173; 335.115]	2.131 [1.183; 3.542]	0.988	0.971	0.944	
31 or more	9 (7.563)	246.629 [71.665; 689.182]	3.596 [1.509; 7.285]	0.996	0.993	0.989	

Do you smoke more frequently during the first hours after waking than during the rest of the day?							
No	94 (74.603)	100.229 [78.315; 126.200]	-	-	-	-	0.400
Yes	32 (25.397)	129.548 [62.353; 251.337]	1.293 [0.796; 1.992]	0.726	0.585	0.434	
Do you smoke when you are so ill that you are in bed most of the day?							0.273
No	95 (76.000)	100.682 [79.319; 125.952]	-	-	-	-	
Yes	30 (24.000)	118.703 [56.016; 232.959]	1.179 [0.706; 1.850]	0.565	0.430	0.304	
			Exclusive e-cig users (with nicotine)				
Overall	164	358.843 [272.737; 457.752]	-	-	-	-	
How soon after you wake up do you smoke your first cigarette?							4.954
After 60 minutes	55 (33.537)	265.609 [169.142; 398.534]	-	-	-	-	
31 to 60 minutes	25 (15.244)	346.607 [91.117; 1046.173]	1.305 [0.539; 2.625]	0.598	0.513	0.424	
6 to 30 minutes	58 (35.366)	430.823 [139.280; 1137.705]	1.622 [0.823; 2.855]	0.850	0.776	0.697	
Within 5 minutes	26 (15.854)	681.639 [188.158; 2084.375]	2.566 [1.112; 5.23]	0.976	0.959	0.936	
Do you find it difficult to refrain from smoking in places where it is forbidden?							0.643
No	141 (85.976)	349.964 [259.147; 458.057]	-	-	-	-	
Yes	23 (14.024)	441.354 [143.189; 1133.234]	1.261 [0.553; 2.474]	0.557	0.472	0.390	
Which cigarette would you hate most to give up?							0.476
Any other	121 (73.780)	320.911 [235.447; 432.800]	-	-	-	-	
The first one in the morning	43 (26.220)	514.950 [201.216; 1172.087]	1.605 [0.855; 2.708]	0.862	0.793	0.715	

How many cigarettes per day do you smoke?								0.382
	10 or less	80 (49.383)	196.582 [137.542; 272.252]	-	-	-	-	
	11 to 20	46 (28.395)	582.043 [221.914; 1348.882]	2.961 [1.613; 4.955]	1.000	0.999	0.997	
	21 to 30	21 (12.963)	833.218 [248.880; 2347.326]	4.239 [1.809; 8.622]	1.000	0.999	0.998	
	31 or more	15 (9.259)	1042.419 [282.882; 3072.437]	5.303 [2.057; 11.285]	0.999	0.999	0.997	
Do you smoke more frequently during the first hours after waking than during the rest of the day?								0.424
	No	133 (81.098)	314.662 [234.900; 411.252]	-	-	-	-	
	Yes	31 (18.902)	678.701 [249.229; 1655.703]	2.157 [1.061; 4.026]	0.968	0.943	0.912	
Do you smoke when you are so ill that you are in bed most of the day?								0.798
	No	121 (73.780)	374.567 [273.990; 500.700]	-	-	-	-	
	Yes	43 (26.220)	336.671 [130.145; 791.870]	0.899 [0.475; 1.582]	0.200	0.133	0.085	

Table 2: Results of the Bayesian hierarchical model for cotinine concentration, both overall and for each level of each item of the Fagerström test for nicotine dependence in Exclusive cigarette smokers and Exclusive e-cig users (with nicotine) . n: absolute frequency, %: relative frequency as a percentage, GM: Geometric Mean, 95% CI: 95% credibility intervals, BF: Bayes Factor.

	Cotinine						
	Exclusive cigarette smokers						
	n (%)	**GM [95% CI]**	**exp(beta) [95% CI]**	**P (beta > 1.1)**	**P (beta > 1.2)**	**P (beta > 1.3)**	**BF**
Overall	128	193.650 [156.147; 238.652]	-	-	-	-	
How soon after you wake up do you smoke your first cigarette?							65.328
After 60 minutes	42 (32.812)	206.927 [169.247; 250.505]	-	-	-	-	
31 to 60 minutes	23 (17.969)	216.962 [126.155; 364.986]	1.048 [0.745; 1.457]	0.359	0.181	0.086	
6 to 30 minutes	47 (36.719)	315.134 [194.207; 497.528]	1.523 [1.147; 1.986]	0.987	0.947	0.858	
Within 5 minutes	16 (12.500)	420.170 [233.811; 722.099]	2.031 [1.381; 2.883]	1.000	0.997	0.989	
Do you find it difficult to refrain from smoking in places where it is forbidden?							0.542
No	108 (84.375)	254.246 [221.547; 289.205]	-	-	-	-	
Yes	20 (15.625)	323.979 [202.967; 504.525]	1.274 [0.916; 1.745]	0.779	0.605	0.414	
Which cigarette would you hate most to give up?							0.395
Any other	71 (57.724)	246.134 [209.595; 287.915]	-	-	-	-	
The first one in the morning	52 (42.276)	290.857 [191.704; 430.872]	1.182 [0.915; 1.497]	0.694	0.427	0.206	
How many cigarettes per day do you smoke?							5.427
10 or less	38 (31.933)	204.101 [163.900; 249.552]	-	-	-	-	
11 to 20	47 (39.496)	291.929 [175.770; 467.911]	1.430 [1.072; 1.875]	0.961	0.881	0.730	
21 to 30	25 (21.008)	297.973 [169.550; 503.846]	1.460 [1.034; 2.019]	0.947	0.864	0.732	
31 or more	9 (7.563)	434.197 [210.525; 824.521]	2.127 [1.284; 3.304]	0.994	0.985	0.972	

Do you smoke more frequently during the first hours after waking than during the rest of the day?							0.302
No	94 (74.603)	257.610 [224.600; 294.805]	-	-	-	-	
Yes	32 (25.397)	293.387 [194.545; 442.067]	1.139 [0.866; 1.500]	0.554	0.329	0.167	
Do you smoke when you are so ill that you are in bed most of the day?							0.286
No	95 (76.000)	255.434 [219.900; 295.200]	-	-	-	-	
Yes	30 (24.000)	289.239 [185.378; 437.501]	1.132 [0.843; 1.482]	0.548	0.325	0.152	

Exclusive e-cig users (with nicotine)

Overall	164	176.917 [143.247; 215.952]	-	-	-	-	
How soon after you wake up do you smoke your first cigarette?							1.098
After 60 minutes	55 (33.537)	120.199 [82.280; 170.805]	-	-	-	-	
31 to 60 minutes	25 (15.244)	204.235 [70.863; 499.442]	1.699 [0.861; 2.924]	0.879	0.823	0.754	
6 to 30 minutes	58 (35.366)	218.306 [87.749; 488.942]	1.816 [1.066; 2.863]	0.970	0.937	0.886	
Within 5 minutes	26 (15.854)	281.695 [99.013; 714.300]	2.344 [1.203; 4.182]	0.989	0.975	0.956	
Do you find it difficult to refrain from smoking in places where it is forbidden?							0.346
No	141 (85.976)	170.590 [134.447; 211.100]	-	-	-	-	
Yes	23 (14.024)	237.215 [100.715; 506.872]	1.391 [0.749; 2.401]	0.726	0.623	0.520	
Which cigarette would you hate most to give up?							0.299
Any other	121 (73.780)	167.548 [129.447; 213.052]	-	-	-	-	
The first one in the morning	43 (26.220)	219.705 [102.295; 439.213]	1.311 [0.790; 2.062]	0.719	0.593	0.467	
How many cigarettes per day do you smoke?							0.254

10 or less	80 (49.383)	117.798 [88.644; 156.357]	-	-	-	-	
11 to 20	46 (28.395)	126.162 [34.305; 549.751]	1.331 [0.387; 3.516]	0.522	0.461	0.406	
21 to 30	21 (12.963)	434.439 [31.469; 2289.379]	3.688 [0.355; 14.642]	0.792	0.763	0.734	
31 or more	15 (9.259)	126.162 [40.599; 339.764]	1.071 [0.458; 2.173]	0.386	0.302	0.238	
Do you smoke more frequently during the first hours after waking than during the rest of the day?							0.505
No	133 (81.098)	165.978 [130.600; 209.652]	-	-	-	-	
Yes	31 (18.902)	249.576 [109.755; 513.979]	1.504 [0.840; 2.452]	0.846	0.756	0.663	
Do you smoke when you are so ill that you are in bed most of the day?							0.211
No	121 (73.780)	182.812 [142.647; 231.352]	-	-	-	-	
Yes	43 (26.220)	168.129 [78.940; 330.956]	0.920 [0.553; 1.431]	0.199	0.119	0.064	

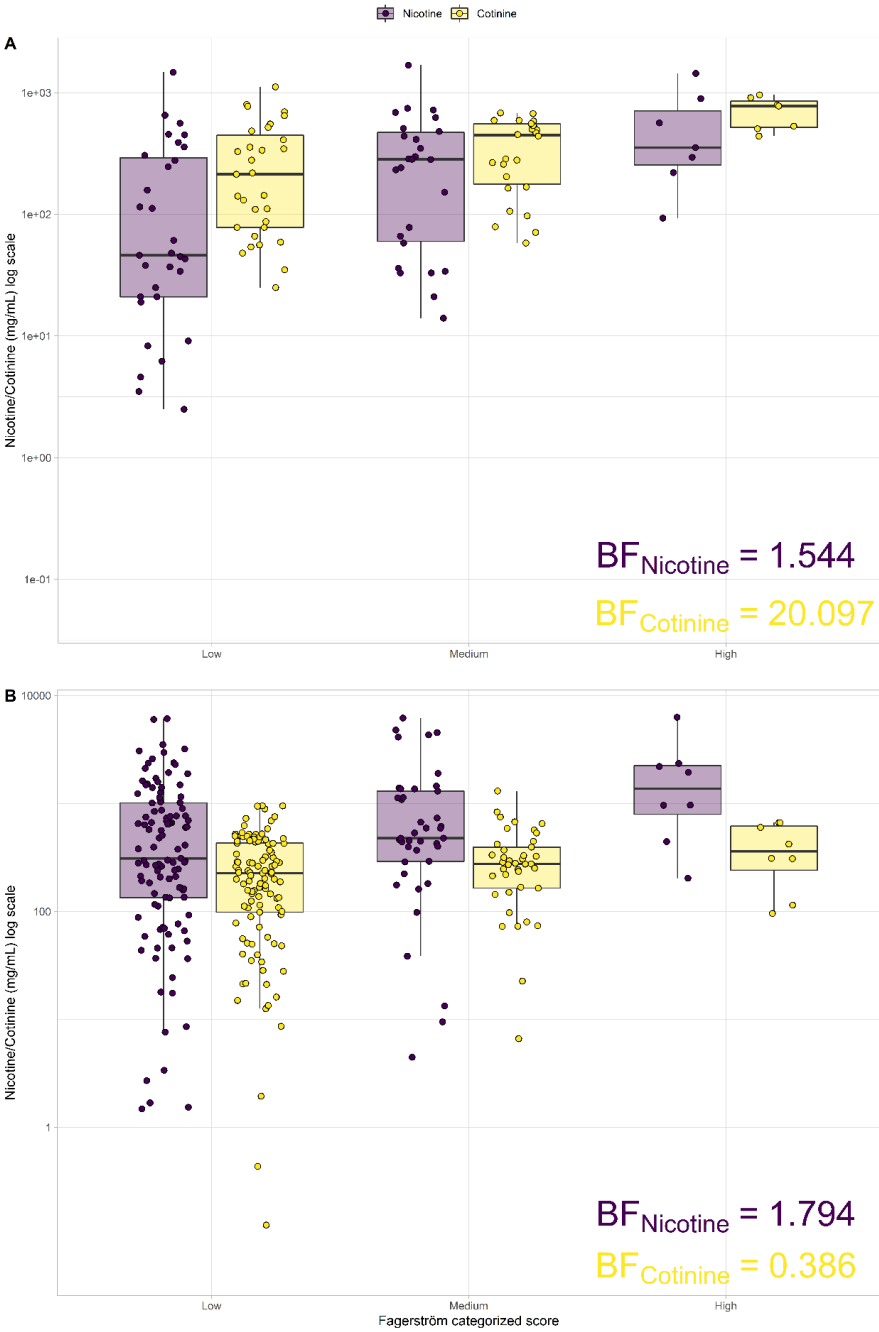

Figure 1: Boxplot of log nicotine and cotinine in saliva (ng/mL) according to the categorized total score of the Fagerström test of A) Exclusive cigarette smokers, B) Exclusive e-cig users (with nicotine). BF: Bayes Factor.

DISCUSSION

We have observed a negative association between the concentration of nicotine or cotinine metabolites in both type of smokers and the TTF. Particularly, people who smoke within the first 31 minutes after waking up are more likely to have higher levels of these metabolites than those who do not. We also found a positive association between the concentration of nicotine and cotinine and the CPD smoked by an Exclusive cigarette smoker, but this is not the case for Exclusive e-cig users (with nicotine).

Broadly, our results are in line with the original study proposing the FTCD (Heatherton et al., 1991), where the authors outlined the two most important questions on the FTCD ("How soon after you wake up do you smoke your first cigarette?" and "How many cigarettes per day do you smoke?"). That study concluded that although both questions are the only contributors to the prediction at biochemical levels, the other items may provide relevant information on smoking cessation, which perfectly fits with our results for Exclusive cigarette smokers but not for Exclusive e-cig users (with nicotine) as the number of CPD in our study was not related to the concentration of nicotine or cotinine, neither did we found association between cotinine concentration and CPD in Exclusive e-cig users (with nicotine). A possible reason for this is because the CPD in our sample was estimated to equate the consumption of traditional cigarettes to that of e-cigarettes. This is a comparison in which there is no gold-standard conversion protocol, and in recent years several alternative methods have been proposed for this conversion. An example of this is the e-cigarette Fagerström Test of Cigarette Dependence (Rahman et al., 2020), where the authors modified the original FTCD question by the query "how many times a day do you vape?", where they considered adopted one-time of vape session consists of an average puff up to 15. The same puff approximation was used in the Penn State Electronic Cigarette (Foulds et al., 2015), although they also assumed one "time" of the smoking lasts around 10 minutes. Both approaches may suffer from the same bias as neither of them provide a justification for this conversion and the nicotine content in e-cigarettes is very heterogeneous (Rahman et al., 2020; Schroeder y Hoffman, 2014), thus the nicotine intake per vape session may vary greatly and therefore potentially bias the results. Since our proposal takes into account the amount of nicotine in the e-cigarette, it should be a better equivalence when looking for scale relationships with metabolites. As we have found no association between the "number of e-cigarettes" and nicotine or cotinine concentrations, we believe that neither our adaptation nor those proposed above to approximate e-cigarettes and traditional cigarettes in this question are optimal and further research on the topic is needed —although they may be impossible to compare—.

Interestingly, in Exclusive cigarette smokers the data suggest that it is 65 times more likely to find differences in cotinine based on the TTF than mot finding any differences, yet when looking at nicotine this hypothesis is only 11 times more likely. Furthermore, this does not occur with the CPD, being approximately 5 times more likely to find differences in both metabolites. One possible explanation is that since the half-life of nicotine (1-2 hours) is much shorter than that of cotinine (13-19 hours) (Benowitz y Jacob, 1994), it is much easier for us to find evidence to support that there are differences in these questions; Moreover the fact that the BF of CPD is similar for nicotine and cotinine in Exclusive cigarette smokers

and that TTF is much higher for cotinine is inferior to 1 may be hinting that although both are measuring the Heaviness of smoking in Exclusive cigarette smokers (Heatherton et al., 1989), CPD is much more influenced by non-biochemical causes than TTF. What is more, considering that in Exclusive e-cig users (with nicotine) we just found a weak association between the concentration of nicotine and TTF, TTF and CPD may not be as good measures of Heaviness of Smoking for e-cigarettes as they are for traditional cigarettes. This fact is reaffirmed if we consider that the probability that people with a CPD greater than 30 have a cotinine concentration at least 10% higher than those who smoke 10 or less is approximately 40%, which is reduced to 24% if we look for differences of at least 30%. To the best of our knowledge this is the first study assessing a biochemical validation of the FTCD in e-cigarettes employing both nicotine and cotinine and further research is needed, however the previous statement is reinforced if we consider the results of the analyses that take into account the categorized FTCD score. These results indicate that the probability of a relationship between FTCD score and nicotine concentration is very low, whereas the probability of such a relationship with cotinine is very high in the case of Exclusive cigarette smokers. In addition, we have found considerable evidence that there is no relationship between cotinine and the categorized FTCD score in Exclusive e-cig users (with nicotine).

This study has several limitations. First, our results may be biased from the use of self-reporting data, which may lead to underestimation or overestimation of actual TTF and CPD. Withal, there is sufficient evidence that self-reported data on smoking performs well when working with trained interviewers. Also, our data came from two different studies (a longitudinal one whose population is aged and another one which used a non-probabilistic sampling technique) the pooled sample is not representative of the general population. In addition, the temporal difference between both studies (2014 and 2018) may affect our results, since the population trend in traditional cigarettes is tending to a decrease in smoking and we have not controlled for pollution to environmental smoke. Besides, perhaps the way in which we have approximated the CPD for Exclusive e-cig users (with nicotine) is not correct. However, the way in which other studies have approximated a CPD equivalent for e-cigarettes has been discussed previously, and we consider the conversion we have employed to be less biased. In addition, there has been no follow-up on whether the questionnaire can be used to monitor smoking cessation, so we did not assess the psychometric properties of the questionnaire. Nonetheless, future studies should consider these aspects.

In conclusion, we have found evidence to support that FTCD is not as optimal in associating nicotine or cotinine concentrations in users of electronic cigarettes with nicotine as it is for traditional cigarettes. If this is confirmed in further studies, the need for the creation of an alternative questionnaire to capture both dependences to biochemical substances and smoking habits in users of electronic cigarettes is imperative. This would be useful both for monitoring the degree of addiction of the population and for evaluating cessation treatments for this type of product.

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APPENDIX

Appendix 1: Results of the Bayesian hierarchical model for nicotine and cotinine concentration adjusted by Sex, Age and BMI, both overall and for each level of each item of the Fagerström test for nicotine dependence in Exclusive cigarette smokers and Exclusive e-cig users (with nicotine) . GM: Geometric Mean, 95% CI: 95% credibility intervals.

	Exclusive cigarette smokers		**Exclusive e-cig users (with nicotine)**	
	Nicotine GM [95% CI]	**Cotinine GM [95% CI]**	**Nicotine GM [95% CI]**	**Cotinine GM [95% CI]**
Overall (n = 292)	91.567 [38.619;181.7]	194.565 [155.947;236.952]	407.064 [130.1;976.259]	287.775 [121.795;570.335]
How soon after you wake up do you smoke your first cigarette?				
After 60 minutes	82.349 [29.839;182.999]	173.594 [99.198;285.382]	275.447 [81.853;695.997]	192.994 [74.018;419.167]
31 to 60 minutes	102.349 [19.872;397.04]	190.418 [74.504;432.218]	309.94 [40.979;1546.574]	298.066 [59.65;1133.649]
6 to 30 minutes	156.343 [34.401;541.958]	274.037 [116.359;593.187]	370.331 [57.194;1650.379]	304.345 [70.066;1048.138]
Within 5 minutes	239.186 [41.878;995.719]	351.111 [132.43;831.617]	750.159 [98.422;3828.452]	436.893 [83.489;1678.95]
Do you find it difficult to refrain from smoking in places where it is forbidden?				
No	118.923 [41.725;269.557]	217.187 [120.8;365.325]	379.61 [113.037;926.659]	269.607 [106.447;566.739]
Yes	181.961 [34.726;694.407]	278.789 [108.29;642.99]	504.686 [64.407;2384.78]	352.431 [73.057;1283.721]
Which cigarette would you hate most to give up?				
Any other	143.708 [53.026;314.852]	238.713 [133.737;391.757]	356.267 [115.542;838.931]	272.221 [113.69;544.41]
The first one in the morning	194.691 [45.783;647.578]	275.982 [119.485;581.015]	589.103 [106.61;2371.822]	376.966 [96.265;1180.022]
How many cigarettes per day do you smoke?				
10 or less	33.223 [12.783;74.701]	152.921 [85.555;258.134]	432.269 [83.43;1309.574]	272.883 [130.747;494.252]
11 to 20	66.039 [15.78;228.999]	228.93 [95.475;506.201]	812.666 [7.425;12144.989]	365.663 [-97.014;384.528]
21 to 30	72.594 [16.106;258.852]	225.01 [89.788;514.855]	916.41 [11.096;412754.152]	294.168 [52.953;1758.549]
31 or more	163.536 [26.35;738.116]	367.529 [122.208;964.55]	1461.069 [41.632;15506.666]	361.451 [50.338;7255.619]
Do you smoke more frequently during the first hours after waking than during the rest of the day?				
No	133.218 [49.8;283.877]	232.545 [131.047;386.425]	288.764 [91.451;720.952]	252.093 [100.016;510.485]
Yes	170.905 [38.307;596.142]	268.747 [112.535;590.863]	634.843 [102.882;2792.875]	369.542 [86.159;1195.044]
Do you smoke when you are so ill that you are in bed most of the day?				
No	133.933 [47.003;293.472]	262.475 [146.4;434.21]	410.068 [123.942;992.492]	300.937 [118.147;620.711]
Yes	165.622 [34.849;577.26]	307.136 [127.115;664.341]	423.102 [68.565;1739.888]	297.56 [72.622;964.911]

Part 6

Discussion

Validation of biomarkers is a crucial step in translating basic research findings into effective public health interventions. In epidemiology, biomarkers are utilized to diagnose, monitor, and predict the progression of diseases, as well as to evaluate the effectiveness and safety of interventions. However, not all biomarkers are suitable for epidemiological use, and the validation process is both complex and time-consuming. This doctoral thesis presents the results of a comprehensive validation study of a panel of potential biomarkers for tobacco use and exposure for measuring the success of anti-smoking legislation. Through a series of analytical and epidemiological evaluations, we demonstrate the reliability, accuracy, and reproducibility of these biomarkers, providing concrete evidence for their usefulness in monitoring the state and changes of the tobacco epidemic. The following discussion will explore the key findings and implications of this thesis.

Chapter 6.1 Changes in tobacco-specific biomarkers

The results of Article I in this doctoral thesis[45] demonstrate a marked decline in the salivary cotinine concentration cut-off point utilized to differentiate between smokers and non-smokers in Barcelona, following the implementation of Law 42/2010. This decline was observed across age and gender groups and was at least 80%. Furthermore, we discovered significant reductions in salivary cotinine concentration cut-off points when contrasting non-smokers with daily smokers (87% reduction) and non-smokers with occasional smokers (94% reduction).

Before the Law 28/2005 it was estimated that the salivary cotinine cut-off for the Spanish population was 20 ng/mL[46]. However, a study conducted in 2005 found that the salivary cotinine cut-off for this cohort was lower, at 9.2 ng/mL. It is important to note that this estimation was made prior to the implementation of Law 42/2010[46]. Article I shows that the general cotinine cut-off in the population of Barcelona has decreased from 12.7 to 5.6 ng/mL after the enforcement of the latest anti-smoking legislation. All the optimal cut-off point post-2010 had higher sensitivity, specificity, and AUC (all were over 90%) values than pre-2005 cut-off points.

This serves as evidence of the efficacy of the implementation of the anti-smoking legislation in reducing passive exposure to tobacco smoke in Spain[47, 48]. Additionally, we observed an increase (from 250 to 650 ng/mL) in the mean salivary cotinine concentration of smokers, as noted in a previous study using data from this same cohort[49]. Despite this improvement in public health, the cut-off point for adult non-smokers in Barcelona is still higher than other populations in advanced stages of the tobacco epidemic (i.e., studies conducted in the past on the adult population in the United States of America have reported cut-off points to be around 3-4 ng/mL[29, 50, 51]).

Optimal cut-off points varied between different types of smokers and non-smokers. Non-smokers had a lower cut-off point when compared to occasional smokers and daily smokers (which was to be expected). Males also had a higher cut-off point than females when comparing non-smokers and smokers. However, females had a higher cut-off point than males when comparing non-smokers versus daily smokers and versus occasional smokers. This could be attributed to the rise in smoking rates and cigarette consumption among women aged 40 to 64 years in the last two decades, as they have become more susceptible to the tobacco epidemic[52, 53]. Consequently, this has led to an increase in their cotinine levels as reported

previously[49].

To sum up, after the implementation of Spanish smoke-free legislation, there was a significant decrease in the salivary cotinine cut-off points used to differentiate between smokers and non-smokers, as well as between non-smokers and daily or occasional smokers. These decreases are likely due to the reduced exposure to SHS and the increased awareness of the harmful effects of smoking[48]. However, it is important to note that cotinine is just one of several tobacco-specific biomarkers, and that other tobacco-specific biomarkers, such as TSNAs, have not been thoroughly studied in the context of anti-smoking policies. Therefore, in Article II, we examine the potential impact of Spain's anti-smoking legislation on TSNAs levels, providing a more comprehensive understanding of the effects of anti-smoking policies on tobacco exposure. These study findings suggest that as previously seen in salivary cotinine, smoking cessation can result in a significant reduction in salivary TSNAs concentration, while continued smoking can increase it.

To our knowledge, our study was the first to describe the variation of TSNAs before and after the implementation of tobacco control legislation in Spain. We found that the salivary concentration of TSNAs in smokers decreased significantly at baseline. Specifically, there was

a decrease of -90.5% [-95.0; -82.2], -48.7% [-60.1; -34.0], and -86.2% [-90.1; -80.9] for NNN, NNK, and NNAL, respectively. However, continuing smokers showed a substantial increase in the concentration of NNN by 149.8% [36.8; 356.1]. Conversely, there was no significant change observed in the TSNAs concentration of continuing non-smokers.

Our findings are in line with our prior studies using salivary cotinine in this same cohort[45, 49]. Additionally, other studies examining various cohorts of continuing non-smokers in Ireland[54], Scotland[55], and the US[29] have also reported comparable reductions in cotinine concentration following the implementation of tobacco-related legislation, corroborating our study's outcomes. However, it is crucial to approach the findings of our study on salivary TSNAs with caution, as more extensive research is required to validate them. Nonetheless, the significant parallels we observed between salivary cotinine and TSNAs among smokers at baseline, continuing non-smokers, and continuing smokers cannot be overlooked. These similarities enable us to extrapolate cotinine's observed patterns to TSNAs. TSNAs are directly correlated with DNA damage and are considered a superior biomarker of tobacco-related health hazards[33, 56]. Therefore, using TSNAs in examining changes in their concentrations can aid in evaluating the impact of

tobacco control legislation and posterior extensions.

There are several possible explanations for the significant increase in salivary NNN levels among Continuing smokers (which should be further contemplated in future studies), and one of them is that some smokers may have switched to cigarette brands with higher levels of TSNAs[57]. Differences in cultivation and curing practices used by various cigarette brands could account for this variation. Such a shift in smoking behavior could lead to increased exposure to TSNAs and other harmful chemicals, including NNN. Furthermore, other unmeasured factors may be contributing to the observed increase in NNN levels among Continuing smokers. Changes in the composition or processing of tobacco products over time could also contribute to the observed increase in NNN levels. For example, changes in the curing process of tobacco leaves or the addition of new flavorings or additives could affect the levels of TSNAs in cigarette smoke. Additionally, some smokers may have increased their smoking or puff frequency over time, which could lead to greater exposure to various tobacco biomarkers, including NNN[58, 59].

It is also possible that the observed increase in salivary cotinine and NNN levels among Continuing smokers is attributed to their high tobacco dependence, which

is consistent with the findings by the different FTCD categories during follow-up. TSNAs can serve as useful biomarkers of tobacco use[33], and they are also indicative of tobacco dependence[60]. The hardening hypothesis, which is controversial, suggests that smokers can be categorized into groups based on their level of dependence, with highly dependent smokers finding it more difficult to quit than those with lower levels of dependence[61]. This hypothesis fits with the increases we observed both in salivary cotinine and TSNAs. However, this hypothesis applies to the population as a whole[62, 63], and we cannot determine how it applies to smokers outside of our sample or new smokers who are not yet part of our study. Of course, there is a possibility that the observed rise in NNN levels among Continuing smokers may be due to chance rather than a genuine trend. Nonetheless, additional research is necessary to investigate all these possibilities and identify the underlying reasons for this phenomenon.

To sum up, after the implementation of Spanish smoke-free legislation, there was a significant decrease in both salivary tobacco-specific biomarkers (cotinine and TSNAs) in those who quit smoking and in non-smokers. This reduction prompted our investigation into whether nicotine metabolism differed among users of different tobacco products. Thus, the third article (Article III) of this thesis explores this topic[64]. We found that users of e-cigs, with or without nicotine, as well as nonusers of any product in our sample, were moderate metabolizers. In contrast, dual users were found to be fast metabolizers, exhibiting a higher NMR. This finding was unexpected since NMR is typically lower in consumers of tobacco products[65]. It is possible that the differences noted among dual users and e-cig users could be due to variations in tobacco consumption topography[66, 67]. Conversely, with regards to RNM, the findings indicate that e-cigs users without nicotine are slow metabolizers, nonusers are moderate metabolizers, while smokers, e-cigarette users with nicotine, and dual users are fast metabolizers. As fast metabolizers have a higher nicotine dependence[68], our results provide support for the hypothesis that faster metabolizers consume a larger

nicotine dose to alleviate withdrawal symptoms.

We also found significant differences in NMR according to sex, with females being fast metabolizers and males being moderate metabolizers. However, no significant differences were found when comparing males and females by RNM. This is in line with previous studies which suggested that females are more likely to have faster nicotine metabolism and higher dependence on nicotine products due to the effect of sex hormones on CYP23A[36, 69].

Regarding BMI we observed a negative association between RNM and BMI, while no association was found between NMR and BMI, although overweight and underweight individuals were categorized as fast metabolizers based on quartile analysis. Previous studies with similar sample sizes reported that BMI is negatively associated with NMR[70–72]. Our study also suggests that accumulated exposure to nicotine and natural metabolic changes associated with age may enhance nicotine metabolization rates. We observed a negative association between RNM and age, with older individuals being faster metabolizers than younger ones. NMR showed a positive association with age, but only between participants under 44 years (moderate metabolizers) and participants 65-89 years (fast metabolizers). These findings align with previous

studies, which reported similar trends concerning age and rates of nicotine metabolism[36, 69].

The differences between the two ratios may be explained by the fact that nicotine metabolization, as indicated by NMR, is not dependent on the number of cigarettes smoked[37]. Thus, NMR provides an unbiased measure of nicotine metabolism. Although both ratios could be reported together to estimate smoking quantities, using them in combination allows for detecting differences between individuals.

Chapter 6.3 Cigarette dependence and nicotine

With the tobacco market being inundated by new products, there is a growing need to develop new tools for validating their impact on the population. In Article IV, we sought to address this by validating the use of the FTCD scale for measuring the impact of e-cig on nicotine and cotinine concentration.

Our study revealed a significant negative association between the concentration of nicotine or cotinine metabolites and the time-to-first cigarette in both types of smokers (Exclusive cigarette smokers and Exclusive e-cig users (with nicotine)). Specifically, individuals who smoke within the first 31 minutes after waking up are more likely to have higher levels of these metabolites compared to those who do not. Furthermore, we observed a positive correlation between the concentration of nicotine and cotinine and the number of cigarettes smoked per day among exclusive cigarette smokers. However, this association was not observed in exclusive e-cigarette users who used nicotine-containing e-cigarettes.

Our findings are largely consistent with the original study by Heatherton et al.[24] proposing the FTCD, which highlighted the two most critical questions ("How soon after you wake up do you smoke your first cigarette?"

and "How many cigarettes per day do you smoke?") for predicting nicotine dependence at the biochemical level. However, our results show that while the number of cigarettes smoked per day is an important predictor of nicotine and cotinine concentrations in Exclusive cigarette smokers, this is not the case for Exclusive e-cig users (with nicotine). In our study, we found no significant association between the concentration of nicotine or cotinine and the number of CPD in Exclusive e-cig users, suggesting that the FTCD may not be as useful in predicting nicotine dependence in this group. Nonetheless, other items in the FTCD may still provide valuable information on smoking cessation, as observed in our results for Exclusive cigarette smokers.

Part 7

Limitations

Our studies have several limitations that should be mentioned.

Firstly, although the baseline of the dCOT3 study sample was representative of the city of Barcelona, working with cohort data overestimates the representation of the elderly population. In addition, in the follow-up of this sample there is female underrepresentation, and as females are faster metabolizers than males due to the role of sex hormones, our results may present an underestimation bias.

Secondly, in the e-cig Study the sample was not representative of the city of Barcelona, as we used a non-probabilistic sampling technique. This may limit the generalizability of our findings.

Thirdly, as baseline data of the dCOT3 Study and the sample of the e-cig Study were taken in different years, the generalizability of our findings is also compromised as differences between different tobacco products could be due to changes in the general population.

Fourthly, all of our analysis relied on self-reported data, which may be subjected to information bias and affect the validity of the results. However, the use of different biomarkers should minimize this bias.

Fifthly, we did not analyze nicotine concentration in

e-cigs to compare with the self-reported data. Furthermore, the categorization of different covariates in the sample according to quartiles has the downside that the selection of the sample influences that classification.

Sixthly, the use of metabolite ratios as a measure of nicotine metabolism has some significant concerns. The NMR may not be reliable when calculated using values below the limit of quantification, and the RNM is highly dependent on time since last use which is why it is not commonly used as a measure of nicotine metabolism rate.

Seventhly, our salivary-based TSNA analysis has not been validated against the more commonly used urinary TSNA analysis.

Eightly, our study may have been underpowered to detect small changes in tobacco-specific biomarkers levels.

Lastly, there was a lack of stratification by SHS among our data sources, which may limit the control of potential tobacco exposure.

Part 8

Conclusions

Chapter 8.1 Conclusión

- La introducción en España de la Ley 28/2005 y la Ley 42/2010 ha llevado a un aumento en los biomarcadores relacionados con el tabaco entre los fumadores, como indican los niveles de cotinina en saliva y los niveles de TSNAs salivares. Sin embargo, este aumento solo fue significativo para la cotinina y NNN.
- La introducción en España de la Ley 28/2005 y la Ley 42/2010 ha llevado a una disminución en los biomarcadores relacionados con el tabaco entre los no fumadores, como indican los niveles de cotinina en saliva y los niveles de TSNAs salivares.
- Tanto la tasa de metabolismo de la nicotina como la relación de metabolitos de nicotina pueden servir como biomarcadores confiables de la dependencia de la nicotina cuando se informan juntos.
- Los fumadores y los usuarios de e-cig con nicotina después de las legislaciones sin humo en España (Ley 28/2005 y Ley 42/2010) tienen una tasa de metabolismo de nicotina más alta que los no fumadores.
- Los fumadores y los usuarios de e-cig con nicotina después de las legislaciones sin humo en España (Ley 28/2005 y Ley 42/2010) tienen la misma relación de metabolitos de nicotina que los no fumadores.

- El FTCD es un predictor confiable de la concentración de nicotina y cotinina en los e-cig, similar a su capacidad predictiva en los cigarrillos tradicionales.

Chapter 8.2 Conclusion

- The enforcement of Law 28/2005 and Law 42/2010 have not led to an overall increase in tobacco-related biomarkers among smokers. However, there was a significant increase in cotinine and NNN.
- The enforcement of Law 28/2005 and Law 42/2010 have led to a decrease in tobacco-related biomarkers among non-smokers, as indicated by both saliva cotinine levels and salivary TSNAs levels.
- Both the Nicotine Metabolite Ratio and the Rate of Nicotine Metabolism may serve as reliable biomarkers of nicotine dependence when reported together.
- Smokers and e-cig with nicotine users after the smoke-free legislations (Law 28/2005 and Law 42/2010) have a higher Rate of nicotine metabolism than non-smokers.
- Smokers and e-cig with nicotine users after the smoke-free legislation (Law 28/2005 and Law 42/2010) have the same Nicotine metabolite ratio than non-smokers.
- The FTCD is a reliable predictor of nicotine and cotinine concentration in e-cig, similar to its predictive ability in traditional cigarettes.

Part 9

Public health implications

- The evaluation of the impact of current and future anti-tobacco laws in Spain and other countries should move beyond measuring tobacco consumption prevalence alone. It is important to consider a comprehensive set of biomarkers that can provide a holistic view of the population's health status, enabling a more accurate assessment of the effectiveness of these laws.
- Future initiatives to promote smoking cessation should recognize that the metabolism of various substances found in both traditional and new tobacco products can differ based on factors such as gender and age. Therefore, tailored programs should be developed to address the specific needs and challenges faced by different demographic groups, maximizing the effectiveness of cessation efforts.
- There is a pressing need for extensive research on specific biomarkers associated with the use of new tobacco products. As the tobacco industry continues to introduce novel products, it is crucial to understand their unique health risks and develop reliable biomarkers that can help monitor and assess the impact of these products on public health.
- It is essential to maintain a strong commitment to implementing comprehensive tobacco control measures, with a particular emphasis on emerging tobacco products. While addressing traditional tobacco use remains

important, it is equally critical to stay vigilant and regulate newer tobacco products effectively, as they may present distinct health risks and challenges.

- Continued monitoring and surveillance of tobacco biomarkers, use patterns, and associated health outcomes are crucial. By regularly collecting and analyzing data on tobacco consumption, prevalence, different biomarkers, and related diseases, public health authorities can identify emerging trends, evaluate the effectiveness of interventions, and adjust strategies accordingly, ultimately leading to better health outcomes for communities.

Part 10

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Part 11

Annexes

Chapter 11.1 Annex I. Acronyms

SHS	Second-hand smoke
WHO	World Health Organization
WHA	World Health Assembly
FCTC	Framework Convention on Tobacco Contro
TSNA	Tobacco-Specific Nitrosamine
NNN	N-Nitrosonornicotine
NNK	4 - (methylnitrosamino) - 1 - (3 - pyridyl) - 1 - butanone
NNAL	4 - (methylnitrosamino) - 1 - (3 - pyridyl) - 1 - butanol
e-cig	Electronic cigarette
HNB	Heat-Not-Burn Product
ENDS	Electronic Nicotine Delivery System
ENNDS	Electronic Non-Nicotine Delivery System
3-HC	trans - 3' - hydroxycotinine
GC-MS	Chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry

NRT	Nicotine replacement therapy
BMI	Body Mass Index
VOC	Volatile Organic Compound
RNM	Rate of Nicotine Metabolism
NMR	Nicotine Metabolite Ratio
CYP2A6	Enzyme cytochrome P450 2A6
CAPI	Computer-Assisted Personal Interview
FTCD	Fagerström Test for Cigarette Dependence

Changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers before and after Spanish smoke-free legislation

Cover letter to the editor of Cancer Epidemiology

Dear Editor,

Please find enclosed our manuscript titled "Changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers before and after Spanish smoke-free legislation".

We believe that in this specific moment, in which the possible implementation of more restrictive tobacco regulations in the European Union is being discussed within the scientific community, the conclusions arising from this manuscript may add more evidence to guide this decision.

All of the authors have read and approved the paper and I confirm it has not been published previously nor is it being considered by any other peer-reviewed journal.

If you deem it necessary, we will be pleased to provide more information on our data and methods.

Thank you for your attention

Adrián González-Marrón
E-mail: agonzalezm@uic.es

Editor's response and comments from the Cancer Epidemiology reviewers

De: Cancer Epidemiology <em@editorialmanager.com>
Asunto: Your Submission CANEP-D-22-00096
Fecha: 26 de abril de 2022, 20:35:54 CEST
Para: "Adrián González-Marrón" <agonzalezm@uic.es>
Responder a: Cancer Epidemiology <support@elsevier.com>

Ms. Ref. No.: CANEP-D-22-00096
Title: Changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers before and after Spanish smoke-free legislation
Cancer Epidemiology

Dear Mr. Adrián González-Marrón,

Your paper "Changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers before and after Spanish smoke-free legislation" submitted to Cancer Epidemiology has been reviewed. It requires revision before being considered for publication.

If you feel that you can suitably address the comments (included below), I invite you to revise and resubmit your manuscript. Please note that we cannot assure acceptance of a revised manuscript and, occasionally, your manuscript will be sent out for review again.

Please note that your revised manuscript must be submitted by Jun 25, 2022. If it is delayed any longer than this, the revised manuscript will need to be reconsidered as a new manuscript.

Please carefully address the issues raised in the comments.

If you are submitting a revised manuscript, please also:

a) outline each change made (point by point) as raised in the reviewer comments

AND/OR

b) provide a suitable rebuttal to each reviewer comment not addressed

To submit your revision, please do the following:

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When submitting your revised paper, we ask that you include the following items:

Manuscript and Figure Source Files (mandatory)

We cannot accommodate PDF manuscript files for production purposes. We also ask that when submitting your revision you follow the journal formatting guidelines. Figures and tables may be embedded within the source file for the submission as long as they are of sufficient resolution for Production. For any figure that cannot be embedded within the source file (such as *.PSD Photoshop files), the original figure needs to be uploaded separately. Refer to the Guide for Authors for additional information.
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Highlights consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See the following website for more information
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Graphical Abstract (optional)

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I look forward to receiving your revised manuscript.

Yours sincerely,

Jelle Vlaanderen
Associate Editor
Cancer Epidemiology

Reviewers' comments:

Reviewer #2: Spanish salivary cotinine cutpoint review 2022

The authors describe changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers in Barcelona before and after Spanish smoke-free legislation.
1. The finding is as expected: lower SHS exposure after the law and thus the cutpoint is lower. I suggest adding text to the abstract and discussion section to highlight the shift in the NS distribution as documentation of effective public health policy. Perhaps a statement like this to connect the dots: "The implementation of the Spanish smoke-free legislation was effective in terms of reducing SHS exposure and thus also

a reduction of the salivary cotinine concentration cut-off point."
2. The manuscript would be strengthened by describing sample collection more fully. If the sample was collected by the study participants: How did you control for poorly collected saliva or Recent smoking right before saliva collection? Alternatively, if the saliva was collected by study staff in a controlled fashion then please mention that fact.
3. Table 1. the 95% confidence intervals include "1.0", which would be more appropriately expressed as "100.0" given the lower bounds being expressed as "96.0"
4. Figure 2 documents that the changes in CP in the second sampling are because the SHS exposure of non-smokers decreased dramatically. The mean cotinine in smokers actually increased for the second sampling. The text would be stronger if it mentioned these observations.
5. Bottom of page 14: the authors talk about DNA methylation as a longer term marker of occasional smoking. However, DNA methylation is not a selective marker. More effective long term markers include acrylamide-hemoglobin adducts and urinary NNAL. I urge you to also cite these biomarkers instead, for example:
a. A correlation study applied to biomarkers of internal and effective dose for acrylonitrile and 4-aminobiphenyl in smokers - PubMed (nih.gov)
b. Tobacco-Specific Nitrosamines (NNAL, NNN, NAT, and NAB) Exposures in the US Population Assessment of Tobacco and Health (PATH) Study Wave 1 (2013-2014) - PubMed (nih.gov)
6. Figure 2 could be simplified and made into an excellent graphical abstract. I looked for the graphical abstract but didn't find it.
7. The authors describe the sampling as "representative" but provide no details as to how the representative sampling was done.

Reviewer #3: This paper evaluates cotinine cut-points that distinguish smokers form non-smokers among a representative adult sample in Barcelona, Spain pre and post enactment of smoke-free laws. I think this paper is well-written. Some additional information is needed describing how significance was determine when comparing the pre- and post- cut-point. Additional feedback below:

Highlights-
In the last bullet the authors note the success of the law in terms of success in changing cotinine cut-points. But the success is really in lowering passive smoke exposure (lower average cotinine levels in the population). The change in cut-points is a byproduct of the lower systemic nicotine exposure. Since the former is not evaluated, this need clarification.
Introduction-
First paragraph, pg. 5- Not clear what is meant by "supply-side issues". Laws do not come into "force", replace with "effect". You note what the first law's effect was but do not actually describe what the law did.
Overall very good, but could use editorial review for English grammar/word choice.
Figure 1- Exclusions say both 24 and 36 were missing "baseline" sample, but the 36 should say no sample at "follow-up".
Results-
Would be helpful to know the breakdown of smokers vs. non-smokers by sex and age since they are so different but the overall cut-point matches the male cut-point.

Pg 12. The analyses in Table 2 are hard to understand. How is % change calculated? I would have thought it would have been a 48.15% change for row 1? It appears this is the result of this analysis described in the methods section "We used linear mixed-effects models, with individuals as random effects, adjusted to model the percentage change (and 95% confidence intervals) of salivary cotinine concentrations (after log 10 transformation) for the baseline and follow-up." But I was expecting this analysis to assess significant changes in cotinine, not changes in cotinine cut-point. Should update methods text to reflect that.
Discussion-
More discussion needed around sex differences when stratified by daily use. Did smoking among females really increase?
Do you have any measures around smoking intensity (cigarettes per day)?
Would be interesting to add, if space permits, some discussion around how to determine when cut-points have significantly changed. The methods you proposed are not familiar to me for this purpose but could be important for the field to better understand why they were selected.
How was other tobacco product use controlled for? Could no users have used e-cigarettes or cigars?

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Data in Brief (optional):

We invite you to convert your supplementary data (or a part of it) into an additional journal publication in Data in Brief, a multi-disciplinary open access journal. Data in Brief articles are a fantastic way to describe supplementary data and associated metadata, or full raw datasets deposited in an external

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Response to the Cancer Epidemiology reviewer's comments

Submission CANEP-D-22-00096
Response to Reviewers

Dear Dr. Vlaanderen,
Thank you for giving us the opportunity to submit a revised draft of the manuscript "Changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers before and after Spanish smoke-free legislation" for publication in the Journal of Cancer Epidemiology. We appreciate the time and effort that you and the reviewers dedicated to providing feedback on our manuscript and are grateful for the insightful comments on and valuable improvements to our paper. We have incorporated most of the suggestions made by the reviewers. Those changes are highlighted using the "Track Changes" tool within the manuscript. Please see below, in blue, for a point-by-point response to the reviewers' comments and concerns. All page numbers refer to the revised manuscript file with tracked changes.

Comments from Reviewer 2

- **Summary comment:** *The authors describe changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers in Barcelona before and after Spanish smoke-free legislation.*
  Response: Thank you very much for the time you have taken to review our work. We believe that the manuscript has improved considerably thanks to your comments and the subsequent discussions they have generated.

- **Comment 1:** *The finding is as expected: lower SHS exposure after the law and thus the cutpoint is lower. I suggest adding text to the abstract and discussion section to highlight the shift in the NS distribution as documentation of effective public health policy. Perhaps a statement like this to connect the dots: "The implementation of the Spanish smoke-free legislation was effective in terms of reducing SHS exposure and thus also a reduction of the salivary cotinine concentration cut-off point."*
  Response: Thank you very much for this comment. We fully agree with you that the result is as expected under the hypothesis that non-smokers will have lower cotinine levels due to decreased exposure to secondhand smoke. Therefore, we have now reflected in the abstract (page 4, lines 19-21), in the discussion (page 15, lines 17-19), and in the highlights the findings on secondhand smoke exposure.

- **Comment 2:** *The manuscript would be strengthened by describing sample collection more fully. If the sample was collected by the study participants: How did you control for poorly collected saliva or Recent smoking right before saliva collection? Alternatively, if the saliva was collected by study staff in a controlled fashion then please mention that fact.*
  Response: Saliva samples were collected by trained personnel using the same protocol in both studies. Thank you for this timely comment. We

now make it notice in the methods section (page 8, line 6) and have also mentioned (page 8, line 7) some articles employing these same data sets where we have discussed the methodology in depth in case you would still like to learn more about the sampling procedure.

- **Comment 3:** *Table 1. the 95% confidence intervals include "1.0", which would be more appropriately expressed as "100.0" given the lower bounds being expressed as "96.0".*
  Response: **Thank you very much for this comment. We agree that writing the confidence intervals as suggested will greatly improve the comprehensibility and cohesiveness of the table. The appropriate improvements have been made in accordance with your comment (page 10, table 1).**

- **Comment 4:** *Figure 2 documents that the changes in CP in the second sampling are because the SHS exposure of non-smokers decreased dramatically. The mean cotinine in smokers actually increased for the second sampling. The text would be stronger if it mentioned these observations.*
  Response: **We fully agree with you, as you rightly pointed out in Comment 1, it was to be expected to find a lower cut-off point in nonsmokers because the exposure they receive to secondhand smoke is lower; Again, we must concede that this observation should appear in the text and if possible mentioning graph 2, since as you rightly point out, this graph perfectly summarizes the approach of the article. Thank you very much for this comment. This observation mentioning Figure 2 now appears in the discussion (page 15, lines 17-19). In addition, it remains for us to comment on the increase in cotinine in smokers from this same sample, which had already been described previously, which we now mention in our text (page 15, lines 21-22).**

- **Comment 5:** *Bottom of page 14: the authors talk about DNA methylation as a longer term marker of occasional smoking. However, DNA methylation is not a selective marker. More effective long term markers include acrylamide-hemoglobin adducts and urinary NNAL. I urge you to also cite these biomarkers instead, for example:*
  a. A correlation study applied to biomarkers of internal and effective dose for acrylonitrile and 4-aminobiphenyl in smokers - PubMed (nih.gov)
  b. Tobacco-Specific Nitrosamines (NNAL, NNN, NAT, and NAB) Exposures in the US Population Assessment of Tobacco and Health (PATH) Study Wave 1 (2013-2014) - PubMed (nih.gov)
  Response: **DNA methylation is a biochemical process involved in a wide range of bodily functions and as such its presence can be an indicator of some wide variety of alterations in the human body, for example, it can also be used as a biomarker for the diagnosis and prognosis of cancer. You are absolutely right, thank you for your kind reminder. We have decided to modify this example to one that mentions tobacco-specific nitrosamines; since these are formed during the growing, curing and**

processing of tobacco leaves their presence in human saliva will be a much more selective biomarker than DNA methylation. The discussion has been modified accordingly (page 17, lines 5-6).

- **Comment 6:** *Figure 2 could be simplified and made into an excellent graphical abstract. I looked for the graphical abstract but didn't find it.*
  Response: Thank you for your comment. We agree with you that a graphical abstract is an important part of conveying the relevant content of an article in a simple way and that Figure 2 fits this definition perfectly. We have modified the figure slightly and have proposed it as a graphical abstract for the editor to consider.

- **Comment 7:** *The authors describe the sampling as "representative" but provide no details as to how the representative sampling was done.*
  Response: Thank you very much for the comment. This document is intended to inform about a new cut-off point in the Spanish population and to encourage its use. We believe that practical and easy to understand methods are better than complicated and tedious ones. In addition, we worked with a database that has been extensively studied before and which sampling and treatment has been widely discussed. This is why we had omitted information about the sampling in the methodology section. However, we have improved the discussion to make clear in which articles can be found all the necessary information on the database (page 17, lines 17-18). Of course, if the editor deems it appropriate, we will be happy to develop it further.

Comments from Reviewer 3

- **Summary comment:** *This paper evaluates cotinine cut-points that distinguish smokers form non-smokers among a representative adult sample in Barcelona, Spain pre and post enactment of smoke-free laws. I think this paper is well-written. Some additional information is needed describing how significance was determine when comparing the pre- and post- cut-point.*
  Response: Thank you for your valuable and insightful comments that led to possible improvements in the current version. We have carefully considered the comments and tried our best to address every one of them. We hope the manuscript after careful revisions meet your high standards.

- **Highlights comment:** *In the last bullet the authors note the success of the law in terms of success in changing cotinine cut-points. But the success is really in lowering passive smoke exposure (lower average cotinine levels in the population). The change in cut-points is a byproduct of the lower systemic nicotine exposure. Since the former is not evaluated, this need clarification.*
  Response: Thank you for pointing this out. Reviewer 2 also mentioned it, we agree that non-smokers will have lower cotinine levels due to decreased exposure to secondhand smoke, which leads to a decrease in cut-points, and, we have corrected it accordingly. The revised text has

been modified in the highlights document and reads as follows: "The implementation of the Spanish smoke-free legislation was effective in terms of reducing secondhand smoke exposure."

- **Comment 1:** *First paragraph, pg. 5- Not clear what is meant by "supply-side issues".*
  Response: **Thank you for drawing our attention to this. By supply-side issues we mean regulations imposed on producers and/or sellers in order to reduce the prevalence of tobacco use, but which do not act directly on consumers. We have included examples of supply-side issues (page 5, line 12).**

- **Comment 2:** *Laws do not come into "force", replace with "effect". You note what the first law's effect was but do not actually describe what the law did.*
  Response: **Thank you very much, we have amended this.**

- **Comment 3:** *Overall very good, but could use editorial review for English grammar/word choice.*
  Response: **Thank you very much for your kind comment, we have carefully revised the entire manuscript and made edits throughout.**

- **Comment 4:** *Figure 1- Exclusions say both 24 and 36 were missing "baseline" sample, but the 36 should say no sample at "follow-up".*
  Response: **Thank you, this error has been corrected in Figure 1.**

- **Comment 5:** *Would be helpful to know the breakdown of smokers vs. non-smokers by sex and age since they are so different but the overall cut-point matches the male cut-point.*
  Response: **Thank you very much for your accurate observation. This has already been discussed internally in our group, when calculating the cut-off point, the algorithms try to capture "intermediate" cotinine values. However as can be seen in Figure 2 these types of values are rare, that is why the overall cutoff point coincides with those stratified by sex and age in some cases.**

- **Comment 6:** *Pg 12. The analyses in Table 2 are hard to understand. How is % change calculated? I would have thought it would have been a 48.15% change for row 1? It appears this is the result of this analysis described in the methods section "We used linear mixed-effects models, with individuals as random effects, adjusted to model the percentage change (and 95% confidence intervals) of salivary cotinine concentrations (after log 10 transformation) for the baseline and follow-up." But I was expecting this analysis to assess significant changes in cotinine, not changes in cotinine cut-point. Should update methods text to reflect that.*
  Response: **Thank you very much for your thorough review. It is true that the analyses performed can be unintuitive if you are unfamiliar with**

them. As you rightly suggest, we have updated the methods to make it clear that the change percentage is done to model changes in cotinine and not its cut-off point (page 10, line 1). The percentage of change was calculated with the usual formula [((XPOST/XPRE)-1)*100] but with values obtained in two different ways — with the geometric mean (GM) and with the coefficients obtained from the model—. Comparison of the values obtained showed that they were the same.

- **Comment 7:** *More discussion needed around sex differences when stratified by daily use. Did smoking among females really increase?*
  Response: **Thank you very much for this very interesting comment. While it is true that the smoking prevalence global trend is toward a decrease in both men and women —and that Spain is following this trend— it has been observed that smoking prevalence in women between the ages of 40 and 64 years has increased. We have modified the text so that this is now reflected (page 16, line 16). As we rightly mentioned in the discussion, one of the limitations of our study is that it overestimates the older population —although we have tried to limit it by weighting the sample— and thus may affect our results. I refer you to the article where this trend is observed in case you would like to know more:**

  Martín-Sánchez, J. C., Martinez-Sanchez, J. M., Bilal, U., Cleries, R., Fu, M., Lidón-Moyano, C., ... & Fernandez, E. (2018). Sex and age specific projections of smoking prevalence in Spain: a Bayesian approach. Nicotine and Tobacco Research, 20(6), 725-730.

- **Comment 8:** *Do you have any measures around smoking intensity (cigarettes per day)?*
  Response: **We collected data on smoking intensity through the questions: On average, how many cigarettes per day do you usually smoke? How many cigarettes have you smoked in the last 24h?, and How many cigarettes have you smoked in the last 48h? Although these questions were only asked to daily smokers. In general, all indicators seem to show that the intensity of smoking has decreased after the implementation of the anti-smoking laws in Spain.**
  Here are some tables that we have made:

	Cig per day		NºCig 24h		NºCig 48h	
	DCOT1	DCOT3	DCOT1	DCOT3	DCOT1	DCOT3
N	163.0	141.0	163.0	96.0	163.0	96.0
Mean	25.6	15.3	14.9	11.8	30.1	23.6
Median	14.8	10.2	15.0	10.0	30.0	20.0

- **Comment 9:** *Would be interesting to add, if space permits, some discussion around how to determine when cut-points have significantly changed. The methods you proposed are not familiar to me for this purpose but could be important for the field to better understand why they were selected.*

Response: **Thank you very much for your comment. We are not aware of any method by which we can compare cut-off points to detect significant differences as if it were a normal numerical variable. However, the changes in the cut-off points are subject to both the quality of the cut-off points and the changes in the cotinine concentration (reflected in the %change). That is why we have calculated the geometric mean of cotinine for smokers and non-smokers before and after the anti-smoking laws and found that it has indeed decreased. In addition, we compared the sensitivity, specificity and AUC of the cut-off points, being much lower before the first law was passed (page 16, lines 22-23). With all this we can both assure that the quality of the new cut-off points is better and that they are a faithful reflection of the change in cotinine concentration in non-smokers. Here are a couple of preliminary tables that we made in case you want to dig deeper:**

		Pre-2005 law				**Post-2010 law**			
	n	Cut-Point	Se (%)	Sp (%)	AUC (%)	Cut-Point	Se (%)	Sp (%)	AUC (%)
Todos	676	10.80	79.04	82.94	85.99	5.60	92.77	98.48	97.31
Sexo									
Hombres	310	25.10	78.65	85.23	84.50	5.60	95.45	97.84	98.31
Mujeres	366	10.80	78.21	84.09	86.60	1.90	93.59	96.96	96.13
Edad (años)									
17-44	311	12.40	72.12	85.05	82.81	1.30	97.12	94.31	97.97
> 44	365	10.80	83.64	82.41	87.37	14.00	90.57	99.07	95.28

Geometric Mean of Cotinine

	Pre-2005 law	Post-2010 law
Smokers	53.078	158.180
Non-smokers	2.710	0.146

Comparar sensibilidad y especificidad para ver que no cambia entre uno y otro.
Añadir GM cotinine total en fumadores y no fumadores porque no Podemos comparar el punto de corte como una variable numérica normal.

- **Comment 10:** *How was other tobacco product use controlled for? Could no users have used e-cigarettes or cigars?*
  Response: **We thank the reviewer for his timely comment. We had a concern about how the type of tobacco would affect cotinine levels. That is why we passed daily, occasional and ex-smokers the following question**

(translated from Spanish): Do you use other types of tobacco or electronic cigarettes? However, the prevalence of users of this type of tobacco product in our sample was very low —8 daily smokers who used this type of product, 4 occasional smokers, and 1 ex-smoker— so we couldn't control according to the type of tobacco. We have added this to our limitations (page 17, lines 13-14).

Additional clarifications

In addition to the above comments, references where updated and other minor errors not pointed out by the reviewers have been corrected.

We look forward to hearing from you in due time regarding our submission and to respond to any further questions and comments you may have.

Sincerely,

Hipólito Pérez-Martín

Letter of acceptance of Cancer Epidemiology journal

De: Cancer Epidemiology <em@editorialmanager.com>
Fecha: 15 de julio de 2022, 13:45:15 CEST
Para: Adrián González-Marrón <agonzalezm@uic.es>
Asunto: Your Submission
Responder a: Cancer Epidemiology <support@elsevier.com>

Ms. Ref. No.: CANEP-D-22-00096R1
Title: Changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers before and after Spanish smoke-free legislation
Cancer Epidemiology

Dear Mr. Adrián González-Marrón,

I am pleased to inform you that your paper "Changes in the salivary cotinine cut-offs to discriminate smokers and non-smokers before and after Spanish smoke-free legislation" has been accepted for publication in Cancer Epidemiology.

Thank you for submitting your work to Cancer Epidemiology.

In case there are further suggestions from the editor and/or reviewers, please find them below.

We appreciate and value your contribution to Cancer Epidemiology. We regularly invite authors of recently published manuscript to participate in the peer review process. If you were not already part of the journal's reviewer pool, you have now been added to it. We look forward to your continued participation in our journal, and we hope you will consider us again for future submissions.

Yours sincerely,

Jelle Vlaanderen
Associate Editor
Cancer Epidemiology

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Variation in Nicotine Metabolization According to Biological Factors and Type of Nicotine Consumer

Cover letter to the editor of Healthcare

Dear Editor,

Please find enclosed our manuscript “Variation in nicotine metabolization according to biological factors and type of nicotine consumer” for your consideration in Healthcare.

All the authors carefully read the manuscript and fully approve of it. In their name, I also declare that the manuscript is original and is not submitted anywhere other than your journal. The authors declare there are no conflicts of interest. Our study received approval by the ethics committee of the Bellvitge University Hospital (PR118/11 y PR133/15, respectively) and all participants signed informed consent prior to conducting the study. We would of course be ready to provide further information about our data and methods you desire. Correspondence about the manuscript should be addressed to me since the indicated corresponding authors in the manuscript text are on sick leave. Thank you very much for your kind attention.

Yours faithfully,

Hipólito Pérez-Martín, MSc, BSc

E-mail: hperez@uic.es

Editor's response and comments from the Healthcare reviewers

Healthcare Editorial Office <healthcare@mdpi.com> 19 de diciembre de 2022, 1:13

Responder a: vivia.jiang@mdpi.com
Para: Hipólito Pérez-Martín <hipolito.perez.martin@gmail.com>
Cc: Hipólito Pérez-Martín <hperez@uic.es>, Cristina Lidón-Moyano <clidon@uic.es>, Adrián González-Marrón <agonzalezm@uic.es>, Marcela Fu <mfu@iconcologia.net>, Raúl Pérez-Ortuño <rperez@imim.es>, Montse Ballbè <mballbe@iconcologia.net>, Juan Carlos Martín-Sánchez <jcmartin@uic.es>, "José A. Pascual" <jap@imim.es>, Esteve Fernandez <efernandez@iconcologia.net>, "Jose M. Martínez-Sánchez" <jmmartinez@uic.es>, Healthcare Editorial Office <healthcare@mdpi.com>

Dear Mr. Pérez-Martín,

Thank you again for your manuscript submission:

Please use the latest version of your manuscript found at the behind link for your revisions, as the editorial office may have made formatting changes or added comments to your original submission.

Please note that author names, affiliations, e-mails and correspondence could not be changed if paper accepted, so please check it carefully when revising your manuscript.

Manuscript ID: healthcare-2097048
Type of manuscript: Article
Title: Variation in nicotine metabolization according to biological factors and type of nicotine consumer
Authors: Hipólito Pérez-Martín, Cristina Lidón-Moyano *, Adrián González-Marrón *, Marcela Fu, Raúl Pérez-Ortuño, Montse Ballbè, Juan Carlos Martín-Sánchez, José A. Pascual, Esteve Fernandez, Jose M. Martínez-Sánchez
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Present and Future Challenges in Tobacco Control
https://www.mdpi.com/journal/healthcare/special_issues/tobaccocontrol_future

Your manuscript has now been reviewed by experts in the field. Please find your manuscript with the referee reports at this link:

https://susy.mdpi.com/user/manuscripts/resubmit/6ed5253c5097275ddd4bfaba2a81bec6

Please revise the manuscript according to the referees' comments and upload the revised file by 29 December.

Please use the version of your manuscript found at the above link for your revisions.

(I) Please check that all references are relevant to the contents of the manuscript.
(II) Any revisions to the manuscript should be marked up using the “Track Changes” function if you are using MS Word/LaTeX, such that any changes can be easily viewed by the editors and reviewers.

(III) Please provide a cover letter to explain, point by point, the details of the revisions to the manuscript and your responses to the referees' comments.
(IV) If you found it impossible to address certain comments in the review reports, please include an explanation in your appeal.
(V) The revised version will be sent to the editors and reviewers.

If one of the referees has suggested that your manuscript should undergo extensive English revisions, please address this issue during revision. We propose that you use one of the editing services listed at https://www.mdpi.com/authors/english or have your manuscript checked by a native English-speaking colleague.

Do not hesitate to contact us if you have any questions regarding the revision of your manuscript. We look forward to hearing from you soon.

Kind regards,
Ms. Vivia Jiang
E-Mail: vivia.jiang@mdpi.com

--
MDPI Office

MDPI Healthcare Editorial Office
St. Alban-Anlage 66, 4052 Basel, Switzerland
E-Mail: healthcare@mdpi.com
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Response to the Healthcare reviewer's comments

Ms. No.: healthcare-2097048
Response to the reviewers' comments

Academic editor:

Dear authors, the manuscript had mixed feedback, very favorable and unfavorable. Therefore, please try to address all the comments of the second reviewer thoroughly.

We thank the Academic editor for allowing us to revise and reply to the reviewers' comments.

Moreover, we would also like to thank the reviewers for all the comments that helped us improve our manuscript and its contribution to the field. We have modified our manuscript to cover all reviewers' suggestions. All amendments are detailed below and appear highlighted in the revised version of the manuscript. Here is our point-by-point response to all those comments.

Reviewer #1:

Congratulations to authors. From my point of view it is an excellent work.

Thank you very much for praising our work and supporting its publication in Healthcare.

Only some erratum to be considered in order to improve the manuscript:

Thank you very much for your comments. All your contributions have been taken into account and we consider that the overall quality of the manuscript has improved.

Line 95 "(≥16 years)" when in line 103 authors mention "adult (≥18 years old)".

Thank you very much for your comment. This manuscript has been prepared with data from two different studies. One of them was the DCOT study. This study collected information on the use of traditional cigarettes in a sample (n = 1245) of the adult population (≥16) of Barcelona. A 10-year follow-up of this sample was carried out, managing to recontact 736 people. Therefore, a bias is added to the results in this study due to the increased age in the sample. The second study focused on older users (18 years of age or older).

We have clarified this in the methodology:

> "This is a pooled analysis carried out with data retrieved from two different studies. The first one was a longitudinal study on tobacco smoking patterns "Determinants of Cotinine project-phase 3 (dCOT3 study)". This was a cohort study of a sample of adults (≥16 years at baseline) from the general population of Barcelona (Catalonia, Spain). The baseline was carried out during the years 2004-2005 (n=1,245) and one follow-up was carried out during 2013-2014 (n=736). The study included self-reported information about smoking patterns

and tobacco exposure, and a saliva sample was collected at the follow up for the determination of various biomarkers of tobacco exposure. After cleansing the follow-up[26], removing subjects that did not have available saliva samples (n=44) and subjects without available nicotine, cotinine or trans-3´-hydroxycotinine information (n=11), this sample retained data of 681 individuals. The second study was a cross-sectional study (n = 302) conducted in 2017-2018 containing data on adult (≥18 years old) users of e-cigarettes living in Barcelona. As an alternative to a probabilistic sampling technique, the consumer panels technique[27] was used in order to enroll users of e-cigarettes. Although this technique renders the sample unrepresentative of the general population, it minimizes the limitations of the reduced sample size, given the low prevalence of use in this population. Individuals who declared to be current users of e-cigarettes were asked to take part in the study. A questionnaire on e-cigarette use patterns was used and a saliva sample was also collected to determine nicotine, cotinine, and trans-3´-hydroxycotinine. Seven nonusers whose cotinine concentrations were incompatible with being a nonuser (>10 ng/mL)[28] and two individuals whose information was missing and could not be categorized were excluded, rendering a final sample of 300 e-cigarette users. Thus, the final merged sample retained data of 974 individuals (Figure 1). The design and methodology of both studies are detailed elsewhere[27,28]. Both studies received approval by the ethics committee of the Bellvitge University Hospital (PR118/11 y PR133/15, respectively) and all participants signed informed consent"

And in the limitations of the study:

"Our study has some limitations that should be mentioned. Although the questionnaire has been self-declared and could represent a reporting bias, there is sufficient evidence that self-reported data on smoking performs well when working with trained interviewers. In addition, as one portion of the pooled data was taken from a longitudinal study (this sample is aged) and the other portion used a non-probabilistic sampling technique, our sample is not representative of the general population. We did not analyze nicotine concentration in the e-cigarettes to compare with the self-reported data. Neither we controlled for passive exposure. Furthermore, classifying individuals in the sample by quartiles has the downside that the selection of the sample influences that classification. Also, there is female underrepresentation in our sample, as female are faster metabolizers than male our results may present underestimation bias. Lastly there are some significant concerns about the use of metabolite ratios that must be mentioned. One of them is that the NMR may not be reliable when calculated using values that are below the limit of quantification, not only because small measurement errors can lead to large differences in ratios but also because such low values indicate that cotinine and trans-3´-hydroxycotinine are not at steady state in the body. When not at steady state, the concentrations of trans-3´-hydroxycotinine are not solely formation-dependent and therefore the assumptions underlying the use of the NMR as a measure of nicotine metabolism are not met[42]. However, we performed a sensitivity analysis between the complete sample size and those participants with a cotinine level greater than the limit of quantification and could not find significant differences between groups. Other potential issue related to the use of metabolite ratios is that the RNM is highly dependent on time since last use

which is why it is not commonly used as a measure of nicotine metabolism rate. Regrettably, time since last use is not found among the data obtained through the questionnaires passed."

Line 99 "After cleansing the final sample (29,30)" It looks like erratum.

Thank you very much for pointing it out, we have taken it into account and modified it as follows:

> "After cleansing the follow-up[26], removing subjects that did not have available saliva samples (n=44) and subjects without available nicotine, cotinine or trans-3´-hydroxycotinine information (n=11), this sample retained data of 681 individuals."

Line 145. About nonusers, if nonuser include non smokers but include people who are passive smokers, I sugges to to clarify it. This may be a limitation as authors recognize at the end of discussion.

As you rightly comment, exposure to secondhand smoke has not been controlled. We have modified the manuscript according to your comment in the following section:

> "According to self-reported information, we classified the participants into the five following groups: a) dual users (participants who were both current cigarette smokers and users of e-cigarettes), b) cigarette smokers, c) e-cigarette exclusive users with nicotine, d) e-cigarette exclusive users without nicotine, and e) nonusers. The inclusion of users of e-cigarettes without nicotine and nonusers of any products is because they can have low levels of nicotine metabolites, generally be attributable to passive exposure to nicotine[31]. Inclusion of non-users is justified since exposure to tobacco smoke was not controlled for."

Table 1. I miss data in the 21-25, 26-30 and 31-60 BMI groups.

Thank you very much for the note, we have already corrected it and table 1:

Table 1. Geometric mean (GM) and Geometric Standard Deviation (GSD) of Rate of Nicotine Metabolism (RNM) and Nicotine Metabolite Ratio (NMR) in oral fluid samples according to smoking status and use of e-cigarette, sex, age and body mass index (BMI).

	n (%)	**RNM GM (GSD)**	**NMR GM (GSD)**
Overall	974	0.43 (4.27)	0.22 (2.09)
Smoking and e-cigarette status[a]			
Nonusers of any product [a5]	508 (52.16)	0.27 (2.30) [(a1, a2, a3, a4)***]	0.23 (1.99) [a2*]
E-cigarette exclusive users without nicotine [a4]	41 (4.21)	0.08 (8.12) [(a1, a2, a3, a5)***]	0.23 (1.80)
E-cigarette exclusive users with nicotine [a3]	164 (16.84)	0.49 (3.45) [(a2, a4, a5)***]	0.22 (1.90)
Dual users[a1]	95 (9.75)	0.48 (4.70) [(a2, a4, a5)***]	0.24 (1.80)
Cigarette smokers[a2]	166 (17.04)	2.08 (4.90) [(a1, a3, a4, a5)***]	0.18 (2.61) [a5*]
Sex[b]			
Female[b1]	442 (45.38)	0.43 (3.98)	0.24 (2.11) [b2***]
Male[b2]	532 (54.62)	0.43 (4.52)	0.21 (2.06) [b1***]

Age[c]			
18-44[c1]	371 (38.09)	0.53 (4.97) [c3***]	0.21 (1.96) [c3***]
45-64[c2]	363 (37.27)	0.42 (4.17) [c3**]	0.22 (2.13)
65-89[c3]	240 (24.64)	0.31 (3.18) [c1***, c2**]	0.25 (2.20) [c1***]
BMI[d]			
10-20[d1]	64 (6.57)	0.54 (4.93)	0.26 (2.07)
21-25[d2]	378 (38.81)	0.52 (4.56) [d3***, d4**]	0.22 (2.12)
26-30[d3]	336 (34.50)	0.35 (3.93) [d2***]	0.22 (2.09)
31-60[d4]	186 (49.21)	0.36 (3.86) [d2**]	0.23 (2.01)

The superscripts for statistical significance (* significant at p<0.050; ** significant at p<0.010; *** significant at p<0.001) report on the significant p-values when comparing the subcategory to which that row corresponds with the rest of the subcategories, always within the same category.

Reviewer #2:

On line 56, you mention that cotinine can be measured in the different human fluids but do not talk about trans-3'-hydroxycotinine

Thank you very much for your thoughtful comment. Broadly speaking, this metabolite shows the same characteristics as its predecessors in saliva, blood and urine (please see doi:10.1007/s00213-011-2341-1). In order not to extend the length of the document too much this information had been omitted. Thanks to your comment we have reconsidered this decision and assessed the measurement of trans-3'-hydroxycotinine in different biological matrices in our manuscript according to the literature. This change is reflected in our manuscript as follows:

> "Cotinine and trans-3'-hydroxycotinine can be measured in human fluids such as saliva, plasma, blood, or urine for ten days after nicotine metabolization[10,11]. About 80% of nicotine is metabolized to cotinine, turning cotinine into a well-fitted biomarker of tobacco consumption and exposure[5,11]. However, different biological matrixes represent measurements of the same biomarker taken at different points in the pharmacokinetic pathway, as seen with the higher concentration of cotinine in urine in comparison with that in saliva or plasma[12,13]."

We have added the following reference:

doi:10.1007/s00213-011-2341-1

On line 57, if 80% of nicotine is metabolized to cotinine, then how does that make trans-3'-hydroxycotinine a consistent metabolite to measure? Less than 20% of trans-3'-hydroxycotinine would be present and would provide a low estimate of nicotine consumption.

Thank you for your timely comment. Nicotine is almost entirely degraded into cotinine, and cotinine is the precursor of trans-3'-hydroxycotinine, the compound into which most of the cotinine is degraded. And since trans-3'-hydroxycotinineis generated directly from cotinine, it has the same half-life because the elimination of trans-3'-hydroxycotinineis formation limited. The trans-3'-hydroxycotinine/cotinine ratio is thus fairly stable over time (please see doi:10.1007/s00213-011-2341-1). We have clarified this in the text as follows:

> "High-dose nicotine absorption in the body happens mainly through smoking or exposure to tobacco smoke. Once the tobacco smoke is inhaled, nicotine is absorbed into the organism. After this, the clearance of the compound results in its conversion into better assimilable metabolites. Nicotine is almost entirely degraded to cotinine and cotinine is mostly degraded to trans-3'-hydroxycotinine, both transformations are catalyzed by the enzyme CYP2A6[5–7]. Both compounds have been well studied due to their relative *in vivo* long half-life (16-20 hours and 6 hours, respectively)[8,9]. "

We have added the following reference:

10.1007/s00213-011-2341-1

On line 65, it would be beneficial to include the half-life of nicotine to compare it see why it is not recommended to use the RNM

Thank you very much for your contribution. The half-life of nicotine is now shown in the manuscript:

> "Even so, as the half-life of nicotine is so short (2 hours)[17], these ratios are heavily reliant on the time since the last cigarette was smoked and the number of cigarettes smoked, so the metabolic ratio of nicotine is highly variable during the day[18,20]."

On line 75, I am unclear by what is meant by "consistent over a year time". Do you mean it is a more consistent measure over the others? If so, why include over a year time?

Thank you very much for your comment. When we talked about the consistency of the ratio we were referring to the stability of the measurement. This quality has been previously described by Tanner (doi: 10.1158/1055-9965.EPI-14-1381) in a sample of people who were quitting smoking through nicotine replacement therapy, in which he observed that the NMR maintained similar levels for one year. All this indicates that a reliable estimate of the nicotine clearance rate can be obtained from a single sample and that the rate of nicotine metabolism is not substantially altered over an extended period for regular smokers. We have clarified this as follows:

> "Besides, it is more stable than the RNM, meaning that it has minimal variation during the day, lower dependence on time since the last cigarette in smokers and it is stable over a year time (the NMR continues to give very similar values even a year after a reduction in tobacco consumption has begun. This quality also applies to people who have recently quit smoking)[19,21]."

On line 79, you should move up how trans-3'-hydroxycotinine is made to line 57 where it says hoe cotinine is made.

Thank you very much for your kind comment. The authors agree that the coherence and cohesion of the text are significantly improved by applying your suggestion. The paragraph is now as follows:

> "High-dose nicotine absorption in the body happens mainly through smoking or exposure to tobacco smoke. Once the tobacco smoke is inhaled, nicotine is absorbed into the organism. After this, the clearance of the compound results in its conversion into better assimilable metabolites. Nicotine is almost entirely degraded to cotinine and cotinine is mostly degraded to trans-3'-hydroxycotinine, both transformations are catalyzed by the enzyme CYP2A6[5–7]. Both compounds have been well studied due to their relative *in vivo* long half-life (16-20 hours and 6 hours, respectively)[8,9]. Cotinine and trans-3'-hydroxycotinine can be measured in human fluids such as saliva, plasma, blood, or urine for ten days after nicotine metabolization[10,11]. About 80% of nicotine is metabolized to cotinine, turning cotinine into a well-fitted biomarker of tobacco consumption and exposure[5,11]. However, different biological matrixes represent measurements of the same biomarker taken at different points in the pharmacokinetic pathway, as seen with the higher concentration of cotinine in urine in comparison with that in saliva or plasma[12,13]. In addition, the metabolization of nicotine can be affected by

various factors, some of the most common in the literature are gender, age and BMI. In the case of sex, it has been found that sex hormones play a role in the metabolism of cotinine and that women have a faster nicotine metabolism than men. In the case of age and BMI, the bibliography is not so clear in this regard. This is because there are countless factors that can influence the liver (and consequently the CYP2A6 enzyme), sometimes even in contradictory ways[14–16]."

On line 147, you should clarify what is meant by occasionally by the # or range of cigarettes a user would use.

Thank you very much for your suggestion. People who smoked regularly within a week but did not smoke every day of the week were categorized as occasional smokers. We have now clarified this in the text:

> "Subjects were considered current cigarette smokers if they declared smoking cigarettes daily or occasionally (people who smoked regularly within a week but did not smoke every day of the week) at the moment of the survey."

For line 154, it would be nice to know the rationale of making these different groups. No scientific validation is given and does not make sense why these groups were chosen.

Thank you very much for your comment. In the case of age, the tertiles of the unrefined sample (the 736 individuals in the traditional cigarette study and the 302 in the e-cigarette study) were calculated. This was done in order to make a distinction in nicotine metabolization between young adults, mature adults and older adults. In the case of BMI, the categorization proposed by the WHO was followed [World Health Organization. Obesity : Preventing and Managing the Global Epidemic : Report of a WHO Consultation; WHO technical report series ; 894; World Health Organization, 2000.], although it should be clarified that the underweight category was slightly expanded from <18.5 to 20 (and consequently the normal weight range was reduced) since the number of individuals who fell into this category was very small. This is now stated in the text:

> "Our study also included information on self-reported biological variables, namely sex, age, and body mass index (BMI). We categorized the individuals' age into 3 groups (according to the sample tertiles): a) between 18 and 44 years old, b) between 45 and 64 years old, and c) between 65 and 89 years old. Similarly, we also categorized the individuals' BMI in a total of 4 groups (in accordance to WHO guidelines[32], although the underweight range was extended to increase the sample size): a) between 10 and 20 kg/m^2, b) between 20 and 25 kg/m^2, c) between 25 and 30 kg/m^2, and d) between 30 and 60 kg/m^2."

We have added the following reference:

World Health Organization. Obesity: Preventing and Managing the Global Epidemic: Report of a WHO Consultation; WHO technical report series; 894; World Health Organization, 2000.

On line 220, this is the first mention of what variables affect nicotine metabolism. There should be a paragraph introducing why there may be differences in nicotine metabolism in the introduction. It came out of nowhere.

Thank you very much for your comment. We have taken this into account and have added information on the variables that affect nicotine metabolism to the introduction:

> "In addition, the metabolization of nicotine can be affected by various factors, some of the most common in the literature are gender, age and BMI. In the case of sex, it has been found that sex hormones play a role in the metabolism of cotinine and that women have a faster nicotine metabolism than men. In the case of age and BMI, the bibliography is not so clear in this regard. This is because there are countless factors that can influence the liver (and consequently the CYP2A6 enzyme), sometimes even in contradictory ways[14–16]."

We have added the following references:

10.1016/j.clpt.2006.01.008, 10.1016/j.clpt.2006.06.011, 10.1093/hmg/ddy434

For line 238, that is a bold statement to say since there is no mention of controlling for when the last cigarette or ecigarette was mentioned in the study. That is important to mention.

Thank you very much for your comment. It is true that we have not controlled the time since the last cigarette because we did not have that information. However, we do not believe that these contrary results are due to the time since the last cigarette as much as to the topography of smoking habits, as we discussed in that same paragraph. Furthermore, in the introduction we have already discussed NMR is independent of time since last cigarette (please see 12 and doi: 10.1016/j.clpt.2004.02.011):

> "The NMR is consistent in different biological fluids (e.g., a person who is determined to be a slow metabolizer by one method is highly likely to be below the cut point for slow metabolism in other fluids, but the cut points may be different between biological matrixes) and captures both inter-individual and environmental differences. Besides, it is more stable than the RNM, meaning that it has minimal variation during the day, lower dependence on time since the last cigarette in smokers and it is stable over a year time (the NMR continues to give very similar values even a year after a reduction in tobacco consumption has begun. This quality also applies to people who have recently quit smoking)[19,21]."

And in the discusión:

> "Discrepancies between both ratios could be attributed to NMR with independence of the number of cigarettes smoked[19]. Based on this, we should use the most stable ratio (NMR) as a good unbiased indicator of nicotine metabolization. However, if reported together, they could be used to determine the differences and have a rough estimation of smoking quantities."

Regardless of this, thanks to your input we have mentioned that we do not monitor the time since the last cigarette in methodology:

> "According to self-reported information, we classified the participants into the five following groups: a) dual users (participants who were both current cigarette smokers and users of e-cigarettes), b) cigarette smokers, c) e-cigarette exclusive users with nicotine, d) e-cigarette exclusive users without nicotine, and e) nonusers. The inclusion of users of e-cigarettes without

nicotine and nonusers of any products is because they can have low levels of nicotine metabolites, generally be attributable to passive exposure to nicotine[31]. Inclusion of non-users is justified since exposure to tobacco smoke was not controlled for."

Although we mentioned it in limitations:

"Regrettably, time since last use is not found among the data obtained through the questionnaires passed."

I am not sure why RNM is included in the paper as this was explained as the more unreliable measure of nicotine use in the study. Since nicotine is metabolized so quickly, I was told it is not the best and that the NMR is the better of the two. Yet, the NMR showed not as many significant differences between the groups as compared to the RNM.

Thank you very much for stating your concerns about our research so clearly in this question. As we discuss throughout the manuscript it is true that RNM alone is much more susceptible to being affected by non-tobacco factors (gender, age, weight, etc.) than NMR, however we consider that since the quantification of cotinine and trans-3 hydroxycotinine is usually also accompanied by nicotine, it does not involve much work to calculate and should be presented together with NMR, since as we concluded in the manuscript it is a way to "control" for the consumption of this substance, which can be especially interesting in the case of electronic cigarettes.

For this reason, we would like to keep it in the manuscript. However, if the editor considers more appropriate to delete it, we are open to do it.

In the discussion, there is no mention of why there would be differences between nicotine metabolism in the populations. Why are females faster metabolizers or older people?

Thank you very much for your thoughtful comment. In his paper Johnstone [doi:10.1016/j.clpt.2006.06.011] obtained similar results to ours and highlighted the fact that it was a constant in the literature that nicotine metabolism was faster in women. This was complemented by the work of Benowitz [10.1016/j.clpt.2006.01.008] in which he concludes that sex hormones affect nicotine metabolism, in particular cotinine metabolism is significantly reduced in women taking oral contraceptives. In addition, these same studies also obtained results similar to ours with regard to age, possibly due to changes in the liver that modify the behavior of the CYP2A6. As for BMI, Taylor [10.1093/hmg/ddy434] did a very detailed study on the relationship between nicotine metabolism and BMI, concluding that there is a causal effect of BMI but that it is too complex to be attributable to a single cause (sometimes even being able to produce contradictory effects). Since the objective of our study was to report on the variations in metabolization and we do not have information to delve further into this relationship, we have not been able to delve further into these differences. The discussion was modified as follows:

"We found significant differences in nicotine metabolism according to sex in NMR (p-values < 0.001); the quartile division in our sample suggests that female are fast metabolizers and male are moderate metabolizers. However, no differences were found when comparing the GM of RNM between males and females. Another study suggests that female are more likely to have faster nicotine metabolism and have higher dependence on nicotine products, possibly due to the effect of sex hormones on CYP2A3[14,15]. Nevertheless, the GM of NMR for female (0.24) and male (0.21) obtained in our study is

lower than the one reported in that study (0.43 and 0.35, respectively)[15]. Discrepancies be-tween both studies may be due to the differences between the two populations, as their sample was composed of failed quitters and ours of cigarette smokers, users of e-cigarettes, and nonusers, and, as previously reported, when the NMR is higher, nicotine metabolization is faster, and faster metabolizers may have a much harder time quitting smoking[39].

When comparing the GM of RNM by age, a negative association was clearly ob-served, being older individuals (65-89 years) faster nicotine metabolizers than younger ones (<64 years). NMR showed a positive association with age, but just between partici-pants <44 years (moderate metabolizers) and participants 65-89 years (fast metabolizers) individuals. A previous study reported a nicotine clearance similar to the one we observed in RNM[40]. However, that study counted with limited subjects (n = 40) and was con-ducted introducing nicotine via intravenous infusion and measured in plasma and urine, so differences in nicotine metabolization between different biological matrixes are to be expected. As previous studies reported similar trends concerning age and rates of nicotine metabolism, our results suggest that accumulated exposure to nicotine and natural metabolic changes associated with age may enhance its metabolization rate[14,15]. In this case, the differences observed are negatively associated with age, being the value for older indi-viduals (65-89 years) practically the same as nonusers.

Our results also shows that there is a negative association between the RNM and the BMI, while no association was found between the NMR and the BMI, although the quartile analysis categorized overweight and underweight individuals as fast metabolizers (> 0.23), while the rest were categorized as moderate metabolizers. Previous studies with a similar sample size reported BMI being negatively associated with NMR[16,33,41]. How-ever, understanding the effect and metabolic rates of novel forms of nicotine intake in smoking status, use of e-cigarette, and biological factors needs further investigation.

Discrepancies between both ratios could be attributed to NMR with independence of the number of cigarettes smoked[19]. Based on this, we should use the most stable ratio (NMR) as a good unbiased indicator of nicotine metabolization. However, if reported to-gether, they could be used to determine the differences and have a rough estimation of smoking quantities."

We have added the following references:

10.1016/j.clpt.2006.01.008, 10.1016/j.clpt.2006.06.011

Letter of acceptance of Healthcare journal

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Dear Mr. Pérez-Martín,

Congratulations on the acceptance of your manuscript, and thank you for submitting your work to Healthcare:

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Kind regards,
Ms. Vivia Jiang
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Determination of soluble angiotensin-converting enzyme 2 in saliva samples and its association with nicotine

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Determination of soluble angiotensin-converting enzyme 2 in saliva samples and its association with nicotine

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ABSTRACT

Introduction: The Angiotensin-Converting Enzyme 2 (ACE2) is the main receptor of the SARS-CoV-2. There is contradictory evidence on how the exposure to nicotine may module the concentration of soluble ACE2 (sACE2). The aim of this study was to assess the association between nicotine and sACE2 concentrations in saliva samples.
Methods: Pooled analysis performed with data retrieved from two studies (n = 634 and n = 302). Geometric mean (GM) concentrations of sACE2, both total and relative to the total amount of protein in the sample, were compared according to sociodemographic variables and variables associated to nicotine. Multivariable linear regression models were fitted to explore the associations of sACE2 with nicotine adjusting for sex, age and body mass index. Spearman's rank-correlation coefficients were estimated between the concentrations of nicotine and cotinine, and pack-years, the concentration of relative sACE2 and the isoforms of sACE2.
Results: We observed a significant increase of 0.108‰ and 0.087 ng/μl in the relative and absolute salivary sACE2 GM concentrations, respectively, between the lowest and highest nicotine levels. Similar results were observed for cotinine. These associations did not change in the multivariable linear models. There was a low correlation of nicotine and cotinine concentration with the concentration of relative salivary sACE2 ($r_s = 0.153$ and $r_s = 0.132$, respectively), pack-years ($r_s = 0.222$ and $r_s = 0.235$, respectively) and with the concentration of isoform 40 KDa ($r_s = 0.193$ and $r_s = 0.140$, respectively).
Conclusion: Salivary nicotine concentration seems to be limitedly associated with the concentration of sACE2.

1. Introduction

Since the SARS-CoV-2 was isolated for the first time at the beginning of 2020, over 250 million cases of Coronavirus Disease (2019) (COVID-19) have been confirmed worldwide, leading to more than 5 million deaths (WHO Coronavirus, 2021).

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The Angiotensin-Converting Enzyme 2 (ACE2), the main receptor for the SARS-CoV-2 infection of human cells (Hoffmann et al., 2020) (Walls et al., 2020), is an integral membrane glycoprotein that contains a single extracellular catalytic domain (Tipnis et al., 2000) (Donoghue et al., 2000). ACE2 acts as a negative regulator of the renin-angiotensin system (RAS), maintaining homeostasis and protecting organs from vasoconstriction, fibrosis, and thrombosis (Imai et al., 2005) (Santos et al., 2018) (Paz Ocaranza et al., 2020). ACE2 is widely expressed in different organs like the heart, lungs, or kidneys (Hamming et al., 2004) (Harmer et al., 2002) and in the oral cavity and salivary glands (Xu et al., 2020) (Hikmet et al., 2020).

ACE2 is mainly bound to the cell membrane, but it is also present as a soluble form (sACE2) in extracellular fluids (Yamaleyeva et al., 2012). The metalloproteinases 10 and 17 (ADAM 10, ADAM17) are the responsible of the ectodomain shedding of ACE2, so releasing a circulating form of the receptor (Niehues et al., 2022). This sACE2 has a dual paradoxical behavior on the COVID-19 prognosis. On the one hand, it is known that high sACE2 levels are associated with poor prognosis in COVID-19 patients, probably due to an imbalance of ACE/ACE2 homeostasis (Kragstrup et al., 2021) (Rysz et al., 2021). On the other hand, high sACE2 circulating levels could act as a neutralizing factor against the infection by SARS-CoV-2 (Batlle et al., 2020). Accordingly, recombinant ACE2 as a treatment for COVID-19 is being investigated (Monteil et al., 2020). It is unknown whether the different isoforms of sACE2 might explain this apparent paradox, pointing the need for more accurate analysis of these isoforms.

Lifestyle factors like diet or cigarette smoking would impact receptor expression (Li et al., 2020). Thus, nicotine, as the main tobacco alkaloid of cigarettes, has been shown to upregulate the expression of ACE2 through α7-nAChR (nicotinic cholinergic) receptors (Russo et al., 2020) (Leung et al., 2020). Although most published studies are aligned with this finding, other works describe an inverse effect, triggering a homeostasis disequilibrium of the RAS (Oakes et al., 2018). While data suggest that nicotine uptake can play a role in the ACE2 expression, it is totally unknown if nicotine also stimulates the proteolysis of the receptor.

Epidemiologically, although results from most observational studies support the fact that the severity of the COVID-19 is directly associated with smoking (Patanavanich and Glantz, 2020) (Jiménez-Ruiz et al., 2021), there are discrepant results concerning the risk of infection. While a significantly higher prevalence of smoking among COVID-19 patients has been reported (Jackson et al., 2021), claims of the protection of smoking against the COVID-19 have also been raised (van Westen-Lagerweij et al., 2021).

The role that nicotine uptake may have on the ACE2 expression, and subsequently on the epidemiology of the COVID-19, is still a matter of debate, also due to the potential interference of the tobacco industry supporting the claim that smoking is a protective factor against COVID-19 infection (Burki, 2021). Although we do not have the capacity to measure the therapeutic effect, we hypothesize that exposure to nicotine may increase the concentration of sACE2, which may subsequently block the SARS-CoV-2 and avoid epithelial fusion. The objective of this work is to assess the association between salivary nicotine and sACE2 concentrations and its isoforms.

2. Materials & methods

This is a pooled analysis using saliva samples collected from two different studies. The first one was a cohort study of a sample of adults (≥16 years) from the general population of Barcelona (Catalonia, Spain) (Lidón-Moyano et al., 2018). The follow-up wave in 2013–2014 included self-reported information about smoking patterns and tobacco exposure, and a sample of saliva (6 ml) was collected for the determination of various biomarkers of tobacco exposure. The sample was separated into 3 ml aliquots and frozen to −20 °C for storage. The frozen samples were sent to the Bioanalysis Research Group of the Municipal Institute for Medical Research (IMIM-Hospital del Mar) in Barcelona to analyze tobacco biomarkers (nicotine, cotinine, and tobacco specific nitrosamines [TSNAs]). After that, the remaining aliquots were frozen to −80 °C for storage to preserve for future studies. We used the self-reported information, salivary nicotine concentration, and a remaining 3 ml aliquot of frozen saliva to analyze ACE2. After cleansing the final sample of participants, removing subjects that did not have available saliva samples (n = 44), subjects without available ACE2 concentration (n = 47) and subjects without available nicotine and cotinine information (n = 11), the final sample retained data of 634 individuals. The second study was a cross-sectional study (n = 302) conducted in 2017–2018 with users of e-cigarettes (e-cigs) living in Barcelona (Bunch et al., 2018). A questionnaire on e-cig use patterns was used and a saliva sample was also collected to determine nicotine and cotinine concentrations. The sample was separated into 3 ml aliquots and frozen to −20 °C for storage. The frozen samples were sent to the Bioanalysis Research Group of the Municipal Institute for Medical Research (IMIM-Hospital del Mar) in Barcelona to analyze tobacco biomarkers (nicotine and cotinine). After that, the remaining aliquots were frozen to −80 °C for storage to preserve for future studies. A frozen aliquot of 3 ml was available for ACE2 analysis from all participants (n = 302). After cleansing the final sample of participants as previously described (there were 44 individuals without available ACE2 concentration and 12 without nicotine and cotinine information), the sample for this study retained data of 246 individuals. Two individuals whose information was missing and could not be categorized in any of the EC user groups were excluded, rendering a final sample of 244 e-cig users. Thus, the final merged sample retained data of 878 individuals. The design and methodology of both studies are detailed elsewhere (Lidón-Moyano et al., 2018) (Bunch et al., 2018). Both studies received approval by the ethics committee of the Bellvitge University Hospital (PR118/11 and PR133/15, respectively) and all participants signed an informed consent, including the use of frozen saliva samples for further research use. Moreover, the present project also received approval by the ethics committee of the Bellvitge University Hospital (PR303/20).

2.1. Determination of salivary nicotine and cotinine

High-dose nicotine absorption in the body happens mainly through inhaling tobacco smoke. Once the tobacco smoke is inhaled, nicotine is absorbed into the organism. After this, the clearance of the compound results in its conversion into better assimilable metabolites. Nicotine's main metabolite, cotinine, has been well studied due to its relative *in vivo* long half-life (16–20 h) (Benowitz et al., 2009). About 80% of nicotine is metabolized to cotinine and its measurement in human fluids such as saliva, plasma, blood or urine, has become a well-fitted biomarker of tobacco consumption and involuntary exposure (Benowitz, 1996). In order to determine nicotine and cotinine concentrations, we analyzed salivary samples employing a common protocol in 2013–2014 (Pérez-Ortuño et al., 2015). The frozen samples were analyzed by the Group of Integrative Pharmacology and Neuroscience of the Municipal Institute for Medical Research (IMIM-Hospital del Mar) in Barcelona. All biomarkers were determined by LC/MS/MS after a single alkaline liquid-liquid extraction with dichloromethane/isopropanol. The limit of quantification of this method was 0.5 ng/mL for nicotine and 0.1 ng/mL for cotinine. The number of values below the limit of quantification was 269 (30.6%) for nicotine and 16 (1.8%) for cotinine. Values under the limit of quantification were halved to avoid overestimation or underestimation bias.

2.2. Determination of salivary ACE2 and protein quantification

ACE2 was quantified using Western blot analysis. 1 ml of saliva was centrifuged at 14.000 rpm for 1 min at 4 °C. 30 μl of the supernatant was mixed with 6 μl of 125 mM Tris-HCl at pH 6.8, 50% glycerol, 5% SDS, 0.25M DTT, and bromophenol blue. Samples were boiled at 90 °C for 5

min. 7.5% Midi-PROTEAN® TGX™ Precast Protein Gels (BioRad) were employed for separation of proteins at 150–250V. Protein transfer to PVDF membranes was performed at 400 mA for 1 h. Membranes were then incubated with the blocking solution (Tris Buffered Saline with Tween 20, pH = 8 and 5% dry milk) for 30 min at room temperature with gentle rocking). Membranes were incubated with a diluted solution (1:1000) of ACE2 antibody (66,699-1-1G, Proteintech) at 4 °C overnight. After primary antibody incubation, membranes were washed with 50 ml of TBS-T three times 10 min with gentle rocking followed by incubation with a diluted solution (1:33,000–1:50,000) of Goat Anti-Mouse light chain Antibody, HRP conjugate (AP200P, Proteintech) for 60 min at room temperature. After secondary antibody incubation, membranes were washed with 50 ml of TBS-T for at least, five times 10 min with gentle rocking.

Immunoblots were developed using SuperSignal™ West Femto Maximum Sensitivity Substrate and images were taken using ChemiDoc Imaging System (BioRad). Protein quantification was performed using Image Lab Software (BioRad). To estimate the quantity of ACE2, different amounts of overexpressed cell extract were loaded into the gel. The total protein concentration of saliva samples was calculated using the Bradford protein assay (Bradford, 1976). The limit of detection of this method was 1 ng per 30 μl of saliva. The number of values below the limit of quantification was 1 (0.1%) for ACE2. Values under the limit of detection were halved to avoid overestimation or underestimation bias.

2.3. Self-reported variables

According to self-reported information in the study on tobacco use (Lidón-Moyano et al., 2018), we classified the participants into the following groups: a) current smokers, those who declared current smoking every day or occasionally, b) former smokers, those who declared smoking in the past (>6 months) every day or occasionally, c) never smokers, those who declared never smoking. Pack-years (i.e., number of packs smoked daily multiplied by the number of years smoking) were also computed for participants in this study, using the current reported number of cigarettes smoked as constant since smoking onset, and categorized into 0, above 0 and below 5, above 5 and below 10 and over 10 pack-years. According to self-reported information in the study on e-cig use (Bunch et al., 2018), we classified as d) e-cig users, those who declared using e-cigs. Moreover, e-cig users were classified as: a) dual users, those declaring smoking in addition to using e-cig, b) exclusive e-cig with nicotine users, those declaring using e-cigs with nicotine, and c) exclusive e-cig without nicotine users, those declaring using e-cigs without nicotine.

Our study also included information from both studies (Lidón--Moyano et al., 2018) (Bunch et al., 2018) on self-reported biological variables, namely sex, age, weight and height, and the body mass index (BMI) was calculated. We categorized the individuals' age into 3 groups: a) between 18 and 44 years old, b) between 45 and 64 years old, and c) between 65 and 89 years old. Similarly, we also categorized the individuals' BMI in a total of 4 groups: a) between 10 and 20 kg/m^2, b) between 20 and 25 kg/m^2, c) between 25 and 30 kg/m^2, and d) between 30 and 60 kg/m^2.

2.4. Statistical analysis

Because of the skewness in the distribution of all compounds determined in saliva, we calculated geometric means (GM) with 95% confidence intervals (95% CI). We compared GM through Mann-Whitney tests and Kruskal-Wallis tests with Bonferroni correction according to sex, age, BMI, smoking status, type of e-cig users (only e-cig users), pack-years (only participants in the study of tobacco use), nicotine level, and cotinine level. We stratified the analyses which explored the association between the relative concentration of sACE2 and cotinine and nicotine according to the categories of smoking status. We fitted eight multiple linear regression models of relative log-concentration sACE2 using nicotine concentration (continuous and discretized), cotinine concentration (continuous and discretized), pack years (continuous and discretized), smoking status, and type of e-cig users as the main independent variables, adjusted for sex, age and BMI. In addition, we estimated the pairwise Spearman's rank-correlation coefficients between the concentrations of nicotine and cotinine, and pack-years (only dCOT3 participants), the concentration of relative sACE2 and the isoforms of sACE2. The proportion of the different isoforms, overall and according to smoking status and type of e-cig used, were estimated. Statistical analyses were performed using R version 4.0.4.

3. Results

The final sample of this study included 878 individuals, classified according to the smoking status as current smokers (n = 152; 17.3%), former smokers (n = 232; 26.4%), never smokers (n = 250; 28.5%) and e-cig users (all) (n = 244; 27.8%) (Table 1). According to the pattern of e-cig use, there were 30.3% (n = 74) dual (cigarette and e-cig) users, 56.6% (n = 138) exclusive e-cig with nicotine users, and 13.1% (n = 32) exclusive e-cig without nicotine users (Table 1).

We found statistically higher salivary sACE2 GM concentrations in men (0.160 ng/μl [95% CI 0.143; 0.180]) and individuals younger than 45 years (0.152 ng/μl [95% CI 0.133; 0.174]) (Table 1). The salivary sACE2 GM concentration also showed statistically significant differences according to the smoking status, having current, former and never smokers relative GM concentrations of 0.094 ng/μl (95% CI 0.076; 0.115), 0.111 ng/μl (95% CI 0.093; 0.132) and 0.093 ng/μl (95% CI 0.079; 0.109), respectively, while the group of e-cig users showed a total salivary sACE2 GM concentration of 0.264 ng/μl (95% CI 0.230; 0.302) (Table 1) (Fig. 1). There were not significant differences in salivary sACE2 GM concentrations according to neither the pattern of e-cig use nor the pack-years smoked in dCOT3 participants (Table 1).

We observed significantly higher GM concentrations of salivary sACE2 in individuals with higher levels of nicotine and cotinine (Table 1). When stratifying according to smoking status, trends were similar in both current smokers and e-cig users although not statistically significant (Table 2). The associations did not change in the multivariable models after adjusting for sex, age and BMI (Table 3).

The concentrations of nicotine and cotinine had a low correlation with the concentration of relative salivary sACE2 ($r_s = 0.153$ and $r_s = 0.132$, respectively), pack-years ($r_s = 0.222$ and $r_s = 0.235$, respectively) and with the concentration of isoform 40 KDa ($r_s = 0.193$ and $r_s = 0.140$, respectively) (Table 4).

The most frequent isoforms among the total sample were isoform 55 KDa (63.1% of individuals), 50 KDa (41.2%) and 65 KDa (17.7%). Moreover, the most frequent isoform among never, current and former smokers and e-cig users was isoform 55 KDa (66.8%, 55.9%, 62.5% and 64.3%, respectively). Among e-cig users, the most prevalent isoform was isoform 55 KDa for users of exclusive e-cig without nicotine (2.5%), exclusive e-cig users with nicotine (9.8%) and dual users with conventional cigarettes (5.6%), too.

4. Discussion

We found low correlations between nicotine/cotinine and sACE2 concentrations; nevertheless, we found significantly higher sACE2 GM concentrations in the group of participants with over 50 ng/mL of salivary GM cotinine and nicotine concentrations in comparison to participants having 0–10 ng/mL. However, after stratifying by smoking status, we did not find statistically significant differences in sACE2 GM according to cotinine and nicotine levels. This could be partially due because the sample size was reduced among categories and the statistical power was reduced. One of the pathways proposed by some authors that may explain how nicotine exposure protect against the COVID-19 infection is that nicotine exposure may increase the concentration of sACE2, which would subsequently block the spike protein of the SARS-

Table 1
Geometric mean concentrations of salivary nicotine, cotinine, relative sACE2 and absolute sACE2 (and 95% confidence intervals) according to covariates.

	n	Nicotine (ng/mL)	p-value	Cotinine (ng/mL)	p-value	sACE2 (relative) (‰)	p-value	sACE2 (total) (ng/μl)	p-value
Sex			≤0.001*		≤0.001*		0.002*		≤0.001*
Men	474	15.334 [11.173; 21.045]		6.836 [4.850; 9.636]		0.247 [0.217; 0.280]		0.160 [0.143; 0.180]	
Women	404	2.480 [1.871; 3.289]		1.068 [0.765; 1.492]		0.180 [0.156; 0.207]		0.102 [0.09; 0.116]	
Age			≤0.001**		≤0.001**		0.012**		0.016**
(18,44]	331	19.404 [13.535; 27.819]		10.618 [7.110; 15.856]		0.247 [0.214; 0.284]		0.152 [0.133; 0.174]	
(45,64]	326	7.383 [5.047; 10.798]		3.19 [2.112; 4.817]		0.198 [0.169; 0.232]		0.119 [0.103; 0.138]	
≥65	221	1.134 [0.832; 1.546]		0.366 [0.256; 0.522]		0.191 [0.156; 0.235]		0.118 [0.098; 0.142]	
BMI			0.002**		≤0.001**		0.117**		0.037**
(10,20]	56	17.083 [7.007; 41.644]		8.543 [3.036; 24.040]		0.300 [0.196; 0.460]		0.152 [0.103; 0.225]	
(20,25]	342	9.465 [6.66; 13.453]		5.011 [3.369; 7.454]		0.210 [0.183; 0.241]		0.126 [0.111; 0.143]	
(25,30]	306	6.339 [4.251; 9.451]		2.395 [1.555; 3.687]		0.229 [0.193; 0.273]		0.15 [0.127; 0.176]	
≥30	164	2.702 [1.714; 4.258]		0.998 [0.602; 1.654]		0.184 [0.149; 0.228]		0.109 [0.089; 0.134]	
Smoking status			≤0.001**		≤0.001**		≤0.001**		≤0.001**
Current smokers	152	80.355 [52.216; 123.657]		161.493 [114.037; 228.699]		0.171 [0.135; 0.217]		0.094 [0.076; 0.115]	
Former smokers	232	0.548 [0.470; 0.638]		0.182 [0.153; 0.218]		0.185 [0.152; 0.225]		0.111 [0.093; 0.132]	
Never smokers	250	0.481 [0.429; 0.540]		0.116 [0.103; 0.131]		0.166 [0.137; 0.200]		0.093 [0.079; 0.109]	
e-cig users (all)	244	220.732 [167.556; 290.784]		89.738 [67.632; 119.069]		0.362 [0.315; 0.414]		0.264 [0.230; 0.302]	
Type of e-cig users			≤0.001**		≤0.001**		0.907**		0.846**
Dual users with conventional cigarettes	74	257.561 [153.792; 431.346]		142.212 [97.442; 207.553]		0.352 [0.269; 0.456]		0.263 [0.202; 0.341]	
Exclusive e-cig (with nicotine)	138	351.846 [263.374; 470.039]		173.742 [137.626; 219.337]		0.368 [0.305; 0.443]		0.265 [0.219; 0.320]	
Exclusive e-cig (without nicotine)	32	20.686 [8.622; 49.631]		1.791 [0.718; 4.468]		0.357 [0.263; 0.484]		0.264 [0.202; 0.344]	
Pack years (only dCOT3)			≤0.001**		≤0.001**		0.171**		0.388**
0	452	0.494 [0.449; 0.542]		0.133 [0.121; 0.147]		0.174 [0.151; 0.200]		0.100 [0.088; 0.112]	
(0,5]	119	42.571 [24.272; 74.665]		71.642 [41.085; 124.925]		0.190 [0.141; 0.255]		0.101 [0.078; 0.130]	
(5,10]	22	14.007 [3.361; 58.373]		26.55 [5.509; 127.954]		0.223 [0.113; 0.437]		0.118 [0.064; 0.217]	
>10	20	146.759 [48.817; 441.200]		132.079 [37.055; 470.777]		0.090 [0.058; 0.139]		0.064 [0.039; 0.106]	
Nicotine level							≤0.001**		≤0.001**
(0,10]	534	–		–		0.178 [0.157; 0.202]		0.103 [0.092; 0.115]	
(10,50]	52	–		–		0.259 [0.192; 0.350]		0.174 [0.130; 0.232]	
>50	292	–		–		0.286 [0.244; 0.335]		0.190 [0.163; 0.222]	
Cotinine level							≤0.001**		≤0.001**
(0,10]	520	–		–		0.177 [0.156; 0.201]		0.105 [0.094; 0.117]	
(10,50]	48	–		–		0.254 [0.174; 0.371]		0.171 [0.119; 0.247]	
>50	310	–		–		0.283 [0.244; 0.329]		0.179 [0.155; 0.208]	

GM: Geometric mean; CI: Confidence interval; * Wilcoxon test; ** Kruskal Wallis test.

CoV-2 and avoid epithelial fusions (Batlle et al., 2020). A phase II clinical trial is currently ongoing to assess the effect of recombinant ACE2 in all cause-mortality or invasive mechanical ventilation in COVID-19 confirmed patients (van Lier et al., 2021). Moreover, our observed decrease of ACE2 levels due to ageing has been previously described and it was postulated as one of the causes for the increased mortality rate in elder patients (Xie et al., 2006; Berni Canani et al., 2021). However, other authors have hypothesized that COVID-19 susceptibility in elderly can be related to the increase of ACE2 expression (Narula et al., 2020; Gu et al., 2022).

Other studies have reported differences in ACE2 concentration according to BMI or age, being higher for older individuals and BMI (Narula et al., 2020). However, these results are given when measuring ACE2 in plasma and not in saliva as in our case. It would be interesting to study the relationship between ACE2 in plasma and saliva to try to understand why our results are inversely proportional to those obtained in plasma. In this sense, the differences observed in ACE2 among e-cig users in comparison with smokers and no-smokers (e-cig users showed

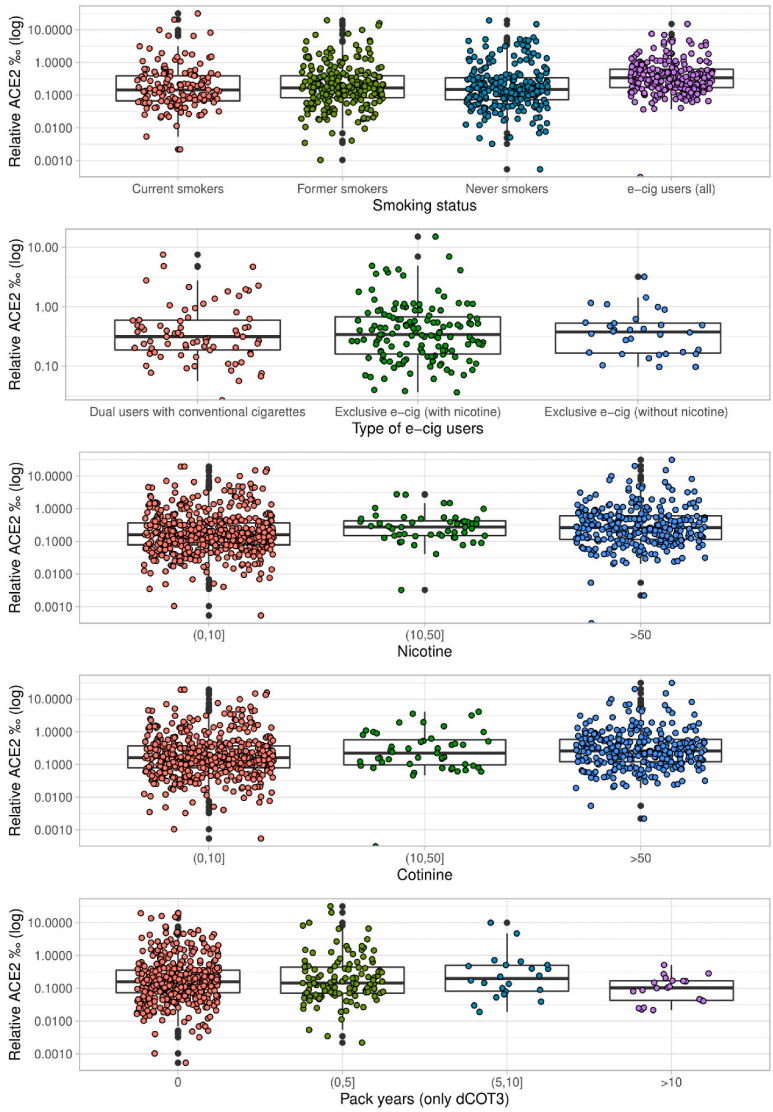

Fig. 1. Boxplots representing the salivary relative sACE2 concentration (in ‰) according to smoking status, type of e-cig user, nicotine and cotinine concentrations and pack-years smoked.

remarkably higher sACE2 concentrations) could be partially due to the fact that there were statistically significant differences between e-cig users and smokers and non-smokers by sex, age, and BMI. The e-cig users were more frequently men, were younger, and with lower BMI than smokers and non-smokers (data not shown).

4.1. Determination of sACE2 in saliva and its association with nicotine uptake

Recently, high sACE2 levels in plasma have been observed in critically ill patients with COVID-19 disease (van Lier et al., 2021), pointing to sACE2 in extracellular fluids as a new biomarker of COVID-19 prognosis. Here, we propose the analysis of sACE2 levels in saliva samples, a less invasive and cheaper method than blood testing (Tabak, 2001) that may be used for those purposes. In fact, a recent publication describes a high membrane and cytoplasmatic expression of ACE2 in parotid and submandibular epithelial cells (Zhu et al., 2022). Moreover, saliva is a protein enriched tissue, containing approximately 2000 unique proteins including secretions from the salivary gland, proteins coming from blood or factors released by epithelial cells (Hu et al., 2010), used for the diagnosis of several pathologies like cancer or infectious diseases (Katsani and Sakellari, 2019).

There are several articles supporting the induction of ACE2 expression on small airway epithelia under nicotine exposure (Smith et al., 2020). This is consistent with our finding that individuals who exhibit a higher concentration of nicotine in saliva have around 85% higher concentration of sACE2. Specifically, the highest concentration is that of ~55 kDa isoform, which was previously described as a short ACE2

Table 2
Geometric mean concentrations of relative sACE2 (and 95% confidence intervals) according to cotinine and nicotine levels in saliva, stratified according to smoking status.

Current smokers			
	n	Relative sACE2 GM [95% CI]	p-value
Overall	152	0.171 [0.135; 0.217]	
Nicotine level			
(0,10]	37	0.157 [0.106; 0.234]	0.472**
(10,50]	19	0.194 [0.136; 0.277]	
>50	96	0.173 [0.123; 0.243]	
Cotinine level			
(0,10]	14	0.122 [0.063; 0.236]	0.535**
(10,50]	15	0.127 [0.074; 0.218]	
>50	123	0.185 [0.140; 0.244]	
Former smokers			
	n	**Relative sACE2 GM [95% CI]**	**p-value**
Overall	232	0.185 [0.152; 0.225]	
Nicotine level			
(0,10]	224	0.181 [0.148; 0.221]	0.230**
(10,50]	6	0.476 [0.130; 1.735]	
>50	2	0.163 [0.000; 161.634]	
Cotinine level			
(0,10]	227	0.186 [0.152; 0.226]	0.586**
(10,50]	2	0.328 [0.150; 0.719]	
>50	3	0.109 [0.003; 4.678]	
Never smokers			
	n	**Relative sACE2 GM [95% CI]**	**p-value**
Overall	250	0.166 [0.137; 0.200]	
Nicotine level			
(0,10]	246	0.168 [0.139; 0.203]	0.836**
(10,50]	3	0.053 [0.000; 24.145]	
>50	1	0.167 [NA; NA]	
Cotinine level			
(0,10]	249	0.166 [0.137; 0.200]	0.841*
(10,50]	0	- [-; -]	
>50	1	0.127 [-; -]	
e-cig users (all)			
	n	**Relative sACE2 GM [95% CI]**	**p-value**
Overall	244	0.372 [0.324; 0.427]	
Nicotine level			
(0,10]	27	0.317 [0.215; 0.467]	0.787**
(10,50]	24	0.342 [0.239; 0.490]	
>50	193	0.384 [0.327; 0.451]	
Cotinine level			
(0,10]	30	0.252 [0.195; 0.326]	0.139**
(10,50]	31	0.442 [0.268; 0.731]	
>50	183	0.385 [0.329; 0.451]	

variant (Blume et al., 2021). Considering that saliva is a protease enriched fluid (Thomadaki et al., 2011), we can speculate that nicotine may, firstly, induce a high expression of the receptor also on oral mucosa and salivary glands that overcome the proteolytic activity in saliva. In fact, it has been described that nicotine exposure may increase ACE2 expression via activation of α7-nAChR receptor in airway epithelial cells (Tizabi et al., 2020). Secondly, it could induce proteolytic shedding of specific isoforms that are more stable after secretion. Interestingly, it has been demonstrated that smoking habit leads to the activation of the EGFR-ADAM17 pathway in airway epithelial cells, probably being the cause of ACE2 proteolysis, and the resulting increase of soluble ACE2 in saliva (Stolarczyk et al., 2016).

4.2. Possible therapeutic role of sACE2 isoforms against COVID-19

ACE2 high expression levels are needed to keep the RAS system balance, protecting the organs from inflammation or vasoconstriction (Gheblawi et al., 2020). SARS-CoV-2 infection downregulates ACE2 receptor expression, triggering the severe acute respiratory distress syndrome (ARDS) or severe acute respiratory syndrome (SARS) (Banu et al., 2020). Thus, this is the main reason why ACE2 has been pointed as a potential target for COVID-19 treatment, using different approaches (Singh et al., 2022). As an example, treatments based on ATBlockers/ACEI have been shown to promote ACE2 expression increase while improving COVID-19 patients' recovery (Ferrario et al., 2005; Choksi et al., 2021). These results suggest that drugs increasing circulating ACE2 levels can have therapeutic potential for COVID-19 patients. These findings, summed to our results showing that individuals with a higher salivary nicotine and cotinine concentration have around 85% higher relative concentration of sACE2 than those with lower nicotine levels (0.286 μg/ml vs 0.178 μg/ml), could indicate that nicotine might play a role against COVID-19 disease.

In addition to the increase in ACE2 expression, on the one hand, nicotine promotes an anti-inflammatory response by inhibiting the pro-inflamatory cytokine-involved in cytokine storm-expression (Kloc et al., 2020; Farsalinos et al., 2020). On the other hand, nicotine is involved in α7-nAChR blockage, described as a possible epithelial cell entry point for SARS-CoV-2 (Oliveira et al., 2021). Finally, it is worthy to highlight that different clinical trials have been carried out (NCT04429815, NCT04583410) to empirically demonstrate the nicotine therapeutic role. However, more studies are needed to confirm this potential association. Also, this finding should be taken with caution from a public health perspective, since this should not mean a gateway for the tobacco industry to interfere and promote smoking, since tobacco smoking is one of the first avoidable causes of morbimortality worldwide (GBD, 2019 Tobacco Collaborators et al., 2021).

Another approach that has been studied for COVID-19 treatment consists on the administration of recombinant ACE2 (Krishnamurthy et al., 2021). Also, to improve treatment efficiency, ACE2 mutant variants have been engineered, to increase receptor affinity for spike protein while maintaining catalytic activity (Chan et al., 2020). However, recent published results indicate possible major side effects for this strategy, due to SARS-CoV-2 ability to infect cells through sACE2 and vasopressin *in vitro* (Yeung et al., 2021). Hence, our manuscript can be considered as a breakthrough in the field, as we describe new ACE2 receptor soluble isoforms. The detailed study of these isoforms could identify which ones have higher virus affinity and which are the ones allowing the virus to infect new cells. In summary, isoform characterization can be the key to unravel if nicotine administration can be a therapeutic strategy, by increasing soluble ACE2 isoforms in saliva with virus neutralization capacity.

Recently, a case report has been published where human recombinant soluble ACE2 (hrsACE2) has been administrated as COVID-19 treatment in symptomatic patients, resulting in a significant improvement of the disease course (Zoufaly et al., 2020). Although a high dose (0.4 mg/kg twice a day) was administered during 7 days, the concentration of hrsACE2 in plasma reaches a weak plateau of 1 μg/ml after 36 h. In addition, these levels are drastically reduced 48h after stopping treatment, suggesting a low stability of the recombinant protein in plasma. Moreover, considering that administration of hrACE2 has two clinical objectives -on the one hand, reducing the angiotensin II circulating levels responsible of inflammatory processes observed during COVID-19 disease, and on the other side, neutralizing circulating viral particles, some of the isoforms we have described would be a good target to increase treatment effectivity. We based this hypothesis on the fact that the 55 kDa isoform is predominant and it seems to display increased stability in extracellular fluids, pointing this isoform as an improved recombinant sACE2 for COVID-19 treatment.

4.3. Limitations and strengths

The results of this study should be considered in the light of some limitations. Regarding nicotine, its half-life is very short and we may be underestimating the immediate effect of nicotine exposure on sACE2 concentration. Nevertheless, some molecules, such as growth factor like signaling molecules and hormones, are characterized by an acute secretion peak and a short half-life (just a few minutes), but their effects can last for hours or even days. In this sense, we also used in our analyses cotinine concentrations, which has a longer half-life than nicotine

Table 3
Multiple linear regression models of salivary relativized ACE2 log-concentrations.

		Beta	SE	95% CI	p-value	R^2
Model 1[a]						0.023
	Constant	0.233	1.103	0.193; 0.283	<0.001	
	Nicotine level (for each 100 ng/mL)	1.037	1.017	1.004; 1.071	0.022	
Model 2[a]						0.029
	Constant	0.178	1.063	0.158; 0.201	<0.001	
	Nicotine level					
	(0–10]			1	1	
	(10–50]	1.456	1.228	0.972; 2.180	0.172	
	>50	1.604	1.109	1.310; 1.963	<0.001	
Model 3[a]						0.020
	Constant	0.239	1.104	0.197; 0.291	<0.001	
	Cotinine level (for each 100 ng/mL)	1.037	1.022	0.993; 1.083	0.021	
Model 4[a]						0.029
	Constant	0.177	1.064	0.157; 0.200	<0.001	
	Cotinine level					
	(0–10]			1	1	
	(10–50]	1.435	1.238	0.943; 2.182	0.050	
	>50	1.600	1.107	1.311; 1.953	0.001	
Model 5[a]						0.054
	Constant	0.192	1.132	0.151; 0.245	<0.001	
	Smoking status					
	Current smokers	1.038	1.161	0.775; 1.390	0.825	
	Former smokers	1.121	1.139	0.867; 1.448	0.387	
	Never smokers			1	1	
	e-cig users (all)	2.063	1.152	1.52; 2.725	<0.001	
Model 6[a]						0.028
	Constant	0.352	1.282	0.216; 0.574	<0.001	
	Type of e-cig users (only e-cig users)					
	e-cig (without nicotine)			1	1	
	e-cig (with nicotine)	1.019	1.244	0.663; 1.565	0.94	
	Dual users with conventional cigarettes	0.959	1.269	0.599; 1.534	0.8	
Model 7[a]						0.008
	Constant	0.263	1.209	0.181; 0.383	<0.001	
	Pack years (only dCOT3)	0.983	1.016	0.952; 1.015	0.289	
Model 8[a]						0.009
	Constant	0.217	1.131	0.171; 0.277	<0.001	
	Pack years (only dCOT3)					
	0			1	1	
	(0–5]	1.141	1.177	0.828; 1.573	0.418	
	(5–10]	1.187	1.392	0.620; 2.274	0.604	
	>10	0.503	1.418	0.253; 0.997	0.049	

[a] Adjusted for sex, age and BMI.

(17–24 h), obtaining similar results. Further studies should be conducted to explore the sustained effect of nicotine on sACE2. Other potential limitation was to assume half of the limit of quantification for the samples with concentration below this limit (0.1% for sACE2, 30.6% for nicotine and 1.8% for cotinine). However, we have performed a sensitivity analysis without this data and only the nicotine concentration changes (increasing), but the correlation and trend do not change.

On the other hand, two analytical limitations must be considered. Firstly, the sensitivity of the method to determine sACE2 just allows the detection of amounts of protein above 1 ng, limiting the quantification of isoforms below this concentration. Secondly, quantification by Western blot requires considering some important parameters that favor reproducibility, like buffer temperature, small variations or the level of acrylamide polymerization. Thus, a study like ours, even though revealing much more information than routine clinical methods, should be carried out in well-trained molecular biology specialized laboratories.

As far as the strengths of this study are concerned, the sample size is large since we have a good number of saliva samples which have been properly stored at −80 °C, thus reducing the degradation of the samples. Also, epidemiological data were available for all the subjects, useful to explore the associations between sACE2 concentrations and covariates. Regarding the determination of sACE2, the majority of sACE2 studies were done analyzing ACE2 catalytic activity using fluorometric methods (Wysocki et al., 2010). These methods have two critical limitations that could only be overpassed with our detection approach. First, they rely on the preservation of the activity on the samples, that sometimes may be lost after long periods of freezing. And second, they do not detect truncated inactive isoforms that still could have a role in some physiological processes. In this article, we have validated the determination of several isoforms of sACE2 even in biological fluids collected long time ago. Also, we detected for the first time the presence of several isoforms of sACE2 in saliva, indicating that this fluid has similar species of sACE2 than other corporal fluids, like plasma (García-Ayllón et al., 2021) or urine (Gutta et al., 2018) (Lew et al., 2006). Furthermore, we have detected many minority bands that exhibit slight differences in their molecular weight, probably due to the high proteolytic activity in saliva (Katsani and Sakellari, 2019), although we cannot rule out the effect of post-translational modifications on the migration pattern of the protein (Gong et al., 2021). The ACE2 immunoreactivity bands larger than 130 kDa, could be the highly glycosylated isoforms (Gong et al., 2021), although further studies are needed to elucidate the origin of this larger species.

5. Conclusion

Salivary nicotine concentration seems to have a limited association on the concentration of sACE2. Further research should be conducted to fully understand the mechanisms through which nicotine may play a role on the pathogenesis of COVID-19.

Table 4
Spearman's rank correlation coefficients between salivary nicotine and cotinine concentrations, pack-years, relative sACE2 concentration and all the sACE2 isoforms.

	Nicotine	Cotinine	Pack-years	Relative sACE2 concentration
Nicotine	1			
Cotinine	0.877 *	1		
Pack-years	0.222 [a]	0.235 [a]	1	
Relative sACE2 concentration	0.153 *	0.132 [a]	−0.057	1
Isoform 20 KDa (ng/μl)	0.043	0.037	NA	0.051
Isoform 34 KDa (ng/μl)	0.037	0.048	NA	0.028
Isoform 37 KDa (ng/μl)	0.05	0.03	NA	0.064
Isoform 40 KDa (ng/μl)	0.193 *	0.140 **	−0.056	0.110
Isofor 45 KDa (ng/μl)	−0.001	0.001	0.1	0.044
Isoform 50 KDa (ng/μl)	−0.064	−0.092	0.052	0.074
Isoform 55 KDa (ng/μl)	0.069	0.06	−0.06	0.392 *
Isoform 57 KDa (ng/μl)	0.107	0.081	NA	0.097
Isoform 60 KDa (ng/μl)	0.025	0.003	−0.011	0.264 *
Isoform 65 KDa (ng/μl)	0.091	0.06	−0.007	0.23 *
Isoform 70 KDa (ng/μl)	0.087	0.051	0.044	0.050
Isoform 73 KDa (ng/μl)	0.097	0.101	-	0.087
Isoform 75 KDa (ng/μl)	0.05	0.056	−0.041	0.159 *
Isoform 80 KDa (ng/μl)	−0.048	−0.063	−0.07	0.114
Isoform 87 KDa (ng/μl)	0.035	0.025	−0.099	0.038
Isoform 100 KDa (ng/μl)	−0.001	−0.027	-	−0.068
Isoform 110 KDa (ng/μl)	−0.042	−0.021	-	−0.003
Isoform 120 KDa (ng/μl)	−0.007	−0.006	-	−0.023
Isoform 125 KDa (ng/μl)	−0.042	−0.047	0.018	0.039
Isoform 145 KDa (ng/μl)	−0.042	−0.047	-	0.016
Isoform 150 KDa (ng/μl)	−0.026	−0.035	−0.041	0.031
Isoform 160 KDa (ng/μl)	−0.042	−0.018	-	0.019
Isoform 175 KDa (ng/μl)	0.065	0.041	0.005	−0.013
Isoform 200 KDa (ng/μl)	−0.027	−0.037	0.031	0.055
Isoform 250 KDa (ng/μl)	0.019	0.018	−0.104	0.074
Isoform 260 KDa (ng/μl)	0.076	0.051	-	0.044

All p-values have been adjusted for Bonferroni correction. *: p-value < 0.05. **: p-value < 0.01.
[a] p-value < 0.001. Pack-years were estimated only for dCOT3 participants.

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Authors contributions

JC and JMMS conceived the study. SM and JC developed the analytical methods to determinate and quantify the ACE2. RC and JMML performed the determinations of ACE2 with the supervision of SM and JC. RPO and JAP developed the analytical methods to determinate and quantify the nicotine and cotinine and performed the determinations. CL and HPM prepared the database and analysed the data. MF, MB, EF, and JMMS contributed in the design and coordination of the two previous studies used in this work. SB, AGM and CLM drafted the first manuscript with the supervision of JC and JMMS. All authors contributed substantially to the interpretation of the data and the successive versions of the manuscript. All authors contributed to the manuscript and approved its final version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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