

DETERMINATION OF SOLUBLE ANGIOTENSIN-CONVERTING ENZYME 2 **IN SALIVA SAMPLES AND ITS ASSOCIATION WITH NICOTINE**

Samuel Bru^{a*}, Adrián González-Marrón^{b*}, Cristina Lidón-Moyano^{b*}, Reyes Carballar^a,
 Joan Marc Martínez-Láinez^a, Hipólito Pérez-Martín^b, Marcela Fu^{c,d,e,f}, Raúl Pérez-
 Ortuño^g, Montse Ballbè^{c,d,f,i}, Jose A Pascual^{g,j**}, Esteve Fernández^{c,d,e,f**}, Josep
 Clotet^{a**}, Jose M Martínez-Sánchez^{b**}

a. Department of Basic Sciences, Universitat Internacional de Catalunya, 08195 Sant Cugat del Vallès, Barcelona, Spain.

b. Group of Evaluation of Health Determinants and Health Policies, Department of Basic Sciences, Universitat Internacional de Catalunya, Sant Cugat del Vallès, Barcelona, Spain.

c. Tobacco Control Research Group, Epidemiology, public health, cancer prevention and palliative care program, Institut d'Investigació Biomèdica de Bellvitge - IDIBELL, L'Hospitalet de Llobregat, Spain

d. Cancer Prevention and Control Group, Institut d'Investigació Biomèdica de Bellvitge - IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain.

e. School of Medicine and Health Sciences, Universitat de Barcelona, Barcelona, Spain.

f. Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES), Madrid, España.

g. Group of Integrated Pharmacology and Neurosciences. IMIM_Hospital del Mar Medical Research Institute, Barcelona Biomedical Research Park (PRBB), Barcelona, Spain.

h. Group of Integrative Pharmacology and Systems Neuroscience, Neurosciences Programme, IMIM (Hospital del Mar Medical Research Institute), Parc de Recerca Biomèdica de Barcelona, Barcelona, Spain.

i. Addictions Unit, Institute of Neurosciences, Hospital Clínic de Barcelona, Barcelona, Spain.

j. Department of Medicine and Life Sciences, Universitat Pompeu Fabra, Barcelona Biomedical Research Park, Barcelona, Spain.

* *Equally contribution*

** *Equally last author*

Corresponding authors

Josep Clotet & Jose M. Martínez Sanchez

Department of Basic Sciences

Universitat Internacional de Catalunya

Carrer de Josep Trueta s/n

08195 Sant Cugat del Vallès (Barcelona)

E-mail: jclotet@uic.es, jmmartinez@uic.es

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 40KDa ($r_s=0.193$ and $r_s=0.140$, respectively).

Conclusion

60 Salivary nicotine concentration seems to be limitedly associated with the concentration
61 of sACE2.

62 **Keywords**

63 Angiotensin-Converting Enzyme 2; cotinine; COVID-19; nicotine.

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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[1].

The Angiotensin-Converting Enzyme 2 (ACE2), the main receptor for the SARS-CoV-2 infection of human cells [2][3], is an integral membrane glycoprotein that contains a single extracellular catalytic domain [4][5]. ACE2 acts as a negative regulator of the renin-angiotensin system (RAS), maintaining homeostasis and protecting organs from vasoconstriction, fibrosis, and thrombosis [6][7][8]. ACE2 is widely expressed in different organs like the heart, lungs, or kidneys [9][10] and in the oral cavity and salivary glands [11][12].

ACE2 is mainly bound to the cell membrane, but it is also present as a soluble form (sACE2) in extracellular fluids [13]. 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 [14]. 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 [15][16]. On the other hand, high sACE2 circulating levels could act as a neutralizing factor against the infection by SARS-CoV-2 [17]. Accordingly, recombinant ACE2 as a treatment for COVID-19 is being investigated [18]. 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 [19]. Thus, nicotine, as the main tobacco alkaloid of cigarettes, has been shown to upregulate the expression of ACE2 through $\alpha 7$ -nAChR (nicotinic cholinergic) receptors [20][21]. Although most published studies are aligned with this finding, other works describe an

inverse effect, triggering a homeostasis disequilibrium of the RAS [22]. 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 [23][24], there are discrepant results concerning the risk of infection. While a significantly higher prevalence of smoking among COVID-19 patients has been reported [25], claims of the protection of smoking against the COVID-19 have also been raised [26].

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 [27]. 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.

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)[28]. 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 TSNA). 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 [29]. 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 [28][29]. 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).

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 hours) [30]. 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 [31]. In order to determine nicotine and cotinine concentrations, we analyzed salivary samples employing a common protocol in 2013-2014 [32]. 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.

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 125mM 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 (1:1,000) of ACE2 antibody (66699-1-1G, 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 taking 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 [33]. The limit of detection of this method was 1ng 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.

Self-reported variables "

According to self-reported information in the study on tobacco use [28], 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 [29], 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[28][29] 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/m², b) between 20 and 25 kg/m², c) between 25 and 30 kg/m², and d) between 30 and 60 kg/m².

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.

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) (Figure 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 40KDa ($r_s=0.193$ 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.

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 stratified to smoking status we do not found statistically significant differences of sACE2 GM according to cotinine and nicotine levels. This could be partially due because the sample size was reduced among categories and reduce the statistically potential. 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-CoV-2 and avoid epithelial fusions [17]. 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 [34]. 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 [35,36]. However, other authors have hypothesized that COVID-19 susceptibility in elderly can be related to the increase of ACE2 expression [37,38].

Other studies have reported differences in ACE2 concentration according to BMI or age, being higher for older individuals and BMI [37]. 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 remarkably higher sACE2 concentrations) could be partially due because 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 frequent men, were younger, and with lower BMI than smokers and non-smokers (data not shown).

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 [34], 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 [39] 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 [40]. Moreover, saliva

is a protein enriched tissue, containing approximately 2,000 unique proteins including secretions from the salivary gland, proteins coming from blood or factors released by epithelial cells [41], used for the diagnosis of several pathologies like cancer or infectious diseases [42].

There are several articles supporting the induction of ACE2 expression on small airway epithelia under nicotine exposure [43]. 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 variant [44]. Considering that saliva is a protease enriched fluid [45], 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 $\alpha 7$ -nAChR receptor in airway epithelial cells [46]. 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 [47].

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 [48]. SARS-CoV-2 infection downregulates ACE2 receptor expression, triggering the severe acute respiratory distress syndrome (ARDS) or severe acute respiratory syndrome (SARS) [49]. Thus, this is the main reason why ACE2 has been pointed as a potential target for COVID-19 treatment, using different approaches [50]. As an example, treatments based on ATBlockers/ACEI have been shown to promote ACE2 expression increase while

improving COVID-19 patients' recovery [51,52]. 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 ug/ml vs 0.178 ug/ml), could indicate that nicotine might play a role against COVID-19 disease. Besides the increase of ACE2 expression, on the one hand, nicotine promotes an anti-inflammatory response by inhibiting the pro-inflammatory cytokine- involved in cytokine storm- expression [53,54]. On the other hand, nicotine is involved in $\alpha 7$ -nAChR blockage, described as a possible epithelial cell entry point for SARS-CoV-2 [55]. Finally, it is worthy to highlight that different clinical trials have been carried out ([NCT04429815](#), NCT04583410) to empirically demonstrate 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 [56]. Another approach that has been studied for COVID-19 treatment consists on the administration of recombinant ACE2 [57]. Also, to improve treatment efficiency, ACE2 mutant variants have been engineered, to increase receptor affinity for spike protein while maintaining catalytic activity [58]. 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* [59]. 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[60]. 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 ug/ml after 36 hours. 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.

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 (17-24 hours), 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 analyze (without this data) and only change the nicotine (with high value) 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 [61]. 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 [62] or urine [63] [64]. Furthermore, we have detected many minority bands that exhibit slight differences in their molecular weight, probably due to the high proteolytic activity in saliva [42], although we cannot rule out the effect of post-translational modifications on the migration pattern of the protein [65]. The ACE2 immunoreactivity bands larger than 130 kDa, could be the highly glycosylated isoforms [65], although further studies are needed to elucidate the origin of this larger species.

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.

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DECLARATION OF INTERESTS

Authors declare that they have no conflicts of interest.

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

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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 [4.85; 9.636]		0.247 [0.217; 0.280]		0.160 [0.143; 0.18]	
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 [7.11; 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; 24.04]		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 [0.47; 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; 0.54]		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 [0.23; 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; 441.2]		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 [0.13; 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 ** Kruskal Wallis

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	NaN [NaN; NaN]	
	>50	1	0.127 [NA; NA]	
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]	

CI: Confidence interval; GM: Geometric mean.

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

		Beta	SE	95% CI	p-value	R ²
Model 1 [^]						0.023
	Constant	0.233	1.103	0.193;0.283	<0.001	
	Nicotine level (for each 100ng/mL)	1.037	1.017	1.004;1.071	0.022	
Model 2 [^]						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 [^]						0.020
	Constant	0.239	1.104	0.197;0.291	<0.001	
	Cotinine level (for each 100ng/mL)	1.037	1.022	0.993 ;1.083	0.021	
Model 4 [^]						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	0.05	
	>50	1.600	1.107	1.311;1.953	0.001	
Model 5 [^]						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	1.15 2	1.52;2.725	<0.001	
Model 6^						0.02 8
	Constant	0.352	1.28 2	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	1.24 4	0.663;1.565	0.94	
	Dual users with conventional cigarettes	0.959	1.26 9	0.599;1.534	0.8	
Model 7^						0.00 8
	Constant	0.263	1.20 9	0.181;0.383	<0.001	
	Pack years (only dCOT3)	0.983	1.01 6	0.952;1.015	0.289	
Model 8^						0.00 9
	Constant	0.217	1.13 1	0.171;0.277	<0.001	
	Pack years (only dCOT3)					
	0			1	1	
	(0-5]	1.141	1.17 7	0.828;1.573	0.418	
	(5-10]	1.187	1.39 2	0.620;2.274	0.604	
	>10	0.503	1.41 8	0.253;0.997	0.049	

^ Adjusted for sex, age and BMI

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 *	0.877 *	0.222	0.153 *
Cotinine	0.877 *	1 *	0.235	0.132 ***
Pack-years	0.222 ***	0.235 ***	1 *	-0.057
Relative sACE2 concentration	0.153 *	0.132 ***	-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	0.11
Isoform 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	0.05
Isoform 73 KDa (ng/μl)	0.097	0.101	NA	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	NA	-0.068
Isoform 110 KDa (ng/μl)	-0.042	-0.021	NA	-0.003
Isoform 120 KDa (ng/μl)	-0.007	-0.006	NA	-0.023
Isoform 125 KDa (ng/μl)	-0.042	-0.047	0.018	0.039
Isoform 145 KDa (ng/μl)	-0.042	-0.047	NA	0.016
Isoform 150 KDa (ng/μl)	-0.026	-0.035	-0.041	0.031
Isoform 160 KDa (ng/μl)	-0.042	-0.018	NA	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	NA	0.044

All p-values has been adjusted by Bonferroni correction. *: p-value < 0.05. **: p-value < 0.01

***: p-value < 0.001. Pack-years were estimated only for dCOT3 participants

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

