Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Epigenetic mechanisms and host factors impact *ACE2* gene expression: Implications in COVID-19 susceptibility

González Daniel [a], Ayala-Ramírez Paola [a], Gelvez Nancy [a], Suarez-Obando Fernando [a], Ariza Beatriz [b], Ruiz Zulema [b], Arcila Julieth [b], Cardozo Claudia [b], Rojas Adriana [a,*]

[a] Institute of Human Genetics, School of Medicine, Pontificia Universidad Javeriana, Bogotá, Colombia
[b] Clinical Laboratory, Hospital Universitario San Ignacio, Bogotá, Colombia

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ABSTRACT

Background: The *ACE2* protein acts as a gateway for SARS-CoV-2 in the host cell, playing an essential role in susceptibility to infection by this virus. Genetics and epigenetic mechanisms related to the *ACE2* gene are associated with changes in its expression and, therefore, linked to increased susceptibility to infection. Although some variables such as sex, age, and obesity have been described as risk factors for COVID-19, the molecular causes involved in the disease susceptibility are still unknown.

Aim: To evaluate the *ACE2* gene expression profiles and their association with epigenetic mechanisms and demographic or clinical variables.

Methods: In 500 adult volunteers, the mRNA expression levels of the *ACE2* gene in nasopharyngeal swab samples and its methylation status in peripheral blood samples were quantified by RT-qPCR and qMSP, respectively. The existence of significant differences in the *ACE2* gene expression and its determinants were evaluated in different study groups according to several demographic or clinical variables such as sex, age, body mass index (BMI), smoking, SARS-CoV-2 infection, and presence of underlying diseases such as type II diabetes mellitus (DM2), asthma and arterial hypertension (AHT).

Results: Our results show that *ACE2* gene overexpression, directly involved in susceptibility to SARS-CoV-2 infection, depends on multiple host factors such as male sex, age over 30 years, smoking, the presence of obesity, and DM2. Likewise, it was determined that the *ACE2* gene expression is regulated by changes in the DNA methylation patterns in its promoter region.

Conclusions: The *ACE2* gene expression is highly variable, and this variability is related to habits such as smoking and demographic or clinical variables, which details the impact of environmental and host factors on our epigenome and, therefore, in susceptibility to SARS-CoV-2 infection.

1. Introduction

Coronavirus disease 2019 (COVID-19) is a severe acute respiratory infection caused by the SARS-CoV-2 virus. In March 2022, two years after being declared a pandemic, more than six million deaths and 482 million confirmed cases have been reported worldwide (World Health Organization, 2022). More than six million cases have been reported in Colombia, with a mortality rate near 2.5% (Instituto Nacional de Salud, 2021).

The COVID-19 clinical presentation has proven to be highly variable between different populations, with mild courses of the disease up to some severe cases where it can cause death. Although susceptibility to COVID-19 has been related to age, sex, immune response, genetic variants, and the presence of comorbidities, among others (Mahamat-Saleh et al., 2021; Parasher, 2021), there is still a lack of knowledge about the molecular basis behind the susceptibility to SARS-CoV-2 infection.

The SARS-CoV-2 virus enters to the host cell through the interaction of its Spike or S protein with the *ACE2* receptors of the host cell (Hoffmann et al., 2020; Ni et al., 2020). Therefore, the *ACE2* gene is a critical protein in susceptibility to SARS-CoV-2 infection, acting as its gateway to the host cell.

The *ACE2* gene encodes the angiotensin-converting enzyme 2 (*ACE2*), a highly expressed protein in the upper and lower respiratory tract cells, which function under physiological conditions is to

* Corresponding author at: Institute of Human Genetics, School of Medicine, Pontificia Universidad Javeriana, Carrera 7 No. 40-62, Bogotá 110231, Colombia.
E-mail address: rojas-adriana@javeriana.edu.co (R. Adriana).

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counteract the Renin-Angiotensin System (RAS) and reduce blood pressure by catalyzing the hydrolysis of AT2 (a vasoconstrictor peptide) into AT1–7 and AT1–9 (vasodilators) (Datta et al., 2020; Han et al., 2021a; Tan et al., 2020). In recent in vitro experiments, it has been shown that a higher expression of the *ACE2* gene is associated with a higher risk of SARS-CoV-2 infection (Corley and Ndhlovu, 2020; Devaux et al., 2020; Han et al., 2021b; Hou et al., 2020).

It is known that susceptibility to diseases and certain viral infections can be influenced by certain genetic or epigenetic factors specific to everyone (Ghafouri-Fard et al., 2020). Epigenetics is the science that studies reversible changes in the expression of specific genes without altering the DNA sequence. This regulation occurs in response to environmental stimuli and is mediated by a series of chemical changes that alter accessibility to chromatin, among which DNA methylation stands out. When the aberrant expression occurs, it is usually caused by abnormal changes in its epigenetic regulation (David Allis et al., 2015).

In this sense, there is great interest in evaluating the *ACE2* gene expression dynamics, knowing those epigenetic mechanisms responsible for controlling the expression of the *ACE2* gene and whether host variables increase its expression and thus could be linked to greater susceptibility to infection (Wu et al., 2021). Therefore, this research aimed to determine the *ACE2* gene expression profiles, assess whether there are associations with the host's different demographic and clinical variables, and finally, to know the epigenetic mechanism responsible for its regulation.

2. Methods

2.1. Samples and data collection

A total of five hundred adult volunteers from Bogotá city were included, without any exclusion criteria, through non-probabilistic convenience sampling. The subjects were invited to participate voluntarily at the time of taking the occupational screening test for COVID-19 at the facilities of the Pontificia Universidad Javeriana, where they were informed about the object of the investigation, risks, and data protection. After written and signed informed consent was obtained nasopharyngeal swab and peripheral blood in EDTA samples were taken. The inclusion and sampling period was conducted between February 10, 2021, and March 17, 2021.

The nasopharyngeal swab samples were collected from all subjects following the CDC guidelines as described in Suppl. Methods. Likewise, the peripheral blood samples were taken before taking the swab to reduce risks of exposure by trained health professionals from the clinical laboratory of the Hospital Universitario San Ignacio in EDTA tubes.

A questionnaire was applied to all the volunteers, which inquired about different demographic variables of the subject and their relevant clinical records such as sex, age, occupation, blood group, weight, height, smoking, SARS-CoV-2 infection and symptoms, and autoreferred presence of underlying diseases as DM2, asthma, AHT, myocardial infarction, peripheral vascular disease, stroke, chronic kidney disease, dyslipidemia, cirrhosis, hepatitis, gastroesophageal reflux, neoplasia, leukemia, connective tissue diseases, HIV, COPD, obstructive sleep apnea, pulmonary hypertension, and depression. Study data were collected and managed using REDCap (Research Electronic Data Capture), a tool hosted at Hospital Universitario San Ignacio (Harris et al., 2019, 2009), with restricted access to researchers, ensuring their security and confidentiality for purely academic use and under the current data protection and treatment policies. This research was performed under the Colombian Ministry of Health Guidelines (008430–1993) and approved by the Pontificia Universidad Javeriana School of Medicine Ethics Committee (FM-CIE-1171-20).

2.2. RNA extraction and quantitative reverse transcription PCR [RT-qPCR]

RNA extraction was performed using the GeneJET Viral DNA and RNA Purification Kit (Thermo Fisher, #K0821) by trained personnel from the Hospital Universitario San Ignacio clinical laboratory, following the biosafety protocols for COVID-19 samples.

A diagnostic test for COVID-19 was performed to determine the presence of SARS-CoV-2 infection at the time of recruitment using the VIASURE SARS-CoV-2 Real-Time PCR Detection Kit (CerTest Biotec, #VS-NCO212L) following the manufacturer's indications in the Cobas z 480 analyzer (Roche).

To determine the mRNA expression profiles of the *ACE2* gene in the evaluated population, an RT-qPCR was performed with the specific TaqMan probes and primers detailed in the Supplementary Table 2. All RT-qPCR assays were run in the Cobas z 480 analyzer (Roche) employing the LightCycler® Multiplex RNA Virus Master kit (Roche, #06754155001). Results were analyzed through the ΔCt method for relative quantification using 18S as a reference gene (housekeeping). Since a lower ΔCt indicates a minor difference with a high-expression gene, therefore, a higher expression level, the Y-axis in the mRNA expression graphs is inverted for a more intuitive interpretation. The expression results are graphed as the median and 95% CI.

2.3. DNA methylation analysis

Genomic DNA extraction was performed using GenElute Blood Genomic DNA kit (Sigma-Aldrich, NA2020-1KT), as described in Supplementary Methods. Quantitative methylation-specific PCR (qMSP) analysis was performed to evaluate the methylation percentage of the *ACE2* gene promoter. DNA was first treated with sodium bisulfite using 500 ng of the extracted DNA using the EZ DNA Methylation kit (Zymo Research, #D5002) following the manufacturer's instructions. Subsequently, the methylation level was quantitatively evaluated by qPCR of the bisulfite-treated DNA, using primers specifically targeted to the methylated sequence of the *ACE2* gene promoter created using the MethPrimer 2.0 tool as detailed in Supplementary Methods and which sequences are provided in the Supplementary Table 2 (Li and Dahiya, 2002). QMSP methylation analyzes were performed on 10% of the total population ($n = 50$), who were selected by partitioning the expression data and taking the highest and lowest 5% of *ACE2* expression data. The qMSP assays were validated using the methylated (M) and unmethylated (N) set of controls, Human Methylated & Non-methylated DNA (Zymo Research, #D5014). CT data was normalized and analyzed as described by Li et al. in 2013 (Li et al., 2013).

2.4. Statistical analysis

Before analyzing the mRNA expression data, a Shapiro-Wilk test was performed to determine the *ACE2* mRNA expression levels (ΔCt) distribution, obtaining that the *ACE2* mRNA expression levels do not follow a normal distribution. Therefore, only non-parametric tests were employed for all analyses. Most expression analyses were made by matching each subject of the compared groups with the closest subject in the parameters of sex, age, BMI and excluded patients with all types of comorbidities or any smoking history (Supplementary Table 1). These matching and exclusion criteria were applied to analyze data to avoid possible bias and confounding variables since a difference in *ACE2* expression by sex, age, BMI, smoking, and comorbidities presence has been reported (Al Heialy et al., 2020; Peng et al., 2021; Pinto et al., 2020; Stelzig et al., 2020).

Two main tests were applied to determine statistically significant differences in *ACE2* mRNA expression: the non-parametric Mann-Whitney *U* test to compare two independent groups and the Kruskal-Wallis test when comparing three independent groups or more. The Spearman correlation test was performed for each variable to assess the

correlation between the age, BMI, and *ACE2* mRNA expression and binomial, multinomial and stepwise logistic regression analysis to identify the determining factors to present *ACE2* gene overexpression in all five hundred subjects.

For all logistic regression analyses, the outcome variable "High *ACE2* gene expression" was defined as the top 25% of expression data (lowest ΔCt data), distributing the expression data by quartiles (Q1-Q2). On the other hand, independent variables were defined as follows: Sex (Male/Female); Age groups (18–29, 30–49, 50–64, 64–71 years); Smoking (Smoker (has consumed >100 cigarettes in his life, the last one was <6 months ago)/Non-smoker (has never smoked)); BMI (Normal weight [18.5–24.9 kg/m^2]/Overweight [25–29.9 kg/m^2]/Obesity [≥30 kg/m^2]); DM2 (Yes/No); Asthma (Yes/No); AHT (Yes/No).

All statistical analyses and variable associations were performed in the Stata Statistical Software: Release 16 (StataCorp LLC.). All graphs were made using GraphPad Prism version 8.01 (GraphPad Software).

3. Results

A total of 500 volunteers were analyzed in this study. Table 1 describes study subjects with their demographic and relevant clinical variables such as sex, age, BMI, smoking, SARS-CoV-2 infection, and presence of underlying diseases such as asthma, DM2, and AHT.

3.1. Age and sex-related differences in ACE2 expression profiles

RT-qPCR assays were performed to assess the *ACE2* gene expression. Although all subjects express the *ACE2* gene, there is high variability in their gene expression profiles throughout the population (Fig. 1A). When dividing the expression by quartiles, 25% of the highest expression data was characterized as the group with high *ACE2* gene expression; likewise, the 25% with the lowest expression data was defined as the group with low *ACE2* expression in the evaluated sample.

ACE2 gene expression was evaluated divided by sex to identify the causes behind this gene expression variability. After comparing 184 matched subjects (Table 2), statistically significant differences in the expression of the *ACE2* gene between female and male subjects were found, being higher in men (Fig. 1B).

Regarding age, when comparing the *ACE2* gene expression profiles by age groups consistent with those defined and used across CDC COVID-19 surveillance pages, it was observed that subjects within 30–49 years have higher *ACE2* gene expression ($p = 0.000191$) compared with subjects within the 18–30 years age range (Fig. 2A). Similarly, when comparing the age distribution between subjects with high and low *ACE2* expression, it was observed that those subjects with high expression have a higher median age, about ten years older, thus associating high *ACE2* expression with individuals older than 30 years (Fig. 2B). Finally, after Spearman's rank correlation coefficient analysis between age and *ACE2* expression, a correlation was found that, although low ($r = -0.162$), is statistically significant ($p = 0.0003$) (Fig. 2C).

These results show a relationship between *ACE2* overexpression and demographic variables, such as sex and age.

Table 1
Description of the studied population. This study included a total of 500 voluntaries of both sex and age ranging from 18 to 71 years and recompiled the most relevant features of its medical record. Absolute and relative frequency are shown for each variable.

Variable	N (%)
Sex	
Female	296 (59.2%)
Male	204 (40.8%)
Age	
18–29 years	184 (36.8%)
30–49 years	224 (44.8%)
50–64 years	85 (17.0%)
65–71 years	7 (1.4%)
Body Mass Index (BMI)	
Normal weight (18.5–24.9 kg/m^2)	289 (57.8%)
Overweight (25–29.9 kg/m^2)	169 (33.8%)
Obesity (≥30 kg/m^2)	42 (8.4%)
Comorbidities	
Asthma	23 (4.6%)
Arterial hypertension	16 (3.2%)
Diabetes	7 (1.4%)
Smoking	
Smoker	61 (12.2%)
Former	84 (16.8%)
Passive	4 (0.8%)
COVID-19	
Positive	67 (13.4%)
Vaccinated	116 (23.2%)

3.2. ACE2 overexpression is related to habits

A comparison was made to identify whether smoking and BMI influence *ACE2* gene expression. After comparing 62 matched subjects (Table 2), significantly higher *ACE2* gene expression in smokers than non-smokers were found (Fig. 3).

On the other hand, differences were observed when contrasting the *ACE2* gene expression in the different BMI categories such as normal weight (BMI 18.5–24.9 kg/m^2), overweight (BMI 25–29.9 kg/m^2), and obesity (BMI ≥30 kg/m^2) in the entire population, observing an upward trend as ascends the BMI category, specifically, there is a statistically significant higher expression in subjects with obesity when compared to those with normal weight ($p = 0.007434$) and overweight (0.007120) (Fig. 4A). Likewise, after comparing 36 matched subjects (Table 2), an *ACE2* gene overexpression was statistically significant in obese subjects compared with those within the normal weight BMI (Fig. 4B). Supporting these results, after comparing the BMI distribution of subjects in the different *ACE2* expression groups, BMI differences are observed in the *ACE2* high expression group compared with those with *ACE2* low expression. Thus, we found an association between high *ACE2* expression with a higher BMI (Fig. 4C).

Spearman's rank correlation coefficient analysis between BMI and the *ACE2* gene expression was performed to assess this association. This analysis showed a correlation that although is low ($r = -0.124$), is statistically significant ($p = 0.0054$) (Fig. 4D). These results indicate a influence of environmental cues such as smoking and nutritional habits of an individual and its *ACE2* expression levels, thus influencing SARS-CoV-2 infection susceptibility.

3.3. ACE2 gene expression changes in patients with comorbidities

The population with comorbidities has been of great interest due to its particular risk evidenced during the COVID-19 pandemic; their *ACE2* gene expression was evaluated separately to identify whether some medical conditions are associated with a different *ACE2* gene expression profile. Following a comparison of fourteen matched subjects (Table 2), a higher *ACE2* gene expression in individuals with type II diabetes mellitus (DM2) compared with matched subjects without the disease was found (Fig. 5A).

In contrast, no significant differences were found in 12 individuals with asthma (Table 2) (Fig. 5B). Similarly, no significant differences in *ACE2* gene expression were found comparing 15 individuals with diagnosed arterial hypertension (AHT) against normotensive matched subjects (Fig. 5C, Table 2). Subsequently, we analyzed the AHT individuals according to the treatment type. Our results show a statistically significant higher *ACE2* gene expression in the hypertensive patient's group treated with ACEIs instead of other treatment types (Fig. 5D, Table 2).

These results evidence how certain medical conditions and clinical conduct in hypertensive are associated with specific *ACE2* gene expression profiles, thus probably representing one factor within the

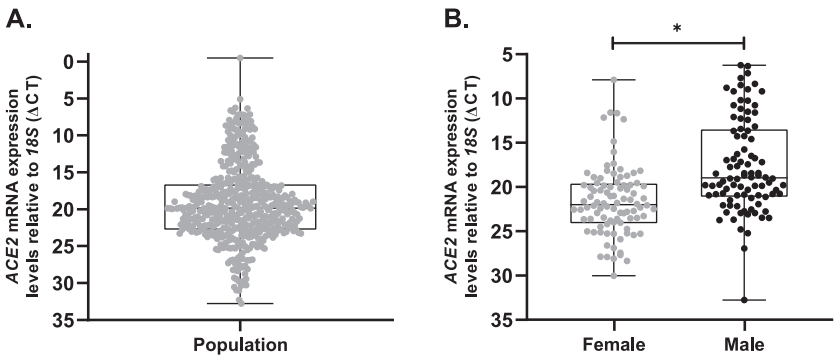

Fig. 1. *ACE2* gene expression levels differ by sex (A.) *ACE2* mRNA expression levels across the studied population ($n = 500$). (B.) Comparison of *ACE2* mRNA expression levels by sex. Patients with comorbidities or smoking history were excluded from the analysis and were matched by age and BMI to avoid bias. Data is presented as median and 95% CI of each group, and significance *p*-values are showed as * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

Table 2
Summary of *ACE2* differential expression analysis. Some demographic and medical variables were assessed to seek for differences in the *ACE2* mRNA expression levels in those groups. For most analyses subjects were matched by age, sex, and BMI to avoid bias. Patients with comorbidities or smoking history were excluded from all except their respective analysis. Significance (S) is defined by the respective adjusted p-value of each analysis.

Variable	*ACE2* mRNA expression relative to 18S (ΔCT)				
	N	Median	Min	Max	p-value
Sex					
Female	92	21.99	7.90	30.01	**0.0000**
Male	92	18.98	6.25	32.76	
Age					
18–29 years	184	20.36	6.75	30.56	**0.0026**
30–49 years	224	19.07	−0.51	32.76	
50–64 years	85	19.00	8.01	30.29	
65–71 years	7	20.76	9.97	29.90	
Smoking					
No	31	21.22	16.72	27.66	**0.0218**
Yes	31	19.08	8.64	29.70	
Body Mass Index (BMI)					
Normal weight (18.5–24.9 kg/m^2)	289	19.85	−0.51	32.76	**0.0386**
Overweight (25–29.9 kg/m^2)	169	19.97	6.35	30.56	
Obesity (≥30 kg/m^2)	42	17.82	6.25	30.33	
Obesity					
No	18	21.03	13.64	28.35	**0.0379**
Yes	18	17.75	6.25	30.33	
Diabetes Type II					
No	7	22.29	20.72	28.19	**0.0175**
Yes	7	15.97	10.63	21.70	
Asthma					
No	12	19.66	7.90	25.32	0.7125
Yes	12	19.79	9.55	27.13	
Arterial hypertension (AHT)					
No	15	18.87	7.16	29.90	0.8195
Yes	15	18.12	9.97	23.59	
AHT treated with ACEIs					
No	4	22.41	16.68	23.18	**0.0433**
Yes	4	12.15	9.97	18.12	

complex mechanisms mediating the higher risk evidenced in this population.

3.4. *ACE2 gene expression profiles in patients with SARS-CoV-2 infection*

An association between *ACE2* gene expression and SARS-CoV-2 infection susceptibility has been reported in several in vitro experiments (Corley and Ndhlovu, 2020; Devaux et al., 2020; Han et al., 2021b; Hou et al., 2020). We evaluated the *ACE2* gene expression in individuals who reported SARS-CoV-2 infection after close contact compared to matched subjects who did not report symptoms or tested positive after close contact (Table 2). Statistically significant differences were found in the *ACE2* gene expression in these groups, being higher *ACE2* expression in those subjects infected by SARS-CoV-2 after close contact (Fig. 6). Despite the sample size limitations in this analysis, these results indicate a relation between expression of the *ACE2* receptor and susceptibility to infection by SARS-CoV-2.

3.5. *Analysis of determining factors for ACE2 gene overexpression*

Logistic regression analysis was performed with some demographic and medical variables in the *ACE2* gene high expression group to identify the factors that increase the probability of *ACE2* gene overexpression. Results from a bivariate analysis remark age between 30 and 64 years and obesity (BMI ≥30 kg/m^2) as variables associated with a high *ACE2* gene expression (Table 3). The same results were found in the multivariate analysis, except for sex, where the adjusted odds ratio also marks the male sex among the variables associated with a high *ACE2* gene expression profile (Table 3). Thus, these factors may influence higher host susceptibility to SARS-CoV-2 infection.

Regarding stepwise analysis, our results show that males have a 1.55-fold more probability of presenting *ACE2* gene overexpression rather than females, and age exhibits an impact on this probability; subjects between 30 and 49 years and 50 to 64 years have a 3.46 and 2.65-fold more probability to overexpress the *ACE2* gene, respectively (Table 4). Moreover, subjects with obesity (BMI ≥30 kg/m^2) have 2.39-fold more chances to present *ACE2* gene overexpression rather than subjects within the normal weight BMI range (Table 4).

These results reinforce those seen previously, demonstrating that factors such as male sex, age over 30 years or obesity influence the *ACE2* gene expression and thus contribute to the complex mechanisms that mediate greater host susceptibility to SARS-CoV-2 infection in these populations.

3.6. *ACE2 gene expression is regulated by DNA methylation*

qMSP assays were performed with specific primers to the *ACE2* gene promoter region to determine if DNA methylation is the epigenetic mechanism responsible for *ACE2* expression regulation. The results obtained evidenced the existence of statistically significant differences ($p \leq 0.0001$) in the levels of methylation of the *ACE2* gene promoter between those subjects with high and low *ACE2* gene expression, thus associating *ACE2* promoter hypomethylation with an increase in its gene expression (Fig. 7A). On the other hand, in those subjects with low *ACE2* gene expression, no significant differences were found with the methylated control ($p = 0.9246$), confirming that promoter hypermethylation determines *ACE2* gene repression (Fig. 7A).

Given the inverse relationship evidenced between promoter

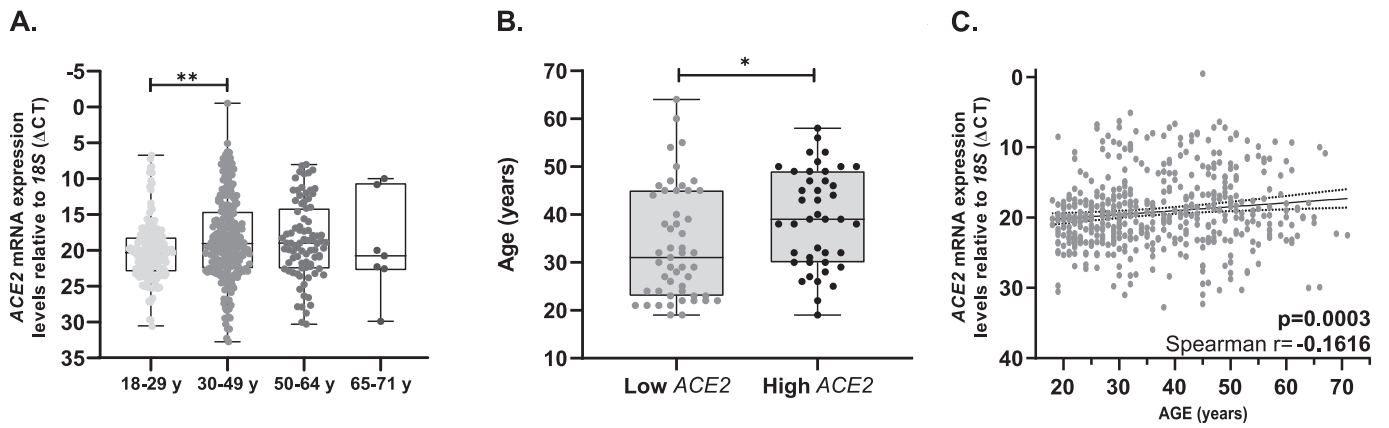

Fig. 2. *ACE2* gene expression levels differ by age. (A.) Comparison of *ACE2* mRNA expression levels by age-groups consistent with those defined and used across CDC COVID-19 surveillance pages. (B.) Age distribution comparison in population with high and low *ACE2* gene expression levels. (C.) Spearman correlation of *ACE2* mRNA expression levels and age. Patients with comorbidities or smoking history were excluded from the analysis to avoid bias. Data is presented as median and 95% CI of each group, and significance p-values are showed as * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

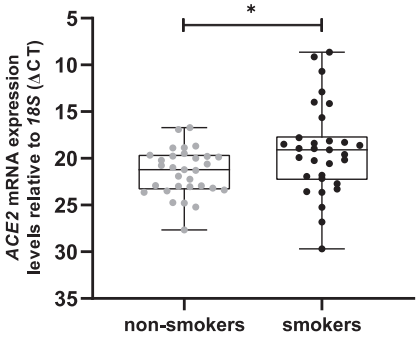

Fig. 3. *ACE2* gene is overexpressed in smokers. Comparison of *ACE2* gene mRNA expression levels in smokers against non-smokers matched by age, sex, and BMI. Patients with comorbidities were excluded from the analysis and to avoid bias. Data is presented as median and 95% CI of each group, and significance p-values are showed as * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

methylation and *ACE2* gene expression, a Spearman correlation was performed between both variables to estimate the strength of this association. As a result, it was found that there is a moderate ($r = -0.5729$) and statistically significant ($p \leq 0.0001$) negative correlation between both variables (Fig. 7B). Simple linear regression analysis between both variables allows us to estimate this relationship since, with each relative methylation unit of the *ACE2* gene promoter, its expression decreases by 9.04 relative expression units, consistent with the repressive role of DNA methylation in gene regulation.

To identify whether *ACE2* gene promoter methylation participates in the previously observed differences in its expression by sex, age, BMI, smoking, and DM2, median analyzes segmented by these variables were performed. As a result, no significant differences were found with any of the above, except by sex, where are differences ($p = 0.0047$) in the *ACE2* promoter relative methylation, with lower levels of methylation in the male sex (Fig. 7C), which is consistent with its higher expression profile seen in previous analyzes compared to females. These results demonstrate that DNA methylation in the *ACE2* gene promoter is an epigenetic mechanism involved in controlling *ACE2* gene expression.

4. Discussion

In this study, we found that the expression of the *ACE2* gene, directly involved in susceptibility to SARS-CoV-2 infection, depends on multiple variables of the individual, such as gender, age, BMI, smoking and presence of comorbidities. Likewise, it was determined that these changes in gene expression are regulated by DNA methylation.

The epigenetic regulation of an organism's genes is variable throughout life and can be influenced by environmental exposures and consumption or eating habits, among others (David Allis et al., 2015; Pinto et al., 2020). These epigenetic changes may alter the expression of genes important in susceptibility to different diseases, thus increasing or decreasing their risk (Ghafouri-Fard et al., 2020).

Susceptibility to certain viral infections is influenced by genetic and epigenetic factors specific to everyone; thus, each person's genome and environment play an essential role in interacting and responding to SARS-CoV-2 infection (Cao et al., 2020; Chen et al., 2020; Chlamydas et al., 2020; Ghafouri-Fard et al., 2020; Pruimboom, 2020). Several in vitro studies have demonstrated that *ACE2* acts as the gateway to SARS-CoV-2 infection; thereby, its higher expression is considered a significant risk factor in the susceptibility to infection (Han et al., 2021a; Hou et al., 2020; Tan et al., 2020).

It has been widely discussed whether sex is involved in the susceptibility or severity of COVID-19, and which is more predisposed to being infected or presenting severe forms of the disease. To date, world statistics indicate that, although the number of cases in males is higher than in females, these differences are not significant. Nevertheless, the male sex has been associated with a risk factor for presenting severe forms of the disease and management in the intensive care unit in several reports (Klein et al., 2020). Accordingly to most studies (Bwire, 2020; Farshbafnadi et al., 2021), the male sex was associated with a higher *ACE2* gene expression. These findings are confirmed by the epigenetic differences in DNA methylation herein evidenced in the promoter of the *ACE2* gene, where males show less methylation of the *ACE2* promoter than females. *ACE2* locus is found among the genes that escape X inactivation, which “per se” can cause women to express more *ACE2* than men; nevertheless, there is evidence of more open chromatin marks in the *ACE2* gene in most male tissues (Chlamydas et al., 2020; Tukiainen et al., 2017). Besides, one in vitro study shows that the 17β-estradiol (E_2), a primarily female sex steroid, can downregulate the *ACE2* gene expression (Stelzig et al., 2020), providing another hypothesis for these sex-based expression differences.

Since the pandemic began, the population aged 60 years and older was reported as one of the central risk populations for SARS-CoV-2 infection, complications, and mortality (Amber L. Mueller et al., 2020; Farshbafnadi et al., 2021). Herein, we report an association between age and *ACE2* gene expression, higher in the population within 30 to 49 years. For all the rest of the age groups we did not find statistically significant differences, which may be the product of the differences in each group size. Nevertheless, it is clear that there is an increasing trend

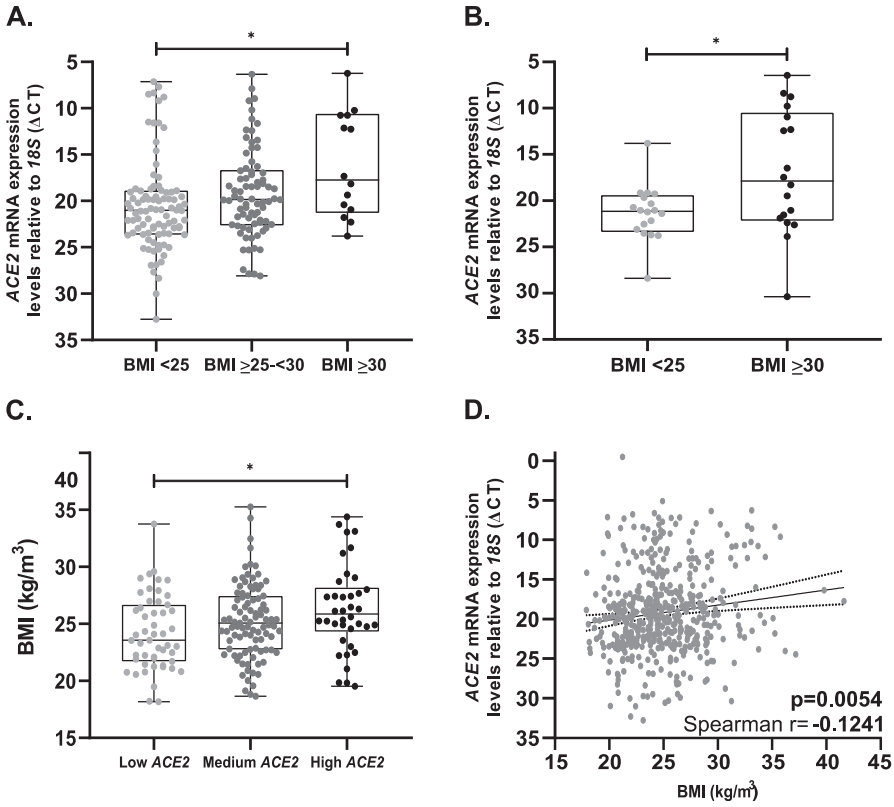

Fig. 4. *ACE2* gene is overexpressed in obese subjects. (A.) Comparison of the mRNA expression levels of *ACE2* gene by BMI category in the entire population and (B.) matched analysis of obesity and normal weight BMI categories. (C.) BMI distribution comparison in population within the different *ACE2* gene expression categories. (D.) Spearman correlation of *ACE2* mRNA expression levels and BMI. Patients with comorbidities or smoking history were excluded from all analysis and were matched by age, sex, and BMI in (B.) to avoid bias. Data is presented as median and 95% CI of each group, and significance p-values are showed as * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

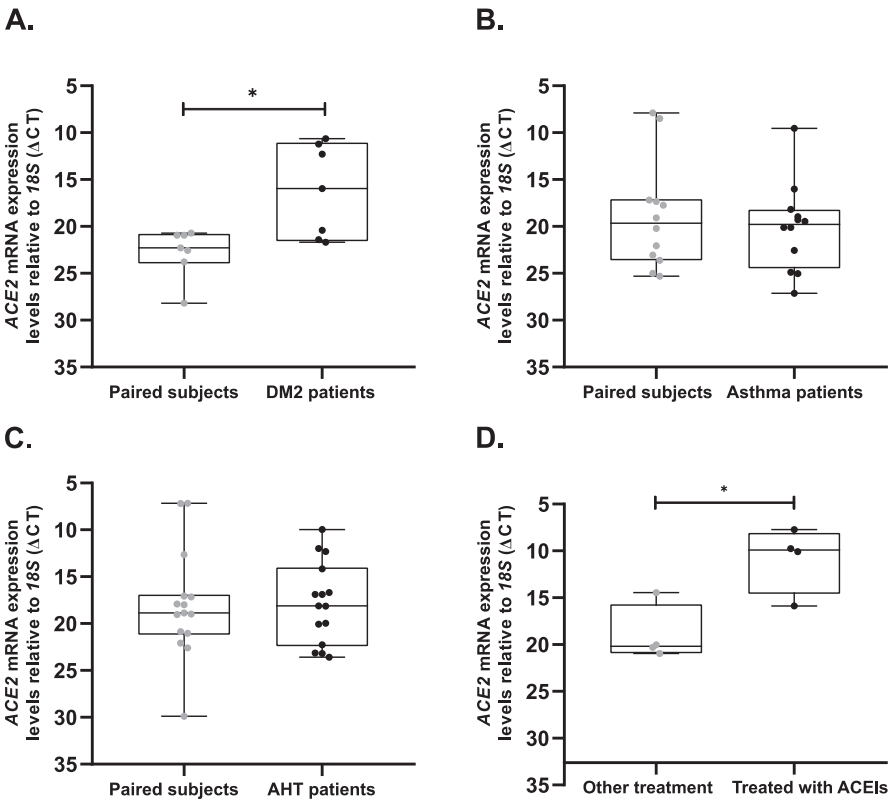

Fig. 5. *ACE2* gene expression profiles associated with certain diseases. (A.) Comparison of *ACE2* gene mRNA expression levels in patients with type II diabetes mellitus, (B.) asthma and (C.) arterial hypertension against matched subjects. (D.) Comparison of *ACE2* gene mRNA expression levels in hypertensive patients by treatment type. Subjects were matched by age, sex, and BMI. Also, patients with comorbidities or smoking history were excluded from the analysis to avoid bias. Data is presented as median and 95% CI of each group, and significance p-values are showed as * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

in *ACE2* expression as age grows, as confirmed by correlation analysis.

Importantly, age-related DNA methylation changes in the *ACE2* promoter have been seen, mediating the observed increases in expression levels in the older population (Farshbafnadi et al., 2021; Pruimboom, 2020). Further, the age-linked severity has been attributed at least partly to the age-associated immune system dysregulation; states

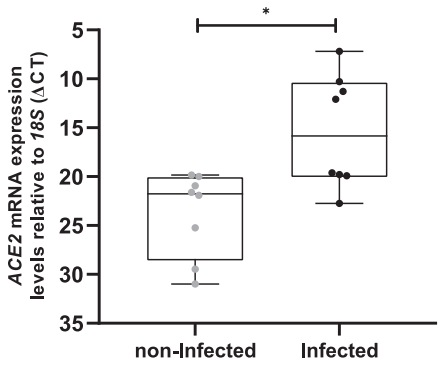

Fig. 6. Differences of *ACE2* gene expression profiles in COVID-19 patients. Comparison of *ACE2* mRNA expression levels in patients infected by SARS-CoV-2 against closely exposed non-infected matched subjects. Patients with comorbidities, vaccinated, or smoking history were excluded from the analysis and were matched by age, sex, and BMI to avoid bias. Data is presented as median and 95% CI of each group, and significance p-values are showed as * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

termed immunosenescence and inflammaging, characterized by a chronic pro-inflammatory response, are more prone to develop a cytokine storm thus severe COVID-19 (Amber L. Mueller et al., 2020; Franceschi et al., 2017).

On the other hand, smoking and obesity (BMI ≥30 kg/m^2) independently increased the *ACE2* gene expression in this research. Although first smokers were thought to have lower levels of *ACE2* (Corley and Ndhlovu, 2020), some recent studies otherwise confirmed that *ACE2* is overexpressed in smokers (Aliee et al., 2020; Cai, 2020; Leung et al., 2020; Pruimboom, 2020), our results being consistent with these latest findings. Cigarette smoke exposure can induce several epigenetic changes, inducing a global hypomethylation, probably due to the smoking-associated oxidative stress which can lead to DNA demethylation, via the established sequential oxidation of which 5hmC is the first step (Ringh et al., 2019). While some specific loci instead tend to hypermethylation (Zong et al., 2019). Thus, the *ACE2* gene upregulation observed in smokers may be mediated by these smoking-associated epigenetic changes.

Concerning obesity, Al Heialy et al. in 2020 (Al Heialy et al., 2020) showed in a small group ($n = 7$) that lung epithelial cells from obese subjects in vitro have higher expression of *ACE2* than non-obese subjects. Concomitantly, researchers found through in silico analysis that *ACE2* expression in human subcutaneous adipose tissue can be regulated through changes in diet and highlight the repression of Srbp1, a gene that codes sterol response element-binding protein 1 (SREBP) as a potential mediator of *ACE2* expression increment in obesity (Al Heialy et al., 2020). Our results reinforce these latter findings giving evidence of in vivo expression profiles from a more considerable number of matched subjects, remarking that *ACE2* overexpression might be a part of the mechanism underlying the increased risk for severe complications in those groups with COVID-19.

There is evidence of a role in the severity of patients with COVID-19. Since *ACE2* is highly expressed in a wide variety of organs other than the lung, such as the brain, heart, kidneys, liver, skeletal muscle, and vasculature, there is a possible implication of its high expression with a higher risk of infection at these organs, leading to multiple organ injury as seen in severe COVID-19 (Ni et al., 2020). However, given that the expression data reported here are from a single sample site, we cannot verify or deny whether these expression patterns are maintained in other tissues and if there are associated with worse outcomes.

Underlying diseases since the pandemic began were risk groups of increasing interest; however, the cause behind this increased risk is not yet fully understood. Here we describe through a matched analysis that DM2 individuals have higher *ACE2* expression levels than non-DM2

Table 4
Stepwise logistic regression analysis of high *ACE2* expression levels. Stepwise logistic regression analysis results aiming to eliminate all confounding factors, thus only significant factors that increase the probability of *ACE2* gene overexpression are shown.

Variables	Stepwise analysis			
	OR	95% CI		P > z
Sex				
Male	1.55	1.01	2.38	**0.045**
Age				
30–49 years	3.46	2.07	5.80	**0.000**
50–64 years	2.65	1.39	5.03	**0.003**
Body Mass Index (BMI)				
Obesity (≥30 kg/m^2)	2.39	1.23	4.67	**0.010**

Table 3
Logistic regression analysis of high *ACE2* expression levels. Bivariate and multivariate logistic regression analysis results aiming to identify the factors that increase the probability of *ACE2* gene overexpression.

Variables	Bivariate analysis				Multivariate analysis			
	OR	95% CI		P > z	Adjusted OR	95% CI		P > z
Sex								
Female	Reference							
Male	1.48	0.98	2.23	0.059	1.56	1.01	2.39	**0.043**
Age								
18–29 years	Reference							
30–49 years	3.78	2.24	6.39	**0.000**	3.63	2.12	6.19	**0.000**
50–64 years	3.07	1.61	5.85	**0.001**	2,71	1.39	5.27	**0.003**
65–71 years	2.95	0.54	16.11	0.213	2.31	0.39	1.38	0.357
Smoking								
Non-smoker	Reference							
Smoker	1.08	0.58	1.98	0.559	0.97	0.51	1.84	0.929
Body Mass Index (BMI)								
Normal weight (18.5–24.9 kg/m^2)	Reference							
Overweight (25–29.9 kg/m^2)	1.05	0.67	1.64	0.839	0.88	0.55	1.40	0.585
Obesity (≥30 kg/m^2)	2.79	1.43	5.44	**0.003**	2.09	1.03	4.25	**0.042**
Underlying diseases								
Non-DM2 subjects	Reference							
DM2 patients	4.10	0.90	1.86	0.067	2.74	0.54	1.39	0.224
Non-asthmatic subjects	Reference							
Asthma patients	0.59	0.13	2.75	0.504	0.82	0.17	3.97	0.802
Non-AHT subjects	Reference							
AHT patients	1.38	0.47	4.05	0.559	1.19	0.77	3.73	0.765

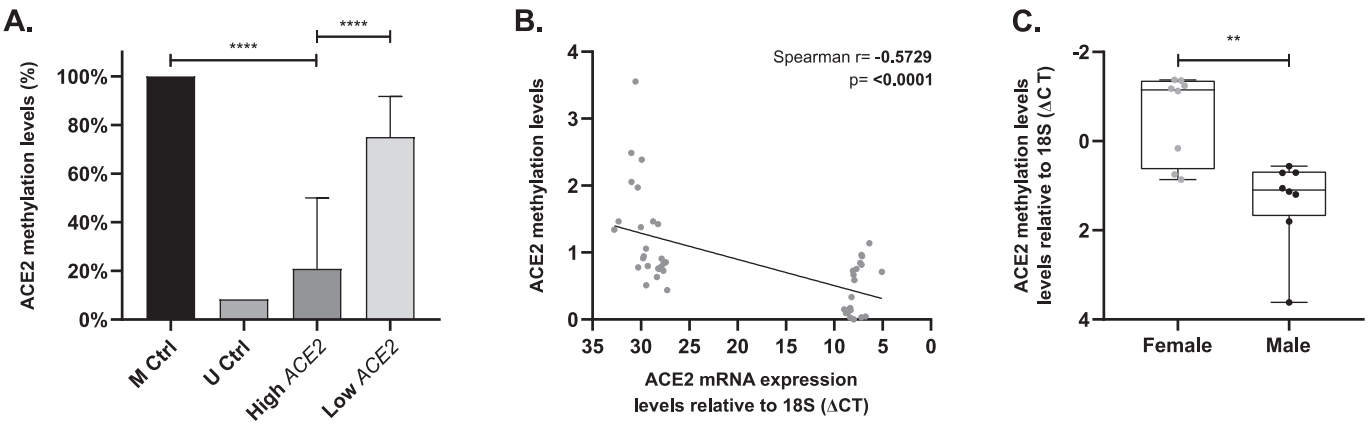

Fig. 7. *ACE2* gene expression is regulated by DNA methylation. (A.) Relative methylation percentages of the *ACE2* gene promoter in subjects with high and low levels of *ACE2* expression. (B.) Spearman's correlation between relative methylation levels of the *ACE2* gene promoter and *ACE2* mRNA expression. (C.) Comparison of relative methylation levels of the *ACE2* gene promoter in female and male subjects. Significance p-values are shown as * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

individuals, supporting the expression patterns reported by some studies in kidney, liver, and lungs of DM2 patients (Rao et al., 2020; Roberts et al., 2021; Soldo et al., 2020; Wysocki et al., 2006). Otherwise, no significant expression differences were found in asthma or hypertensive individuals, even though the latter was considered a risk factor in COVID-19 (Clark et al., 2021). Regarding our results about asthma, even though the sample size is small to reach to any conclusion, it is important to mark out that are consistent to those findings from other studies that have not reported it as a risk factor to infection, and the mortality rate is similar in non-asthmatic COVID-19 patients (Branco et al., 2020; Guan et al., 2020; Wang et al., 2020). Regarding hypertension, in the AHT individuals treated with ACEIs, a higher *ACE2* mRNA expression was found. Consistently, some studies have reported that within AHT patients, those treated with Angiotensin-converting enzyme inhibitors (ACEIs) have higher *ACE2* expression levels than those treated with other drugs (Bean et al., 2020; Chung et al., 2020; Li et al., 2020). Nevertheless, these results must be carefully interpreted because even though ACEIs treatment is associated with an increase in *ACE2* gene expression, which theoretically may enhance viral infection, several studies reviewed by Chung et al. show that ACEIs use was not associated with a higher risk of severe COVID-19 or mortality (Bean et al., 2020; Chung et al., 2020; Li et al., 2020). Moreover, *ACE2* overexpression by ACEIs in hypertensive patients might also have a protective role, given that *ACE2* protect against Ang II effects by promoting vasodilation, as well as anti-inflammatory, anti-oxidant, anti-thrombotic, and anti-fibrotic activity via the receptors Mas1 (a G-protein coupled receptor [GPCR]) and AT2R, respectively (Chung et al., 2020; Patel et al., 2016). This may explain why ACEIs have not shown a relevant impact in COVID-19 patients until now.

This study shows that *ACE2* gene expression is higher in COVID-19 individuals when matched to non-infected or asymptomatic subjects. Even if the compared subjects are few, several studies support this finding, showing that SARS-CoV-2 infection is closely related to the expression levels of its gateway, *ACE2* (Han et al., 2021b; Ni et al., 2020; Tan et al., 2020). Besides susceptibility, *ACE2* might be implicated in the severity of the disease, as aforementioned, given that in severe COVID-19 patients, higher *ACE2* levels were observed (Kragstrup et al., 2021). Nonetheless, the mechanism behind *ACE2* can modify the severity risk is not yet fully understood, and more studies are required to amend these knowledge gaps.

One of the main difficulties in epigenetic studies, unlike genetic studies, is sample obtaining. Since each cell of our organism has a different expression and epigenetic profile adapted to its functions, the specific cell of interest is required to evaluate its epigenome (Cacabelos et al., 2019; David Allis et al., 2015). DNA methylation regulates gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA (Moore et al., 2013). As one of the most important findings of this work, our results show an inverse relationship between the ACE2 promoter methylation and its gene expression through correlation and linear regression analysis. As mentioned above, to evidence the effect of DNA methylation on expression in a more noticeable way for the methylation analyzes two groups of 25 subjects each were selected, those with the highest and lowest *ACE2* expression levels. The correlation graph shows on the left those patients with the lowest *ACE2* expression and highest methylation levels and, on the right, the ACE2 hypomethylation correlates with the ACE2 overexpression. These results demonstrate that DNA methylation in the *ACE2* gene promoter is an epigenetic mechanism involved in the control of *ACE2* gene expression in humans and can also be detected in peripheral blood samples, which lays the theoretical foundations for future search for markers of host susceptibility to SARS-CoV-2 infection as part of precision medicine (Cacabelos et al., 2019).

The study limitations include a small sample size in some comparison groups, a small COVID-19 positive population, and the fact that expression was measured only in nasal epithelial cells obtained by nasopharyngeal swab, being aware that these *ACE2* expression patterns can vary between tissues. However, this sample constitutes a valid approximation to the expression of the *ACE2* receptor in the respiratory tract used in previous investigations.

This study also has some strengths since the data were analyzed through differences in the median, correlations and regression analysis which yielded equivalent results. In addition, this is the first study of *ACE2* receptor expression in a sample of subjects matched by sex, age, and BMI excluding those with comorbidities and smoking history, thus eliminating most of the possible confounding variables described to date and giving greater weight to the associations reported here. Likewise, most of the associations and trends described here are currently supported in the in vitro and in vivo assay literature.

Knowledge acquisition of host factors that influence the SARS-CoV-2 infection and replication in the lungs allows a better understanding of the pathogenic mechanisms of the virus in humans. The results described here expand and reinforce what has been described in the literature, pointing out the vital role of the environment on the *ACE2* gene expression and in understanding the susceptibility and severity of COVID-19, highlighting a influence of lifestyle on the risk of infection.

5. Conclusion

To date, still unclear which genes or elements of the genome can influence the susceptibility or development of COVID-19. Our results

hint at the impact of our environment, habits, and demographic and clinical variables on the SARS-CoV-2 infection susceptibility through expression changes on its gateway receptor encoded by the *ACE2* gene.

Altogether, our results show that *ACE2* gene overexpression, directly involved in susceptibility to SARS-CoV-2 infection, depends on multiple host factors such as male sex, age over 30 years, smoking, the presence of obesity, and DM2. Likewise, it was determined that the *ACE2* gene expression is regulated by changes in the DNA methylation patterns in its promoter region.

Given the *ACE2* role as a gateway in SARS-CoV-2 infection, its genetic variants and epigenetic regulation can influence the modulation of the susceptibility of the infection across populations by increasing or decreasing the virus's capacity to interact with the host cell. These results suggest that each person's genome and epigenome may play an essential role in interacting and responding to the SARS-CoV-2 infection. This study lays some foundations for further investigation as a marker of SARS-CoV-2 infection susceptibility or modulating this risk through epigenome editing.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2022.105357.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the ethical committee of Pontificia Universidad Javeriana (FM-CIE-1171-20). All methods were conducted under relevant guidelines and regulations. The selected hospital was a teaching hospital, and at the time of admission, informed consent was obtained from all participants.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

DG performed experimental assays, analyzed, and interpreted the results and patient data. BA, JA and CC helped researchers gather data in the hospital. DG, PA, and FS performed the statistical analysis. AR, PA, and NG supervised the research and analyzed the data. All authors read and approve the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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Not applicable.

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