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Coronavirus Pandemic

Expression analysis of *DDC*, *dACE2*, and *EPO* genes in Moroccan COVID-19 patients: links to viral load and demographics

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Abstract

Introduction: Interactions between host and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are incompletely understood. Studies have highlighted the roles of L-dopa decarboxylase (*DDC*), interferon-inducible truncated isoform of angiotensin-converting enzyme 2 (*dACE2*), and immunomodulatory hypoxia-regulated gene erythropoietin (*EPO*) in viral infections. This study investigated the expression levels of *DDC*, *dACE2*, and *EPO* in 136 coronavirus disease 2019 (COVID-19) patients and 88 controls.

Methodology: Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to quantify mRNA levels of *DDC*, *dACE2*, and *EPO*; and the SARS-CoV-2 viral load in nasopharyngeal swabs.

Results: Significantly elevated levels of *dACE2* ($p = 0.003$), *DDC* ($p = 0.004$), and *EPO* ($p = 0.006$) were observed in patients compared to controls. No correlation with the viral load (*DDC*: $r = 0.12$, $p = 0.136$; *EPO*: $r = 0.02$, $p = 0.802$; *dACE2*: $r = 0.05$, $p = 0.491$), and no associations with age or gender (all $p > 0.05$) were noted. There were positive correlations between *DDC* and *dACE2* mRNA levels in infected ($r = 0.31$, $p = 0.0002$) and uninfected individuals ($r = 0.25$, $p = 0.015$); and between *DDC* and *EPO* in infected ($r = 0.22$, $p = 0.008$) and uninfected individuals ($r = 0.27$, $p = 0.010$). There was a positive correlation between *dACE2* and *EPO* mRNA levels in both groups (infected: $r = 0.22$, $p = 0.007$; uninfected: $r = 0.38$, $p = 0.0002$).

Conclusions: *DDC*, *dACE2*, and *EPO* may contribute to COVID-19 pathogenesis through mechanisms independent of viral load, age, or gender.

Key words: COVID-19; SARS-CoV-2; *DDC*; *dACE2*; *EPO*.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified as the etiological agent of coronavirus disease 2019 (COVID-19), in Wuhan, China, in December 2019. Since then, the virus has spread rapidly around the world, leading to a pandemic with significant mortality. SARS-CoV-2 is an enveloped, non-segmented, positive-sense RNA virus that enters host cells via the transmembrane glycoprotein spike (S), which forms homotrimers extending outward from the surface of the virus [1]. The S protein binds directly to angiotensin-converting enzyme 2 (ACE2) as a functional receptor, facilitating the S protein-dependent entry of SARS-CoV-2 into cells [2,3]. A novel interferon (IFN)-inducible truncated isoform of ACE2, deltaACE2 (dACE2), has been identified in various cell types, particularly in airway epithelial cells [4]. dACE2 is a primate-specific isoform of ACE2 encoding a protein of 459 amino acids, including the first 10 amino acids encoded by Ex1c [4]. Unlike ACE2, the dACE2 protein shows no affinity to the viral spike protein and is induced following viral infection as part of the IFN-stimulated gene (ISG) response [5]. Several binding motifs for transcription factors related to IFN signaling, required for dACE2 expression, have been predicted in the *dACE2-Ex1c* promoter which is an intronic regulatory element within the *ACE2* gene, activated in response to IFN [6].

L-Dopa decarboxylase (*DDC*) is the gene that is the most co-expressed gene with *ACE2* [7]. It encodes for an enzyme responsible for catalyzing decarboxylation reactions, which include the transformation of L-3,4-dihydroxyphenylalanine (L-Dopa) into dopamine, an immunomodulatory catecholamine [8]; and 5-hydroxytryptophan into serotonin, a regulator of inflammation and immunity [7]. DDC has been demonstrated to have physiological functions in both cell proliferation and apoptosis [9], and its expression has been associated with various viral infections [10]. However, research investigating the impact of SARS-CoV-2 on the expression of *DDC* is lacking. It is possible that the impact of SARS-CoV-2 infection on the immune system indirectly affects the expression of *DDC*, given that DDC plays a role in immunomodulatory processes [11,12]. Therefore, additional investigation is required to comprehensively grasp the potential influence of SARS-CoV-2 on the expression of *DDC*.

The COVID-19 pandemic was characterized by a broad spectrum of clinical manifestations, spanning from asymptomatic instances to severe acute respiratory distress syndrome (ARDS) and multi-organ failure [13]. Neurological symptoms, including anosmia, encephalopathy, and hypoxemia, have also been reported [14]. While most patients present with mild flu-like symptoms and recover without complications, a subset of patients develop severe viral pneumonia, multi-organ dysfunction, and hypoxia [14]. Acute systemic hypoxia is a major pathophysiological consequence of lung injury and the primary cause of death in severe cases of COVID-19. Lung injury stabilizes the hypoxia-inducible factor 1α (HIF-1α) and causes an increase in hypoxia-inducible genes [15]. A recent investigation showed that expression levels of *erythropoietin* (*EPO*), an immunomodulatory gene regulated by hypoxia, were significantly higher among COVID-19 patients with severe illness compared to those with mild or moderate symptoms; suggesting that *EPO* may play a role in the pathogenesis of the severe form of COVID-19 [5]. The objective of this study was to evaluate the expression levels of the *DDC*, *dACE2*, and *EPO* genes in individuals diagnosed with COVID-19.

Methodology

Study design and participants

This was a cross-sectional case-control study. A total of 136 nasopharyngeal (NP) swab samples were collected from patients with COVID-19, and 88 NP swab samples were collected from SARS-CoV-2-uninfected participants between 20 December 2020 and 31 December 2021. The samples were collected from multiple sites to ensure the representativeness of the study sample and to capture a diverse population. These sites included the Institut Pasteur du Maroc, Moulay Youssef Hospital, and Morizgo Medical Analysis Laboratory in Casablanca. In addition, the study included individuals across the full spectrum of COVID-19 severity, ranging from asymptomatic to severe cases; thereby reflecting the diverse clinical presentations of the disease. Controls were selected based on reverse transcriptase polymerase chain reaction (RT-PCR)-negative results and were chosen to match the patients in terms of key demographics (age, gender), ensuring comparability between the groups.

In order to be included in the study, the participants had to be adult men or women who tested RT-PCR positive for SARS-CoV-2 RNA. This included both asymptomatic individuals and those with mild symptoms such as cough, sore throat, mild fever below 38 °C, or loss of smell. Participants with severe COVID-19 cases were also included. Controls were chosen from individuals presenting at the same institution for routine testing during the study period to minimize selection bias. None of the participants had received the anti-COVID-19 vaccination prior to sample collection.

Viral RNA was automatically extracted from NP swabs using commercial kits according to the manufacturer's instructions. SARS-CoV-2 was detected using real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) with commercial kits. A complete demographic and clinic-biological information sheet was meticulously

Table 1. Primer sequences used for real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis.

Gene	Orientation	Sequence (5'-3')	Reference
dACE2	Forward	GTGAGAGCCTTAGGTTGGATTC	[1-3]
	Reverse	TAAGGATCCTCCCTCCTTTGT	
DDC	Forward	AGAGGGAAGGAGATGGTGGATTA	[2-4]
	Reverse	GGGGCTGTGCCTGTGCGT	
EPO	Forward	GCCCCACCACGCCTCATCTGT	[2,3,5]
	Reverse	CTTCCAGGCATAGAAATTAAC	
YWHAZ	Forward	GCTGGTGATGACAAGAAAGG	
	Reverse	GGATGTGTTGGTTGCATTTCCT	

completed for each participant.

RNA isolation and cDNA synthesis

RNA was extracted from all the SARS-CoV-2 positive and SARS-CoV-2 negative NP samples using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in 40 µL of RNase-free water and stored at – 80 °C. The RNA samples were quantified using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. 250 ng/µL of RNA from each sample was reverse transcribed into cDNA using Tetro reverse transcriptase (Bioline, Paris, France) following the manufacturer's instructions.

Relative quantification of dACE2, DDC, and EPO gene expression

Relative quantification of gene expression was assessed through real-time PCR using the SensiFAST SYBR Lo-ROX Master Mix (Bioline, Paris, France). qPCR assay of the cDNA samples was used to determine the expression levels of *dACE2*, *DDC*, and *EPO* genes using gene-specific primers (Table 1) as described previously [5,16]. The *14-3-3-zeta polypeptide* (*YWHAZ*) gene was used as a housekeeping gene to normalize the gene expression. Negative controls were consistently included in each assay. The qPCR was performed using the following thermal cycling conditions: 95 °C for 6 minutes; followed by 40 cycles of 95 °C for 10 seconds, 60 °C for 15 seconds, and 72 °C for 45 seconds; with a melt curve at 65–95 °C. The delta Ct method was utilized to plot and compare the expression of each gene in both groups. Meanwhile, the 2^(-ΔΔCt) method was employed to estimate the relative expression of each gene in the positive versus negative group [17].

Statistical analyses

The data distribution was assessed using the Kolmogorov-Smirnov (KS) test, and the appropriate statistical tests were chosen accordingly. The Mann-Whitney test (unpaired) was employed to compare gene expression between positive and control groups. Spearman's correlation coefficient (r) was utilized to ascertain correlations between gene mRNA levels and viral RNA data. The statistical association between gene expression and demographics (age and gender) was assessed using Spearman's r. Missing data were addressed by excluding samples with insufficient RNA quality or quantity, ensuring robustness of the analyses. All statistical analyses were conducted using GraphPad Prism 6.0 (GraphPad Software, Inc.), and statistical significance was determined at $p < 0.05$ (two-tailed).

Results

Demographic characteristics of study participants

A total of 136 NP swab samples were collected from COVID-19 patients exhibiting mild or no clinical symptoms, including cough, sore throat, low-grade fever below 38 °C, and loss of smell; as well as from 88 uninfected individuals. The demographics of the study participants are presented in Table 2. The COVID-19 patient group had a median age of 45 years, while the negative control group had a median age of 31.5 years. Of the COVID-19 patients, 40.44% were male and 59.55% were female; compared to 44.31% male and 55.68% female in the control group (Table 2).

Expression of dACE2 mRNA in patients infected with SARS-CoV-2

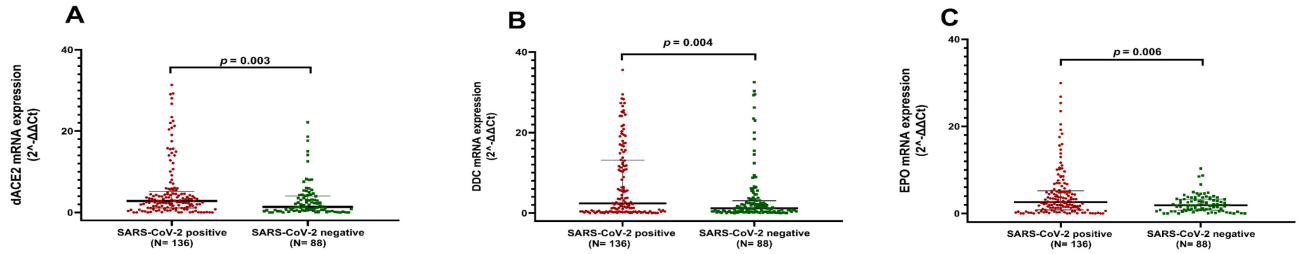
The ISG *dACE2* mRNA levels were significantly higher in COVID-19 patients ($p = 0.003$) with a median of 2.53 (IQR = 1.06–4.53); compared to uninfected individuals, who had a median of 1.33 (IQR = 0.39–3.36) (Figure 1A).

Table 2. Demographic data of SARS-CoV-2 positive and negative subjects.

Demographic feature	SARS-CoV-2 positive (n = 136) (%)	SARS-CoV-2 negative (n = 88) (%)	*p* value
Median age, years (IQR)	45 (33–61)	31.5 (25.75–47.25)	0.0001[a]
Age group, years			
0–17	10 (7.35%)	15 (18.75%)	
18–39	46 (33.82%)	39 (44.31%)	
40–64	53 (38.97%)	29 (32.95%)	
65+	27 (19.85%)	5 (6.25%)	
Gender (%)			
Male	55 (40.44%)	39 (44.31%)	0.581[b]
Female	81 (59.55%)	49 (55.68%)	
Median CT			
DDC	28.92	29.78	
dACE2	27.80	28.62	< 0.0001[c]
EPO	26.84	27.45	

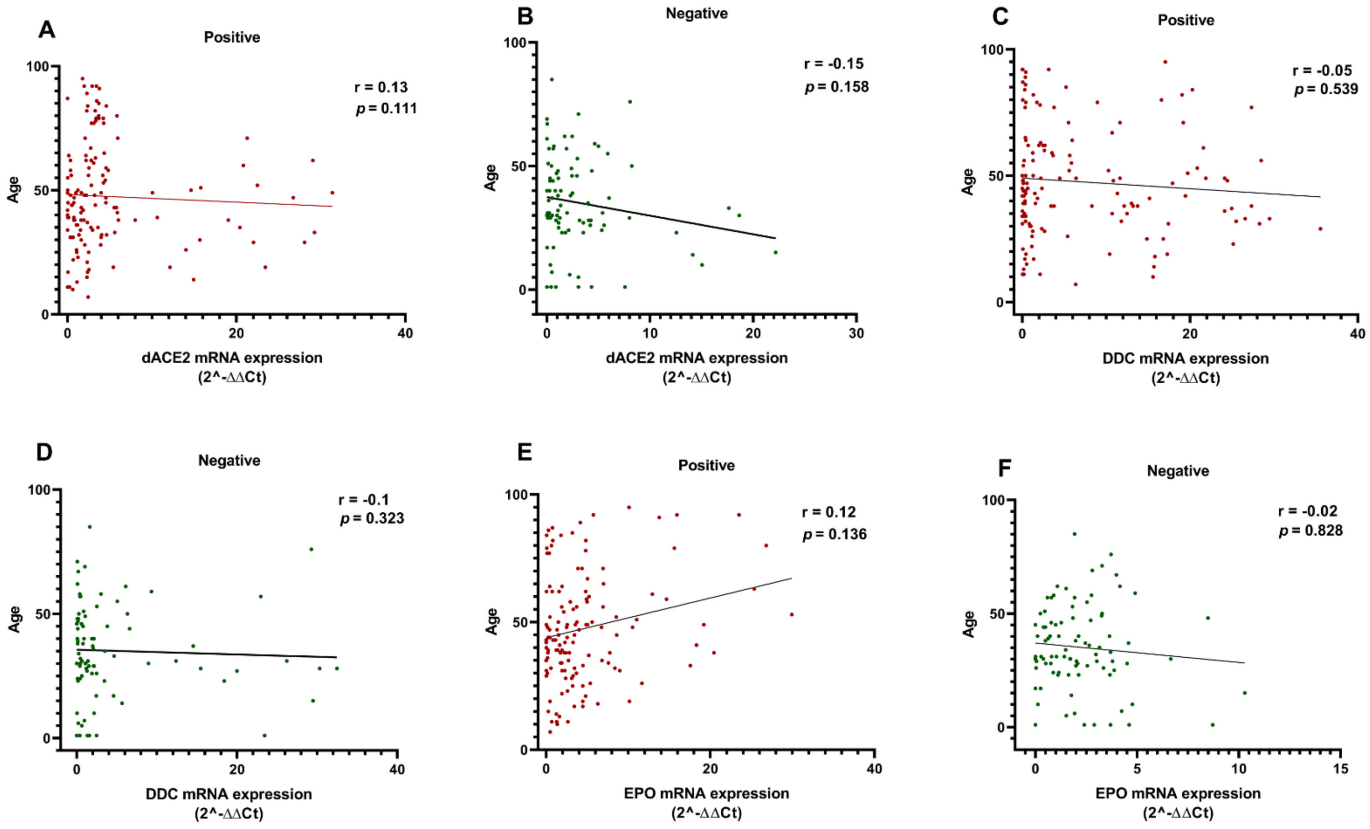
[a] Mann-Whitney U test; [b] Fisher's exact test; [c] Wilcoxon test. SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; IQR: interquartile range.

Figure 1. Comparison of *dACE2*, *DDC*, and *EPO* mRNA expression levels in nasopharyngeal swab samples from COVID-19 patients showing no or mild symptoms, and uninfected individuals.



Relative mRNA values (2-ΔΔCt) of *dACE2* (A), *DDC* (B), and *EPO* (C) from the patient (n = 136) and control (n = 88) groups are presented. p values were calculated with the Mann-Whitney test (data are presented as median with interquartile range). *dACE2*: the interferon-inducible truncated isoform of angiotensin-converting enzyme 2; *DDC*: L-Dopa decarboxylase; *EPO*: immunomodulatory hypoxia-regulated gene erythropoietin; COVID-19: coronavirus disease 2019.

Figure 2. Correlation of *DDC*, *dACE2* and *EPO* gene expression levels and age in SARS-CoV-2-positive and negative nasopharyngeal swab samples.



XY scatter plot of the mRNA levels of *dACE2* (A, B), *DDC* (C, D) and *EPO* (E, F), in SARS-CoV-2-positive (A, C, E) and negative (B, D, F) samples. Spearman's correlation coefficient (r) and p values (p) were calculated. *dACE2*: the interferon-inducible truncated isoform of angiotensin-converting enzyme 2; *DDC*: L-Dopa decarboxylase; *EPO*: immunomodulatory hypoxia-regulated gene erythropoietin; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

The Spearman's correlation coefficient (r) of 0.13 in patients indicated a positive correlation between age and *dACE2* mRNA levels; however, the *p* value of 0.111 suggested that this correlation was not statistically significant (Figure 2A). The Spearman's correlation coefficient (r) of – 0.15 in controls suggested a negative correlation between age and *dACE2* expression levels, but the *p* value of 0.158 indicated that this correlation was also not statistically significant (Figure 2B). Additionally, *dACE2* mRNA levels revealed no correlation with gender in both infected (p = 0.055) (Figure 3A), and uninfected individuals (p = 0.214) (Figure 3B).

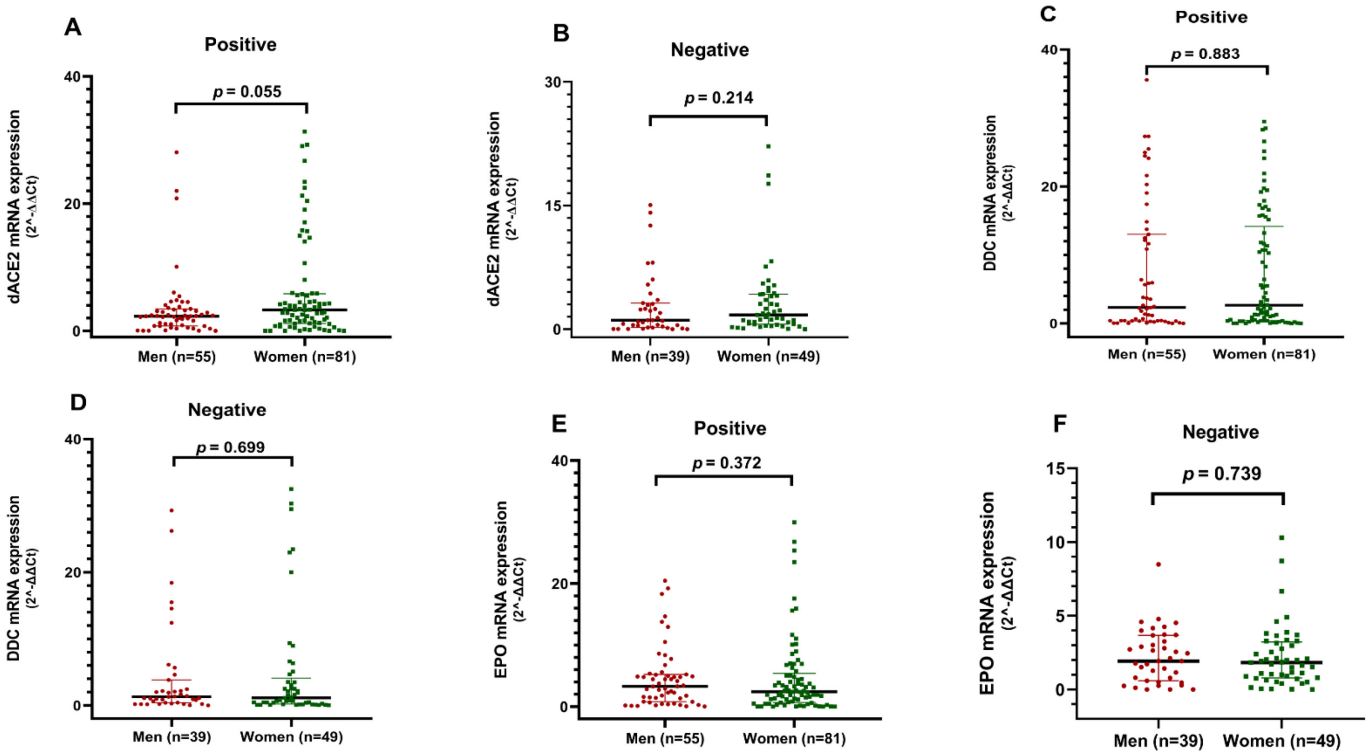
Expression of DDC mRNA in patients infected with SARS-CoV-2

A significant difference in the expression levels of *DDC* between patients and controls was observed (p = 0.004) in the samples collected from NP swabs, with SARS-CoV-2 patients generally exhibiting higher levels of *DDC* mRNA (median = 2.40; IQR = 0.40–13.06), compared to uninfected subjects (median = 1.15; IQR = 0.36–3.58; Figure 1B). Additionally, Spearman correlation analysis suggested a negative correlation (Spearman's r = – 0.05) between age and *DDC* mRNA levels in patients; however, this correlation was not statistically significant (p = 0.539; Figure 2C). Similarly, no significant correlation (Spearman's r = – 0.1; p = 0.323) was observed in uninfected individuals (Figure 2D). Finally, no correlation was observed with gender in both infected (p = 0.883; Figure 3C) and uninfected (p = 0.699; Figure 3D) individuals.

EPO mRNA expression in patients infected with SARS-CoV-2

Given that the expression of *DDC* and *dACE2* is influenced by hypoxia [10]; and that *EPO*, an immunomodulatory gene, is a target of HIF [13]; the expression pattern of *EPO* was also analyzed in COVID-19 patients and compared with the uninfected individuals. *EPO* mRNA expression levels exhibited a significant difference (p = 0.006) between SARS-CoV-2 patients (median = 2.68; IQR = 0.79–5.19) and the control group (median = 1.88; IQR = 0.77–3.26; Figure 1C). The Spearman's correlation coefficient (r) of 0.12 in COVID-19 patients indicated a positive correlation between age and *EPO* mRNA levels; however, the *p* value of 0.136 suggested that this correlation was not statistically significant (Figure 2E). The Spearman's

Figure 3. Relative mRNA expression in men as compared to women in uninfected and SARS-CoV-2-infected groups.



dACE2 (A, B), *DDC* (C, D) and *EPO* (E, F), in positive (A, C, E) and negative (B, D, F) samples. p values were calculated with Mann-Whitney test (data are presented as median with interquartile range). *dACE2*: the interferon-inducible truncated isoform of angiotensin-converting enzyme 2; *DDC*: L-Dopa decarboxylase; *EPO*: immunomodulatory hypoxia-regulated gene erythropoietin; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

correlation coefficient (r) of – 0.02 in controls suggested a negative correlation between age and *EPO* expression level; and the *p* value of 0.828 indicated that this correlation was not statistically significant (Figure 2F). No correlation was observed with gender in both infected (p = 0.372; Figure. 3E) and uninfected individuals (p = 0.739; Figure 3F).

Correlation of SARS-CoV-2 viral load with DDC, dACE2, and EPO gene expression levels

The correlation between the mRNA levels of *DDC*, *dACE2*, and *EPO*; and the viral load was investigated, as indicated by the quantification of viral RNA levels. *DDC* (Spearman's r = 0.12, p = 0.136; Figure. 4B); *EPO* (Spearman's r = 0.02, p = 0.802; Figure 4C); and *dACE2* (Spearman's r = 0.05, p = 0.491; Figure. 4A), all showed no correlation with the viral load.

Correlation analysis of DDC, EPO, and dACE2 mRNA levels

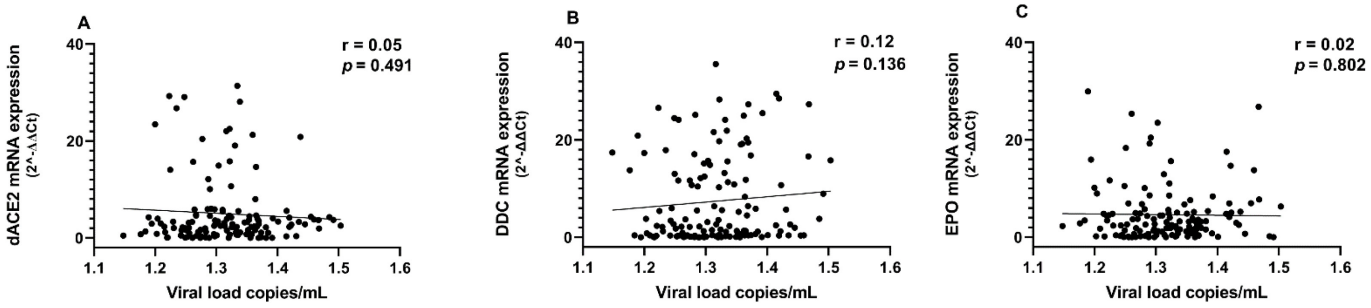
The correlation between mRNA expression levels of the genes examined in COVID-19-patients and SARS-CoV-2 uninfected individuals was investigated. A positive significant correlation was noted between *DDC* and *dACE2* mRNA levels (Spearman's r = 0.31, p = 0.0002; Figure 5A) in the group of infected cases. Moreover, a positive correlation (Spearman's r = 0.25, p = 0.015) was found in the uninfected group (Figure 5B). Additionally, a positive correlation was detected between *DDC* and *EPO* mRNA levels in both COVID-19 cases (Spearman's r = 0.22, p = 0.008; Figure. 5C) and the uninfected group (Spearman's r = 0.27, p = 0.010; Figure 5D). Furthermore, a positive correlation was found between *dACE2* and *EPO* mRNA levels in infected individuals (Spearman's r = 0.22, p = 0.007; Figure. 5E). Similarly, a positive correlation was identified (Spearman's r = 0.38, p = 0.0002; Figure 5F) in the uninfected individuals.

Discussion

The results demonstrated that mRNA expression levels of the newly identified IFN-inducible truncated isoform *dACE2* [4,18] and *DDC* [7,10,19] were notably elevated in NP swab samples collected from COVID-19 patients exhibiting either no symptoms or mild symptoms, in contrast to uninfected individuals. In addition, a comparable expression pattern was noted for the hypoxia-regulated immunomodulatory gene *EPO* between NP swab samples obtained from symptom-free or mildly symptomatic COVID-19 patients and those obtained from controls. Similar expression patterns have been reported previously [5].

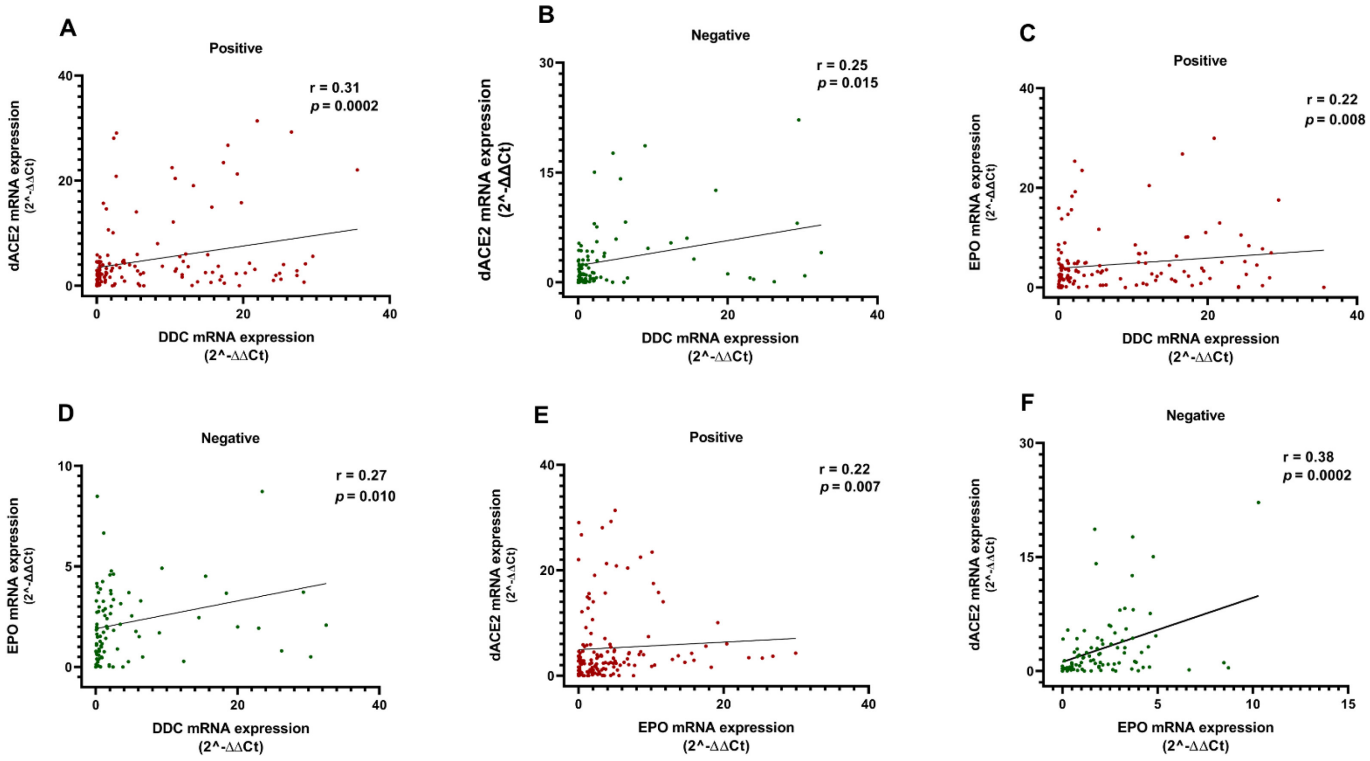
Previous studies showed that SARS-CoV-2 downregulated the expression of *DDC*, *dACE2*, and *EPO* in infected tissues [5]. It was previously emphasized that SARS-CoV-2 downregulates *DDC* expression, given that the enzyme catechol-O-methyltransferase (COMT), necessary for SARS-CoV-2 infection, relies on *DDC* downregulation [20]. Likewise, the decrease in *dACE2* expression in NP tissue due to SARS-CoV-2 has been documented previously; and attributed to the role of *dACE2* as an ISG [3,6] and the virus's suppression of IFN signaling [18]. SARS-CoV-2 is notably adept at inhibiting IFN expression and signaling via the type I IFN receptor, compared to other respiratory viruses [21,22]. Furthermore, both *DDC* and *dACE2* are regulated by hypoxia [10,23] and the HIF-target immune-modulatory gene *EPO* [13]. Similarly, *EPO* has been reported to be downregulated by SARS-CoV-2 in infected tissues [5], potentially serving as a mechanism for the virus to diminish the immune-modulatory function of erythropoietin [21,22,24] or to exploit its anti-apoptotic properties, considering that SARS-CoV-2 induces cell death [5,25–27]. Consequently, SARS-CoV-2 may benefit from the reduced levels of these genes in specific individuals to facilitate efficient

Figure 4. XY scatter plot of SARS-CoV-2 N RNA levels versus *dACE2* (A), *DDC* (B), and *EPO* (C) mRNA expression.



The viral load was log transformed (base 10) prior to calculation of the Spearman's correlation coefficient (r) and p values (p). SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; *dACE2*: the interferon-inducible truncated isoform of angiotensin-converting enzyme 2; *DDC*: L-Dopa decarboxylase; *EPO*: immunomodulatory hypoxia-regulated gene erythropoietin.

Figure 5. Correlation among *DDC*, *dACE2* and *EPO* gene expression levels in SARS-CoV-2-positive and negative nasopharyngeal swab samples.



XY scatter plot of the mRNA levels of *dACE2* versus *DDC* (A, B), *EPO* versus *DDC* (C, D), and *dACE2* versus *EPO* (E, F), in positive (A, C, E) and negative (B, D, F) samples. Spearman's correlation coefficient (r) and p values (p) were calculated. *dACE2*: the interferon-inducible truncated isoform of angiotensin-converting enzyme 2; *DDC*: L-Dopa decarboxylase; *EPO*: immunomodulatory hypoxia-regulated gene erythropoietin; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

propagation. Therefore, the plausible explanation for the upregulation of *DDC*, *dACE2*, and *EPO* is that COVID-19 patients may undergo an alteration in the expression of these genes compared to controls, which could potentially represent an antiviral immune strategy employed by the infected host.

In this study, the statistical relationship between *DDC*, *dACE2*, and *EPO* gene expression and demographic data were investigated. The findings revealed no correlation with age for *DDC*, *dACE2*, and *EPO* in both patients and uninfected individuals. There is concordance between these results and the outcomes documented in preceding research for *DDC* and *dACE2* [5]. However, the results diverge from the conclusions drawn in an earlier study where an overall decrease in *EPO* mRNA levels was observed with increasing age [28,29]. Moreover, no correlation with gender was observed for *DDC*, *EPO*, and *dACE2* in both patients and uninfected individuals. The results of this study did not align with a previous study where a correlation with gender was noted for *DDC* [5].

Further, the relationship between the mRNA levels of the respective genes and viral load were assessed. The observations revealed no significant correlation between *DDC*, *dACE2*, and *EPO* mRNA levels, and viral load in infected individuals. The absence of correlation between viral load and *DDC*, *dACE2*, and *EPO* gene expression suggests that their regulation is independent of the level of viral presence and that the replication of the SARS-CoV-2 genome and virus spread occurs independently of the expression of these genes. In contrast, a previous study showed a significant negative correlation between *DDC*, *dACE2*, and *EPO* mRNA and the viral load [5]; insinuating that SARS-CoV-2 genome replication and virus spread occur in parallel with the suppression of these genes.

Finally, this study revealed a notable and statistically significant expression correlation between *dACE2* and *DDC*, as well as between *dACE2* and *EPO*, in NP swab samples of both infected and uninfected individuals. The findings support and echo those of previous research by Mpekoulis *et al*. [5]. Additionally, the data unveiled a considerable and statistically significant correlation between *EPO* and *DDC* in NP swab samples of both patients and controls. The results corroborate the findings reported in prior research, as

correlations were observed between the expression of *EPO* and *DDC* in virus-infected individuals [5]. However, there exists a contrast between the findings of this study and the conclusions drawn in the previous study where the controls presented no correlation of *DDC* with *EPO* [5]. These findings complement the previous findings on the co-expression of *DDC* with *dACE2*.

The results of this study, while insightful, may be limited in their generalizability to broader populations. The sample was primarily obtained from a specific geographic region, and gene expression patterns may differ in individuals from other regions or ethnic backgrounds. Further research involving more diverse populations is needed to validate and expand upon these findings.

Conclusions

This study compared the gene expression profiles of *DDC*, *dACE2*, and *EPO* in COVID-19 patients and uninfected individuals; and investigated their correlation with viral load and demographic factors. The findings suggest that *DDC*, *dACE2*, and *EPO* are elevated in patients experiencing mild or asymptomatic symptoms in response to SARS-CoV-2 infection. However, no correlation between the expression of the three genes and age, gender, or viral RNA levels was observed. Additionally, a correlation between *dACE2*, *DDC*, and *EPO* mRNA levels in COVID-19 NP samples was established. These findings underscore the potential roles of the three genes in SARS-CoV-2 infection pathogenesis, highlighting the need for further investigation to understand the mechanisms underlying COVID-19, especially gene expression modulation.

Ethical considerations

This study was approved by the Ethics Committee of Ibn Rochd Hospital in Casablanca (2020-2022) and the Ethics Committee at the Rabat Faculty of Medicine (No. S/22). The research was conducted ethically, and all study procedures were performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki. Informed consent was obtained from all subjects involved in the study.

Data availability statement

The data presented in this study are available upon request from the corresponding author. The data are not publicly available following the decision of the ethical committee on the conduct of this study.

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Authors' contributions

SE, ML, study conception and acquisition of funding; OL, manuscript draft; OL, HA, RN, SA, AP, sample collection, laboratory experiments, statistical analyses, and data interpretation; ML, supervision; SE, MS, AM, manuscript review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of interests

No conflict of interests is declared.

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