Nasopharyngeal Expression of Angiotensin-Converting Enzyme 2 (ACE2) and Transmembrane Serine Protease 2 (TMPRSS2) in Children Compared to Adults Within Family Clusters Exposed to COVID-19

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Article

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Abstract

There is accumulating evidence that the lower levels of angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) in the nasal epithelium of children may be related to a lower incidence of COVID-19 compared to adults. However, no direct evidence is available to support this hypothesis. In this study, we compared the transcript levels of ACE2 and TMPRSS2 in nasopharyngeal swabs (n=207) from children and adult members within COVID-19-exposed families and assessed their association with SARS-CoV-2 infection status. The expression of both genes was higher in adults compared to the children (n=115 adults and 92 children, $p<0.05$), but was not significantly different between COVID-19 positive and negative patients of all ages or within the same age groups. Using paired data, expression of both genes was significantly higher in COVID-19 positive adults compared to COVID-19 negative children (n=47 pairs; $p<0.001$) within the same families. ACE2 and TMPRSS2 expression is positively associated [OR:1.16(1.06-1.3) and 1.14(1.04-1.26) for ACE2 and TMPRSS2, respectively, $p<0.001$)] with SARS-CoV-2 infection status in the sub-group of families with COVID-19 positive adults and COVID-19 negative children, suggesting that children with lower levels of nasal ACE2 and TMPRSS2 are more likely to remain COVID-19 negative despite being exposed to a COVID-19-positive adult family member.

Main Text

The global pandemic of coronavirus disease-2019 (COVID-19)[1] caused by the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV2) triggered urgent research on the pathogenesis and transmission of the virus including elucidation of the mechanisms by which SARS-CoV-2 binds and enters host cells. SARS-CoV-2 is genetically and structurally related to SARS-CoV and both share the same cell surface receptor, angiotensin-converting enzyme 2 (ACE2), for binding and entry into the host cells through the spike (S) glycoprotein [2-4]. However, mutations in the receptor binding domain of the S gene providing higher binding affinity for ACE2 have led to increased infectivity and enhanced transmissibility of SARS-CoV-2. The SARS-CoV-2 spike also exhibits a unique furin cleavage site that is proteolytically cleaved by a transmembrane protease serine 2 (TMPRSS2) which may enhance the human-to-human transmission [3,4]. The expression of both ACE2 and TMPRSS2 has been reported in various human body organs including the lungs, heart, kidneys, ileum and bladder[5]. Within the respiratory tract, based on both single-cell RNA sequencing and immunohistochemistry, ACE2 expression was shown to be the highest in the sinonasal epithelium and alveolar type II cells. TMPRSS2 is also co-localized with ACE2 on the apical surface of a small subset of alveolar type II cells in the lung parenchyma suggesting a role in regulating disease severity [6].

The clinical manifestation of SARS-CoV-2 infection is heterogeneous, from asymptomatic to moderate to highly severe, life-threatening disease. Although individuals of any age can acquire SARS-CoV-2 infection, most cases of COVID-19 have been reported in adults and elderly people[7]. The incidence of COVID-19 is much lower (10-13% of laboratory confirmed cases in children <18 years of age) in children compared to

the adults[8]. Also, the severe forms of COVID-19 and mortality associated with SARS-CoV-2 infection are much lower among children compared to the adults. In most cases, children remain asymptomatic, or mildly or moderately symptomatic[9]. The reason for the differences in the rates of infection and severity among adult versus pediatric patients is not well known. However, a number of theories exist, including differences in the immune response and cytokine regulation between adult and children, the presence of pre-existing cross-reactive antibodies, and off-target vaccine derived immunity in children. The role of ACE2 receptors expressed in the airways and lungs has also been discussed[9,10]. A recent study of ACE2 gene expression in the nasal epithelium of children and adults, using samples from a pre-COVID-19 asthma cohort, showed that ACE2 expression in nasal epithelium correlates with age and suggested that the lower expression of the gene in the nasal epithelium of children may explain why children are less affected by COVID-19[11]. Similarly, in cultured cells, it was shown that TMPRSS2-expressing cell lines are highly susceptible to SARS-CoV-2 infection[12]. Using a public gene expression dataset, it was also shown that the nasal and bronchial expression of ACE2 and TMPRSS2 is lower in children than adults[13]. However, there is no direct evidence that correlates nasal ACE2 and TMPRSS2 expression with SARS-CoV-2 infection or symptoms associated with COVID-19.

We hypothesized that lower levels of nasopharyngeal ACE2 and TMPRSS2 in children may protect children from acquiring SARS-CoV-2 infection despite being exposed to COVID-19 positive adult family members. In this study, we have analyzed the transcript levels of ACE2 and TMPRSS2 in nasopharyngeal swab (NPS) specimens from COVID-19 positive and negative adult and children from families with COVID-19 and assessed their association with SARS-CoV-2 infection status.

Results

Patient demographics and clinical characteristics

NPS specimens were collected from children and adults from families with a history of laboratory-confirmed SARS-CoV-2 infection in at least one of the family members. A total of 207 NPS specimens were collected of which 115 were from adults and 92 were from children from a total of 92 families (Table 1). NPS specimens from more than one adult members were available from 16 families. Among the study subjects, 62.6% and 46.7% were of female sex in the adult and pediatric groups, respectively. The mean age of adults and children were 34.7±7.9 years and 5.1±4.4 years, respectively, and 61.7% adults and 48.9% children were COVID-19 positive by RT-qPCR. For paired data analysis, each of the children were paired with their mothers with the exception of such cases where mother was negative for COVID-19 or no specimen was available from the mother.

All patients and visitors presenting to Sidra Medicine are triaged for a prior history of COVID-19 and COVID-19 associated symptoms, including temperature assessment. As a designated COVID-19 free facility, only patients with no history of COVID-19 within the last two weeks or patients having no COVID-19 related symptoms are eligible for admission to the hospital. Therefore, the children and adults

included in this study are asymptomatic at the time of NPS collection. No other clinical data on COVID-19 associated symptoms were collected.

Relative gene expression of ACE2 and TMPRSS2 in nasopharyngeal specimens

For gene expression analysis, reaction conditions for pre-designed TaqMan assays for two targets (Hs01085333_m1and Hs00222343_m1) for ACE2 and one target for each of TMPRSS2 (Hs01122322_m1) and b-actin (ACTB) (HS01060665) were first optimized using a set of 10 NPS specimens (5 COVID-19 positive 5 COVID-19 negative) (data not shown). TaqMan assay for one of the ACE2 targets (Hs01085333_m1) was consistently higher (mean Ct, 32.8±2.6 vs 34.7±2.2, p=0.00022 by paired, T-test) than the other, and was therefore chosen for the study. The chosen ACE2 target is located at exon 17-18 boundary (NCBI reference sequence, NM_021804.2) within the transmembrane domain of ACE2 (https://www.thermofisher.com/order/genome-database/?pearUXVerSuffix=pearUX2&elcanoForm=true#!/ge/assays/ge_all/?keyword=Hs01085333_m1). While nasopharyngeal gene expression of the endogenous b-actin was detected in all specimens, the RT-qPCR Ct values for ACE2 and TMPRSS2 remained undetermined for a number of specimens. Therefore, for quantitative analysis, a Ct value of 40 was assigned to data representing these specimens. Based on RT-qPCR Ct values, the relative transcript levels of ACE2 (mean Ct±SD, 37.7±3.6) and TMPRSS2 (mean Ct±SD, 35.5±3.4) were weaker and more widely variable than b-actin (mean Ct±SD, 26± 2.8) (Fig 1A). In order to account for variation in b-actin gene expression or the number of cells present in the NPS samples, the raw Ct values for ACE2 and TMPRSS2 were normalized by subtracting them for the Ct values for b-actin and Ct values relative to b-actin (DCt) were used as a measure of gene expression for all comparisons. When the transcript levels of ACE2 and TMPRSS2 were compared for the overall study population, they were both significantly higher (p=0.0045 and 0.0189, for ACE2 and TMPRSS2, respectively) in the adult population (Fig 1B). However, expression of these genes was not significantly (p>0.05) different in COVID-19 positive versus negative subjects in the overall population or in either the adult population or the pediatric population (Supplemental table 1). The expression of these genes was also not significantly different between COVID-19 positive versus negative children from families where at least one adult member was positive for COVID-19. Also, the expression of these genes was also not significantly different between COVID-19 positive versus negative adults from families when the child was positive for COVID-19.

By paired analysis of COVID-19 positive adults versus COVID-19 negative children from the same families, the expression of both genes was significantly (p=0.0004 and 0.0049, for ACE2 and TMPRSS2, respectively) higher in the adult members of the family compared to the children (Fig. 2A). However, the expression of the same genes was not significantly different (p>0.05) between COVID-19 positive adults versus COVID-19 positive children and COVID-19 negative adults versus COVID-19 positive children (Fig. 2B-C). To assess whether age differences among the COVID-19 positive and negative children are responsible for such difference, we compared the age distribution of the subjects in these groups. However, the mean age of COVID-19 positive children (4.4±4.2 years) and COVID-19 negative children (5.3±4.5 years) were not significantly different from each other (Fig. 2D).

Association of nasopharyngeal ACE2 and TMPRSS2 expression with SARS-CoV-2 infection status

By multivariate analysis of the overall study population using random effect logistic regression, age, sex and nasopharyngeal expression levels of ACE2 and TMPRSS2 were not significantly (p<0.05) associated with SARS-CoV-2 infection status (data not shown). However, by simple linear regression analysis the odds of SARS-CoV-2 positivity was higher (odds ratio, OR: 1.07, 95%CI: 1.002-1.152, p=0.044) with higher expression of ACE2 (Table 2). By sub-group analysis for families with at least one adult member positive for COVID-19, the odds of SARS-CoV-2 positivity was higher, with OR: 1.118 and 1.105, with higher expression of ACE2 and TMPRSS2, respectively. The odds of SARS-CoV-2 positivity is even higher, with OR: 1.163 and 1.14, with higher expression of ACE2 and TMPRSS2 in the subgroup of COVID-19 positive adults and negative children only. Conversely, in the subgroup of families with at least one children positive for COVID-19, or in the subgroup of COVID-19 negative adults and positive children only, SARS-CoV-2 infection status was not significantly associated with the expression of ACE2 and TMPRSS2 genes (data not shown). Apart from SARS-CoV-2 infection status, we have also noted that ACE2 and TMPRSS2 is significantly associated (OR: 1.115, 95%CI: 1.037-1.2, p=0.003 for ACE2 and OR: 1.112, 95%CI: 1.032-1.1983, p=0.005 for TMPRSS2) with the adult population, consistent with the gene expression data (Fig. 1B). However, no significant association of ACE2 and TMPRSS2 expression was found with gender.

Discussion

Although the role of ACE2 in SARS-CoV-2 infection was suggested soon after the declaration of COVID-19 pandemic and has been well described in the literature, little is known about the role of the protein in the nasopharyngeal epithelium. Considering the respiratory and olfactory epithelium in the nasal mucosa as one of the first contact points for the virus, ACE2 expression in nasal epithelium may have an important role in SARS-CoV-2 infection and transmission. We have conducted a case-control study to test the recently described hypothesis that lower nasal or nasopharyngeal expression of ACE2 is associated with a lower incidence of COVID-19 in children. To obtain more insight into the role of nasopharyngeal ACE2 in SARS-CoV-2 infection and transmission, we have also included the other viral entry factor, TMPRSS2, which is known to be involved in the proteolytic activation of ACE2, facilitating the cellular entry of SARS-CoV-2 virus. We included families where members were differentially affected by COVID-19 so that COVID-19 negative members in this setting would serve as an appropriate case controls for the study, because these individuals remained negative despite being exposed to the virus.

For gene expression analysis of ACE2, transmembrane ACE2 was specifically targeted because the soluble form of the protein may have a contrasting role in SARS-CoV-2 infection and no role in viral entry into the cells[14,15]. Consistent with earlier reports, nasopharyngeal ACE2 expression in our study was higher in the adults compared to the children[11,13]. In addition, our study provides direct evidence that the nasopharyngeal expression of TMPRSS2 is also higher in the adults than in children. ACE2 and TMPRSS2 expression was not significantly different between COVID-19 positive and negative groups in the overall population. However, by logistic regression analysis ACE2 expression was positively

associated with SARS-CoV-2 infection. This result is consistent with findings from an independent study conducted in British Columbia, Canada, which is currently in a pre-print status[16].

ACE2 and TMPRSS2 expression were not different between COVID-19 positive and negative patients in different age groups. However, using paired data, we demonstrate that the expression of both genes was significantly higher in the COVID-19 positive adults compared to the COVID-19 negative children from the same families. On the other hand, the expression of these genes was not significantly different between COVID-19 positive adult and COVID-19 positive children or between COVID-19 negative adult and COVID-19 positive children from the same families. These results were further supported by the fact that the associations of ACE2 and TMPRSS2 expression were stronger in the subgroup of COVID-19 positive adults and COVID-19 negative children compared to the overall population.

Our study has several limitations, first the COVID-19 positive patients in our setting were mostly asymptomatic. A stronger age-related association of nasopharyngeal ACE2 and TMPRSS2 expression with SARS-CoV-2 infection status might be found if this study was performed in symptomatic family clusters. In our study, ACE2 and TMPRSS2 expression was not significantly different between the COVID-19 positive and negative groups. However, mean ACE2 and TMPRSS2 transcript levels were consistently higher in the COVID-19 positive groups (Supplemental table 1). These results may become statistically significant with a larger sample number.

In conclusion, our results support the previously described hypothesis that children have lower transcript levels of nasopharyngeal ACE2 and TMPRSS2, which may protect them against infection from SARS-CoV-2 when exposed to COVID-19 positive adult family members. These results also provide new insights into the mechanisms by which adults and children within the same families are differentially affected by COVID-19, which may have important prognostic implications for the disease.

Methods

Study design and collection of specimens

Sidra Medicine is a tertiary care, 400 bed pediatric tertiary care hospital in Qatar. In 2020, as part of an integrated, national pandemic management plan the hospital was designated as a COVID-19-free facility. Since April 16, 2020 all patients and accompanying adult family members visiting the hospital were actively screened for COVID-19 by RT-qPCR testing of nasopharyngeal swab (NPS) specimens. During the period of June to December 2020, residual NPS specimens that met the inclusion criteria of the study were deidentified and saved at -80^{o}C for further analysis. The inclusion criteria were: i) at least one member of the family was positive for COVID-19 by RT-qPCR; ii) paired NPS specimens were available for the child and at least one of the accompanying adult family members; and iii) at least 0.5 ml of specimen was available for gene expression analysis. Paired specimens from COVID-19 positive and negative children and their adult companions were identified from the hospital infection prevention and control records. Data on the age, gender and SARS-CoV-2 RT-qPCR results for the selected patients were extracted

from the laboratory information systems. Ethics approval for the study and a waiver of informed consent was obtained from the Institutional Review Board of Sidra Medicine. All methods were carried out in accordance with relevant guidelines and regulations.

Gene expression analysis

Nucleic acid from NPS specimens were extracted on a Kingfisher Flex system using MagMAX Viral/Pathogen Nucleic Acid Isolation Kits according to the manufacturer instructions (Thermofisher). Extracted nucleic acids were assessed to determine the transcript levels of ACE2, TMPRSS2 and human b-actin (ACTB) genes using pre-designed TaqMan gene expression assays (Assay ID: Hs01085333_m1and Hs00222343_m1 for ACE2, Hs01122322_m1 for TMPRSS2 and HS01060665 for ACTB; Thermofisher). 10 ml of the extracted nucleic acids were tested by the respective TaqMan assays using TaqPath 1-Step RT-qPCR master mix according to the instructions provided by the manufacturer (Thermofisher).

Statistical analysis

Descriptive statistics were used to summarize data on the study population and their COVID-19 infection status. The statistical significance of difference of ACE2 and TMPRSS2 expression between different groups were determined by Mann-Whitney U test and 2-tailed, paired T-test, for unpaired and paired data, respectively. The association of ACE2 and TMPSS2 gene expression with SARS-CoV-2 RT-qPCR results (as the dependent variable) for different age groups were tested by simple logistic regression analysis and by multivariate, random effect logistic regression analysis. All analyses were carried out in SPSS.

Declarations

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Author contributions

MRH and PT coined the idea and designed the study; MNA performed research; MRH, MNA, and SRD analyzed data; AAH, NN helped with the acquisition of the specimens; MRH wrote the paper; HZI, APL, SD, LJAR and PT reviewed the paper and provided critical suggestions. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Tables

Table 1: Participant characteristics

Categories		Adult	Children
No. of samples		115	92
Sex, n (%)	Female	72 (62.6)	43 (46.7)
	Male	43 (37.4)	49 (53.3)
Age, mean ± SD		34.7±7.9	5.1±4.4
COVID-19 result, n (%)	Negative	44 (38.3)	47 (51.1)
	Positive	71 (61.7)	45 (48.9)

Table 2: Association of nasopharyngeal ACE2 and TMPRSS2 gene expression with SARS-CoV-2 infection

Category	Overall (n=207)		Subgroup 1: Families with COVID-19 positive adults only (n=163)		Subgroup 2: Families with COVID-19 positive adult and negative children only (n=97)	
	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value
Age group	1.685 (0.967-2.967)	0.065	-	-	-	-
Gender	1.036 (0.596-1.800)	0.900	0.848 (0.430-1.672)	0.634	0.603 (0.284-1.273)	0.185
ACE2 gene expression	**1.074 (1.002-1.152)**	**0.044**	**1.118 (1.023-1.222)**	**0.014**	**1.163 (1.057-1.295)**	**0.0035**
TMPRSS2 gene expression	1.044 (0.972-1.122)	0.240	**1.105 (1.012-1.206)**	**0.025**	**1.14 (1.035-1.263)**	**0.0093**

OR, odds ratio; CI, confidence intervals. OR calculated from simple logistic regression analysis - reference category is “COVID-19 negative”

Figures

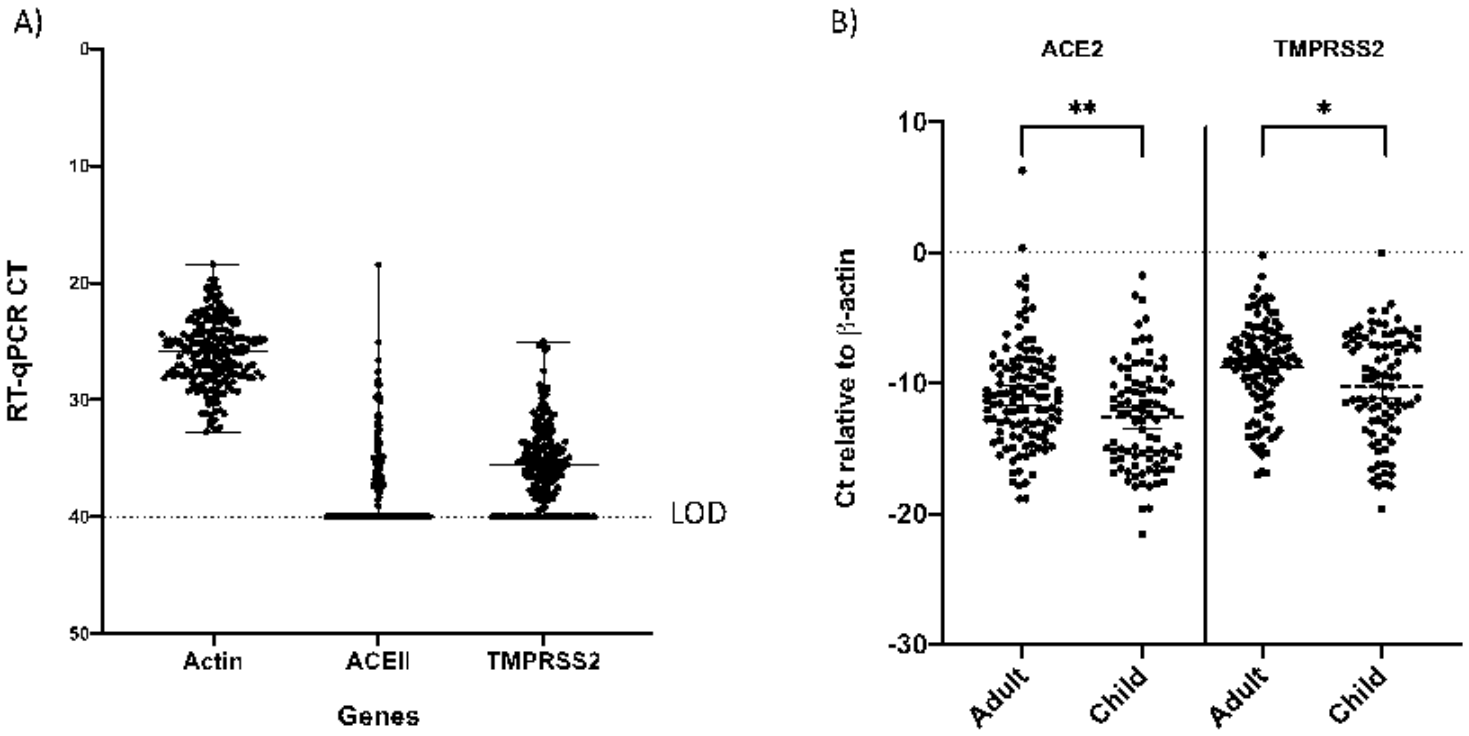

Figure 1

Gene expression of ACE2 and TMPRSS2 in children and adults within family clusters exposed to COVID-19. (A) Variation in the transcript levels of β-actin, ACE2 and TMPRSS2 in nasopharyngeal swab specimens in the study population. Data shows mean Ct values with 95% CI, n = 207 (B) Comparison of transcript levels of ACE2 and TMPRSS2 in nasopharyngeal swab specimens relative to β-actin (ΔCt) between adults and children. ΔCt values were calculated by subtracting the Ct values for ACE2 or TMPRSS2 from the respective Ct values for the house-keeping gene, β-actin. Data shows mean Ct values with 95% CI, n = 115 adult and 92 children. p-values were calculated from Mann Whitney U test; *p=0.0189, **p=0.0045.

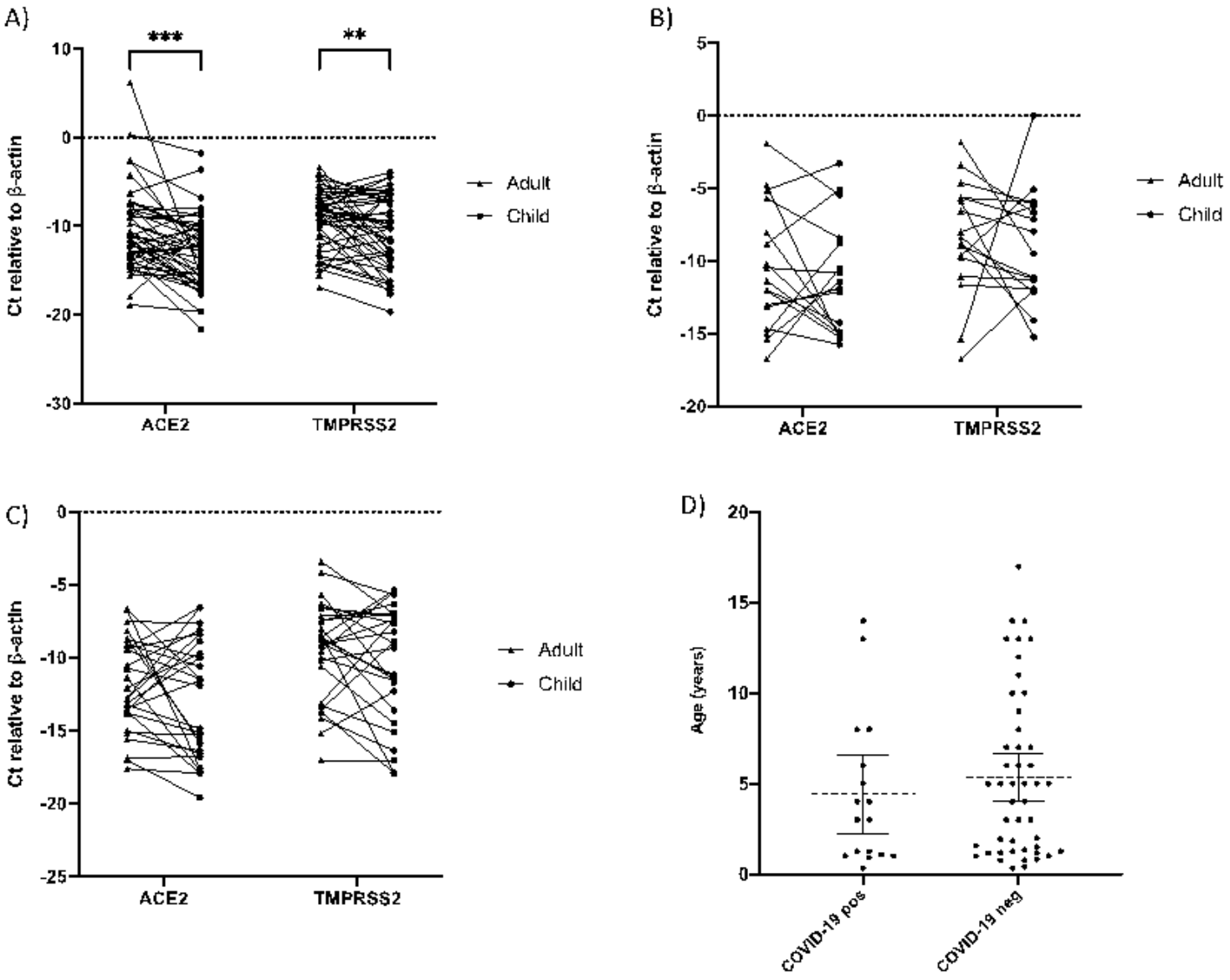

Figure 2

Comparison of transcript levels of ACE2 and TMPRSS2 between children and adults within the same families. ACE2 and TMPRSS2 expression in (A) COVID-19 positive adults versus COVID-19 negative children (n=94; 48 pairs) (B) COVID-19 positive adult versus COVID-19 positive children (n=34; 17 pairs), and (C) COVID-19 negative adult versus COVID-19 positive children (n=56; 28 pairs) within the same families. p-values were calculated from paired, 2-tailed Student's t-test, ***p=0.0004, **p=0.0049. (D) Age distribution of COVID-19 positive and negative children. Data shows mean age with 95% CI, n = 45 COVID-19 positive and 47 COVID-19 negative children. p-values were calculated from Mann-Whitney U test; p=0.4081.

Supplementary Files

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