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Nasopharyngeal ACE2, CD147, and TMPRSS2 expressions in MIS-C patients

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Abstract

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Aim: Multisystem Inflammatory Syndrome in Children (MIS-C) is a disease developed after about eight weeks of the new coronavirus disease (COVID-19) infection in children. The underlying reason for MIS-C is still unknown. This study aims to assess the nasopharyngeal expressions of Angiotensin-converting enzyme 2 (*ACE2*), the differentiation 147 receptor cluster (*CD147*), and the transmembrane serine protease 2 (*TMPRSS2*) receptors in the MIS-C population. Since these receptors are related to COVID-19 infection, children with previous COVID-19 history were included as a control group. This study is essential for future studies to see if there is any significant expression difference between these receptors in control and MIS-C populations.

Materials and Methods: The prospective cohort study took place at Ondokuz Mayıs University. The participants of this study consisted of patients aged 0-18 years who were diagnosed with MIS-C due to COVID-19 infection or patients with only previous COVID-19 pneumonia but without MIS-C development. A total of 79 cases were registered in this study. Nasopharyngeal samples of children were collected. Total RNA was isolated from all samples. The expression of *ACE2*, *CD147*, and *TMPRSS2* genes was accessed by real-time quantitative PCR (RT-qPCR).

Results: Nasopharyngeal expression of *CD147* and *TMPRSS2* were not changed in COVID-19 (n=42) and MIS-C (n=37) cases. Expression of *ACE2* was increased in the under 10-year-old MIS-C group (n=23) (p=0.004381).

Conclusion: Future studies may exclude the nasopharyngeal expression of *CD147* and *TMPRSS2* to develop MIS-C. Further investigations are required to learn how *ACE2* expression contributes to MIS-C development.



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Introduction

The new coronavirus disease (COVID-19) is caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) which was initially reported in December 2019 [1]. The World Health Organization (WHO) reported that about 7 million people have already died from SARS-CoV-2 and more than 700 million individuals had been infected as of July 2023 [2]. COVID-19 has a wide clinical spectrum that ranges from asymptomatic to severe [3]. Even

though most patients usually recover without any complications and/or major interventions, the elderly and people with underlying chronic diseases are more likely to develop a serious disease phenotype [4]. Yet, severe COVID-19 is more common in children with comorbidities [5]. Moreover, a recent study has reported that COVID-19 has indirect consequences on children and adolescents including mental health issues, poorer lifestyle habits, reduced access to health care, and increases in online sexual abuse [6].

Although COVID-19 is rare and asymptomatic in children, a group with similar clinical expressions of Kawasaki disease applied to clinics when the first peak of COVID-19 was seen in Europe around April 2020 [7]. The symptoms are related to COVID-19, yet it was reported that the development took at least 2 to 4 weeks after acute

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COVID-19 infection [8]. Later, it was reported that those children didn't have Kawasaki disease but had COVID-19 associated Multisystem Inflammatory Syndrome in Children (MIS-C) [9, 10].

The UK Centers for Disease Control and Prevention (CDC) defines the following features for MIS-C: -under 21 years of age, -high fever with more than 24 hours, -inflammation, -severe disease requiring hospitalization, -at least two organ involvement, -positive SARS-CoV-2 PCR test result or direct contact with COVID-19 positive case 4 weeks before the development of symptoms [7]. The typical clinical marks of MIS-C include rash, conjunctivitis, GI symptoms, and myocardial dysfunction [11-14]. Understanding COVID-19 and MIS-C epidemiology and progress essential in developing better management strategies for babies, kids, and teenagers. In addition, the determination of the infection potential of asymptomatic children infected with SARS-CoV-2 could create new strategies for protection against viral infection. Most importantly, determination of MIS-C pathogenesis is crucial for managing and treating the disease.

The expression level of SARS-CoV-2 receptors is thought to have a function on the infection of the virus and the severity of pathogenesis. Angiotensin-converting enzyme 2 (*ACE2*) receptor is the initial point for viral infection of the cell, and it is co-expressed with the transmembrane serine protease 2 (*TMPRSS2*) receptor [15, 16]. Additionally, when the virus's S protein binds to the differentiation 147 receptor cluster (*CD147*), *TMPRSS2* makes viral entry easier [17, 18]. The expression level of *ACE2* and *TMPRSS2* receptors increase by age [19, 20]. However, the relationship between *CD147* expression and age is reported to be complicated [21]. *ACE2* and *TMPRSS2* receptors expression is decreased in children's upper and lower respiratory tract, yet this situation is not validated for MIS-C cases. Age-related influences of *ACE2* are still being investigated [22].

This study aims to define the nasopharyngeal *ACE2*, *CD147*, and *TMPRSS2* receptors expression in MIS-C patients compared to children with previous COVID-19 history. In this way, scientists could have more insight into factors influencing MIS-C development.

Materials and Methods

Study population and sample collection

The participants of this research consisted of patients aged 0-18 years who were diagnosed with MIS-C due to COVID-19 infection or only COVID-19 pneumonia in the College of Medicine, Ondokuz Mayıs University. Power analysis was used to estimate the minimum sample size (n=35). Vaccination for COVID-19 was not obligatory for children during the specimen collection period. Therefore, all the cases in this study were unvaccinated. Informed consent was obtained from all subjects and/or their legal guardian(s). Demographic and clinical data for all children were recorded. The study was conducted according to the ethical standards set forth in the Helsinki Declaration of 1964 and subsequent amendments or comparable ethical standards. Approval was granted for the "Comparison of *ACE2*, *TMPRSS2*, and *CD147* surface receptor

expressions in those suffering from MIS-C and COVID-19 pneumonia" study by the Ethics Committee of Ondokuz Mayıs University (No: 2021/191). Nasopharyngeal samples of children aged 0-18 were included in the research. After the nasopharyngeal specimens were collected, they were stored at -80°C in Eppendorf containing 1 ml of Qi-azol until RNA isolation.

RNA extraction of nasopharyngeal samples

RNA isolation was performed by modifying the protocol from the literature [23]. Briefly, the samples stored in Qi-azol were dissolved, vortexed, and incubated for 5-10 min. at room temperature (RT), then vortexed, and 800 µl of the sample was moved into a separate Eppendorf tube. 0.2 ml of chloroform was added into the tubes, and the tubes were inverted 5-10 times and incubated for 5 min. at RT. Then, the tubes were centrifuged at 10,000 g at +4°C for 20 min. and the clear solution was moved into a separate tube. After adding 0.8 ml of cold isopropanol into the tubes, they were vortexed and incubated on ice for 10 min. Later, the tubes were centrifuged at 10,000 g at +4 °C for 20 minutes and the supernatant is discarded. The pellets were washed 2 times with 1 ml of 70% cold ETOH at 10,000 g for 5 min. at +4 °C. Afterward, the pellets were completely dried in air and homogenized with 25 µl of RNAase-free water. The concentration of RNA was computed through Nanodrop2000 spectrophotometer (ThermoScientific, USA). The RNA quantification was obtained in duplicates.

cDNA synthesis and Real-Time qPCR

The expression of target receptors was tested via real-time - quantitative polymerase chain reaction (RT-qPCR) because it was fast and cheap. Moreover, RT-qPCR allows for studying more samples in triplicate at the same time. Complementary DNA (cDNA) was synthesized by using the iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA) along with the manufacturer's instructions. Briefly, a 10 µl RNA sample was mixed with iScript reagents including 4 µl 5X iScript reaction mix, 1 µl iScript reverse transcriptase, and 5 µl nuclease-free water to a final reaction volume of 20 µl. The reaction was incubated for 5 min at 25°C, 20 min at 40°C, and 1 min at 95°C, and then stored at -20°C.

Primer designs were done with the Primer3 software (<http://frodo.wi.mit.edu/primer3>) based on the mRNA sequences from ENSEMBL database (*ACE2*: ENSG00000130234, *CD147*: ENSG00000172270, *TMPRSS2*: ENSG00000184012, and *GAPDH*: ENSG00000111640) (<https://www.ensembl.org/index.html>). The specificity of the primers was confirmed with the BLAST database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The primer sequences are listed in Supplementary Table 1.

A real-time qPCR assay was performed in 96-well white plastic plates with a final volume of 20 µl which contained 10 µl of SsoAdvanced™ Universal Inhibitor Tolerant SYBR® GreenSupermix(2X) (Bio-Rad Laboratories, USA), 1 µl of 5 mM primer solution (250 nM final concentration) containing primers for forward and reverse, 5 µl of cDNA, and 3 µl of nuclease-free water. PCR program was

set as follows: 3 min at 98°C for an initial activation stage followed by 40 cycles including 15 s at 98°C for denaturation and 30 s at 60°C for annealing and elongation by using an AppliedBiosystems 7500 real time machine (Applied Biosystems, USA). The default settings were used to analyze melting curves. *GAPDH* was used for normalization and the fold change was calculated using the 2-ΔΔCT method [31]. The 2-ΔΔCT values were then used for statistical analysis.

Statistical analysis

Graph Pad Prism7 (GraphPad, San Diego, CA, USA) was used for the analysis of data. Each experiment was performed at least thrice, and all data were reported as mean ± SEM except if otherwise noted. Mean ± SD was used to measure data variability around the mean of a sample of the study group. Fold changes were calculated from 2-ΔΔCt values. Analysis was done by using the Mann-Whitney two-tailed test. Age-related gene expressions were done by using an unpaired nonparametric Mann-Whitney test. Statistical signification was calculated with the Mann-Whitney U test and stated as a p<.05.

Results

Demographical features of the cases

A total of 79 children were involved in this study. Among them, 53% were children in which Covid antibody or antigen positivity was detected in the past (at least 2 months ago) but were not diagnosed with MIS-C. 47% of the population were diagnosed with MIS-C who were positive for COVID-19 in the past, either by antibody PCR or an antigen PCR test. While 43% of the cases were female, 57% were male, and 56% were ≤10 years old (Table 1). The mean age of the population was nine years.

Expression level of ACE2, CD147, and TMPRSS2 transcript in nasopharyngeal samples

Nasopharyngeal samples of the COVID-19 group were taken at least 8 weeks after active COVID-19 positivity to exclude MIS-C positivity. Nasopharyngeal samples of MIS-C patients were taken in the course of the disease. A comparison of target genes in the study population revealed no significant change in expression of *ACE2*, *CD147*, and *TMPRSS2* (Supplementary Figure 1).

Recent research has shown that *ACE2* and *TMPRSS2* expressions change with age [19, 20]. Therefore, these findings were tested along with *CD147* expression in this study population. Accordingly, it was examined whether gender and age affect target gene expression in children (Figure 1 and Supplementary Figure 2). No significant differences were found in gender and age-specific expression of *ACE2*, *CD147*, and *TMPRSS2* in children.

Table 1. Demographical data of the study population.

Group	Female	Male	Age≤10 years	Age>10 years	Total Cases
COVID-19	19	23	22	20	42
MIS-C	15	22	23	14	37

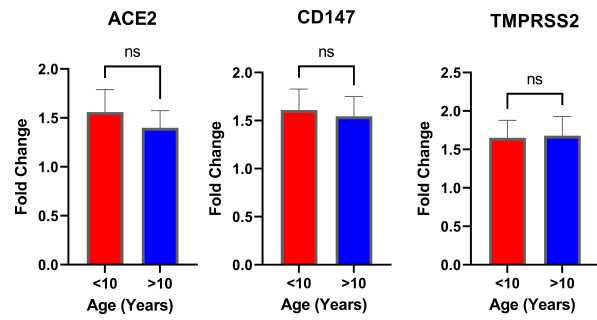


Figure 1. Comparison of *ACE2*, *CD147*, and *TMPRSS2* Expression Fold changes were calculated from 2-ΔΔCt values. Analysis was done by using the Mann-Whitney two-tailed test. Error bars represent mean ± SEM. P values for age comparison of *ACE2*, *CD147*, and *TMPRSS2* genes are 0.9863, 0.9666, and 0.8923 respectively. Aged 10 years were included in the <10 group.

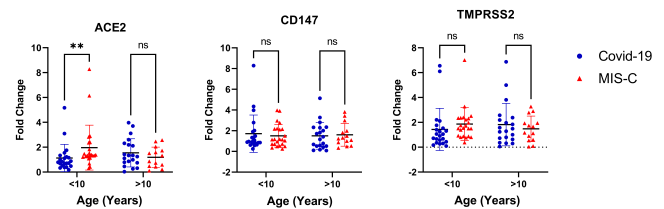


Figure 2. Comparison of the target genes in COVID-19 and MIS-C groups. The analysis was done by using multiple nonparametric Mann-Whitney two-tailed tests. Error bars represent mean ± SDM. Aged 10 years were included in the <10 group. *ACE2* expression is higher in children with MIS-C under 10 years old, Mann-Whitney U test, p=0.004381.

Comparison of ACE2, CD147, and TMPRSS2 expression between COVID-19 and MIS-C groups

According to RT-qPCR data, children have similar *ACE2*, *CD147*, and *TMPRSS2* expression in their nasopharyngeal samples (Figure 1 and Supplementary Figure 2). Next, these data were analyzed between COVID-19 and MIS-C groups to see if there is any statistical significance (Supplementary Figure 3). It is interesting to note that there was no notable change in *ACE2*, *CD147*, and *TMPRSS2* gene expression between COVID-19 and MIS-C groups (Supplementary Figure 3).

Next, gender and age-dependent expression of target genes were tested (Figure 2 and Supplementary Figure 4). Comparison of *ACE2* expression in children under 10 years shows a significant p=0.004381. *ACE2* expression is higher in children with MIS-C under 10 years old. There was no significant difference in the *CD147* and *TMPRSS2* expression in the study groups.

TMPRSS2/ACE2 ratio is reported to be related to respiratory distress severity [20]. In this study, TM-

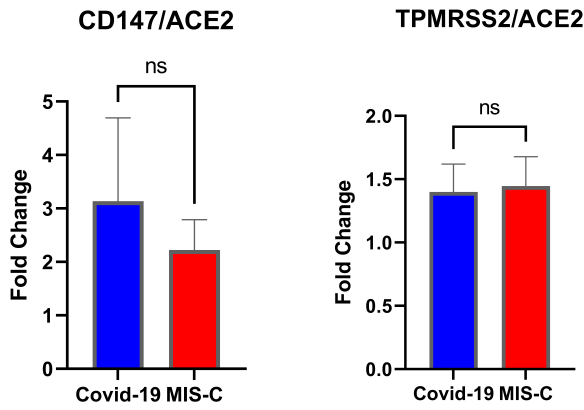


Figure 3. Comparison of *CD147/ACE2* and *Tmprss2/ACE2* ratio in COVID-19 and MIS-C groups. Analysis was done by using the Mann-Whitney two-tailed test. Error bars represent mean ± SEM. P values *CD147/ACE2* and *Tmprss2/ACE2* are 0.3539 and 0.7788, respectively.

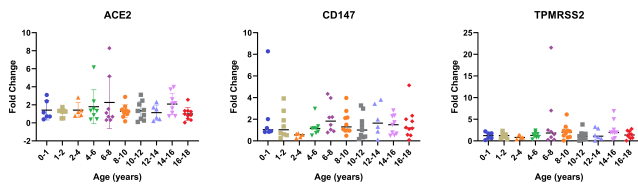


Figure 4. Age-Related Expression of *ACE2*, *CD147*, and *Tmprss2* Analysis was done by using an unpaired non-parametric Mann-Whitney test.

PRSS2/ACE2 and as well as *CD147/ACE2* ratios in two groups were compared. The result shows that no statistical significance was found on the expression ratio of *Tmprss2/ACE2* and *CD147/ACE2* in children with COVID-19 and MIS-C (Figure 3).

Lastly, it was tested to see if the expression of each gene changes by a certain age. For this reason, the study population was divided into ten groups of different ages (Figure 4). Age-related analysis of *ACE2*, *CD147*, and *Tmprss2* RT-qPCR data revealed no significant difference in the target gene expressions at various ages (Figure 4).

Discussion

It was initially believed that healthy children were not affected by the severe COVID-19 disease. Later, it was understood how incalculable COVID-19 could be as MIS-C appeared with findings of mortalities in children [24]. MIS-C in children is a type of disease in which different parts of the body can develop inflammation, the affected organs include the brain, heart, kidneys, lungs, eyes, skin, or gastrointestinal organs [25, 26]. The precise cause of MIS-C is still unidentified. It is only known that many children with MIS-C have previously been infected with the SARS-CoV-2 virus or have been in contact with someone with COVID-19. MIS-C can be mild; however, sometimes, it

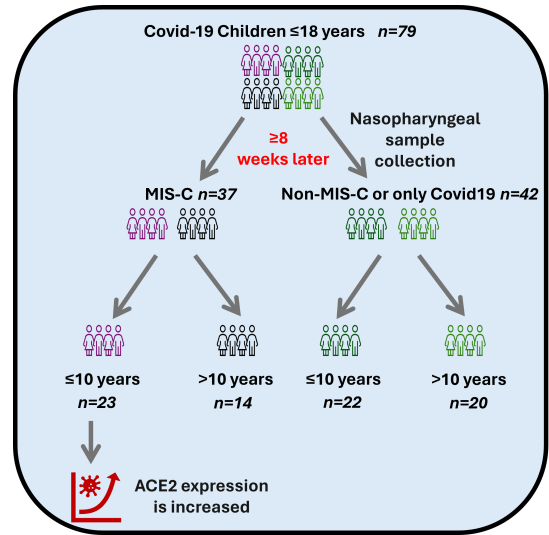


Figure 5. Summary of the study. The study population was divided into MIS-C and non-MIS-C (only COVID-19) groups. Nasopharyngeal samples were taken from the population. After RNA isolation, the expression of receptor genes *ACE2*, *CD147*, and *Tmprss2* was analyzed. *ACE2* expression is increased in MIS-C children ≤10 years old, $p=0.004381$.

can be serious and even fatal [26, 27]. Many of these children can return to normal lives with an on-site medical intervention [26, 28].

In this study, *ACE2*, *CD147*, and *Tmprss2* receptors expression that are entry gates of SARS-CoV-2 were analyzed to see whether it has any correlation with MIS-C development. In the MIS-C group of ≤10 years old, an increase in *ACE2* expression is found (Figure 5). Rossi et al. evaluated the nasopharyngeal expression of *ACE2* and *Tmprss2* in adults with respiratory distress and interestingly they found that *ACE2* and *Tmprss2* levels increase with age [20]. They also evaluated the *Tmprss2/ACE2* ratio and concluded that an increase in this ratio was related to disease severity. In this study, even though *CD147* and *Tmprss2* ratios over *ACE2* were tested, no statistical significance was found. These findings could be related to the age of the study’s population. In addition, Hasan et al. compared the nasopharyngeal expression of *ACE2* and *Tmprss2* in children and adults. They reported that *ACE2* expression increased in adults while expression didn’t change in both groups [19]. Additionally, Bunyavanich et al. evaluated the nasopharyngeal expression of *ACE2* in children and adults and reported an increase in *ACE2* expression with age [29]. The expression of target genes according to age has revealed no statistical significance in this study (Figure 1). However, when the age-dependent expression of these genes was tested in COVID-19 control and MIS-C groups, an increase of *ACE2* expression in children ≤10 years old with MIS-C was shown (Figure 2). Even though Bunyavanich et al. had a bigger cohort, their cohort was not MIS-C or COVID-19 specific. Our study is the first to test the expression of these three receptors in children with MIS-C

and previous COVID-19 history. The complicated interplay among the SARS-CoV-2, *ACE2* receptor, pulmonary tissue, and immune system is still ambiguous [22].

Lastly, in this study, the number of under 10-year-old MIS-C cases are higher than the COVID-19 cases (Table 1). This result is consistent with the literature [30, 31] and may explain why children under 10 years old are more prone to developing MIS-C.

Even though we didn't see any difference in the mRNA expression of *CD147*, *TMPRSS2* in the two groups, a change at the protein level, or post-translational modification could be a reason.

There were some limitations in this study. The first one was that re-infection status was not tested in the COVID-19 control group, due to the no COVID-19-related symptoms. The second one was that the study population was not big due to some parents or children being uncomfortable taking nasopharyngeal samples. The last limitation is that the study results could not be validated with a second method because taking nasopharyngeal samples from children was uncomfortable.

Conclusion

The significance of this study is that for the first time, the expressions of *ACE2*, *CD147*, and *TMPRSS2* were compared in the nasopharyngeal sample of children with MIS-C and previous COVID-19 history. *ACE2* expression was found to increase in children ≤ 10 years of age MIS-C population. This will aid future studies to exclude MIS-C development due to *CD147* and *TMPRSS2* expressions in nasopharyngeal samples of the children. Yet, further studies with a larger population will generate better results.

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Ethical approval

Approval was granted for the study by the Ethics Committee of Ondokuz Mayıs University (No: 2021/191).

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