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NASAL EXPRESSION OF ANIOTENSIN-CONVERTING ENZYME 2 (ACE2) IN CHILDREN AND ADULTS WITH COVID-19

BY

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ABSTRACT

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Title: Nasal Expression of Angiotensin-Converting Enzyme 2 (ACE2) in Children and Adults with COVID-19

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Emerging evidence suggests that the lower expression of SARS-CoV-2 entry factor angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) in the nasal epithelium of children may contribute to lower incidence of COVID-19 in this age group compared to adults, yet no direct evidence is available for this hypothesis. In this study, we compared the transcript levels of ACE2 and TMPRSS2 in nasopharyngeal swabs (NPS) of children and their companion adults within COVID-19 exposed-families (n=207), to assess their association with infection status. Additionally, NPS specimens from COVID-19 positive and symptomatic children (n=24) and adults (n=10) were assessed for associations of ACE2 and TMPRSS2 expression with patients' clinical and laboratory outcomes.

In the paired dataset, the expression of both genes was higher among adults (n=115) compared to children (n=92), but the expression was not significantly different between COVID-19 positive and negative patients of all ages or within the same age group. Within the same families, the expression of ACE2 and TMPRSS2 was higher in COVID-19 positive adults when compared to COVID-19 negative children (p=0.0002 and 0.0061, for ACE2 and TMPRSS2, respectively, by Wilcoxon Signed-Rank test, n=94), but the

expression of these genes was not significantly different between COVID-19 positive adults and children or between COVID-19 negative adults and positive children. Consistently, the expression of both genes was positively associated with SARS-CoV-2 positivity in this subgroup only (OR: 1.146, 95%CI: 1.038-1.284, p=0.0114 for ACE2 and OR: 1.123, 95%CI: 1.012-1.254, p=0.0334 for TMPRSS2). These findings suggest that children with lower expression of nasal ACE2 and TMPRSS2 are likely to remain COVID-19 negative despite being exposed to a COVID-19 positive family member.

Using data from all specimens collected in this study and by grouping them as negative, asymptomatic and symptomatic for COVID-19, no significant association was found between the expression of ACE2 and TMPRSS2 genes and clinical symptoms or laboratory findings.

DEDICATION

I dedicate my thesis work to my family, friends and colleague at molecular infectious disease lab, Sidra Medicine, for their continuous encouragement.

I also dedicate this work and give special thanks to my special colleague and friend Alaa Al Hashemi for helping and supporting me throughout this project, I will always appreciate all she has done.

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Chapter 1: Introduction

In China, in December 2019, a string of pneumonia cases of undetermined etiology were reported [1]. Soon after, the Chinese Centre for Disease Control and Prevention discovered the presence of a new coronavirus in the throat swabs of affected patients. The novel virus resembled SARS-CoV, that caused severe acute respiratory syndrome with elevated fatality during 2002-2003 [2]. Afterward, the newly identified coronavirus was designated as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and the disease is called coronavirus disease 2019 (COVID-19). Subsequently, a global pandemic was declared by World Health Organization in early March 2020. At the time of writing this review (March 08, 2021), the pandemic has affected many countries with more than 116.4 million cases, and more than two million deaths have been associated with COVID-19 were reported [3].

COVID-19 is principally a disease of the respiratory system, although the virus may also affect other organs. Clinical expression of SARS-CoV-2 infection is diverse, varying from asymptomatic to severe, life-threatening infection. Recent data suggests that up to 30 to 40% of the cases could be asymptomatic [4]. Among the symptomatic cases, most of the cases were mild, with flu-like symptoms being common among them, and only few experiencing severe disease with dyspnea, hypoxia, lung involvement, acute respiratory distress syndrome, shock, or multi-organ failure, that could lead to death [5]. Other features such as myalgia, diarrhea, and loss of senses of smell or taste, are also common [6].

The COVID-19 pandemic initiated urgent research on the transmission, pathogenesis, immune response, treatment and prevention of the virus transmission.

Currently there is a large gap in the understanding of the pathophysiology of the disease and the host response to SARS-CoV-2. The available knowledge in these areas so far is mostly derived from previously reported, similar coronaviruses such as MERS-CoV and SARS-CoV. The novel coronavirus, SARS-CoV-2, is one of the zoonotic viruses from beta-coronavirus family that has evolved to transmit between humans, and it has affected large numbers of people worldwide in a short period of time. SARS-CoV-2 has some important structural proteins that are necessary for viral replication, attachment and entry to host cells, which subsequently aid in the spread of the virus. The structural proteins include envelope, membrane, nucleocapsid, and spike proteins. Spike protein, which is the most important protein for virulence, protrudes from the viral surface, and is composed of dual functional subunits, S1 and S2. The S1 subunit functions in attachment to the target cell surface receptors, while the S2 subunit aids in merging the virus and the host cell membrane [7].

Previously, it was reported that SARS-CoV gains access to human cells through the contact of its spike protein with angiotensin converting enzyme 2 (ACE2) receptor [8]. Recent studies demonstrate that the novel SARS-CoV-2 likewise interacts via the ACE2 receptor to gain entry into the human cells. ACE2 is expressed in various body organs involving the lungs, kidney, bladder, ileum and heart. It is expressed at a much higher level in the respiratory tract than other tissues, and this could explain the common respiratory symptoms that are associated with COVID-19 infection [9].

TMPRSS2 codes a protein that belongs to the serine protease family. TMPRSS2 contains a type II transmembrane domain, a scavenger receptor

cysteine-rich domain, a receptor class A domain and a protease domain. The protein remains membrane-bound with a noticeable part being embedded in the extracellular matrix. TMPRSS2 is involved in the proteolytic activation of influenza virus and coronaviruses including MERS-CoV and SARS-CoV [10]. SARS-CoV-2 also uses the TMPRSS2 serine protease for S protein priming, and protease inhibitors have been shown to prevent SARS-CoV-2 entry into the host cells [11].

SARS-CoV-2 can infect individuals of any age. Nevertheless, increased rates of SARS-CoV-2 infection were reported mainly among adults and elderly people. Also, the severe forms of SARS-CoV2 infection and rates of mortalities are much higher among elderly patients than in children, who experience mild to asymptomatic infection most of the time [12]. The factors contributing to the difference in the rates of infection among adults and pediatric patients is not clear yet. In a recent study, based on gene expression data from a non-COVID-19 cohort of asthma patients, it was hypothesized that the lower risk among children is due to differential expression of ACE2. The study shows that the expression levels of ACE2 in the nasal epithelium associates with age and proposed that the lower expression of ACE2 protein in the nasal epithelial cells of children may elucidate why children are less affected by COVID-19 [13]. Again, in cultured cells it was shown that cell lines that express TMPRSS2 are highly vulnerable to SARS-CoV-2 infection [14]. Using a public gene expression dataset, it was also shown that the expression of nasal and bronchial SARS-CoV-2 target receptors, ACE2 and TMPRSS2, is lesser in children when compared to adults [15]. However, there is no direct evidence that correlates ACE2 and TMPRSS2 expression with lower rates of infections or the absence of symptoms or mild symptoms in children.

Worldwide, the total cases and deaths that are associated with COVID-19 are still on rise. In the lack of an effective or specific treatment against the novel SARS-CoV-2 virus, early recognition and contact tracing, physical distancing measures and quarantine of positive cases have remained the principal measures to limit the community transmission of SARS-CoV-2. Understanding the pathogenicity of the virus and host factors in relation to the age of the patients is critical for potential therapeutic development, as well as the management of patients with COVID-19.

Hypothesis:

Nasal expression of ACE2 and TMPRSS2 determine differential SARS-CoV-2 infection status in children and adults within the exposed families.

Aim:

The aim is to analyze the transcript levels of nasal ACE2 and TMPRSS2 in nasopharyngeal (NP) swabs from children and adult members of exposed families and assess their association with the clinical presentation and SARS-CoV-2 RT-qPCR results.

Chapter 2: Literature Review

2.1 Coronavirus disease 2019

In December 2019, a string of pneumonia cases was reported in Wuhan, China. Patients were presented with fever, dry cough, and shortness of breath and radiological evidence of bilateral lung infiltration [1]. The underlying etiology of this clinical phenotype was not known, until the Chinese Center for Disease Control and Prevention took throat swabs from affected individuals, and subsequently identified the presence of a novel coronavirus [2]. The newly identified coronavirus resembled severe acute respiratory syndrome coronavirus (SARS-CoV), which was associated with high morbidity and mortality back in 2002-2003 [16]. Consequently, the novel coronavirus 2019 was named severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and the disease caused by SARS-CoV-2 was named coronavirus disease 2019 (COVID-19). On March 11, 2020, a global pandemic was declared by the WHO and global preventive measures were implemented to prevent worldwide transmission of the disease.

2.2 COVID-19 prevalence

To date, SARS-CoV-2 affected a vast number of people worldwide. As of March 08, 2021, around 116.4 million confirmed cases of COVID-19 have been reported to WHO [17], including 2.6 million deaths globally (figure 1). Of these cases around 93 million recovered from infection. Currently the active infectious cases are approximately 21.9 million with only 0.4% with severe and critical form of infection and the rest with mild condition [3]. The available data to date shows that COVID-19 mortality rate is between 2-3%, which has evolved over the course

of the pandemic. In Qatar, according to the Ministry of Public Health, there were around 167 thousands of positive cases from the beginning of this pandemic to March 08, 2021, with only 10,855 active cases of COVID-19 (figure 2) [18]. SARS-CoV-2 infects individuals of any age group, but many studies showed that children have lower rates of SARS-CoV-2 infection when compared to the adults. For instance, Chinese Center for Disease Control and Prevention reported that among 44,672 confirmed cases of COVID-19, only 1% were children, with the rest being mainly adults and elderly patients [19]. While another study that was conducted in Korea revealed that only 4.8% of confirmed COVID-19 cases were children [20].

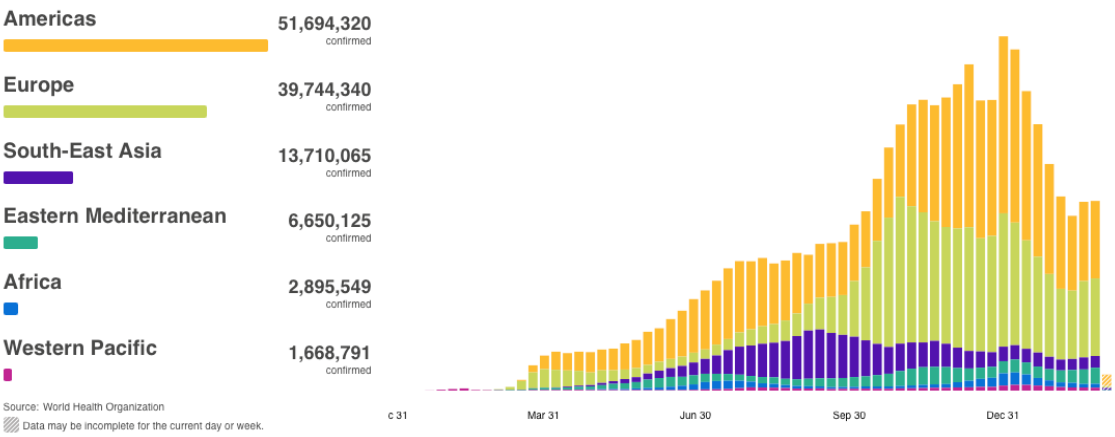

Figure 1: COVID-19 confirmed cases data across regions, as of March 08, 2021. The data on the left of the figure shows cumulative COVID-19 positive cases across WHO regions. While the graph on the right shows COVID-19 weekly data in different regions [17].

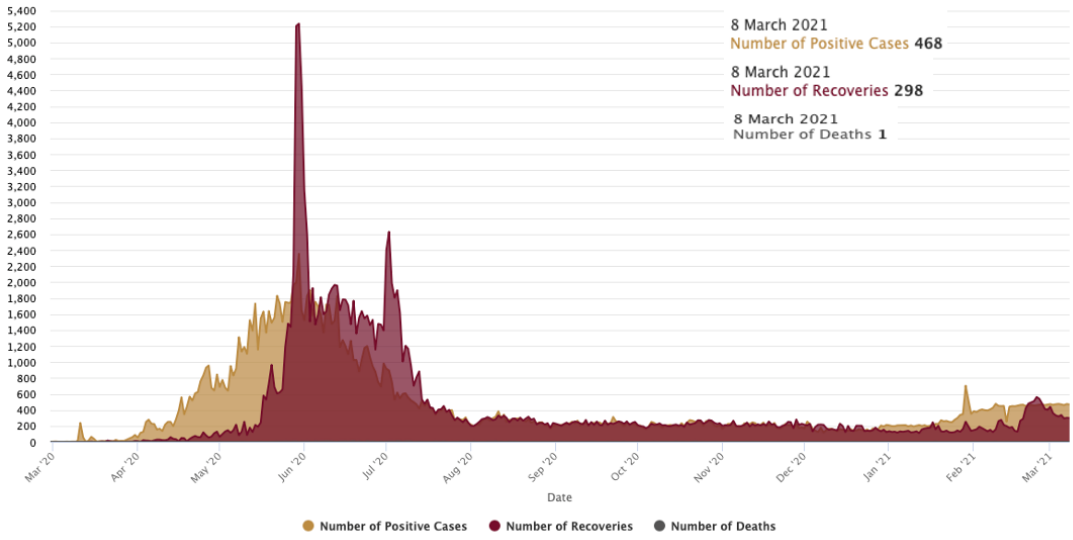

Figure 2: COVID-19 cases in Qatar, As of March 08, 2021.

The Ministry of Public Health in Qatar updates COVID-19 status daily. In March 8, 2021, there were 468 new cases of COVID-19, 298 recoveries and 1 death [18].

2.3 Clinical presentation of COVID-19

Clinical manifestations of COVID-19 are heterogenous. Infection with SARS-CoV-2 could result in asymptomatic infection: in a large cohort of COVID-19 patients, around 30%-40% of positive cases were asymptomatic [4]. On the other hand, some patients experience symptomatic course of infection, that range from mild to moderate disease manifestations to severe and life-threatening forms of the disease that may lead to death. Symptomatic cases of COVID-19 experience flu-like symptoms in most cases. Most common symptoms are fever, dry cough, dyspnea and fever and the involvement of lower respiratory tract, which could eventually lead to acute respiratory distress syndrome (ARDS) [21]. Other symptoms including, diarrhea, vomiting, generalized weakness, and headache were also reported among COVID-19 patients; this suggested that other organ systems are also affected by SARS-CoV-2 infection [22]. Various studies have been

conducted to determine the clinical presentations associated with COVID-19 (Table 1). For instance, in a retrospective study that was conducted on 104 COVID-19 patients from the Diamond Princess cruise ship, in Japan, the majority of COVID-19 patient experienced either an asymptomatic (33/104) course of infection or a mild to moderate (43/104) respiratory infection, with fever, sore throat and dry cough, and these patients were eventually freed of infection and its associated symptoms within a few days. On the other hand, some infected individuals (28/104) experienced serious disease symptoms including, pulmonary edema, severe pneumonia, acute respiratory distress syndrome (ARDS), septic shock, and subsequently multiple organ failure [23]. Also, in a retrospective study conducted on 41 COVID-19 hospitalized patients, other disease complications including acute cardiac injury, acute kidney injury, lymphopenia and secondary infections and thromboembolic events were identified [5]. Furthermore, in a recent systematic review it was shown that there is a high prevalence of smell and taste dysfunction among COVID-19 patients [6]. Several observational studies of laboratory findings in COVID-19 patients were assessed in a systematic review with a meta-analysis. The review included 4,663 patients, and the common abnormal laboratory findings among them were, elevated C-reactive protein (CRP, 73.6%), decreased albumin (62.9%), increased erythrocytes sedimentation rate (ESR, 61.2%), decreased eosinophils (58.4%), increased interleukin-6 (IL-6, 53.1%) lymphopenia (47.9%), and increased lactate dehydrogenase (LDH, 46.2%). Severe cases of the disease were found to be closely associated with increased CRP, LDH, and reduced lymphocyte count. Together these clinical phenotypes and laboratory findings could contribute to the diagnosis of COVID-19 [24].

SARS-CoV-2 infects people of different age groups with a varying degree of disease severity. Symptomatic severe cases of SARS-CoV-2 infection, that required hospitalization and intensive care were common among elderly patients especially those with comorbidities. The clinical characteristics of elderly COVID-19 patients with young and middle-aged patients were compared in a retrospective study. It was found that elderly COVID-19 patients have higher pneumonia severity index (PSI) (grade VI and V), with multiple lobe involvement, and lower lymphocyte count. These data suggest that elderly people with SARS-CoV-2 infection are more likely to develop severe forms of the disease, with high mortality rates. Furthermore, the higher mortality rates among elderly could be also related to variations in the lung anatomy, and muscle weakness, which eventually leads to changes in the normal function of the respiratory tract, such as reduced airway clearance and barrier defense, which render the host more susceptible to infection [12]. Poor prognosis from COVID-19 and higher morbidity and mortality rates have been associated with underlying metabolic conditions, including diabetes and cardiovascular diseases, at any age [25]. Also, it was found that patients with chronic obstructive pulmonary disease are at 4-fold higher risk of being infected with SARS-CoV-2 with severe disease outcome [26]. The association between body mass index (BMI) and clinical presentation of COVID-19 was also studied. It was found that among 124 patients, who required intensive care, 47.6% were obese with a BMI more than 30 kg/m2, and 28.2% were suffering from severe obesity with a BMI of more than 35 kg/m2. Disease severity is enhanced in patients with increased BMI and require invasive mechanical ventilation [27].

On the other hand, fewer cases of COVID-19 have been reported among children, which often presents with an asymptomatic course of infection or mild to moderate disease complications. In a study that was conducted in China in the initial stages of this pandemic, the clinical presentations of a cohort of 1099 patients were assessed: only 0.8% were children, and only one of them experienced a severe course of the disease [28]. Later on, a case series was reported by the Chinese Center for Disease control and prevention, that included 72,314 cases of which only 1% were children under the age of 10 years [19]. Furthermore, another study evaluated confirmed and suspected cases of COVID-19 among 1391 children, and they found that the incidence of SARS-CoV-2 infection increased to 12.3% [29]. In addition, similar to adults, children with COVID-19 experience a wide range of clinical manifestations of the disease, based on their general health, and the presence of other chronic diseases. Dong et al., evaluated the clinical presentation of COVID-19 among 2135 pediatric patients, and they found that 4.4% of the cases were asymptomatic, 51% and 38.7% of the cases experienced mild to moderate disease symptoms, respectively [30]. Those with mild infection had fever, cough, runny nose, and some cases experienced gastrointestinal symptoms like diarrhea and vomiting. The moderate cases had pneumonia with frequent fever, productive cough, and their chest tomography revealed lung lesions [30]. Hence, pediatric symptoms are close to the ones observed among adults, but so far, no cases of anosmia have been reported. Furthermore, in a recent meta-analysis involving 1667 pediatric patients, of which 19% where asymptomatic cases, only 3% of them were severe cases of COVID-19 and the rest experienced mild forms of the disease with fever and cough being the most common symptoms [31]. Furthermore, in another

meta-analysis, in which they included a total of 19 studies with 2855 children and adolescents with SARS-CoV-2 infection. They found that 79% of the cases presented with mild disease symptoms and 4% of the cases were critical. Also, they reported that around 47% experienced fever, 37% were presented with cough, 4% had diarrhea, 2% had nasal congestion, and 1% suffered from dyspnea. While none of the studies cases experienced abdominal discomfort [32]. The clinical presentation of COVID-19 is widely variable among children, adults, and elderly, especially those with underlying health condition, the reason behind this diversity is not well known yet and understanding it would help in disease diagnosis and management.

Table 1. Summary of COVID-19 clinical presentation per published reports

Study	number of patients	% asymptomatic	% symptomatic	Clinical presentation
Diamond Princess cruise ship – Japan [23]	104	31.7%	68.2%	Mild to moderate symptoms (41.3 %): fever, sore throat and dry cough. Severe infection (26.9 %): pulmonary edema, severe pneumonia, ARDS, septic shock and organ failure
Wuhan hospital [5]	41 hospitalized patients	NA	100%	Acute cardiac injury (12 %), secondary infection (10 %) and acute kidney injury (7 %) and
Systematic review [24]	4663	-	-	Increased CRP (73.6%), ESR (61.2%), increased IL-6 (53.1%), decreased albumin (62.9%), decreased eosinophils (58.4%), lymphopenia (47.9%) and increased LDH (46.2%)
Hainan Provincial People's Hospital [12]	56 18 Elderly 38 young and middle-aged	-	100%	Higher PSI, multiple lobe involvement and lower lymphocyte count among elderly.
China - 552 hospitals [28]	1,099 0.9% younger than 15 years of age	-	100% 84.2% non-severe disease 15.7% severe disease	Fever (88.7%), cough (67.8%), vomiting (5%) and diarrhea (3.8%)
Chinese CDC [19]	72,314 1% under the age of 10 years	1.2%	98.7% 81% mild 14% severe: 5% critical	Mild: non-pneumonia and mild pneumonia Severe: shortness of breath, lung infiltration Critical: respiratory failure, septic shock, multiple organ dysfunction
National Notifiable Infectious Disease Surveillance system at the Chinese CDC [30]	2135 pediatric patients	4.4%	89.7% 51% mild 38.7% moderate	Mild: fever, cough, runny nose, diarrhea and vomiting Moderate: pneumonia, fever, cough and lung lesions
Systematic review [32]	2855	17%	83% 79% mild 4% severe	Fever (47%), cough (37%), diarrhea (4%), nasal congestion (2%) and dyspnea (1%)

2.4 Causative agent and COVID-19 transmission

SARS-CoV-2 belongs to the family Coronaviridae, which are enveloped, positive-sense, single-stranded RNA viruses. Coronaviruses are divided into four genera, which include, α, β, γ, and δ coronavirus [33]. The α and β coronaviruses are mainly found to infect mammals and cause pneumonia, acute lung injury, and acute respiratory distress syndrome and organ failure. SARS-CoV-2 belongs to the β coronavirus genus [34], along with previously reported SARS-CoV and Middle East Respiratory Syndrome (MERS-CoV) [35]. Coronaviruses in general, including SARS-CoV-2, have four essential structural proteins, that are necessary for viral attachment and penetration of the host, biosynthesis of viral proteins, maturation of new viral particles, and release of the virus. These structural proteins include spike glycoprotein (S), envelope glycoprotein (E), membrane glycoprotein (M), and nucleocapsid protein (N), in addition to other proteins that are necessary for the viral life cycle [7] (Figure 3). S glycoprotein is a transmembrane protein that protrude from the viral surface, which gives the virus a crown-like appearance, and it aids in the attachment and the fusion of the virus to the host cells. Viral S protein is cleaved to become functionally active by the host's furin-like protease into S1 and S2 subunit. The S1 subunit is found to function in the recognition and binding to host cell receptors and the S2 subunit aids in the subsequent fusion with the host membrane receptor [36]. Following the fusion of the virus with host membrane, N protein, which is found to be bound with RNA, aids in its entry to the host cells, and utilization of host's cellular machinery for viral reproduction [37]. Furthermore, E glycoprotein is a component of viral envelope and it forms viroporins that are required for viral assembly and release [38]. M glycoprotein determines the shape

of the viral envelope, and it has the ability to bind to and stabilize N protein-RNA complex, and it further promotes viral assembly [39].

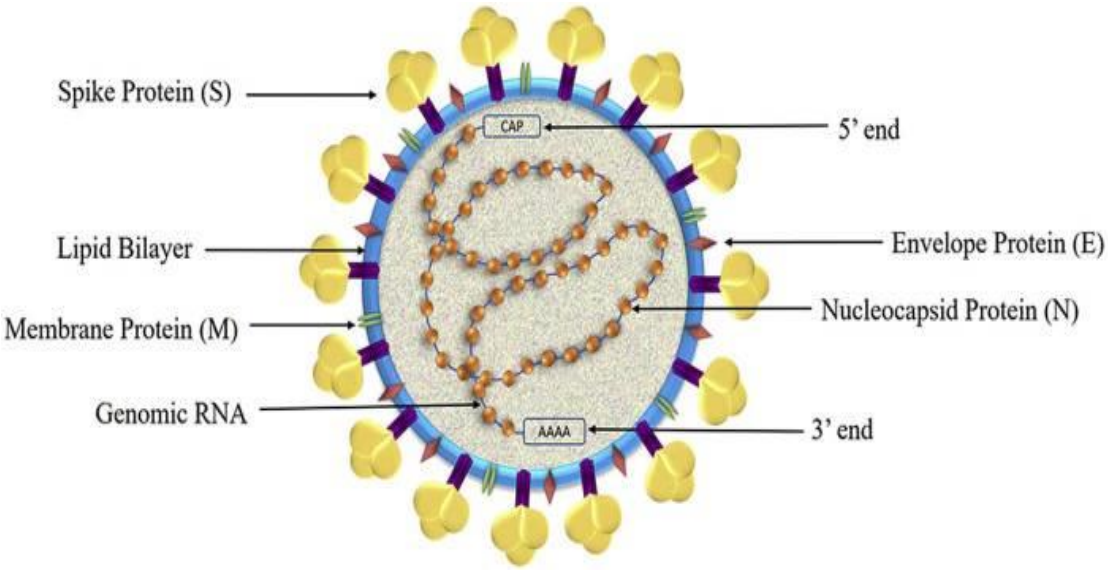

Figure 3: Structural proteins of coronaviruses.
All the structure proteins of SARS-CoV-2 are shown in the figure above and these proteins are essential for viral pathogenesis. Retrieved from [7].

The precise mechanism behind the disease initiation and subsequent transmission is not yet known. But due to the high similarities between SARS-CoV-2 and other coronaviruses that are found in the bats, which is considered as a common reservoir for coronaviruses [40], and due to the previous gained knowledge about other coronaviruses that infected human, it is suggested that SARS-CoV-2 is a zoonotic virus that evolved to become capable of human-to-human transmission. It has been reported that respiratory viruses in general are highly contagious during the symptomatic stages of the infection, while SARS-CoV-2 is found to be infectious and contagious even in the incubation period of the virus when the patients are pre-symptomatic. SARS-CoV-2 can transmit and infect

people both directly and indirectly [41]. Direct transmission of the virus from one individual to another could occur through respiratory droplets that are generated when SARS-CoV-2 infected people sneeze, cough or even when they talk. In one experiment SARS-CoV-2 was viable in the aerosols after 3 hours, but there was a reduction in the TCID50 from 10^3.5 to 10^2.7 per liter of air [42]. SARS-CoV-2 can also be transmitted indirectly via touching contaminated surfaces and objects. Knowing the mode of viral transmission could eventually help in determining effective ways to control and limit the spread of the virus. The virus typically accesses the human body, particularly to the lungs, through the mucosal membrane of the nose, mouth and eyes [43]. The nose is the most common route of internalizing aerosols through inhalation which makes it an important site for the initial stages of SARS-CoV-2 infection [44].

2.5 Angiotensin-converting enzyme-2 (ACE2) and type 2 transmembrane serine protease TMPRSS2

ACE2 is a transmembrane protein that is encoded by a gene located on chromosome X [45]. ACE2 was first identified as a homolog of angiotensin-converting enzyme, with an amino-terminal domain and carboxyl-terminal catalytic domain that contains the active zinc metalloprotein domain [46] [47]. ACE was found to play a role in the classic renin-angiotensin system to maintain blood pressure through catalyzing the conversion of angiotensin I to angiotensin II, the active peptide [48]. Functionally ACE2 is divided into two different groups: peptidase dependent and independent AEC2. Peptidase dependent ACE2 catalyzes the metabolism of angiotensin I and angiotensin II into angiotensin 1-9 and angiotensin 1-7, respectively. Angiotensin 1-7 has a protective opposing role to

angiotensin II, through binding to a G-coupled protein receptor, inducing vasorelaxation and diminishes pulmonary arterial hypertension and inflammation [49]. Peptidase independent ACE2 is mainly involved in the pathogenesis of coronavirus infection (described below), and the absorption of amino acids into gut epithelial cells [50]. ACE2 is found to be differentially expressed on various body organs. For instance, Zou et al., analyzed data from single-cell RNA sequencing of human systems and they reported that ACE2 is highly expressed on respiratory tract epithelial cells, particular on type 2 alveolar cells [51]. Also, ACE2 receptor is found on cardiac muscle cells, kidney tubule cells, urothelial cells and enterocytes [51].

ACE2 expression could be either detrimental or protective during COVID-19 infection. Independent recent studies showed that SARS-CoV-2 requires ACE2 receptor to successfully enter into the host cells and cause tissue damage [11] [52]. Similar to SARS-CoV, where it was found that increased expression of ACE2 induced viral entry, and blocking the receptor protected against viral invasion and subsequent tissue damage [53] [54] [8]. On the other hand, the protective function of ACE has been also reported. ACE2 facilitates the degradation of angiotensin II, which exerts severe effects on the host, and these include myocardial dysfunction, endothelial dysfunction [55]. ACE2 also interferes with adaptive immune response, in such a way that enhance its response and increase the production of inflammatory cytokines, including IL-6, TNF-alpha and other cytokines developing a condition of cytokine storms that could negatively affect host tissue [56]. Furthermore, the generation of angiotensin 1-7 through ACE2 counteracts the function of angiotensin II [57].

ACE2 can be either membrane bound or soluble in body fluids. Membrane-bound ACE2 is essential for SARS-CoV-2 infection, since the virus needs to bind to the receptor active domain to successfully enter into host cells [54]. The expression of cell surface ACE2 is found to be higher in well-developed lung tissues when compared to immature ones, and a gene expression dataset revealed that ACE2 levels are higher among adults when compared to children and its expression increases with age [58] [15]. Angiotensin II is responsible for the formation of soluble ACE2, through increasing the production of transmembrane proteases such as TNF converting enzyme and ADAM-17. This occurs under normal conditions, infection, and acute lung injury [59]. The soluble ACE2 in serum samples from different age individuals was assessed and it was found that soluble ACE2 level increases with age and is associated with COVID-19 severity [60]. It was hypothesized that the high levels of soluble ACE2 is a result of increased membrane-bound ACE2 and ADAM-17, which is associated with an increase in host susceptibility to SARS-CoV-2 infection. The soluble ACE2 does not have an apparent direct role in the pathogenesis of COVID-19 since membrane-bound ACE2 is required for receptor mediated endocytosis of the virus. However, the soluble ACE2 has a protective role which is implicated by the binding of the soluble ACE2 to SARS-CoV-2 and sequestrating it away from the membrane bound ACE2 to prevent viral entry and the initiation of the virus pathogenesis [61]. In this regard recombinant human ACE2 could be used as a therapeutic candidate to control SARS-CoV-2 invasion.

Systemic injuries that were reported during SARS-CoV and SARS-CoV-2 were mostly related to ACE2 function and its expression in different tissues. The

binding of SARS-CoV to ACE2 receptor was found to reduce its expression, which further induces acute lung injury in wild type mice, when compared to ACE2 knockout mice, which indicates that acute lung injury during SARS-CoV is related to ACE2 expression level and this could also apply for SARS-CoV-2 [62]. Moreover, due to the high expression of ACE2 on cardiac cells, cardiac injury was reported among SARS-CoV [63] and SARS-CoV-2 [64] infected patients which was associated with increased disease severity along with high disease mortality. In addition, increased expression of ACE2 receptor in the kidney makes it highly vulnerable to be infected with SARS-CoV and SARS-CoV-2 leading to kidney injury [65]. A study was conducted by Hirsch et al. in New York where they found that acute lung injury among COVID-19 patients could reach up to 36.6% [66]. Other body organs, including the pancreas [67] [68], central nervous system [69], and endothelial cells [70] are also affected by coronavirus infection due to the differential regulation and expression of the ACE2 receptor.

Transmembrane serine protease type 2 (TMPRSS2) is an androgen-dependent serine protease that is encoded by chromosome 21. TMPRSS2 is mainly expressed on prostatic epithelial cells, where they play a role in the pathogenesis of prostate cancer [71]. Furthermore, TMPRSS2 is also expressed on kidney cells, digestive tract, and cardiac cells [10]. In addition, this protein is found to be highly expressed in the nose [72], and the upper respiratory tract [10]. This serine protease is found to have a role in the pathogenesis of certain viruses, including influenza viruses and coronaviruses, mainly through the cleavage of viral envelope glycoprotein which facilitates viral entry into hosts' cells [10, 11]. In a recent cell culture analysis, it was found that cells expressing TMPRSS2 are highly susceptible

to SARS-CoV-2 infection [14]. Furthermore, serine protease inhibitors block S protein priming and subsequently it blocks viral entry and reduces infection, which suggest the importance of TMPRSS2 protease cleavage of S1 glycoprotein for infection [11].

2.6 COVID-19 pathogenesis

The global pandemic of COVID-19 spurred the need to determine the pathogenesis of the disease, which would eventually help in disease management. Currently the pathogenesis of the novel coronavirus is mainly derived from knowledge from previously known coronaviruses including SARS-CoV and MERS-CoV. In 2003, during the SARS-CoV outbreak, it was reported that SARS-CoV entered into human cells through binding of its spike protein to specific hosts' cell surface receptors, particularly ACE2 [73]. Similarly, Zhou et al., found that the novel SARS-CoV-2 only infected cells expressing ACE2 receptors, and the absence of this receptor prevented viral entry into host cells, confirming that ACE2 receptor is required for SARS-CoV-2 pathogenesis [9]. The receptor binding domain of SARS-CoV-2 spike protein interacts with the ACE2 receptor, particularly to subdomain I, activating ACE2 in a peptidase-independent manner [74]. Furthermore, for the successful viral entry into the host cells, the spike protein must be first cleaved at the S1/S2 site for priming and then at S'2 for activation, where S1 is required for recognition and S2 for fusion to the host membrane through mediating irreversible conformational changes. The cleavage of S glycoprotein is found to be achieved through the involvement of transmembrane proteases like transmembrane protease serine 2 (TMPRSS2) [11]. After the successful entry of the

virus to the host cells via ACE2 and TMPRSS2, additional viral proteins are synthesized including the replicase-transcriptase complex, which enables the virus to replicate its genome through RNA-dependent RNA polymerase. Later viral structural proteins are synthesized for assembly and the release of viral particles [75].

2.7 Host response to SARS-CoV-2

Symptomatic cases of SARS-CoV-2 are mainly associated with respiratory symptoms, that range from mild to moderate to severe respiratory distress syndrome with multi-organ failure. The common respiratory symptoms are due the increased expression of its target receptor ACE2 in the respiratory airways, which facilitates viral entry that result in lung damage. Moreover, immune system activation due to the presence of any pathogen would eventually augment the tissue damage and the onset of disease manifestations. The host immune system is activated when any pathogen, a virus in this case, makes it into the cells, which is subsequently recognized by the immune system cells, leading to the activation of both innate and the adaptive immune response. The presence of a virus or viral particles are first recognized by innate immune cells through pathogen recognition receptors, particularly Toll-like receptors (TLR), including TLR-3, TLR-7, and TLR-8, and this will lead to the activation of signaling pathways and the production of interferons (IFNs) [76]. Antiviral IFN is an important mediator to combat infection, through immune modulation, restricting cells proliferation and inducing apoptosis [77]. Moreover, alveolar macrophages and dendritic cells (DC) are the main innate immune cells in the respiratory airways, which upon activation result in the

production of inflammatory cytokines as well as function in antigen presentation that is needed for further activation of adaptive immune cells. At the site of infection, antigen presenting cells (APC) take up the virus and process it, and subsequently express the viral antigen, mainly S and N structural proteins [78], on its surface through MCH-I and MHC-II. Dendritic cells with the viral antigen travel through the lymph to the draining lymph nodes where they present the antigen to naïve T-cells. The interaction between APC peptide-bound MHC-I or MHC-II with T-cell receptor (TCR) aids in the activation of cytotoxic T-cells (CD8+ T-cells), which are able to kill infected cells directly or helper T-cells (CD4+ T-cells) that can induce immune response and activate B-cells to produce antibodies, respectively [79]. Severe cases of COVID-19 among adults were associated with over-activation of immune system and the increased production of inflammatory mediators including, interlukin-6 and 10 (IL-6 and 10), monocyte chemoattractant protein-1 (MCP-1), granulocytes colony stimulating factor (G-CSF), along with tumor necrosis factor-α (TNF-α). The higher the level of IL-6 was associated with increased disease severity. Also, these patients experienced a reduction in the number of lymphocytes in their peripheral blood, which could be the reason behind disease severity [80] [81]. Lung infiltration with inflammatory cells, mainly neutrophils and T lymphocytes, was reported among the severe cases [82]. Furthermore, in SARS-CoV-2 infection, the expression of MHC molecules on APC was found to be altered, which could affect host's immune response. In a recent study, it was found that the increased production of inflammatory mediators, mainly IL-6, is associated with reduced expression of HLA-DR in CD14+ monocytes in the plasma samples of COVID-19 patients [83]. Enhanced inflammatory response

to SARS-CoV-2 affects host immune system negatively, increasing disease severity, and rendering the host more susceptible to bacterial infections. The adaptive immune response to SARS-CoV-2 was assessed among 20 positive cases [84]. They found that COVID-19 patients have an enhanced activity of both T and B cells mainly against S, N, and M proteins of SARS-CoV-2. However, they found that individuals unexposed to SARS-CoV-2 had the same specific CD4+ T-cells response, illustrating cross-reactivity to other coronaviruses. Furthermore, in association to activated CD4+ T-cells they reported an increase in anti-S IgG and IgA antibodies titer eliciting the involvement of humoral immunity. Furthermore, in another study, anti-S IgG was detected in all of the patients tested, while only some had anti-S IgM, anti-N IgG and anti-N IgM [85]. Moreover, Guo et al., assessed the production of SARS-CoV-2 IgM, IgA and IgG antibodies in 208 plasma samples post-infection, using indirect ELISA. They found that IgM and IgA were detected 5-days after the onset of disease symptoms, while IgG was detected 14-days post infection [86]. Non-respiratory symptoms, for instance thrombosis and pulmonary embolism, were also reported, especially among those with severe cases of COVID-19. Since it has been reported that endothelial cells also express ACE2 receptor, this predisposes endothelial lining to SARS-CoV-2, damaging these cells and leading to the loss of the endothelial role in regulating blood clotting, inducing the formation of thrombi. These findings are consistent with the increased level of D-dimer and fibrinogen among COVID-19 patients [87].

2.8 Probable justifications for the differences between adults and children with COVID-19

The rate of COVID-19 cases and its clinical presentations is different in pediatric patients versus adults. Children are having lower rates of infection with SARS-CoV-2 and usually experience an asymptomatic course of the infection. There is no confirmed explanation, but several hypothetical explanations have been considered. The first explanation for this variation could be simply because schools were closed at the start of this pandemic and it was a holiday season in China, which reduced the exposure of the pediatric population to the novel coronavirus. The second hypothesis to this variation could be related to differences in the immune system response of both children and adults. With the continuous exposure to antigens, adult and older individuals experience exhaustion of naïve T-cells and the increase in the number of effector and memory T-cells [88], which is associated with an increase in the production of proinflammatory cytokines causing cytokine storms that worsen the disease condition. Also, aging was reported to be associated with reduction in the expression of costimulatory molecules including CD27 and CD38, that render them more susceptible to infection [89]. On the other hand, the immune response during infancy is strongly regulated by differences in the activation of Toll-like receptor pathways and the production of regulatory mediators and cells. Early after birth, there is a decrease in the expression of proinflammatory cytokines by impaired CD4+ Th1 cells, and reduced expression of cytotoxic mediators by CD8+ T-cells [90]. Moreover, lung immune system in children is suppressed by increased number of regulatory T cells there [91], further reducing disease complication. The third hypothesis is the presence of other respiratory

viruses in the children's airways [92] could compete with SARS-CoV-2 and limit the growth of the virus, subsequently lowering the rate of COVID-19 cases among children.

Furthermore, the differential expression of ACE2, which is the SARS-CoV-2 target receptor, among children and adults could be one of the explanations. ACE2 expression levels among children could be less than that in adults, as it was reported previously that ACE2 expression is higher in well differentiated lung cells and children's lung epithelial cells continue to mature after birth [58]. Furthermore, in a recent study Bunyavanich et al., a retrospective analysis of ACE2 expression in the nasal epithelium of 302 subjects from an asthma cohort, with age ranging from 4 to 60 years old, they found that ACE2 expression levels are lower among children when compared to the adults, and this expression was found to increase with age [13]. The public gene expression dataset was used to explore the expression of hosts' receptor genes in the respiratory tract that have been reported to be involved in the pathogenesis of SARS-CoV-2, namely ACE2 and TMPRSS2. It was found that children have lower expression of ACE2 along with TMPRSS2 in the lung airways [15]. However, Chen et al., combined the public genomics, epigenomics and transcriptomic records of ACE2, and they found that ACE2 receptor is high among children and this expression decreased with age, which is opposing to other studies, suggesting that this receptor could have a protective role instead to SARS-CoV-2 [93].

2.9 Laboratory diagnosis

Upper respiratory tract specimens are routinely collected for COVID-19 real time reverse transcription-polymerase chain reaction (real time RT-PCR). The CDC recommended nasopharyngeal specimens as an ideal specimen for testing [94]. But, if it difficult to obtain, then other respiratory specimen would be appropriate, such as nasal swab, oropharyngeal specimen, and nasal and nasopharyngeal wash or aspirate. The specimens should be collected using a swab with appropriate viral transport media or in a sterile container and transported to the lab as soon as possible for testing. Blood samples can be also collected in case if serological testing is required. Furthermore, it was also found that SARS-CoV-2 is also identified in anal swabs and urine samples apart from respiratory specimens by RT-qPCR, but with varying viral loads [95]. Real time RT-PCR is considered as the most important method among the techniques that are used to detect coronaviruses, because of higher sensitivity and because the amplification and the analysis occur in a closed system which minimizes the risk of contamination and laboratory hazards [96]. There are various PCR assays that detect the presence of SARS-CoV-2 by detecting different viral genetic targets including genes for helicase, nucleocapsid, transmembrane, envelope, spike protein, open reading frames (ORF1a and ORF1b), and RNA-dependent RNA polymerase (RdRp) [97]. Furthermore, it is recommended to use at least two targets, one targeting a conserved region and another targeting a more specific region to avoid the effect of genetic drift since may occur as the virus evolves. The viral RNA is measured through PCR cycle threshold (Ct-value), which represent the number of amplification cycles required for the fluorescent signal to cross the assay threshold and be detectable.

Furthermore, an immunological assay can be used to identify the existence of antibodies against SARS-CoV-2. However, antibodies may not be detectable if tested too early during the symptomatic period [98] and a positive antibody test may rather indicate past infection.

2.10 COVID-19 Treatment

To date there is no definite treatment that is effective for SARS-CoV-2 infection. Symptomatic positive cases are treated with antibiotics, antiviral therapy, and corticosteroids. Moreover many COVID-19 patients are also under oxygen therapy and only the severe cases are managed with mechanical ventilators [99]. The National Health Commission and State Administration of Traditional Chinese Medicine recommended certain antivirals for the management of COVID-19, such as interferon alfa (IFN-α), ribavirin, lopinavir, arbidol chloroquine phosphate and tocilizumab in the presence of increased IL-6 that led to lung injury in critical cases. Furthermore, traditional Chinese medicine was also suggested to be used as a potential treatment for COVID-19, as in vitro studies showed the presence of anti-SARS-CoV-2 substances in Chinese herbs that were previously used for the treatment of other respiratory infections [100]. Furthermore, there are other drugs that are recommended as potential treatment for SARS-CoV-2 infection, including certain antimalarial drugs (chloroquine, hydroxychloroquine), antivirals (remdesivir and favipiravir), along with convalescent sera from recovered individuals and many more, but still they are under investigation and their efficacy should be confirmed by preclinical and clinical trials. Wang et al., and Yao et al., showed that antimalarial chloroquine is able to prevent SARS-CoV-2 infection in vitro studies, yet the mechanism behind this is not known [101] [102]. Clinical trials

of chloroquine have shown that it is effective in inhibiting pneumonia, improving lung image and reducing disease severity among 100 COVID-19 patients [103]. In the same study, none of the subjects experienced adverse reaction, yet the US restricted the use of chloroquine due to the cardiac toxicity concerns. Furthermore, remdesivir is an antiviral drug that was found to limit coronavirus infection in vitro [101], and it showed beneficial responses in a COVID-19 patients in US [104], but clinical trials are on-going to ensure the safety and efficacy of such treatment. Moreover, favipiravir is an antiviral agent that functions in inhibiting RNA-dependent RNA polymerase from RNA viruses [105], therefore this drug could have an therapeutic role against SARS-CoV-2. Furthermore, clinical trials on favipiravir showed that it is more efficient with no side effects compared to patients treated with lopinavir/ritonavir and it is preferred when comparing to arbidol [106]. Convalescent plasma from recovered individuals can be used as treatment. During SARS-CoV infection convalescent plasma was used to treat patients, and it was effective in reducing the viral load one day after transfusion [107]. Shen et al., showed that the treatment of severely diseased COVID-19 patients with plasma from recovered individuals was associated with normalization of body temperature and reduction in the total viral load, yet its safety is under investigation [108].

Vaccination is required to prevent infections and its associated morbidities, by the production of memory B and T cells, so that whenever the body encounters the same infectious agent, the immune system recognize it rapidly and destroy it, before affecting the host. Currently there are accepted vaccines for SARS-CoV-2 pandemic. At the beginning of this pandemic there were evidence that the live attenuated strain, which is known as Bacille Calmette-Guerin of *M. bovis* is used

against tuberculosis, may be useful in preventing COVID-19. In vivo and in-vitro studies showed that it protects against respiratory infections and it has been hypothesized that it would partially protect against SARS-CoV-2 infection [109]. Other vaccines include artificial antigen presenting cells vaccine, which are lentiviral-based vectors that are made to express viral proteins and immune modulatory genes to aid in the T-cells activation, and currently it is in the clinical trial phase I [110]. Recombinant adenovirus-5 vector, it is genetically engineered so that it is replication defective, and it expresses viral S protein. Hyperimmune plasma, is heat-inactivated plasma of people who were previously positive for SARS-CoV-2, were also used as one of the disease management strategies [111]. Moreover, two newly developed vaccines that are based on mRNA sequences that codes for viral protein once inside the host cells showed high efficacy in phase III clinical trials. Pfizer and BioNTech and US pharmaceutical company Moderna both generated mRNA vaccines that codes for the viral spike glycoprotein, as an antigen to trigger host immune responses against the virus [112]. Currently the FDA approved the use of both Pfizer/BioNTech and Moderna COVID-19 vaccines. These two vaccines have shown to protect individuals from SARS-CoV-2 infection, by the formation of antibodies against SARS-CoV-2. Adverse events have been reported with both the vaccine, but higher rates of adverse events have been more reported among those who are vaccinated with Moderna [113]. Moreover, chimpanzee adenovirus-vectored vaccine that expresses SARS-CoV-2 spike protein showed an acceptable cellular and humoral immune responses along with an acceptable safety profile, which predispose this vaccine for phase 3 clinical trials [114].

Chapter 3: Materials and Methods

3.1 Material

3.1.1 kits

All TaqMan assays were purchased from ThermoFisher Scientific for gene expression assays.

- ACE2 TaqMan Gene Expression Assays were obtained (ACE2-1: Catalog number: 4331182, assay ID: Hs01085333_m1, ACE2 target is located at exon 17-18 boundary (NCBI reference sequence, NM_021804.2) within the transmembrane domain of ACE2), and (ACE2-2: Catalog number: 4331182, assay ID: Hs00222343_m1, ACE2 target is located at exon 8-9 boundary (NCBI reference sequence NM_021804.2) within the transmembrane domain of ACE2).
- TMPRSS2 TaqMan Gene Expression Assays (Catalog number: 4331182, assay ID: Hs01122322_m1, TMPRSS2 target is located at exon 10-11 boundary (NCBI reference sequence, NM_001135099.1))
- ACTB TaqMan Gene Expression Assays (Catalog number: 4331182, assay ID: HS01060665_g1, ACTB target is located at exon 2-3 boundary (NCBI reference sequence, NM_001101.3))
- TaqPath 1-Step RT-qPCR MasterMix (REF: A15300)

3.2 Methods

Prior to initiating this study, ethical approval and a waiver of informed consent was obtained from the Institutional Review Board of Sidra Medicine and Qatar University (Appendix). All the research procedures were performed in

compliance with the appropriate ethical guidelines and regulations.

3.2.1 Sample and data collection

At the beginning of COVID-19 pandemic in 2020, Sidra Medicine, which is a pediatric care hospital in Qatar, was deemed a COVID-19 free facility. Therefore, from April 2020 all the patients and their companion adult family members coming to Sidra Medicine were screened for COVID-19 through RT-qPCR testing of nasopharyngeal swab specimens. Subsequently, from June to December 2020, residual nasopharyngeal swab specimens collected in universal viral transport media for COVID-19 testing, and those that met the inclusion criteria of the current study were identified and included in this study. The inclusion criteria included: at least one of the family members tested positive for COVID-19 by RT-qPCR, at least 0.5ml of nasopharyngeal swab specimens were available for the child and the companion adult family member. The required COVID-19 positive and negative nasopharyngeal swab specimens for children and their companion adults were identified by querying the hospital infection prevention and control records and in patient's electronic medical records (Power Chart, Cerner). Additional unpaired COVID-19 positive pediatric and adults who were symptomatic, were identified, and their residual nasopharyngeal swab specimens were obtained for this study. Only residual specimens were used in the study. Specimens were aliquoted from the viral transport media into labelled 2-ml tubes and were stored at -80°C for further analysis. Moreover, since Sidra Medicine is a pediatric hospital, clinical and laboratory data related to positive pediatric patients included in this study were obtained through reviewing patient's charts. A spreadsheet was created on a secure

network drive, accessible only to the research investigators, to save all the required information regarding the study samples; including patient medical record number, specific specimen identification number, assigned specific study ID, the storage location, gene expression data, clinical and laboratory data. To maintain patient anonymity, all patient identifiers were removed from the copy of the spreadsheet that was used for data analysis.

3.2.2 Nucleic acid (RNA) extraction

Total RNA from nasopharyngeal swab specimen was extracted using the Thermo Scientific Kingfisher Flex System platform, that uses magnetic particles for RNA purification. Extraction was performed as per the manufacturer's protocol (ThermoFisher, Catalog no. N07669). All the reagents used in this procedure were obtained from ThermoFisher and are stored at room temperature. This procedure starts with preparing processing plates according to the number of samples that are going to be extracted in one run, and each of these plates has a specific reagent, with specific volume and a position in the Kingfisher machine. First all 96 deep well plates are labelled based on the reagent that will be added to them and they are prepared according to (Table 2).

Table 2. Preparation of KingFisher processing plates.

Plate ID	**Reagent**	**Volume per one well**	**Plate position**
Wash 1 plate	Wash buffer 1	500 μL	2
Wash 2 plate	80% ethanol	1000 μL	3
Elution plate	Elution buffer	50 μL	4
Tip comb plate	A Kingfisher 96 tip comb are place in a kingfisher 96 microplate		5

After preparing the processing plate they are covered with adhesive seal and kept at room temperature until the sample plate is prepared. Another Kingfisher 96 deep well plate is labelled as sample plate and 5 μL of proteinase K is added to each well. Meanwhile, the frozen samples are allowed to thaw at room temperature, vortexed and 200 μL of each sample is added to a specific well. Then binding bead mix is prepared (as shown below in Table 3) and 275 μL of the mix is added to each sample well.

Table 3. Binding bead mix preparation.

Component	**Volume per well**
Binding solution	265 μL
Total Nucleic Acid Magnetic Beads (mix well before using)	10 μL
Total volume per well	275 μL

Then KingFisher Flex instrument is switched on and the RNA extraction protocol is selected. The instrument door is opened by sliding the plastic cover and all the prepared plates are loaded in the instrument after the removal of the adhesive seal into their specific locations as indicated by selected protocol. The sample plate is loaded in position 1, wash 1 plate is in position 2, wash 2 plate is in position 3, elution pate is loaded in position 4 and the tip combs in the microplate are loaded in position 5. Then the instrument door is closed, and the start button is pressed. This platform of extraction takes around 23 minutes and once it is done the elution plate includes the extracted RNA is used to proceed with gene expression analysis.

3.2.3 Real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

The extracted nucleic acids are used for gene expression analysis for ACE2, TMPRSS2 and ACTB genes (Thermofisher) using pre-designed TaqMan gene expression assays. This is performed by real-time RT-qPCR in a MicroAmp Fast 96-well reaction plate (Applied Biosystems) using the Applied Biosystems 7500 Fast Dx system. All the reagents of TaqMan assay are allowed to thaw in room temperature prior to starting. The Master Mix for each of the target genes are prepared according to the number of samples in each run as shown in table 4.

Table 4. Reaction master mix preparation.

Master Mix name	**20x Taqman assay**	**4x Taqpath 1-step**	**Nuclease free water**
ACE2 (Hs01085333_m1)	1 μL x sample no.	5 μL x sample no.	4 μL x sample no.
TMPRSS (Hs01122322_m1)	1 μL x sample no.	5 μL x sample no.	4 μL x sample no.
ACTB (Hs01060665_g1)	1 μL x sample no.	5 μL x sample no.	4 μL x sample no.

Each reaction well has a total volume of 20 μL, which contains 10 μL of prepared reaction master mix (that contain specific gene Taqman assay, 4x Taqpath 1-Step RT-qPCR master mix and nuclease free water) with 10 μL of sample extract. The PCR plate containing the reaction mixture is placed in ABI7500 thermal cycler and the following program was set:

- Holding stage:
    - 25.0 °C for 02:00 minutes
    - 50.0 °C for 15:00 minutes

- 95.0 °C for 02:00 minutes

- Cycling stage (40 cycles)
  - 95.0 °C for 00:03 seconds
  - 60.0 °C for 00:30 seconds

3.2.4 Statistical analysis

Descriptive statistics are used to describe the study population and their COVID-19 infection status**.** Distribution of data was tested by D'Agostino & Pearson test. The mean (±SD) transcript levels for ACE2 and TMPRSS2 for COVID-19 positive adults and paired COVID-19 negative children from the same families was determined and compared by Wilcoxon Signed-Rank Test. Also, the statistical difference of ACE2 and TMPRSS2 expression between unpaired data groups was determined using Mann-Whitney U test. Logistic regression analysis was done to determine i) association between the expression of ACE2 and TMPRSS2 with SARS-CoV-2 RT-qPCR results (as dependent variable) for the overall population and for different age groups ii) association between the expression of ACE2 and TMPRSS2 with patient groups who were negative, asymptomatic or symptomatic with COVID-19 (as dependent variable). All statistical analysis in this study is carried out using SPSS and Graphpad Prism 9 software. For all the performed tests, a p-value less than 0.05 was considered statistically significant.

Chapter 4: Results

4.1 Study subject demographics

Residual nasopharyngeal swab specimens were obtained from children along with their companion adults, with history of SARS-COV-2 infection in at least one of the family members (paired samples). Beside the paired samples that were asymptomatic at the time of COVID-19 testing, additional unpaired samples from COVID-19 positive children and adults with known history of COVID-19 associated symptoms were also obtained and included in the analysis. A total of 241 nasopharyngeal specimens were included in this study, of which 207 samples were paired samples from 92 families in which at least one member was positive for SARS-COV-2 infection, consisting of 92 children and 115 adult companions. Furthermore, 24 and 10 COVID-19 positive unpaired nasopharyngeal swab specimens from pediatric and adults, respectively, were later included in this study. Nasopharyngeal swab specimens were available from more than one adult companions from 23 families. Among the study subjects, 63.2% of the adults and 47.4% of the children were of female sex. The median age of the study children and adults were 4 (IQR: 1.4-7) and 34 (IQR: 30-39), respectively, and 59.5% and 64.8% were COVID-19 positive children and adults, respectively. In most of our paired samples, the child was paired with the mother for paired data analysis, but in some cases, where the mother was negative, or the sample for the mother was not available, then the child is paired with other family member.

Table 5. Study subjects characteristics

Categories		Adult	Children
No. of samples		125	116
Sex, n (%)	**Female**	79 (63.2)	55 (47.4)
	Male	46 (36.8)	61 (52.6)
Age, median (IQR)		34 (30-39)	4 (1.4-7)
COVID-19 result, n (%)	**Negative**	44 (35.2)	47 (40.5)
	Positive	81 (64.8)	69 (59.5)

4.2 Transcript level of β-actin, ACE2 and TMPRSS2 in nasopharyngeal swab specimens

The reaction conditions were optimized for the purchased TaqMan assays, two ACE2 targets, ACE2-1 and ACE2-2 (Hs01085333_m1 and Hs00222343_m1, respectively), one target for TMPRSS2 (Hs01122322_m1) and β-actin (ACTB) (HS01060665), by using 10 nasopharyngeal swab specimens (5 COVID-19 positive and 5 COVID-19 negative). One of the ACE2 targets (ACE2-1, Hs01085333_m1) was consistently giving stronger Ct values than the other ACE2 target (mean Ct, 32.8±2.6 vs 34.7±2.2, $p<0.001$ by paired sample T-test) (Figure 4), therefore ACE2-1 was chosen for the gene expression analysis for this study. The expression of the endogenous control β-actin gene was detected in all of analyzed nasopharyngeal specimens. Furthermore, the transcript levels of β-actin were normally distributed among all the specimens by D'Agostino and Pearson normality test ($K^2 = 0.2395$; $p= 0.8871$). Moreover, the mean Ct value of β-actin were not significantly different among positive COVID-19 cases and negative COVID-19 patients ($p=0.5623$; unpaired 2 tailed T-test). Yet, the mean Ct values of β-actin in nasopharyngeal

specimens from children (25.1±2.9) were significantly stronger than that from adults (26.7±2.5) (p<0.0001; unpaired 2-tailed T-test) (Figure 5). On the other hand, ΔCt for ACE2 and TMPRSS2 are not normally distributed (K^2=18.5; p<0.0001 for ACE2 and K^2=6.3; p<0.042 for TMPRSS2), hence nonparametric tests including Mann Whitney U test and Wilcoxon matched pairs signed ranked test were chosen to compare unpaired and paired data, respectively. In addition, the RT-qPCR Ct value for ACE2 and TMPRSS2 for a number of specimens remained undetermined. Among 241 nasopharyngeal swab specimens, 143 and 45 specimens remained undetermined for ACE2 and TMPRSS2 gene expression, respectively, with 40 samples with undetermined results for both ACE2 and TMPRSS2. Of the 143 specimens that were undetermined for ACE2, 83 were SARS-COV-2 positive, and among the undetermined TMPRSS2, 25 were positive for COVID-19. Therefore, for quantitative data analysis, a Ct value of 40 was assigned to these specimens. For the specimens in which ACE2 and TMPRSS2 transcript levels were not detectable, it was expected that relative gene expression of these genes can still be calculated using the assigned value because of the variation in qPCR Ct for β-actin. Based on the gene expression analysis by RT—qPCR Ct values, the relative transcript levels of ACE2 (mean Ct±SD, 37.7±3.5) and TMPRSS2 (mean Ct±SD, 35.4±3.5) were much weaker and more variable when compared to the transcript levels of β-actin (mean Ct±SD, 25.9± 2.8) (Figure 6).

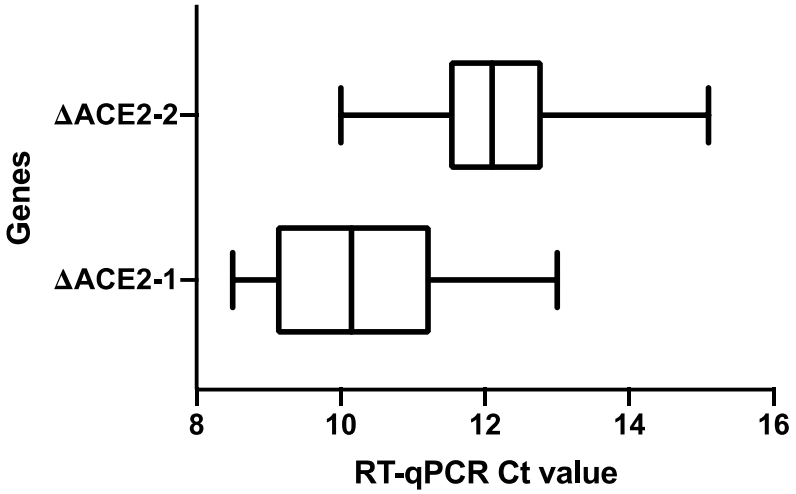

Figure 4: Transcripts level of two ACE2 targets (ACE2-1 and ACE2-2) by RT-qPCR for assay optimization.

Two ACE2 targets were initially evaluated in this study for higher sensitivity and ACE2-1 assay was found to generate stronger Ct values for ACE2 when compared to ACE2-2 assay ($p<0.001$)

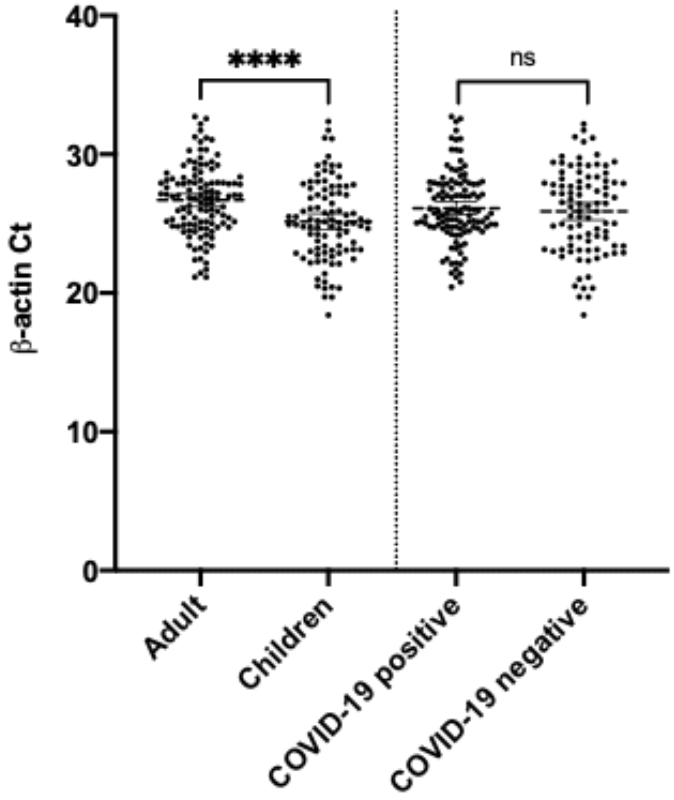

Figure 5: Comparison of β-actin gene expression between adults and children and between COVID-19 positive and negative participants

Mean qPCR Ct values for β-actin gene in different groups with 95% CI. *p*-value are computed by unpaired 2-tailed T-test. ****$p<0.0001$, ns= not significant.

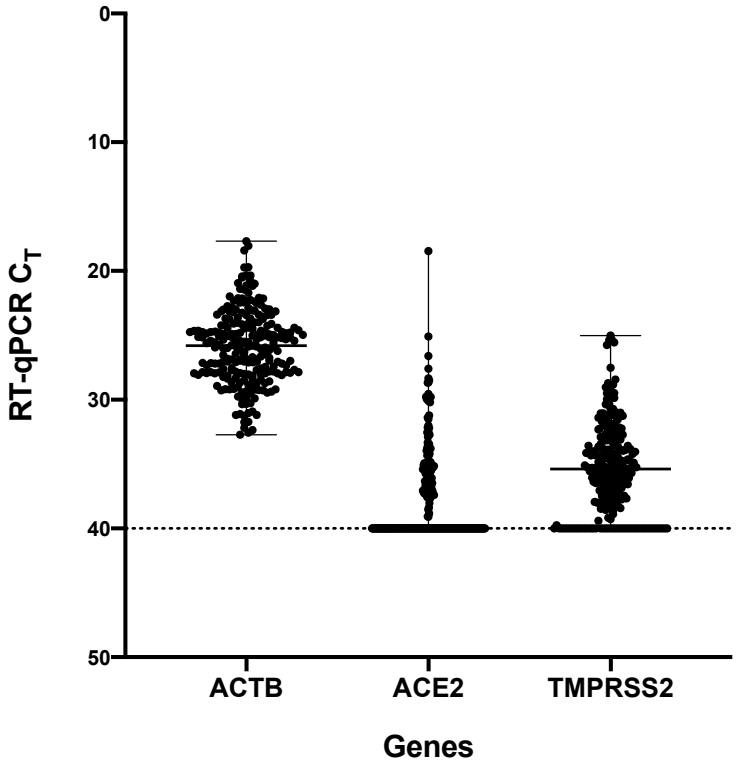

Figure 6: β-actin, ACE2 and TMPRSS2 gene expression in nasopharyngeal swab specimens among all study subjects.

The data show mean Ct values of all three target genes with 95% confidence interval, n=241.

4.3 Transcript levels of ACE2 and TMPRSS2 in SARS-CoV-2 exposed families nasopharyngeal swab specimens relative to β-actin

In this study, 207 paired samples from children and their companion adults, where at least one of them is positive for SARS-CoV-2 infection, were involved in the gene expression analysis for ACE2, TMPRSS2 and the background reference gene β-actin. The raw Ct values obtained from RT-qPCR for both ACE2 and TMPRSS2 were normalized by subtracting the Ct values from ACE2 and TMPRSS2 from the Ct values from the respective β-actin gene's Ct value, in order to account for variation in β-actin gene expression or the number of cells present in

each of the nasopharyngeal samples, subsequently the Ct values for ACE2 and TMPRSS2 relative to β-actin are used in all of the following statistical comparisons. The transcript levels of ACE2 and TMPRSS2 were compared for the paired samples, and the expression of both of the genes were significantly higher (p=0.0034 and 0.0174, for ACE2 and TMPRSS2, respectively, by Mann Whitney U test) in the adults in comparison with the children (Figure 7).

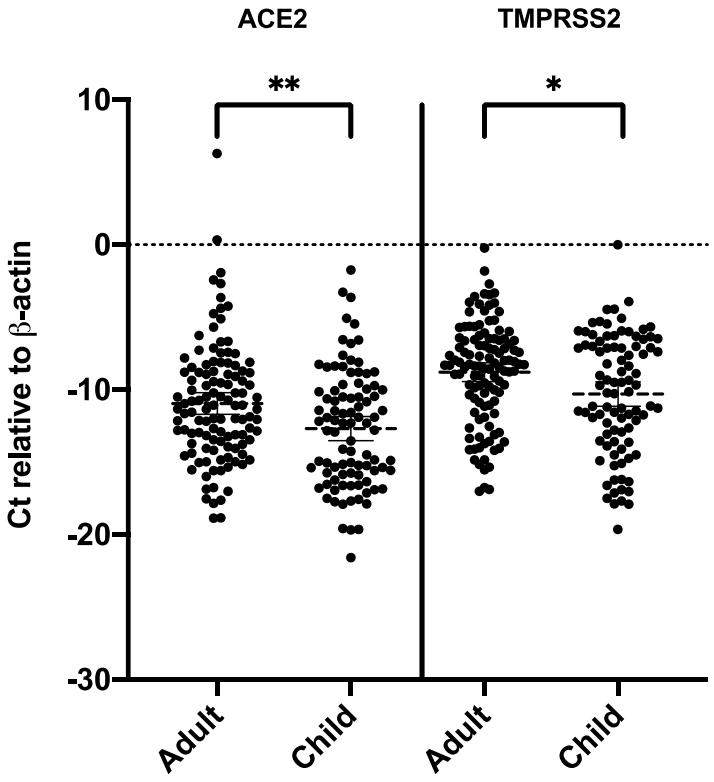

Figure 7: Expression of ACE2 and TMPRSS2 genes in children and adults within families exposed to SARS-CoV-2 relative to β-actin.

Comparison between the transcript levels of ACE2 and TMPRSS2 in paired nasopharyngeal swab specimens relative to β-actin. The data shows mean Ct values with 95% CI, n=115 adult and 92 children. *P*-values were computed by Mann Whitney U test; *p=0.0174 for TMPRSS2 and ** p=0.0034 for ACE2.

Furthermore, among the same 207 paired samples the transcript levels of ACE2 and TMPRSS2 were assessed, and they were not significantly different between positive and negative COVID-19 subjects (p=0.0771 and 0.3518, for ACE2 and TMPRSS2, respectively, by Mann Whitney U test), or among the adult subjects only (p=0.3817 and 0.7288, for ACE2 and TMPRSS2, respectively, by Mann Whitney U test), or pediatric subjects (p=0.2490 and 0.3963, for ACE2 and TMPRSS2, respectively, by Mann Whitney U test). Moreover, no significant difference was obtained between COVID-19 positive versus COVID-19 negative children from families where one of the adult companions is positive for SARS-COV-2 (p=0.1218 and 0.2263, for ACE2 and TMPRSS2, respectively, by Mann Whitney U test), and COVID-19 positive versus COVID-19 negative adults where the child tested positive for SARS-COV-2 (p=0.3120 and 0.3350, for ACE2 and TMPRSS2, respectively, by Mann Whitney U test) (data summarized in table 6, by Mann Whitney U test). No difference (p>0.05) was observed in the ACE2 and TMPRSS2 genes expression when the samples were stratified into various groups.

Table 6. ACE2 and TMPRSS2 gene expression in the nasopharyngeal samples of COVID-19 positive Versus negative patients.

Population	**Gene**	**#Mean ΔCt ± SD**		***p*-value**
		COVID-19 positive	**COVID-19 negative**	
Overall	ACE2	-11.2±4.364	-12.38±3.755	0.0771
	TMPRSS2	-9.186±3.724	-9.818±3.963	0.3518
Adult	ACE2	-10.54±4.466	-11.62±3.250	0.3817
	TMPRSS2	-8.754±3.548	-8.854±3.438	0.7288
Children	ACE2	-12.26±4.024	-13.09±4.080	0.2490
	TMPRSS2	-9.868±3.929	-10.72±4.238	0.3963
Adult in families with at least one positive child	ACE2	-10.49±4.231	-11.86±3.176	0.3120
	TMPRSS2	-8.353±3.929	-9.442±3.349	0.3350
Children in families with at least one COVID-19 positive adult	ACE2	-11.38±3.984	-13.09±4.08	0.1218
	TMPRSS2	-9.028±3.869	-10.72±4.238	0.2263

**p*-values were calculated by two tailed, Mann-Whitney U test

#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.

4.4 Paired analysis of transcripts levels of ACE2 and TMPRSS2 among children and adults from the same families

Additionally, among the paired study subjects, particularly when comparing the relative expression of ACE2 and TMPRSS2 genes of COVID-19 positive adults versus COVID-19 negative children from the same families (n=94, 47 pairs), the transcript levels of both genes are significantly higher (p=0.0002 and 0.0061, for ACE2 and TMPRSS2, respectively, by Wilcoxon Signed-Rank test) in the adult member of the family compared to children (Figure 8). On the other hand, the expression of ACE2 and TMPRSS2 among COVID-19 positive adult versus COVID-19 positive Children (p=0.5477 and 0.2247, for ACE2 and TMPRSS2,

respectively, by Wilcoxon Signed-Rank test) (n=34, 17 pairs) (Figure 9A) and COVID-19 negative adult versus COVID-19 positive children (p=0.2946 and 0.1782, for ACE2 and TMPRSS2, respectively, by Wilcoxon Signed-Rank test) (n=56, 28 pairs) (Figure 9B) within the same families, were not significantly different from each other (p>0.05). Furthermore, to assess if the significant difference in the gene expression was due to differences in the age distribution among the COVID-19 positive adults versus COVID-19 negative children within the same family, we compared the age distribution of the subjects involved in these groups. Yet, there was no significant difference (p=0.4081, by Mann-Whitney U-test) between the mean or the median age of COVID-19 positive children (4.402±4.197 years or 3 (1.04-7)) and COVID-19 negative children (5.346±4.539 years or 5 (1.25-8)) in this study population (Figure 10).

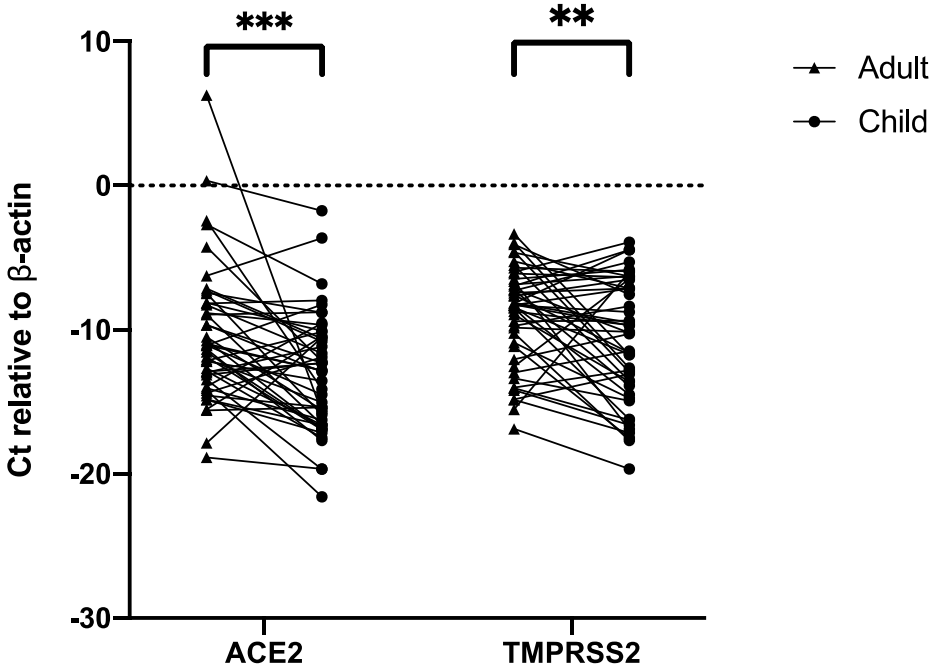

Figure 8: Transcript levels of ACE2 and TMPRSS2 of COVID-19 positive adult versus COVID-19 negative children from the same family.

The transcript levels of ACE2 and TMPRSS2 in the nasopharyngeal swab specimens of COVID-19 positive adults have been compared to negative children within the same family. A total number of 47 families, where the adult is positive, and the child is negative for SRAS-CoV-2. *p*-values were calculated from Wilcoxon Signed-Rank test, ****p*=0.0002, ***p*=0.0061.

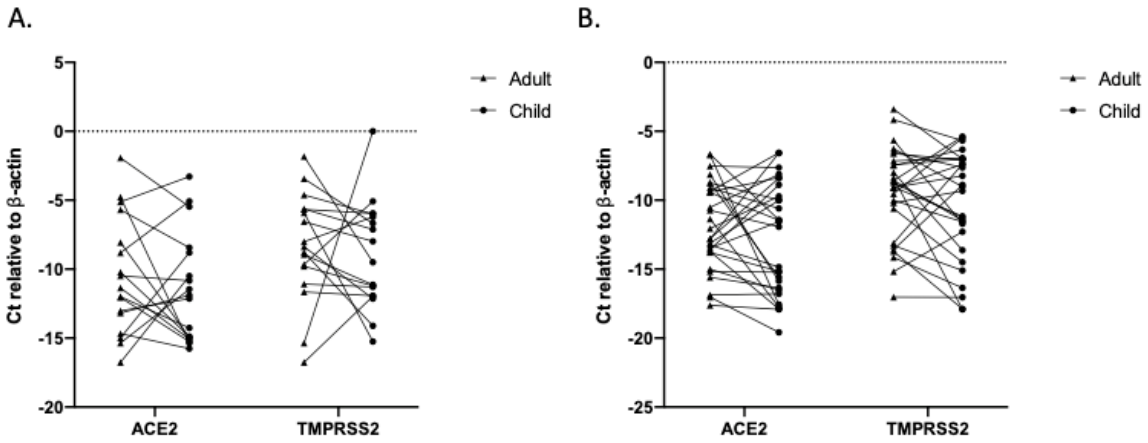

Figure 9: Comparison pf transcript levels of ACE2 and TMPRSS2 among adults versus children from the same family.

Comparison of the transcript levels of both ACE2 and TMPRSS2 in the nasopharyngeal swab specimens of (A.) COVID-19 positive adults versus COVID-19 positive children and (B.) COVID-19 negative adults versus COVID-19 positive children within the same family. A total number of 17 families where the adult is positive, and the child is also positive for SARS-CoV-2 and 28 families where the adult member is negative for COVID-19 and the child is positive for COVID-19.

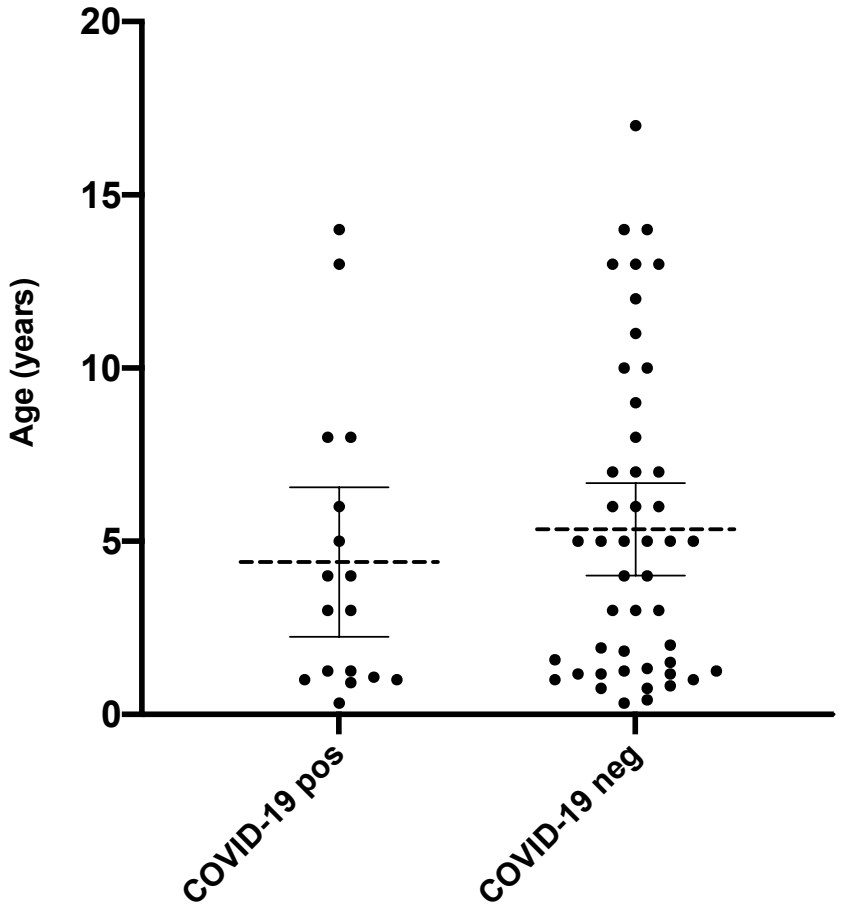

Figure 10: Age distribution of COVID-19 positive and COVID-19 negative children.

Comparison of age distribution among COVID-19 positive adults versus COVID-19 positive children (n= 17) or COVID-19 negative children (n=47). Data shows median age with IQR, *p*-values were calculated from Mann-Whitney U test; p=0.4081.

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

Age, sex and nasopharyngeal expression of ACE2 and TMPRSS2 were not significantly associated with SARS-CoV-2 infection status, by multivariate analysis of the overall paired study population (n=207), through using random effect logistic

regression. However, by performing univariate regression analysis the odds ratio of SARS-CoV-2 positivity status was higher (odds ratio, OR: 1.074, 95%CI: 1.003-1.154, *p*=0.0443) with higher expression of ACE2 (Table 7). Later we divided the entire paired sample population into two subgroups, subgroup 1; families where at least one adult member is positive for COVID-19 with positive or negative children, and subgroup 2; families with positive COVID-19 adults and negative children only. In subgroup 1 the odds of SARS-CoV-2 positivity was higher, with OR: 1.123 and 1.107, with higher expression of ACE2 and TMPRSS2, respectively. Furthermore, in subgroup 2 the odds of SARS-CoV-2 positivity is even higher, with OR: 1.146 and 1.123, with higher expression of ACE2 and TMPRSS2. On the other hand, SARS-CoV-2 infection status was not significantly associated with ACE2 and TMPRSS2 gene expression in the subgroup of families with at least one COVID-19 positive child with negative or positive COVID-19 adults (for ACE2; odds ratio, OR: 0.9712, 95%CI: 0.8669 – 1.085, *p*=0.6062, for TMPRSS2; odds ratio, OR: 1.017, 95%CI: 0.9100 – 1.139, *p*=0.7715, n=96), or in subgroups of families with COVID-19 positive children and negative COVID-19 adults only, (for ACE2; odds ratio, OR: 0.9243, 95%CI: 0.7941-1.069, *p*=0.2950, for TMPRSS2; odds ratio, OR: 0.9012, 95%CI: 0.7762 - 1.034, *p*=0.1498, n=60). In addition to SARS-CoV-2 infection status, these results also showed that ACE2 and TMPRSS2 are significantly associated (OR: 1.115, 95%CI: 1.039-1.203, *p*=0.0034 for ACE2 and OR: 1.112, 95%CI: 1.033-1.200, *p*=0.0055 for TMPRSS2) with the adult population, which is consistent with the data obtained from gene expression analysis (Figure 8). Yet, no significant association was found between gender and ACE2 and TMPRSS2 gene expression.

Table 7. Association of nasopharyngeal ACE2 and TMPRSS2 gene expression with SARS-CoV-2 infection status.

Category	**Overall (n=207)**		**Subgroup 1: Families with COVID-19 positive adults only (n=147)**		**Subgroup 2: Families with COVID-19 positive adult and negative children only (n=99)**	
	OR (95%CI)	***p*-value**	**OR (95%CI)**	***p*-value**	**OR (95%CI)**	***p*-value**
Age group	1.685 (0.9690-2.948)	0.0655	-	-	-	-
Gender	1.036 (0.5962-1.803)	0.9003	0.7311 (0.3746- 1.424)	0.3566	0.6522 (0.2908-1.447)	0.2947
ACE2 gene expression	**1.074 (1.003-1.154)**	**0.0443**	**1.123 (1.034-1.230)**	**0.0084**	**1.146 (1.038-1.284)**	**0.0114**
TMPRSS2 gene expression	1.044 (0.9718-1.123)	0.2396	**1.107 (1.014-1.212)**	**0.0246**	**1.123 (1.012-1.254)**	**0.0334**

Simple logistic regression analysis, reference category is "COVID-19 negative"

4.6 Association of nasopharyngeal ACE2 and TMPRSS2 expression with patient's clinical presentation and laboratory findings

Sidra Medicine is a designated COVID-19 free pediatric facility and 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, all the patients and the visitors presenting to Sidra Medicine are triaged for a prior history of COVID-19 and COVID-19 associated symptoms, including temperature assessment, along with SARS-CoV-2 RT-qPCR testing. Hence, most of the paired children and adults included in this study are asymptomatic, except for 10 children (out of 92 children and 115 adult) that had symptoms associated with SARS-CoV-2 infection, such as high body temperature (7/10), upper respiratory

tract symptoms including sore throat, cough, and runny nose (4/10) and gastrointestinal symptoms such as abdominal discomfort, vomiting and diarrhea (5/10). In order to further assess the association between the transcript levels of ACE2 and TMPRSS2 with the symptomatic cases of SARS-CoV-2 in comparison to the asymptomatic cases, we included additional unpaired COVID-19 positive symptomatic children and adults (n = 24, n = 10, respectively). First, we assessed the expression of ACE2 and TMPRSS2 among COVID-19 negative (n = 91) versus COVID-19 positive symptomatic (n = 44) and asymptomatic (n = 106) in the overall population (Figure 11 A), also we compared the expression of these two genes among COVID-19 negative (n = 47) versus positive symptomatic (n = 34) and asymptomatic (n = 35) children only (Figure 11 B), and in both classifications, there was no significant difference in the expression of these two genes across the groups. Among the total positive symptomatic cases (n=34, including paired and unpaired), 29 only had fever, 19 with upper respiratory tract symptoms, and 13 with gastrointestinal symptoms.

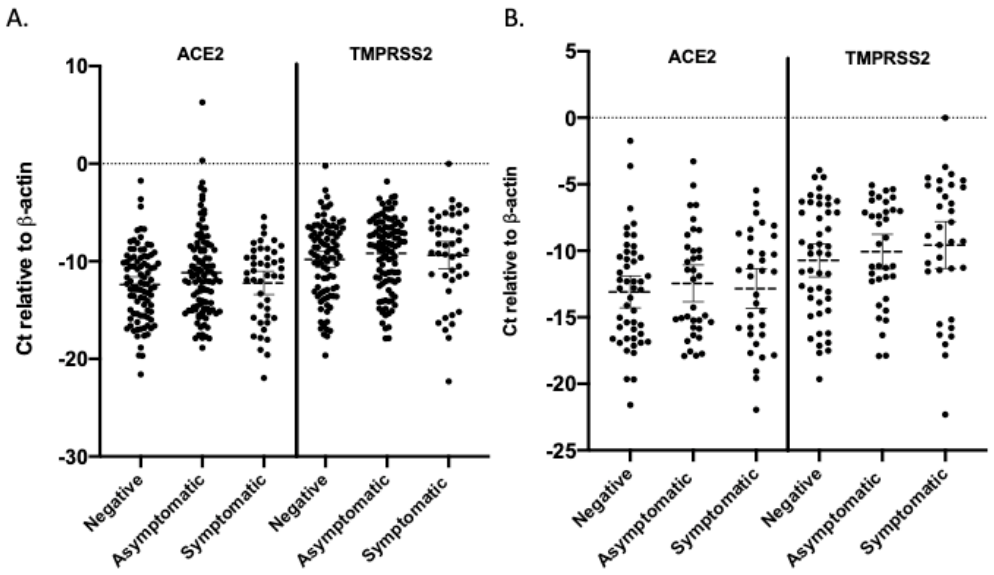

Figure 11: The transcript levels of ACE2 and TMPRSS2 relative to β-actin in COVID-19 negative versus asymptomatic versus symptomatic cases.

A. Transcript level of ACE2 and TMPRSS2 among COVID-19 negative versus asymptomatic versus symptomatic cases; n = 91 negative, 106 positive asymptomatic, and 44 positive symptomatic cases. B. Gene expression among COVID-19 negative versus asymptomatic versus symptomatic pediatric samples only, n = 47 negative, 35 asymptomatic, and 34 symptomatic. The data shows the mean with 95% CI, by One way ANOVA.

Age, sex and nasopharyngeal expression of ACE2 and TMPRSS2 were not significantly associated with patients clinical presentation (symptomatic versus asymptomatic), by multivariate analysis of the overall paired study population Furthermore, we assessed the association of transcript levels of ACE2 and TMPRSS2 with each of the symptoms, including fever, upper respiratory tract symptoms, and gastrointestinal symptoms among the positive pediatric symptomatic patients only, using univariate logistic regression analysis, we excluded the symptomatic adults because the specific symptoms for these adults

were not documented the electronic medical record. There is no significant association between the expression of ACE2 and TMPRSS2 with fever, or upper respiratory tract symptoms, or gastrointestinal symptoms among positive children (n=68, with 34 symptomatic) and the overall positive population (n=150, with 44 symptomatic) (Table 8).

Table 8. Association of nasopharyngeal ACE2 and TMPRSS2 gene expression with specific symtoms associated with COVID-19.

Population	**Outcome variable**	**ACE2 OR (95%CI)**	***p*-value**	**TMPRSS2 OR (95%CI)**	***p*-value**
All positive (n= 150) Children & adults)	All symptoms	0.9419 (0.8611 – 1.025)	0.1757	0.9882 (0.9044 – 1.082)	0.7931
Children (n= 69)	All symptoms	1.024 (0.9115 – 1.153)	0.6885	0.9745 (0.8732 – 1.085)	0.6362
	Fever	1.033 (0.9191 – 1.164)	0.5884	0.9610 (0.8612 – 1.068)	0.4630
	URTS	1.115 (0.9759 – 1.288)	0.1202	1.010 (0.8965 – 1.135)	0.8639
	GI	1.029 (0.8930 – 1.188)	0.6911	0.9656 (0.8512 – 1.097)	0.5832

Simple logistic regression analysis, all positive population (n = 150), and positive children only (n= 68). OR; odds ratio, CI; confidence interval, URTS; upper respiratory tract symptoms, GI; gastrointestinal symptoms.

In addition to the clinical presentations, few of the positive asymptomatic and symptomatic children had specific clinical laboratory test results for D-dimer, C-reactive protein (CRP), procalcitonin (PCT), ferritin, white blood cells (WBCs), platelets, lymphocytes, neutrophil, prothrombin time (PT), fibrinogen, troponin, N-terminal pro b-type natriuretic peptide (NT-proBNP) (Table 9). There was no significant association between the nasopharyngeal expression of ACE2 and TMPRSS2 with at least one abnormal lab finding (n = 69, OR= 0.9807, *p*-value= 0.7557, and OR= 1.035, *p*-value= 0.5485 for ACE2 and TMPRSS2, respectively). Moreover, among 22 COVID-19 positive children who were tested for CRP, 13 were reported with abnormally high levels of CRP, but it was not significantly associated with the transcript levels of ACE2 and TMPRSS2 (*p*-value=0.9373, and 0.7155 for ACE2 and TMPRSS2, respectively). Furthermore, complete blood count was performed for 28 COVID-19 positive children, and no significant association was found between nasopharyngeal ACE2 and TMPRSS2 with abnormal WBC (OR=1.014, *p*-value= 0.8738 for ACE2 and OR=1.010, *p*-value= 0.9162 for TMPRSS2), neutrophil (OR=1.030, *p*-value= 0.7377 for ACE2 and OR=1.107, *p*-value= 0.3107 for TMPRSS2), lymphocyte (OR=1.041, *p*-value= 0.6667 for ACE2 and OR=1.222, *p*-value= 0.0923 for TMPRSS2), and platelets (OR=0.9809, *p*-value= 0.8226 for ACE2 and OR=1.026, *p*-value= 0.7868 for TMPRSS2). For the rest of the laboratory findings, they were tested only for few patients, and they are not significantly associated with transcript levels of ACE2 and TMPRSS2.

Table 9. Summary of patients tested for specific clinical laboratory tests.

Laboratory test	Total no. of patients tested	Normal	High	Low
CRP	22	9	13	0
D-dimer	3	0	3	0
PCT	4	1	3	0
Ferritin	3	0	3	0
WBC	28	17	5	6
Neutrophil	28	18	7	3
Lymphocyte	28	20	0	8
Platelets	28	17	5	6
PT	9	4	4	1
Fibrinogen	8	7	1	0
Troponin	3	1	2	0
NT-proBNP	3	0	3	0

Chapter 5: Discussion

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

Since the start of COVID-19 outbreak, many studies have shown that people at any age can acquire the infection, but they are differentially affected by SARS-CoV-2 infection. Lower rates of infection and milder COVID-19 presentation are reported more among children when compared to adults and elderly patients who experience higher mortality rates [12]. Many hypothetical explanations have been proposed, including the differential expression of specific proteins that are involved in the pathogenesis of SARS-CoV-2 infection. Moreover, several studies have explained the role of respiratory tract ACE2 receptor [9] and TMPRSS2 [11] in SARS-CoV-2 pathogenesis, based on the knowledge that was gained previously from SARS-CoV. However, little attention has been given to the role of ACE2 and TMPRSS2 protein in the nasopharyngeal epithelium. Nasopharyngeal expression of these proteins may have an essential role in SARS-CoV-2 transmission and infection, as the nasal cavity acts as one of the common routes for viral entry into the human body [44]. Therefore, in the current study, we conducted a case-control study to test our hypothesis that decreased expression of nasal or nasopharyngeal ACE2 is associated with a lower incidence of SARS-CoV-2 infection among pediatric population. To further understand the role of ACE2 in COVID-19 pathogenesis, we also analyzed the expression of TMPRSS2, which is a proteolytic enzyme required for the activation of ACE2 receptor, facilitating SARS-CoV-2 entry into host cells via the viral spike glycoprotein [11]. In the presented study, our target population are families where the members are differentially affected by

SARS-CoV-2, so that COVID-19 negative members in this setting would serve as an appropriate case-control for the study, since these individuals remained negative despite being exposed to the virus.

ACE2 receptors appear to be either soluble or membrane bound. In our study we specifically targeted the transmembrane protein, which have a role in viral entry in the host cells, in contrast to the soluble form of ACE2, which is purported to play a protective role in SARS-CoV-2 infection [115, 116]. In our study, nasopharyngeal expression of ACE2 was significantly higher in the adult group in comparison to the children, irrespective of SARS-CoV-2 infection status (Figure 7), which is consistent with the earlier report from a pre-COVID-19 asthma cohort that showed that nasopharyngeal ACE2 expression increases with age [13]. Furthermore, our study provides direct evidence that also the nasopharyngeal expression of TMPRSS2 is also significantly higher in the adults when compared to children (Figure 7). Since our study is particularly targeting families with at least one positive COVID-19 case, these findings together give a direct evidence that lower nasopharyngeal expression of ACE2 and TMPRSS2 is one of the reasons behind lower rates of SARS-CoV-2 infection among children, despite being in close contact to a positive family member. Moreover, these results are also consistent with the findings from a study conducted by Askari et al., where they used public gene expression datasets, and they reported that the expression of ACE2 and TMPRSS2 in the nasal and bronchial airways are lower among children when compared to adults [117]. Furthermore, it was previously reported that increased expression of ACE2 enhances viral entry [53, 54], along with higher expression of TMPRSS2 in cell culture analysis [118], increases the susceptibility of the host cells

to SARS-CoV-2 infection, thus, higher expression of these proteins among adults could render them more susceptible to SARS-CoV-2 infection. On the other hand, one study in the literature reported that by combining the public genomic, epigenomic and transcriptomic records of ACE2, it was found that ACE2 receptor expression is however higher in children and the expression decreases with age [93]. This variation could be explained by the fact that there are different types of ACE2; soluble and membrane bound, that could have different roles during infection, and more attention should be given to the type of ACE2 before interpreting gene expression.

The expression of ACE2 and TMPRSS2 was not significantly different between COVID-19 positive versus COVID-19 negative groups among the overall study subjects, yet the mean transcript levels of both genes were consistently higher in the COVID-19 positive groups (Table 6). Statistical insignificance of these data may be related to the smaller population size of our study, which is also one of the major limitations of our study. Furthermore, apart from the nasopharyngeal expression of ACE2 and TMPRSS2, additional systemic factors may also be involved in SARS-CoV-2 pathogenesis. These factors may also account for the variation in disease presentation from one individual to another. Some examples of such differences may include differences in the immune response and cytokine production and regulation between adults and children [88] [90], the presence of other respiratory viruses in the airways of children, which may compete with SARS-CoV-2 and limit its growth [92], and the presence of pre-existing cross-reactive antibodies [119]. Yet, by logistic regression analysis the expression of ACE2 was positively associated with SARS-CoV-2 infection status, which further

indicates that the higher expression of ACE2 induce the incidence of SARS-CoV-2 infection. This finding is consistent with the findings from an independent study that was conducted in British Columbia, Canada, which is currently in a pre-print status, where the expression of ACE2 was also positively associated with SARS-CoV-2 infection status [120].

Additionally, we compared the expression of ACE2 and TMPRSS2 between children with their companion adult family members, where the adult member was mostly the mother, expect in some families where the mother was negative, or the sample was not available, then we compared the child with other family members. By paired data analysis, we found that the expression of both receptors, ACE2 and TMPRSS2, was significantly greater among positive COVID-19 adults when compared to negative COVID-19 children from the same families (Figure 8). This finding was further supported by the result that was obtained from the association of ACE2 and TMPRSS2 expression, which were stronger in the subgroup of families with COVID-19 positive adults and COVID-19 negative children when compared to the overall study population (Table 7). The fact that the expression of these genes was not significantly different between COVID-19 positive adults versus COVID-19 positive children, although the expression of these genes was significantly different between overall adult and pediatric population further supports the role of nasopharyngeal expression of ACE2 and TMPRSS2 in COVID-19 infection in children. On the other hand, the fact that there was no significant difference of transcript levels of ACE2 and TMPRSS2 in nasopharyngeal specimens from COVID-19 negative adults and COVID-19 positive children

suggests that the role of these proteins in acquiring COVID-19 infection may be more pronounced to the pediatric population. The involvement of other underlying differences in the host cells cannot be ruled out as well. For example, it was reported by Barker et al., that SARS-CoV-2 itself could induce the expression of ACE2 [121], which could mask the differential roles seen between adults and children. Increased levels of interferon triggered by innate immunity that can also act as a transcription factor for ACE2 gene and subsequently it induces its expression during SARS-CoV-2 infection. Further investigates are required to confirm the role of all these factors in acquiring COVID-19 infection.

5.2 Association of ACE2 and TMPRSS2 with COVID-19 clinical and laboratory outcomes

In our study, most of the subjects were asymptomatic. Yet, few symptomatic subjects were involved to be able to compare the expression of these protein among symptomatic cases. There was no significant difference and no association between the expression of ACE2 and TMPRSS2 among COVID-19 negative versus COVID-19 positive asymptomatic versus COVID-19 positive symptomatic cases in the overall population, and specifically among the pediatric population (Figure 11 & Table 8). To our knowledge there is no direct report in the literature correlating the role of nasopharyngeal transmembrane ACE2 and TMPRSS2 expression with COVID-19 clinical presentations. However, in a meta-analysis that was conducted recently, there was no direct association between the prevalence of ACE (I/D) genotype and COVID-19 clinical outcome, but they reported that this allele was associated with an increased recovery rate [122]. It is necessary to understand the

relationship between these receptors and disease outcome for better disease management. Similarly, there was no significant association between the expression of ACE2 and TMPRSS2 with abnormal laboratory findings. Yet, various studies have reported that worse COVID-19 outcome could be associated with increased expression of TMPRSS2 and ACE2 [123-125]. Also, since most of the symptomatic cases have been reported so far are among adults, higher expression of ACE2 and TMPRSS2 could be one of the reasons for more severe infections in adults compared to children. However, our study remains inconclusive on this point because of low number of symptomatic subjects in our cohort but these findings could be significant if additional symptomatic families were involved in the study.

CONCLUSION

In conclusion, COVID-19 pandemic is differentially affecting a vast number of people worldwide and an urgent understanding of the disease pathogenesis, and identifying disease associated host factors and biomarkers is essential for better disease management. Our results support the earlier described hypothesis that children have lesser expression of nasopharyngeal ACE2 and TMPRSS2 proteins, which could contribute to their protection against COVID-19, even when exposed to a COVID-19 positive individual. Furthermore, our study provides indications for future studies with larger sample numbers to further understand the theory behind the differential incidence of SARS-CoV-2. These findings could further help in specifically identifying patients who are likely to benefit more from specific types of therapies. Additionally, the differential expression of viral entry factors could have prognostic implications in COVID-19.

LIMITATION AND FUTURE STUDY

There are several limitations in this study. The COVID-19 positive subjects included in the current study were mostly asymptomatic, where a stronger age-related association of nasopharyngeal expression of ACE2 and TMPRSS2 with SARS-CoV-2 infection status might be found if this study included more symptomatic family clusters. These results may become statistically significant with a larger sample size. Therefore, for future studies, conducting the analysis among larger number of subjects and including more symptomatic subjects would give more reliable and significant results.

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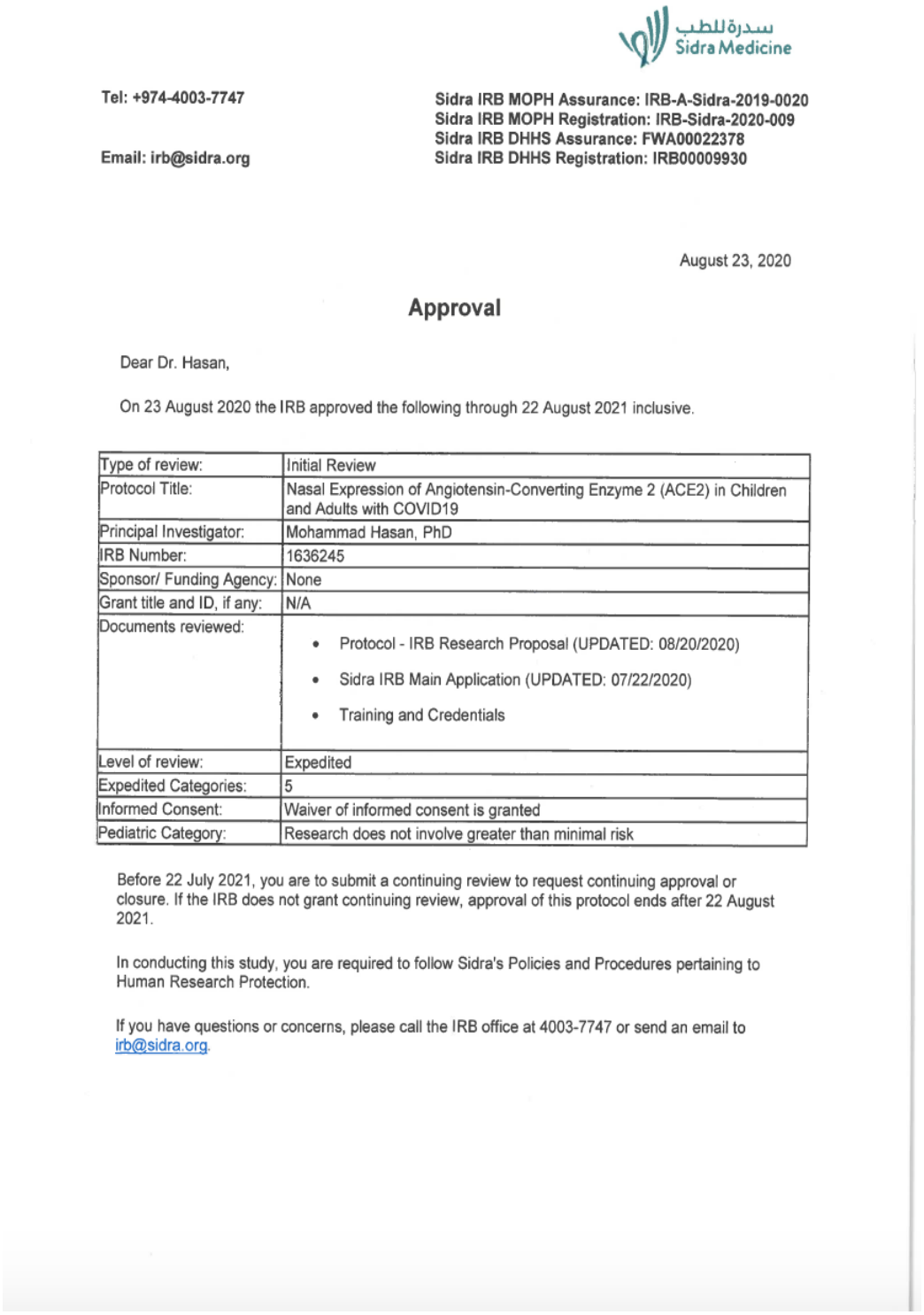
Chapter 7: Appendix

7.1 Appendix A: Sidra Medicine IRB approval



سدرة للطب
Sidra Medicine

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Email: irb@sidra.org

Sidra IRB MOPH Assurance: IRB-A-Sidra-2019-0020
Sidra IRB MOPH Registration: IRB-Sidra-2020-009
Sidra IRB DHHS Assurance: FWA00022378
Sidra IRB DHHS Registration: IRB00009930

August 23, 2020

Approval

Dear Dr. Hasan,

On 23 August 2020 the IRB approved the following through 22 August 2021 inclusive.

Type of review:	Initial Review
Protocol Title:	Nasal Expression of Angiotensin-Converting Enzyme 2 (ACE2) in Children and Adults with COVID19
Principal Investigator:	Mohammad Hasan, PhD
IRB Number:	1636245
Sponsor/ Funding Agency:	None
Grant title and ID, if any:	N/A
Documents reviewed:	• Protocol - IRB Research Proposal (UPDATED: 08/20/2020) • Sidra IRB Main Application (UPDATED: 07/22/2020) • Training and Credentials
Level of review:	Expedited
Expedited Categories:	5
Informed Consent:	Waiver of informed consent is granted
Pediatric Category:	Research does not involve greater than minimal risk

Before 22 July 2021, you are to submit a continuing review to request continuing approval or closure. If the IRB does not grant continuing review, approval of this protocol ends after 22 August 2021.

In conducting this study, you are required to follow Sidra's Policies and Procedures pertaining to Human Research Protection.

If you have questions or concerns, please call the IRB office at 4003-7747 or send an email to irb@sidra.org.

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Sidra IRB MOPH Registration: IRB-Sidra-2020-009
Sidra IRB DHHS Assurance: FWA00022378
Sidra IRB DHHS Registration: IRB00009930

Sincerely yours,

Ayman Saleh, MD, MPH, FAAP
Chair
Institutional review Board
Sidra Medicine
t. +974 40036567

7.2 Appendix B: QU IRB approval

Qatar University Institutional Review Board **QU-IRB**
QU-IRB Registration: IRB-QU-2020-006, QU-IRB, Assurance: IRB-A-QU-2019-0009

October 12th, 2020

Dr. Hatem Zayed
College of Health Sciences
Qatar University
Tel.: 4403 4809
Email: hatem.zayed@qu.edu.qa

Dear Dr. Hatem Zayed,

Sub.: Research Ethics Review Exemption
Ref.: Student, Muneera Naseer Ahmad/ e-mail: ma1403939@student.qu.edu.qa
Project Title: "Nasal Expression of Angiotensin-Converting Enzyme 2 (ACE2) in Children and Adults with COVID19"

We would like to inform you that your application along with the supporting documents provided for the above project, has been reviewed by the QU-IRB, and having met all the requirements, has been granted research ethics **Exemption** based on the following category(ies) listed in the Policies, Regulations and Guidelines provided by MoPH for Research Involving Human Subjects:

Exemption Category 3: Research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified.

Documents Reviewed: 4-10QU-IRB Application Human Subject- MA-HZ-RH-edit, QU-IRB Application Material Check List, Thesis Proposal, Sidra Approval Letter, Data Collection Form, Review Forms, responses to IRB queries and updated documents.

Please note that exempted projects do not require renewal; however, any changes/modifications to the original submitted protocol should be reported to the committee to seek approval prior to continuation.

Your Research Ethics Approval Number is: **QU-IRB 1386-E/20**. Kindly refer to this number in all your future correspondence pertaining to this project. In addition, please submit a closure report to QU-IRB upon completion of the project.

Best wishes,
Dr. Mohamed Emara

Vice Chair, QU-IRB

Institutional Review Board
(IRB)
Office Of Academic Research