Viral stimulation modulates endotype-related ACE2 expression in eosinophilic chronic rhinosinusitis*

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Abstract
Background: Angiotensin-converting enzyme 2 (ACE2), a receptor targeted by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is highly expressed in the nasal mucosa. Chronic rhinosinusitis (CRS) shows diverse endotypes and is aggravated by viral infection. Whether viral stimulation and CRS endotype influence ACE2 expression remains unclear. We investigated the expression of ACE2 and the transmembrane protease, serine 2 (TMPRSS2), which mediate the entry of SARS-CoV-2 into cells, and assessed polyinosinic:polycytidylic acid (poly[I:C])-induced changes based on CRS endotype.

Methodology: ACE2 and TMPRSS2 expression was evaluated based on CRS phenotype, endotype, and tissue type. Correlations between ACE2/TMPRSS2 expression and inflammatory mediators in nasal polyps (NP) were examined. Air-liquid interface culture experiments were performed to assess the effects of major cytokines or poly(I:C) stimulation on ACE2/TMPRSS2 expression in primary epithelial cells from healthy nasal mucosa, eosinophilic NP (ENP), and non-eosinophilic NP (NENP).

Results: In primary nasal epithelial cells, interleukin (IL)-13 decreased ACE2 expression but increased TMPRSS2. Eosinophilic CRS showed lower ACE2 expression than non-eosinophilic CRS, regardless of CRS phenotype. CRS endotype was an independent factor associated with ACE2/TMPRSS2 expression in NP. Serum and tissue eosinophilic marker levels were inversely correlated with ACE2 expression, whereas tissue neutrophilic marker levels and ACE2 expression were positively correlated in NP. ACE2 expression was suppressed in ENP tissues; however, a combination of poly(I:C) and IL-13 induced ACE2/TMPRSS2 upregulation in ENP.

Conclusions: ENP tissues have lower ACE2 expression than NENP; however, viral stimulation promotes ACE2/TMPRSS2 upregulation in ENP.

Key words: angiotensin-converting enzyme 2, chronic rhinosinusitis endotype, COVID-19, SARS-CoV-2, sinusitis

Introduction
Since the outbreak of the coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the rapid viral transmission has resulted in a pandemic and is a global threat to human health(1). Although health authorities around the world are taking actions to prevent the spread of COVID-19, community transmission continues to occur, and the overall burden of the disease is increasing(2). Ongoing studies continue to investigate the mechanisms underlying transmission of the virus in order to develop treatment and prevention strategies(3). The interaction between the viral spike (S) protein and airway epithelial cell receptors is a critical step which allows the SARS-CoV-2 to enter the human airway. Among the various epithelial factors present, the angiotensin-converting enzyme 2 (ACE2), which acts as a receptor for viral entry, and the transmembrane protease serine 2 (TMPRSS2) protein, which primes the S protein, have been suggested as key factors for the entry of coronaviruses into host cells(4). Both factors are considered to determine the host susceptibility toward SARS-CoV-2 infection, and antibodies against these factors may offer protection against COVID-19(5,6). Considering the difference in viral susceptibility between individuals, recent studies have demonstrated that the expression of ACE2 and TMPRSS2 can be modulated by the host airway...
Viral infection modulates ACE2 Expression in CRS

Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Total no. of subjects</th>
<th>Control</th>
<th>CRSsNP</th>
<th>CRSwNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 9</td>
<td>N = 23</td>
<td>N = 22</td>
<td>N = 88</td>
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<tr>
<td>Tissue used</td>
<td>UP</td>
<td>UP</td>
<td>NP</td>
</tr>
<tr>
<td>Age, y (SD)</td>
<td>50.7 (18.7)</td>
<td>46.0 (19.1)</td>
<td>50.2 (13.9)</td>
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<tr>
<td>Atopy, N (%)</td>
<td>2/9 (22.2%)</td>
<td>8/23 (34.8%)</td>
<td>9/22 (40.9%)</td>
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<tr>
<td>Asthma, N (%)</td>
<td>0 (0%)</td>
<td>3/23 (13.0%)</td>
<td>6/22 (27.3%)</td>
</tr>
<tr>
<td>LM CT score (SD)</td>
<td>NA</td>
<td>10.4 (4.7)</td>
<td>14.0 (3.6)</td>
</tr>
</tbody>
</table>

CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps; UP, uncinate process; NP, nasal polyps; LM CT score, Lund-Mackay computed tomography score; SD, standard deviation; NA, not applicable.

Environment, such as the type of inflammation or immune status\(^{(14)}\). Interleukin (IL)-13, a type 2 inflammatory cytokine, has been shown to significantly reduce ACE2 and increase Tmprss2 expression in lower airway epithelial cells\(^{(15)}\). Moreover, IL-4 and IL-13 have been found to downregulate ACE2 expression, whereas tumour necrosis factor alpha, IL-12, and IL-17A upregulate ACE2 expression in a human bronchial epithelial cell line\(^{(16)}\). A study using a lung epithelial cell gene dataset showed that several genes associated with immune functions including innate and antiviral immune functions are correlated with ACE2 expression\(^{(16)}\).

ACE2 and Tmprss2 have been detected in both the lower and upper airways. However, despite the clinical importance of the upper airway as the initial site of entrance for the virus as well as a viral reservoir\(^{(17)}\), most of these reports are based on lower airway studies or dataset studies.

Chronic rhinosinusitis (CRS) is an inflammatory disease affecting the upper airway\(^{(18)}\). CRS is known to be a heterogenous disease, and its endotypes are categorized according to the associated inflammatory pattern\(^{(18)}\). Meanwhile, viral infection is a strong regulator of airway epithelial. A number of genes within the airway epithelial cells can be modulated by respiratory viruses\(^{(19)}\). Therefore, it may be assumed that the expression of the viral entry mediators, ACE2 and Tmprss2, may vary depending on the CRS endotype and the immune response associated with viral infection. The aims of this study were to investigate CRS endotype-related differences in the expression of ACE2 and Tmprss2, along with changes in their expression levels following stimulation with polyinosinic:polycytidylic acid [poly(I:C)].

Materials and methods

Patients and tissue samples

Sinonasal tissues were obtained from patients with bilateral CRS, during routine functional endoscopic sinus surgery, and from control subjects. All subjects provided written informed consent for study participation. The study was approved by the internal review board of our hospital (20-2020-225) and conducted in accordance with the Declaration of Helsinki. Additional information and details of the patient characteristics are listed in Table 1. The diagnosis of CRS was based on personal history, clinical examination, nasal endoscopic examination, and computed tomography (CT) findings of the sinuses according to the 2020 European position paper on rhinosinusitis and nasal polyps guidelines\(^{(15)}\). Exclusion criteria were as follows: 1) below the age of 18 years, 2) previous treatment with antibiotics, systemic or topical corticosteroids, or other immunomodulatory drugs for four weeks before surgery; and 3) conditions such as unilateral rhinosinusitis, allergic fungal sinusitis, antrochoanal polyps, cystic fibrosis, or ciliary dyskinesia. We obtained uncinate process (UP) tissues from control subjects, patients with CRS without nasal polyps (CRSsNP), and from those with CRS with nasal polyps (CRSwNP). Control tissues were acquired from patients without any sinonasal disease during other rhinologic surgeries, such as lacrimal duct, orbital decompression, or skull base surgery. Nasal polyps (NP) were obtained from patients with CRSwNP.

Each tissue sample was divided into four sections for further processing: one section was fixed in 10% formaldehyde and embedded in paraffin for histological analysis; another section was immediately frozen and stored at -80°C for subsequent isolation of mRNA. The third section was submerged in 1 mL of phosphate-buffered saline supplemented with 0.05% Tween-20 (Sigma-Aldrich, St Louis, MO, USA) and 1% protease inhibitor cocktail (Sigma-Aldrich) per 0.1 g of tissue. These samples of the third section were then homogenised using a mechanical homogeniser (Bullet Blender Blue, Next Advance, Averill Park, NY) at setting 7 for 8 min at 4°C. After homogenisation, the homogenate was centrifuged at 1500 g for 20 min at 4°C, and the supernatant was stored at -80°C for further analysis of cytokines and other inflammatory mediators\(^{(16)}\). Lastly, nasal mucosa from study subjects were incubated with 0.1% protease for 1 hour, and then epithelial cells were scraped. After washing with DMEM medium, cells were maintained in BEBM bronchial epithelial cell growth basal medium (CC-3170, Lonza, Basel, Switzerland) at 37°C in a humidified environment containing 5% CO₂. The air-
liquid interface (ALI) culture system was described previously\(^{(17)}\). In brief, nasal epithelial cells were seeded on 0.4-μm, 0.33-cm\(^2\) polyester Transwell inserts (Costar, Corning, NY, USA) at a density of 5 × 10\(^4\) cells per well. ALI culture medium was made from a 1:1 mixture of bronchial-epithelial cell growth medium bullet kit (Lonza, Basel, Switzerland) and DMEM with added antibiotics (1% penicillin and streptomycin), an antifungal agent (0.2% amphotericin B; Life Technologies, Grand Island, NY, USA). ALI culture was maintained until 14 days to induce differentiated epithelium. The allergy sensitisation of the subjects was evaluated by performing an ImmunoCAP assay (Phadia, Uppsala, Sweden) to detect specific immunoglobulin E (IgE) antibodies against six common aeroallergen mixtures (house dust mites, moulds, trees, weeds, grass, and animal dander). Subjects who showed allergen-specific IgE levels > 0.35 IU/mL to one or more allergens were considered as allergic\(^{(18)}\). The CRSwNP endotypes were histologically defined using hematoxylin and eosin staining: eosinophilic CRSwNP: >10% eosinophils per high-power field (HPF), and non-eosinophilic CRSwNP: ≤10% eosinophils per HPF\(^{(19)}\). A patient with asthma was defined as one who experienced chronic airway symptoms (dyspnoea, cough, wheezing, and/or sputum), reversible airflow limitation, and had their forced expiratory volume in 1 sec increased by ≥ 12% or 200 mL after using a bronchodilator, or showed a methacholine provocation test result of PC20 ≤ 16 mg/mL. Additionally, all enrolled patients were classified into five subgroups according to the algorithm from the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis study: control, non-eosinophilic CRS (non-ECRS), mild ECRS, moderate ECRS, and severe ECRS\(^{(19,20)}\). Subgrouping was conducted based on several clinical factors, including bilateral disease sites, NP, sinus CT findings, eosinophilia in peripheral blood, and comorbidity (bronchial asthma and aspirin-exacerbated respiratory disease/steroidal anti-inflammatory drug-exacerbated respiratory disease)\(^{(19)}\).

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis**

Total RNA was extracted from the tissue samples using the TRI reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse transcribed into cDNA using the amfiTRI reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was extracted from the tissue samples using the TRI reagent (Invitrogen, Carlsbad, CA, USA). GAPDH was used as an internal control for normalisation. Cycling conditions were as follows: 95°C for 5 min, followed by 60 cycles at 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s. The Sequence Detection Software version 1.9.1 (Applied Biosystems) was utilised for data analysis. Relative gene expression was calculated using the comparative 2\(^{-\Delta\Delta C_T}\) method.

**Measurement of inflammatory markers**

The protein concentrations of the tissue extracts were determined using the Pierce 660 nm Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the samples were thawed at room temperature and vortexed to ensure a well-mixed sample, as described in previous studies\(^{(13)}\). A multiplex cytokine analysis kit (IL-1β, IL-5, IL-17A, IL-33, chemokine [C-X-C motif] ligand [CXCL]-1, CXCL-8, myeloperoxidase [MPO], and granulocyte-macrophage colony-stimulating factor [GM-CSF]) was obtained from R&D systems (Cat. No. LMSAHM, Minneapolis, MN, USA) and used for cytokine analyses. Data were collected using a Luminex 100 reader (Luminex, Austin, TX, USA). Data analysis was conducted using the MasterPlex QT version 2.0 software (MiraiBio, Alameda, CA, USA). Total IgE levels in the NP tissues were measured using a human IgE enzyme-linked immunosorbent assay (ELISA) kit (K323106; KOMA, Seoul, Korea). Chitinase-3-like protein 1 (YKL-40) levels were measured using a commercial ELISA kit (R&D). The sensitivity of the assays for each cytokine were as follows: IL-1β: 0.8 pg/mL, IL-5: 0.5 pg/mL, IL-17A: 1.8 pg/mL, IL-18: 1.9 pg/mL, IL-33: 1.8 pg/mL, CXCL1: 5.3 pg/mL, CXCL8: 1.8 pg/mL, GM-CSF: 4.1 pg/mL, MPO: 26.2 pg/mL, and YKL-40: 8.1 pg/mL. The serum eosinophil cationic protein (ECP) and total IgE levels were measured using the ImmunoCAP assay (Phadia). All assays were performed in duplicate according to the manufacturer’s protocols. All protein levels in the tissue homogenates were normalised to the level of total protein.

**Air-liquid interface (ALI) cell culture system**

The ALI culture system was set up and used as described previously\(^{(17)}\). Before treatment, the primary nasal epithelial cells were maintained in BEGM in the absence of hydrocortisone for at least two days. The primary nasal epithelial cells were treated with recombinant human IL-4 (100 ng/mL, R&D Systems), IL-5 (100 ng/mL, R&D Systems), IL-6 (100 ng/mL, R&D Systems), IL-13 (100 ng/mL, R&D Systems), IL-17A (100 ng/mL, R&D Systems), and poly(I:C) (25 μg/mL, InvivoGen, San Diego, CA, USA). Supernatants were removed after 24 h of stimulation, after which RNA was extracted from the cells.

**Statistical analysis**

Statistical analyses were performed using the GraphPad Prism software 9.0 (GraphPad Software Inc., La Jolla, CA, USA) and R software 9.0 (GraphPad Software Inc., La Jolla, CA, USA) and R.
Viral infection modulates ACE2 Expression in CRS

version 3.6.1 software (R Foundation for Statistical Computing, Vienna, Austria). The continuous variables of demographics and clinical characteristics were calculated as the median with interquartile range and were analysed using a Mann-Whitney U-test with a two-tailed test for unpaired comparisons. In multiple comparison analyses, the p-value was calculated using the Kruskal-Wallis test with Dunn’s multiple comparison test. Multiple linear regression was performed to evaluate the CRS endotype-related differences in the expression of ACE2 and TMPRSS2 to adjust for confounding factors, and coefficients, standard error (SE), and p-value were calculated. A p-value < 0.05 was considered significant.

Results

Differences in ACE2 and TMPRSS2 expression in ALI cultures of primary nasal epithelial cells following stimulation with IL-4, IL-5, IL-6, IL-13, and IL-17A

It has been reported that Th2 cytokines such as IL-13 and IL-4 suppress ACE2 expression, and that IL-17 upregulates ACE2 expression in an epithelial cell line(9). To confirm the effect of each cytokine on ACE2 expression in primary nasal epithelial cells from healthy mucosa, we utilised an ALI culture system because a previous study clearly identified ACE2 and TMPRSS2 in subsets of secretory cells among the different cell types found in the nasal mucosa in healthy and allergic subjects(10). Using the ALI culture system, we found that stimulation with IL-4 and IL-13 suppressed ACE2 expression while IL-17A upregulated it. IL-13 upregulated TMPRSS2. These findings were consistent with previous studies (Figure 1)(8,9).

Endotype- and phenotype-related ACE2 expression in CRS

Patients with non-ECRS showed higher levels of expression of ACE2 than patients with ECRS, regardless of the CRS phenotype and tissue type; however, no difference was observed in the TMPRSS2 expression (Figure 2). Based on the severity of ECRS, defined using clinical algorithms(19), samples from patients with non-ECRS showed higher expression levels of ACE2 than those with moderate or severe ECRS (Figure 3A and B). Samples from patients with type 2 inflammation-associated CRS phenotypes, such as asthmatic and allergic CRS, showed lower expression levels of ACE2 compared to patients with other phenotypes (Figure 3C and D). There was no difference in ACE2 expression based on age, and the presence of underlying diseases including diabetes mellitus (DM) and hypertension (HT) in patients.

Figure 1. Differences in ACE2 and TMPRSS2 expression based on the cytokine environment in human nasal epithelial cells. (A) ACE2 expression (n=6 for each group) (B) TMPRSS2 expression (n=6 for each group) KW: Kruskal-Wallis test; Dunn’s multiple comparison test *p<0.05, **p<0.01. ACE2, angiotensin-converting enzyme 2; TMPRSS2, transmembrane protease, serine 2; IL-4, interleukin 4; IL-5, interleukin 5; IL-6, interleukin 6; IL-13, interleukin 13; IL-17A, interleukin 17A.

Figure 2. Comparison of the expression of ACE2 and TMPRSS2 based on CRS endotype and phenotype, and tissue type. (A) ACE2 expression in the UP of CRSsNP (n=9 for control, n=6 for ECRSSNP, n=18 for NECRSsNP) (B) ACE2 expression in the UP of CRSwNP (n=9 for control, n=8 for ECRSwNP, n=14 for NECRSwNP) (C) ACE2 expression in the NP of CRSwNP (n=9 for control, n=36 for ECRSwNP, n=52 for NECRSwNP) (D) TMPRSS2 expression in the UP of CRSsNP (n=9 for control, n=6 for ECRSSNP, n=18 for NECRSsNP) (E) TMPRSS2 expression in the UP of CRSwNP (n=9 for control, n=8 for ECRSwNP, n=14 for NECRSwNP) (F) TMPRSS2 expression in the NP of CRSwNP (n=9 for control, n=36 for ECRSwNP, n=52 for NECRSwNP). KW: Kruskal-Wallis test; Dunn’s multiple comparison test; *p<0.05, ***p<0.001. ACE2, angiotensin-converting enzyme 2; TMPRSS2, transmembrane protease, serine 2; C, control tissue; ECRSSNP, eosinophilic chronic rhinosinusitis without nasal polyps; NECRSsNP, non-eosinophilic chronic rhinosinusitis without nasal polyps; ECRSwNP, eosinophilic chronic rhinosinusitis with nasal polyps; NECRSwNP, non-eosinophilic chronic rhinosinusitis with nasal polyps; ENP, eosinophilic nasal polyps; NENP, non-eosinophilic nasal polyps; UP, uncinate process tissue.
Using a multivariate analysis, patients with non-eosinophilic NP (NENP) were found to have significantly higher ACE2 expression levels than those with eosinophilic NP (ENP), when adjusting for age, sex, Lund Mackay CT scores, presence of asthma, presence of allergic sensitisation status, underlying systemic diseases including DM and HT, smoking history, and BMI (β = 0.819, SE = 0.291, p = 0.007). Moreover, patients with NENP were found to have significantly lower expression levels of TMPRSS2 than those with ENP when adjusting for the confounding factors mentioned above (β = -1.536, SE = 0.442, p = 0.001). These findings suggest that the endotype is an independent factor which affects the expression of ACE2 and TMPRSS2 in NP.

Correlation between ACE2 expression and inflammatory mediators in CRS

Blood eosinophil percentage, serum ECP, and serum total IgE levels in patients with CRSwNP were found to be inversely associated with ACE2 expression. Additionally, the tissue eosinophil count, IL-5 levels, and total IgE levels in the NP homogenates of patients with CRSwNP showed inverse correlations with ACE2 expression (Figure 4). There was no significant association between ACE2 expression levels and the percentage of blood neutrophils. However, tissue neutrophil markers, such as CXCL8, IL-17A, GM-CSF, IL-1β, and YKL-40, were positively correlated with ACE2 expression levels (Figure 5). More interestingly, ACE2 expression levels were found to be associated with goblet cell markers such as MUC5AC (encoding mucin 5AC) and MUC5B (Figure S1). Eosinophil inflammation therefore has a different effect on ACE2 expression levels than neutrophil inflammation.

Poly(I:C) in combination with IL-13 induces the upregulation of ACE2 in ALI cultures of primary epithelial cells from NPs

ACE2 expression has been found to be suppressed in eosinophilic tissues; however, it has been positively associated with the expression of TLR3 and antiviral genes. Therefore, we hypothesised that stimulation with poly(I:C), a TLR3 agonist, would affect ACE2 and TMPRSS2 expression levels in CRS tissues. Poly(I:C) in combination with IL-13 enhanced the expression levels of both ACE2 and TMPRSS2 in ALI cultures of primary epithelial cells of ENP, whereas it suppressed ACE2 expression and enhanced TMPRSS2 expression in control and NENP tissues (Figure 7A and B). These findings suggest that viral stimulation offsets the IL-13-induced downregulation of ACE2, and enhances the IL-13-induced upregulation of TMPRSS2 in a type 2-inflammatory environment. As expected, the upregulation of ACE2 was correlated with TLR3 and IFNA1 expression in nasal tissues, especially in ENP (Figure 7C and D). Moreover, the expression of antiviral genes such as MX1, OAS1, RIG1, and IFIH1 was increased by stimulation using IL-13 in combination with poly(I:C) in primary epithelial cells from healthy controls, ENP, and NENP (Figure S2).
Viral infection modulates ACE2 Expression in CRS

Figure 4. Correlation between ACE2 expression and eosinophil-associated markers in nasal polyps. (A) percent of blood eosinophils (n=88), (B) serum ECP (n=30), (C) serum total IgE (n=75), (D) eosinophilic count (/HPF) in nasal polyps (n=54), (E) levels of IL-5 in nasal polyps (n=54), and (F) levels of total IgE in nasal polyps (n=54). ACE2, angiotensin-converting enzyme 2; IgE, immunoglobulin E; IL-5, interleukin 5; ECP, eosinophilic cationic protein; HPF, high-power field; HE, hematoxylin and eosin.

Figure 5. Correlation between ACE2 expression and neutrophil-associated markers in nasal polyps. The levels of (A) IL-23, (B) MPO, (C) CXCL8, (D) IL-17A, (E) IL-18, (F) IL-33, (G) GM-CSF, (H) IL-1β, (I) YKL-40, and (J) IFNγ in nasal polyps (n=54). ACE2, angiotensin-converting enzyme 2; IL-23, interleukin 23; MPO, myeloperoxidase; CXCL8, chemokine ligand 8; IL-17A, interleukin 17A; IL-18, interleukin 18; IL-33, interleukin 33; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1β, interleukin 1 beta; YKL-40, chitinase-3-like protein 1; IFNγ, interferon gamma.
Figure 6. Correlation between the expression of ACE2 and antiviral genes in nasal mucosa and polyps. Expression of (A) RIG1, (B) IFIH1, (C) OAS1, (D) MX1, and (E) TLR3 (n=38 for CRS-UP; n=26 for CRS-NP). ACE2, angiotensin-converting enzyme 2; MX1, myxovirus resistance 1; RIG1, retinoid acid inducible gene 1; OAS1, 2’-5’-oligoadenylate synthetase 1; IFIH1, interferon induced with helicase C domain; CRS, chronic rhinosinusitis; NP, nasal polyp; UP, uncinate process tissue.

Figure 7. Poly(I:C) in combination with IL-13 induces the upregulation of ACE2 and TMPRSS2 in ENP. Expressions of (A) ACE2 and (B) TMPRSS2 were measured when treated with poly (I:C) with/without IL-13/IL-17 in control, ENP and NENP groups (n=6 for each group). Correlations between ACE2 and TLR3 and between ACE2 and IFNA1 (C and D) were calculated in poly (I:C)-treated ENP and NENP samples (n=18 for each group). KW: Kruskal-Wallis test, Dunn’s multiple comparison test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Poly(I:C), polyinosinic-polycytidylic acid; ACE2, angiotensin-converting enzyme 2; ENP, eosinophilic nasal polyp; NENP, non-eosinophilic nasal polyp; TLR3, toll-like receptor 3; IFNA1, interferon alpha 1.
Discussion

Here, we found that in nasal epithelial cells, ACE2 was downregulated by IL-4, IL-13 treatment which was different from the effect of IL-17A treatment. Non-type 2 CRS tissues showed higher expression level of ACE2, but not of TMPRSS2, regardless of the CRS phenotype and tissue type. A positive correlation was observed between neutrophil inflammation and ACE2 expression; however, eosinophil inflammation, including other type 2 mediators, had an inverse correlation with ACE2 expression. The endotype of CRS was found to be associated with low ACE2 expression levels as an independent factor. ACE2 expression was found to be related to the expression of antiviral response genes, and stimulation using poly(I:C) in combination with IL-13 induced higher expression levels of ACE2 and TMPRSS2 in NP, especially ENP including upregulated antiviral genes. To our knowledge, this is the first study to investigate changes in the expression levels of ACE2 and TMPRSS2 in upper airway tissues, and study the effects of CRS endotypes and viral infection, simulated by poly(I:C) stimulation, using ALI cultures.

A certain proportion of patients with COVID-19 have comorbidities that cause severe complications and have poor disease outcomes\(^\text{[9]}\). In spite of the vulnerable conditions of chronic airway disease against COVID-19, recent studies based on COVID-19 epidemiological data suggest that asthma and allergy are not frequently found as negative comorbid conditions associated with COVID-19\(^\text{[36]}\). A study from Wuhan, China demonstrated that allergic diseases, asthma, and chronic obstructive pulmonary disease (COPD) are not risk factors for SARS-CoV-2 infection\(^\text{[29]}\). In fact, the prevalence of asthma and COPD is lower in patients with COVID-19 compared to the general population\(^\text{[28]}\). Asthma and other allergic diseases result in a type 2-induced immune response. The varying prevalence of these co-existing respiratory diseases has been attributed to the different capabilities of viral entry in SARS-CoV-2 infections\(^\text{[20]}\). The entry-mediating factors for SARS-CoV-2 are highly expressed in nasal epithelial cells, and we found that the expression of ACE2 was decreased in the type 2-inflammatory environment (eosinophilic CRS) of the nasal mucosa. This was in accordance with a previous transcriptome analysis that showed significantly increased ACE2 expression in nasal tissues of NECRSwNP patients compared with ECRSwNP and control subjects\(^\text{[27]}\). This inverse correlation between ACE2 expression levels and type 2-inflammatory mediators was very clear, which also reflects the low entry efficiency of SARS-CoV-2 under allergic airway conditions.

However, the expression of TMPRSS2 did not follow this trend and was found to increase after IL-4, IL-13 stimulation, which would be expected to enhance viral entry. Seibold et al also demonstrated that TMPRSS2 was highly upregulated by type 2 (T2) inflammation through the action of interleukin-13 in a recent in vitro study (ALI culture with IL-13) as well as a gene data analysis study\(^\text{[28]}\). Meanwhile, studies have shown a higher susceptibility to COVID-19 in patients with chronic airway disease. A population-based surveillance of laboratory-confirmed COVID-19-associated hospitalisations reported a higher incidence of chronic lung disease (34.6%) among patients with COVID-19\(^\text{[20]}\). ACE2 expression is also increased in the lower airways of smokers and patients with COPD\(^\text{[30]}\). The binding affinity of the virus with ACE2 is a major determinant of the SARS-CoV replication rate and disease severity\(^\text{[31]}\). Therefore, whether these airway conditions have a protective effect remains a controversial subject, and the differential expression of ACE2 and TMPRSS2 under different inflammatory conditions may be one of the major reasons for this discrepancy.

The co-infection of SARS-CoV-2 with other pathogens is of clinical importance since it can delay diagnosis as well as aggravate disease progression\(^\text{[32,33]}\). A more than two-fold population-level increase in SARS-CoV-2 transmission associated with influenza was found to occur during periods of co-circulation\(^\text{[34]}\). Currently, several respiratory virus co-infections with SARS-CoV-2 have been reported, including influenza, coronavirus, and rhinovirus/enterovirus, and the prevalence may be as high as 50% among non-survivors\(^\text{[35]}\). Poly(I:C)-induced TLR3 signalling which simulates viral infection, results in the production of IFN and the upregulation of other antiviral pathway genes\(^\text{[35]}\). In our study, ACE2 expression was linked to the expression of antiviral genes including OSA1, MX1, RIG1, and IFIH1. This is consistent with a previous report that describes ACE2 as an ISG in human respiratory epithelial tissues\(^\text{[36]}\). Notably, after the stimulation of nasal epithelial cells with poly(I:C) in combination with IL-13, the expression of ACE2 along with other anti-viral genes was increased in ENPs. These results highlight the possibility that viral infections in the upper airways of patients with allergic diseases can facilitate the spread of SARS-CoV-2 by increasing the expression of ACE2 via anti-viral genes pathways.

Along the respiratory tree, nasal epithelial cells including goblet and ciliated cells, show the highest levels of expression of ACE2, which reflects the inherent immune function of the nasal cavity against viral infection\(^\text{[17]}\). Because the nasal cavity is a critical site for transmission, manipulating the viral entry proteins in the nasal mucosa may potentially be an effective modality for the management of COVID-19. Animal model studies report a decrease in infectivity and disease severity in the lungs after ACE2 modulation\(^\text{[36]}\). The enhanced expression levels of ACE2 have been found to accelerate the infection and spread of SARS-CoV-1, whereas silencing of ACE2 blocks viral entry into cells\(^\text{[37]}\). A serine protease inhibitor that inhibits TMPRSS2 has been shown to block SARS-CoV-2 infection in lung cells\(^\text{[46]}\). Recently, the use of steroids has been suggested to suppress pulmonary ACE2 expression by downregulating IFN pathways\(^\text{[38]}\). However, besides its role as a site for viral entry, ACE2 has been shown to have important anti-inflammatory and antioxidant roles in protecting the lungs from injury\(^\text{[39]}\). Therefore, prospective trials
aimed at modifying ACE2 are likely to be critical in developing new treatments for COVID-19. This study has potential limitation. As this is a retrospective cross-sectional study and there were no COVID-19 subjects during the enrolment period, we could not provide the actual incidence rate of COVID-19 between endotypes or viral infection state. Currently, there is no epidemiologic data supporting the direct association between ACE expression and the risk of COVID-19.

**Conclusion**

In conclusion, ACE2 and TMPRSS2 have distinct expression patterns in the upper airway epithelium depending on CRS endotype and the presence of viral stimulation. ENP samples showed lower expression levels of ACE2 than NENP samples; however, poly(IC) stimulation upregulated ACE2 and TMPRSS2, which were found to be associated with the expression of antiviral genes. These findings advance our understanding of viral pathogenesis and provide clinical implications for treating COVID-19.

**Conflict of interest**

The authors declare that no conflict of interest exists.

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**References**

Viral infection modulates ACE2 Expression in CRS

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Figure S1. Correlation between ACE2 and MUC5AC/MUC5B expressions in nasal mucosa and polyps. (A) Correlation between the expression of ACE2 and MUC5AC (n=54). (B) Correlation between the expression of ACE2 and MUC5B (n=54). ACE2, angiotensin-converting enzyme 2; MUC5AC, mucin 5AC; MUC5B, mucin 5B; CRS, chronic rhinosinusitis; NP, nasal polyp; UP, uncinate process tissue.
Viral infection modulates ACE2 Expression in CRS

Figure S2. Poly(I:C) in combination with IL-13 induces the upregulation of antiviral genes in ALI cultures of primary epithelial cells. Expression of (A) MX1, (B) RIG1, (C) OAS1, and (D) IFIH1 (n=6 for each groups). KW: Kruskal-Wallis test, Dunn’s multiple comparison test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Poly(I:C), polyinosinic:polycytidylic acid; IL-13, interleukin 13; IL-17A, interleukin 17; ALI, air-liquid interface; MX1, myxovirus resistance 1; RIG1, retinoid acid inducible gene 1; OAS1, 2′-5′-oligoadenylate synthetase 1; IFIH1, interferon induced with helicase C domain; ENP, eosinophilic nasal polyp; NENP, non-eosinophilic nasal polyp.