

# Differential Chloride Secretory Capacity in Transepithelial Ion Transport Properties in Chronic Rhinosinusitis

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## Abstract

**Background:** Epithelial ion transport regulates hydration of airway mucosal surfaces, and thus promotes effective mucociliary clearance (MCC). Decreased transepithelial Cl<sup>-</sup> transport may contribute to epithelial dysfunction by abrogating MCC and increasing mucus viscosity in chronic rhinosinusitis (CRS). The objective of the current study is to evaluate Cl<sup>-</sup> channel transport properties from cultures of human sinonasal epithelia.

**Methods:** Human sinonasal epithelia (HSNE) from patients undergoing sinus surgery were cultured at an air-liquid interface to confluence and full differentiation. The epithelial monolayers were mounted in Ussing Chambers to investigate pharmacological manipulation of ion transport. Epithelial Na<sup>+</sup> channel (via Amiloride), CFTR (via forskolin), and Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel (CaCC, via UTP) transport were investigated among three different patient groups: Control, CRS and CRS with polyposis. CFTR mRNA levels were evaluated with quantitative RT-PCR.

**Results:** HSNE cultures from 18 patients (Control = 9, CRS = 6, CRS with polyposis = 3) were evaluated in 142 experiments. Summary data from the 18 patients demonstrated that stimulated CFTR-mediated anion transport ( $\Delta I_{SC}$ ) was significantly lower with CRS (7.58 ± 2.24  $\mu$ A/cm<sup>2</sup>) compared to control (25.86 ± 3.44  $\mu$ A/cm<sup>2</sup>) and CRS with polyposis (20.16 ± 4.0  $\mu$ A/cm<sup>2</sup>) (p = 0.004). No statistically significant difference was found for CaCC anion transport between groups (p = 0.39). Significantly decreased mRNA (relative expression) was noted in CRS cultures (CRS = 40.83 ± 1.76 vs. control = 116.2 ± 24.27, p = 0.03).

**Conclusions:** A substantial decrease in the Cl<sup>-</sup> secretory capacity of HSNE monolayers was demonstrated in CRS subjects. Data suggest that CFTR may contribute more to abnormal ion transport in CRS than CaCC.

## Keywords

chronic rhinosinusitis, CFTR, cystic fibrosis, potential difference, transepithelial ion transport, sinusitis, chloride secretion, chloride channels, nasal mucosa, ussing chamber analysis

## Introduction

The sinonasal epithelium is comprised of airway surface liquid (ASL) containing a low viscosity periciliary fluid layer (sol) around the respiratory cilia and a superficial

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mucus (gel) layer, which function to trap and sweep inhaled particles into the digestive tract through coordinated mucociliary clearance (MCC).<sup>1</sup> The cystic fibrosis transmembrane conductance regulator (CFTR) functions as a chloride ( $\text{Cl}^-$ ) and bicarbonate ( $\text{HCO}_3^-$ ) channel and is critical for the normal height of the ASL. Decreased CFTR-mediated  $\text{Cl}^-$  secretion across the mucosal surface contributes to the development of airway disease by depletion of ASL, which hinders effective MCC by increasing the viscosity and adhesion of mucus resulting in persistent infection and/or inflammation.<sup>2-6</sup> These effects are pronounced in patients with cystic fibrosis (CF) who exhibit CFTR dysfunction and chronic lower and upper airway infection. An enriched understanding of the role of CFTR in the maintenance of normal epithelial function has revealed that wild-type (WT) CFTR processing, endocytic recycling, and function can also be significantly inhibited by environmental insults, including cigarette smoke exposure, high altitude/hypoxia, inflammation, and infectious agents, and may be a contributing factor in CRS and other disease of MCC such as chronic obstructive pulmonary disease (COPD).<sup>7-12</sup> Our previous study measured functional CFTR-mediated  $\text{Cl}^-$  transport activity and detected a decrease in the stimulatory effect of CFTR mediated  $\text{Cl}^-$  secretion across the sinonasal epithelia from CRS patients compared to normal controls.<sup>2</sup> However, additional apical channels (e.g. Calcium activated Chloride Channel, CaCC also known as TMEM16A) and transporters contribute to transepithelial ion transport in sinonasal epithelium, and could be potential therapeutic targets for sinusitis treatment.<sup>2,13</sup>

Air-liquid interface (ALI) cultures of airway epithelial cells reproduce key aspects of *in vivo* airway epithelial structure and function and have proved to be invaluable for understanding airway diseases.<sup>3,14</sup> Well-differentiated ALI cultures of normal and chronic sinusitis epithelia from surgical specimens are well established models for studying airway ion transport properties.<sup>11,15,16</sup> The objective of the current study is to evaluate apical ion transport characteristics from cultures of human sinonasal epithelia from controls and CRS with and without polyposis by assessing  $\text{Na}^+$  absorptive and  $\text{Cl}^-$  secretory properties. Based on previous data from *ex vivo* specimens, the hypothesis of the current study is that recapitulated HSNE cultures from CRS patients would exhibit decreased CFTR-mediated anion transport.

## Methods

### Study Population

This study was approved by the institutional review board at University of Alabama at Birmingham. All

subjects enrolled in the study provided informed consent. Normal sinonasal mucosa was obtained intraoperatively from patients undergoing endoscopic surgery for pituitary tumors, facial trauma, benign sinonasal tumors, or lacrimal obstruction. Sinonasal tissue was also procured from patients with medically refractory chronic rhinosinusitis (CRS) with and without polyposis undergoing endoscopic sinus surgery (ESS). All CRS patients fulfilled diagnostic criteria endorsed by the American Academy of Otolaryngology.<sup>17</sup>

### Primary Cell Culture

After tissue harvest, human sinonasal epithelial (HSNE) cells were grown on Costar 6.5-mm diameter permeable filter supports (Corning Life Sciences, Lowell, MA), and submerged in culture medium as previously described.<sup>18-21</sup> Media was removed from the monolayers on day 4 after the epithelium reached confluence, and cells fed via the basal chamber. Differentiation and ciliogenesis occurred in all cultures within 10 to 14 days. Cultures were used for experiments when fully differentiated with widespread ciliogenesis and transepithelial resistances ( $R_t$ )  $>300 \Omega\text{-cm}^2$ .

### Ussing Chamber Analysis

Transwell inserts (Costar) were mounted in Ussing chambers to investigate pharmacologic manipulation of vectorial ion transport as previously described.<sup>7,10,11,15,16,22-26</sup> Monolayers were evaluated under short circuit current conditions following fluid resistance compensation using automatic voltage clamping (VCC 600; Instruments, San Diego, CA). Serosal bath solutions contained (in mM): 120 NaCl, 25  $\text{NaHCO}_3$ , 3.3  $\text{KH}_2\text{PO}_4$ , 0.8  $\text{K}_2\text{HPO}_4$ , 1.2  $\text{MgCl}_2$ , 1.2  $\text{CaCl}_2$ , and 10 glucose. Baths for transwell filters were warmed to 37°C and gassed continuously with a 95%  $\text{O}_2$ -5%  $\text{CO}_2$  mixture that provides a pH of 7.4 under conditions studied here. Chemicals were obtained from Sigma (St. Louis, MO). To increase the sensitivity of the assay for detecting chloride currents across the apical membrane, a serosal to mucosal  $\text{Cl}^-$  secretory gradient was applied. The mucosal bathing solution was changed to a low  $\text{Cl}^-$  solution containing (in mM) 1.2 NaCl and 115 Na gluconate. Stock concentrations of the following pharmacologic agents were used: amiloride (100 mM) - blocks epithelial  $\text{Na}^+$  channels, as a means to isolate changes in short-circuit current ( $\Delta I_{\text{SC}}$ ) secondary to effects on  $\text{Cl}^-$  channel activity;<sup>27</sup> forskolin (20 mM) - activates CFTR by elevating intracellular cAMP, which results in protein kinase A (PKA)-dependent phosphorylation of the CFTR regulatory domain; INH-172 (10 mM) - a CFTR inhibitor that permits determination of CFTR-dependent contributions to  $I_{\text{SC}}$ ;<sup>22</sup> uridine

triphosphate (UTP) (150 mM)- stimulates the CaCC, TMEM16A, by triggering P2Y receptor-mediated increases in intracellular  $\text{Ca}^{2+}$ . Each solution was produced as 1000x stock and studied at 1x in the Ussing chamber. The  $I_{\text{SC}}$  was continuously assessed at one current measurement per second. Amiloride was added first to track ENaC response. The total CFTR response was measured as the difference between baseline after the effect of amiloride had stabilized to the peak of the forskolin response. INH-172 was then administered to block CFTR, and finally UTP was added to measure CaCC function. The  $I_{\text{SC}}$  was continuously assessed at one current measurement per second. By convention, a positive deflection in  $I_{\text{SC}}$  under conditions tested here was defined as the net movement of anions from the serosal to mucosal direction. A schematic drawing of ion transport in airway epithelium is demonstrated in Figure 1.

### Gene Expression

**RNA Isolation.** Total RNA was isolated with RNeasy mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions from HSNE cultures. To prevent possible DNA contamination, samples were pretreated with RNase-free DNase (Qiagen, Germantown, MD) and column purified.

**Primers and Probes.** Sequences used for human CFTR and 18S rRNA were purchased from Assays on Demand (ABI); with assay ID for human CFTR, Hs00357011\_m1.

**Quantitative RT-PCR.** A one-step Applied Biosystems PCR protocol was used to quantify CFTR mRNA transcripts on ABI Prism 7500 sequence detection system on six serial dilutions of RNA isolates according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). TaqMan OneStep PCR Master Mix Reagents Kit (ABI) was used for reverse transcription and PCR. The thermocycler conditions were as follows: Stage 1: 48 °C for 30 min; Stage 2: 95 °C for 10 min; Stage 3: 95 °C for 15 sec; Stage 4: 60 °C for 1 min; 40 cycles. All CFTR values were normalized to 18S rRNA (from the same sample) according to the Applied Biosystems relative quantification method as described in ABI manual to establish specific changes in CFTR mRNA levels as opposed to more general effects on widespread suppression of cellular transcription. All experiments were performed in triplicate.

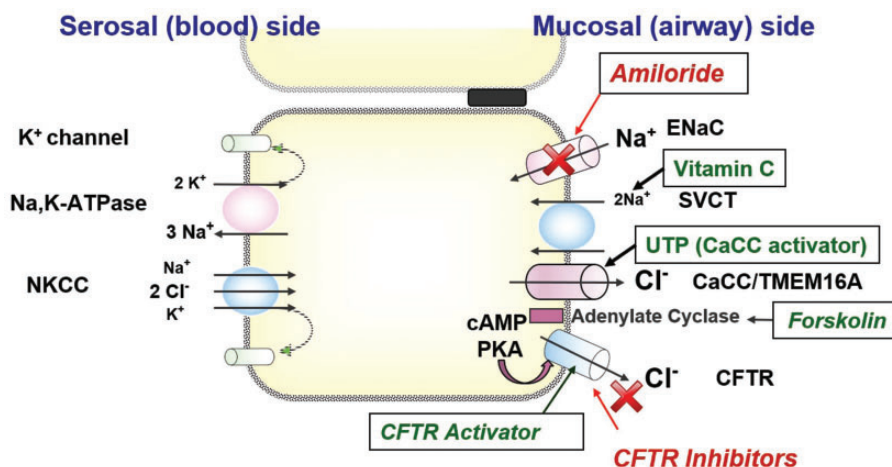
### Statistical Analysis

Statistical analyses were conducted using Microsoft Excel 2016 and GraphPad Prism Version 7.01 and significant statistical difference was set at  $p < 0.05$ . Statistical analyses were performed using paired and unpaired Student's *t*-tests and Tukey-Kramer as appropriate.

## Results

### Patient Characteristics

Sinonasal tissue from 21 patients were included in this study. Mean ages were  $51.7 \pm 15.4$  years in the control



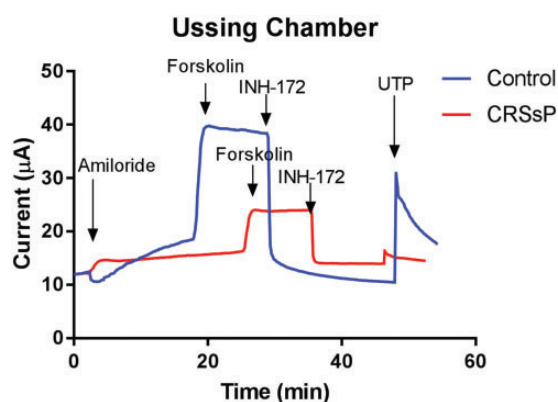
**Figure 1.** Ion transport and pharmacologic manipulation in airway epithelium. Transepithelial  $\text{Na}^+$  reabsorption mediated by concerted activity of apical epithelial  $\text{Na}^+$  channels (ENaC: Blocker = Amiloride) and the basolateral  $\text{Na}^+/\text{K}^+$  ATPase. Apical located sodium dependent vitamin C transporter might also contribute to  $\text{Na}^+$  reabsorption. In addition to  $\text{Na}^+$  reabsorption airway, epithelia display a prominent apical  $\text{Cl}^-$  secretion that is mainly mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) in humans and to a lesser extent by  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels (CaCC) such as the TMEM channels. This secretion is kept up by the basolateral  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  cotransporter (NKCC). In the basolateral membrane, several voltage-dependent  $\text{K}^+$  channels have been identified.

( $n = 11$ , 5 males, 6 females),  $58.8 \pm 7.1$  years in the CRS group ( $n = 8$ , 3 males, 5 females), and  $39.0 \pm 7.1$  years in the CRS group with polyposis ( $n = 3$ , 1 male, 2 females) ( $p > 0.05$ ).

### Ussing Chamber Analysis

HSNE cultures from 18 patients (Control = 9, CRS = 6, CRS with polyposis = 3) were evaluated in 142 experiments. Transepithelial  $\text{Cl}^-$  currents ( $I_{\text{Cl}}$ ) across the monolayer of HSNE were measured in the presence of the sodium channel blocker amiloride and a serosal-to-mucosal directed  $\text{Cl}^-$  gradient ( $-\text{Cl}_{\text{muc}}$ ) developed. Gradient driven  $\text{Cl}^-$  currents ( $I_{\text{Cl}}$ ) stabilized after  $\sim 30$  minutes. Exposure of the apical surface to the cAMP agonist forskolin ( $20 \mu\text{M}$ ) gradually stimulated  $I_{\text{Cl}}$  in a sustained fashion over the course of 15–30 minutes. Subsequent additions of the CFTR transport inhibitor INH-172 significantly reduced forskolin-stimulated  $I_{\text{Cl}}$  to baseline levels indicating that the cAMP agonist stimulated  $\text{Cl}^-$  transport across the epithelium. Exposure of the apical surface to UTP activated  $\text{Cl}^-$  transport across the epithelium and represented CaCC function (Figure 2).

**Enac.** The epithelial sodium channel, ENaC, plays a key role in apical sodium absorption and is important in regulating ASL hydration and mucociliary transport.<sup>13</sup> ENaC activity is upregulated in CF, which leads to  $\text{Na}^+$  hyper-absorption and contributes to ASL dehydration.<sup>28</sup> The goal of this study was to test whether ENaC activity

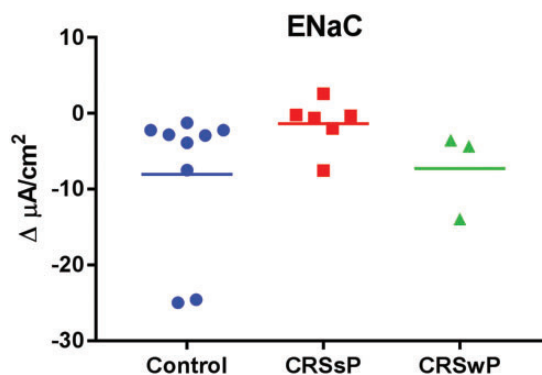


**Figure 2.** Representative Ussing chamber tracings—transepithelial  $\text{Cl}^-$  currents ( $I_{\text{Cl}}$ ) across the monolayer of HSNE were measured in the presence of the sodium channel blocker amiloride and a serosal-to-mucosal directed  $\text{Cl}^-$  gradient ( $-\text{Cl}_{\text{muc}}$ ). Exposure of the apical surface to the cAMP agonist forskolin ( $20 \mu\text{M}$ ) gradually stimulated  $I_{\text{Cl}}$  in a sustained fashion over the course of 15–30 minutes. Subsequent additions of the CFTR transport inhibitor INH-172 significantly reduced forskolin-stimulated  $I_{\text{Cl}}$  to baseline levels indicating that the cAMP agonist stimulated  $\text{Cl}^-$  transport across the epithelium. Exposure of the apical surface to UTP activated  $\text{Cl}^-$  transport across the epithelium.

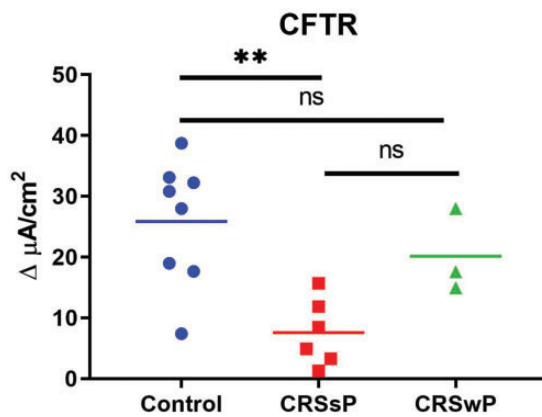
is impaired in CRS. Changes in basal  $I_{\text{SC}}$  after inhibition of  $\text{Na}^+$  transport with  $100 \mu\text{M}$  amiloride in 3 groups are demonstrated in Figure 3. Interestingly, a notable decrease in amiloride-sensitive  $I_{\text{SC}}$  was noted in CRS HSNE compared to control and CRS with NP patients, although statistical significance was lacking ( $\Delta I_{\text{SC}} = \text{Controls}, -8.04 \pm 3.22 \mu\text{A}/\text{cm}^2$  vs. CRS with polyposis,  $-7.27 \pm 3.34 \mu\text{A}/\text{cm}^2$  vs. CRS without polyposis,  $-1.36 \pm 1.38 \mu\text{A}/\text{cm}^2$ ) ( $p = 0.26$ ). ENaC activity was inhibited rather than up-regulated in CRS without polyposis compared to controls.

**Cftr.** Decreased CFTR activity leads to defective apical chloride and bicarbonate transport into the airway lumen. The goal of this experiment was to identify the CFTR-mediated anion transport capacity in CRS with and without polyposis. Stimulated CFTR-mediated anion transport ( $\Delta I_{\text{SC}}$ ) was significantly lower with CRS ( $7.58 \pm 2.24 \mu\text{A}/\text{cm}^2$ ) compared to controls ( $25.86 \pm 3.44 \mu\text{A}/\text{cm}^2$ ) and CRS with polyposis ( $20.16 \pm 4.0 \mu\text{A}/\text{cm}^2$ ) (one way ANOVA,  $p = 0.004$ ; post hoc Tukey-Kramer analysis,  $p = 0.003$  (control vs CRS)) demonstrated in Figure 4.

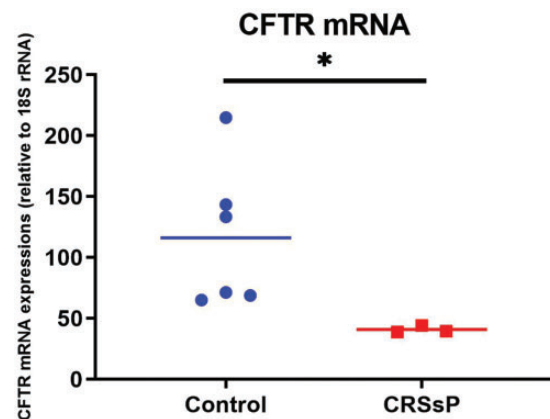
**CaCC.** TMEM16A (ANO1) is a calcium-activated chloride channel (CaCC) expressed in airway epithelium and has shown higher activity in CF airways.<sup>13</sup> Ano1 knockout mice develop a CF-like phenotype, highlighting its importance in  $\text{Cl}^-$  transport and potential role for therapy. Even though cells from CRS with and without polyposis exhibited relatively smaller UTP-mediated CaCC anion transport compared to normal controls, statistical significance was lacking: control =  $15.43 \pm 3.78 \mu\text{A}/\text{cm}^2$ ; CRS without polyposis =  $7.00 \pm 4.70 \mu\text{A}/\text{cm}^2$ ; CRS with polyposis =  $9.39 \pm 1.27 \mu\text{A}/\text{cm}^2$  ( $p = 0.39$ ) (Figure 5).



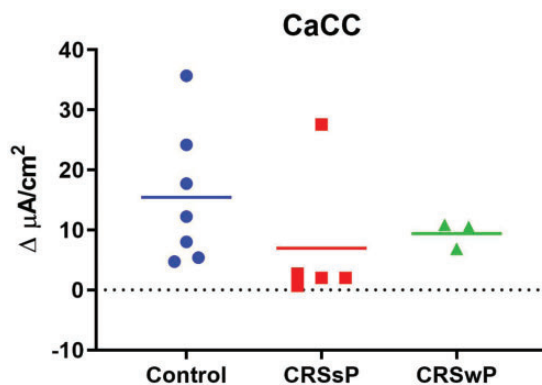
**Figure 3.** No significant difference was observed in ENaC channel function in control, CRS without polyps (CRSsP), and CRS with polyps (CRSwP) ( $p = 0.26$ ). Average amiloride-sensitive  $I_{\text{SC}}$  in controls was due to the contributions of 2 outliers.



**Figure 4.** Stimulated CFTR-mediated anion transport was significantly lower in CRS cultures than those generated from nasal polyps or control patients (\*\* $p=0.004$ ).



**Figure 6.** CFTR mRNA expressions relative to 18S rRNA in HSNE derived from CRS without polyps were significantly decreased compared to controls (\* $p=0.03$ ).



**Figure 5.** No significant difference was observed in stimulated CaCC-mediated anion transport between disease states ( $p=0.39$ ).

**Relative CFTR mRNA expression.** As CFTR-mediated Cl transport was down-regulated in HSNE from CRS without polyposis from above experiments, mRNA expression of CFTR was assessed in normal control ( $n=6$ ) and CRS without polyposis ( $n=3$ ). RT-PCR after RNA isolation from primary HSNE cultures showed significantly decreased CFTR mRNA expression in CRS without polyposis ( $40.83 \pm 1.76$ ) compared to normal controls ( $116.2 \pm 24.3$ ) (t test with Welch's correction  $p=0.03$ ) (Figure 6).

## Discussion

Airway epithelial ion transport regulates the ASL homeostasis, essential for airway mucosal immunity and sinonasal host defense. This study was designed to investigate 3 apical ion channels (ENaC, CFTR, and CaCC) in controls and CRS patients with and without polyposis in recapitulated HSNE cultures to clarify

potential contributions to the pathogenesis of CRS. The markedly diminished Cl<sup>-</sup> secretion across sinonasal epithelial cells from human subjects with CRS suggests acquired CFTR dysfunction may be present in CRS without polyposis. This finding is also consistent with PCR analysis demonstrating decreased mRNA in CRS without polyposis. The decreased CFTR function observed in our analysis could be secondary to decreased transcription. A recent study demonstrated that exoproducts from *P. aeruginosa* impacted CFTR function in human airway epithelial cells isolated from non-CF patients with a decrease in CFTR protein expression, likely in part from enhanced protein degradation and protein synthesis.<sup>29</sup> However, it is still unclear whether the relationship that is suggested between mRNA levels and CFTR function in CRS patients is a result of CRS or a predisposing factor to developing CRS. In addition, there was no accounting for the presence of CFTR heterozygotes since genetic testing was not performed in these subjects.

The precise underlying mechanisms for ENaC upregulation in CF are not fully understood and CFTR dysfunction is thought to be linked to an increase in ENaC activity.<sup>13</sup> This has not been demonstrated consistently in all experimental models and their connection remains in question.<sup>30</sup> Dejima et al. demonstrated that cells from CRS exhibited relatively large  $I_{sc}$  responses to amiloride, suggesting increased Na<sup>+</sup> transport rates.<sup>3</sup> However, in the current study, there was no statistical difference between controls and CRS. Wang et al.'s *in vivo* nasal potential difference (PD) measurement also showed a mean  $\Delta$ PD (changes in PD) that was similar to that of controls after exposure to amiloride.<sup>31</sup> ENaC activation occurs through a number of other mechanisms, including the cleavage of its subunits by endogenous serine proteases.<sup>32</sup> Its expression and function is influenced by diverse factors, such as oxygen tension,

glucocorticoids, and cytoskeletal proteins.<sup>33</sup> In addition, ENaC dysfunction has been shown to be induced by reactive species (such as, in acute lung injury) and neutrophil elastase, which are abundant in inflamed sinusitis.<sup>33,34</sup> Therefore, it may contribute to the various ENaC activities demonstrated in cells from CRS patients in our study. It is important to note that interpreting the effect of amiloride on ENaC-specific  $I_{SC}$  is difficult in the presence of a  $Cl^-$  gradient. Changes in the apical membrane and paracellular driving force, as well as depolarization of the electrochemical potential of  $Cl^-$  close to the potential of  $Na^+$ , will impact amiloride-sensitive  $I_{SC}$ .<sup>35</sup>

Salomon et al. observed substantial differences in the  $Cl^-$  secretory capacity of human nasal epithelial cell monolayers from patients.<sup>36</sup> UTP-induced responses reflecting  $Ca^{2+}$ -activated  $Cl^-$  secretion were substantially lower in CRS compared to normal controls, which was consistent with data in the present study. Pretreatment of cells with IL-13 increased TMEM16A transcript levels in cultured human nasal epithelial cells. Thus, upregulation of CaCC could be associated with allergic inflammation or individuals with CRS with polyposis and T helper type 2 (Th2)-skewed cytokine profiles.<sup>37</sup> These cytokines induce eosinophilic inflammation, which is not typically exhibited in CRS without polyposis. CFTR function appears to be significantly more suppressed in CRS patients indicating non-eosinophilic disease may have greater susceptibility to environmental perturbations that induce acquired CFTR deficiency.

In this study, the CFTR-mediated chloride secretion that was stimulated by the cAMP agonist forskolin was reduced on average by 70% in cultured cells from CRS subjects when compared to controls. The diminished CFTR function demonstrated in CRS subjects could serve as a therapeutic target for small molecules identified in high-throughput screening that act as  $Cl^-$  secretagogues.<sup>38</sup> This strategy has been pursued for therapy of other diseases of mucus clearance, including CF and chronic obstructive pulmonary disease. One such agent, ivacaftor (Vertex Pharmaceuticals, Boston MA), represents a novel CF therapeutic approved for treatment of CF patients with at least one copy of the G551D mutation.<sup>39</sup> In this CF population, ivacaftor improves CFTR channel opening and thereby augments endpoints predictive of benefit, including MCC, sweat  $Cl^-$ , and lung function.<sup>40</sup> Importantly, the same strategy being applied to activate  $Cl^-$  secretion in the lower airways of CF patients is also applicable to acquired CFTR dysfunction in CRS patients with impaired sinus and nasal mucus clearance.<sup>38,40</sup>

There are several shortcomings of this study. The present data included a small number of tissue samples from patients with CRS with polyposis. A larger patient

cohort is required to examine CaCC anion transport effects in this population. The lack of CF genetic testing is another limitation that could skew cultures of CRS patients towards decreased CFTR function if heterozygotes were present in the study population.

## Conclusion

A substantial decrease in the  $Cl^-$  secretory capacity of HSNE monolayers was demonstrated in CRS subjects. Data suggest that CFTR may contribute more to abnormal ion transport in CRS than CaCC and indicates  $Cl^-$  secretagogues could be useful as potential therapy for these individuals.

## Authors' Note

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## Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Bradford A. Woodworth, MD, is a consultant for Olympus and Cook Medical. He also receives grant support from Cook Medical.




## Ethical Approval

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guidelines on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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