Final research report

For fulfillment of the requirements of the long term EAACI research fellowship

Submitted by:

Naiera Zayed, MSc[a,b]

- EAACI long term (12 months) research fellowship winner, 2018 (September 2018 to August 2019).
- Laboratory of Allergy & Clinical Immunology, KU Leuven, Belgium.

Promotors: Peter Hellings [b,c] ,Brecht Steelant [b]

[a] Department of Microbiology and Immunology, faculty of Pharmacy, Menoufia University, Egypt.
[b] Laboratory of Allergy and Clinical Immunology, Department of Microbiology and Immunology, KU Leuven, Belgium.
[c] Clinical Department of Otorhinolaryngology, Head and Neck Surgery, University Hospitals Leuven, Belgium.

- **Addressed research questions:**
  - **Q1**: Are macrolides and tetracyclines able to effectively increase the epithelial barrier integrity?
  - **Q2**: Can macrolides and tetracyclines restore the nasal epithelial barrier integrity in chronic rhinosinusitis patients with nasal polyps (CRSwNP)?

- **Time frame of our research project was divided into 3 main parts according to the following table:**

	Type of tested cells	Measured parameters	Aim
Part I	Primary nasal epithelial cells isolated from healthy subjects (n=4) and CRSwNP patients (n=4).	Trans-epithelial electrical resistance (TEER)	- To study the effect of the tested anti-inflammatory antibiotics on epithelial cells of CRSwNP if they were administered systemically.
Part II	Primary nasal epithelial cells isolated from healthy subjects (n=2) and CRSwNP patients (n=2).	TEER	- To study the effect of the tested anti-inflammatory antibiotics if they were administered locally.
Part III	Calu-3 cell line.	TEER, Paracellular flux	- To study the transepithelial permeability changes caused by the anti-inflammatory antibiotics

- **Methodology:**

1. <u>Selection of macrolides & tetracyclines:</u>

To select the anti-inflammatory antibiotics that will be tested for their effect in our investigations, we took into consideration which ones are recently recommended for managing CRSwNP [1, 2]. Azithromycin and doxycycline were selected as representatives of the "macrolides" and "tetracyclines" classes of antibiotics respectively. Also, the tested concentrations were selected according to the recommended prescribed doses of the drugs, taking into consideration avoiding very low concentrations with no significant effect and very high concentrations which might be toxic to the cells. Fluticasone propionate (FP), a 1st line corticosteroid used for managing CRSwNP was used in our investigations as a positive control to compare the effect of tested antibiotics to.

2. <u>Investigating the effect of tested anti-inflammatory antibiotics on primary nasal epithelial cells:</u>

2.1. Isolation of primary nasal epithelial cells:

Primary nasal epithelial cells (pNECs) were isolated according to the method described by our group [3], from the inferior turbinates of both healthy controls (with no history of rhinosinusitis or allergic rhinitis) and patients with CRSwNP. Collected tissues were washed

in sterile saline then enzymatically digested in 0.1% Pronase (Protease XIV, Sigma) solution in DMEM-F12 culture medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 2% Ultroser G (Pall Life Sciences, Zaventem, Belgium). After overnight shaking incubation at 4°C, the protnase reaction was stopped by the addition of FCS (10%). Cells were washed in culture medium and pelleted by means of centrifugation for 5 minutes at 100g. After that, they were then resuspended in 10 mL of culture medium and incubated in a plastic culture flask for 1 hour at 37°C to remove fibroblasts. One hour later, the cell suspension was collected from the flask then incubated, while shaking, with 2×10^7 prewashed CD45 then CD15 magnetic beads afterwards, each for 20 minutes at 4°C (Dynabeads; Invitrogen, Merelbeke, Belgium). Each time, epithelial cells were purified by means of negative selection, according to the instructions for use. Isolated pNECs were seeded on 0.4-mm3 0.33-cm2 polyester transwell inserts (Greiner Bio-One, Vilvoorde, Belgium) at a density of 10^5 cells per transwell. The culture medium used for pNECs was Dulbecco modified Eagle medium/F12, supplemented with antibiotics and Ultroser G (2%). Culture medium was refreshed every other day. When the cells grew to complete confluence, the apical culture medium was removed to allow further cell differentiation at air-liquid interface (ALI). At day 21 in ALI, the TEER values of the epithelial cell cultures were checked and then they were ready to be used for in-vitro investigations. However, wells not building up sufficiently (TEER < 200 V 3 cm2) were not included in experiments.

2.2. Investigating the effect of tested anti-inflammatory antibiotics on epithelial monolayers integrity:

Different concentrations of tested antibiotics were added either basolaterally or apically to our epithelial cell cultures from day 21 in ALI for the subsequent time points. Culture inserts having only culture media without added antibiotics served as -ve controls for the experiment and other inserts with added FP served as +ve controls for the tested antibiotics. The culture medium with/without a certain concentration of a tested antibiotic or FP was refreshed every 24 hours. Trans-epithelial electric resistance (TEER), as an indicator of epithelial layers integrity, was measured at following time points. The time course change in epithelial integrity in response to added antibiotics was then recorded.

2.3. TEER measurement:

Epithelial integrity of epithelial monolayers was evaluated by TEER measurements using an EVOM/Endohm (World Precision Instruments, Sarasota, Fla) according to Steelant B. *et al*., 2018 [4]. Before each measurement, electrodes were equilibrated and sterilized according to the manufacturer's instructions. Immediately after taking the culture plates out of the incubator, two hundred microliters of pre-warmed culture medium were added in the upper compartment of the transwell inserts. The culture plates were left standing for 5 minutes at room temperature to equilibrate (to eliminate the influence of temperature changes, so that we could get a relatively accurate TEER as possible as it could be). Following that, TEER values were measured. The electrical resistance of a blank (Transwell insert without cells) was measured in parallel. To obtain the sample resistance, the blank value was subtracted from the total resistance of the sample. The final unit area resistance (Ω × cm2) was calculated by multiplying the sample resistance by the effective area of the membrane (0.33 cm2 for 24-well transwell inserts).
TEER values were recorded for each transwell insert at the beginning of the experiment (zero time) representing the base line and at predetermined time points afterwards.

3. **Investigating the effect of tested anti-inflammatory antibiotics on Calu-3 epithelial cells:**

3.1. Growing of Clau-3 epithelial cells:

More laboratory investigations of the tested drugs were performed using Calu-3 epithelial cell line. Calu-3 cells were grown according to the method described by Martens K. *et al.* [5]. Cells were grown in T75 culture flasks in EMEM medium (Lonza), supplemented with 10 % FCS, 1 % L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The culture medium was changed every other day. After reaching confluence (in 7:10 days), cells were split from the T75 flask using 0.25 % trypsin solution in EDTA (Sigma-Aldrich, St Louis, Missouri, USA). Splitted Calu-3 epithelial cells were then seeded on 0.4 µm 0.33 cm^2 polyester transwell inserts (Costar, Corning, NY, USA) at a density of 10^5 cells/transwell. EMEM medium was refreshed every other day. Once the cells grew to complete confluence, the apical culture medium was removed to allow further cell differentiation in air liquid interface (ALI). At day 21 in ALI, epithelial layers integrity was evaluated by TEER measurements using an EVOM/Endohm (WPI Inc, Sarasota, USA). Wells that were not building up sufficiently (TEER < 200 Ω cm^2) were not included in experiments.

3.2. Adding tested antibiotics to the epithelial cell cultures:

At day 21 in ALI, the experiment could start with culture inserts having good TEER values. Different concentrations of tested antibiotics were added basolaterally to the Calu-3 cell cultures from day 21 in ALI for subsequent 3 days. Culture inserts with only culture media without added antibiotics served as -ve controls for the experiment and other inserts with added FP served as +ve controls for our tested antibiotics.

3.3. TEER measurement:

Trans-epithelial electric resistance (TEER), as an indicator of epithelial layers integrity, was measured at time points 0, 24, 48 and 72 hours using an EVOM/Endohm (World Precision Instruments, Sarasota, Fla) as mentioned previously.

3.4. Paracellular flux measurement:

Fluorescein isothiocyanate dextran 4 kDa (FD4) (Sigma-Aldrich, St Louis, Missouri, USA) was used to measure epithelial permeability and follow the time course change in epithelial integrity in response to added antibiotics according to Steelant B. *et al.*,[3] . FD4 (2 mg/mL) was added apically to the ALI cultures and the FITC intensity of basolateral fluid every 24 hours was measured using a fluorescence reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany). FD4 concentration detected in basolateral fluid was calculated and expressed in pmol.

• Results:

1. **Effect of tested anti-inflammatory antibiotics on primary nasal epithelial cells basolaterally:**

The effect of adding anti-inflammatory antibiotics basolateally to the pNECs addressed the potential effect of oral intake of these antibiotics for managing CRSwNP. Tissues from four CRSwNP patients (n=4) and four healthy control (HC) subjects (n=4) were included in the investigation. After isolating the cells from these tissues, they were grown in ALI. TEER values of each transwell as well as a blank one (without cells) were measured at day 21 in ALI to serve as starting values.

Concentrations of 0.1, 1 or 10 ug/ml of either doxycycline or azithromycin and FP (10^{-7}M) were added basolaterally to the transwells containing pNECs of CRSsNP and HCs and the TEER vales were measured at the beginning of the experiment, 24hrs, 48hrs and 72hrs later (Fig. 1).

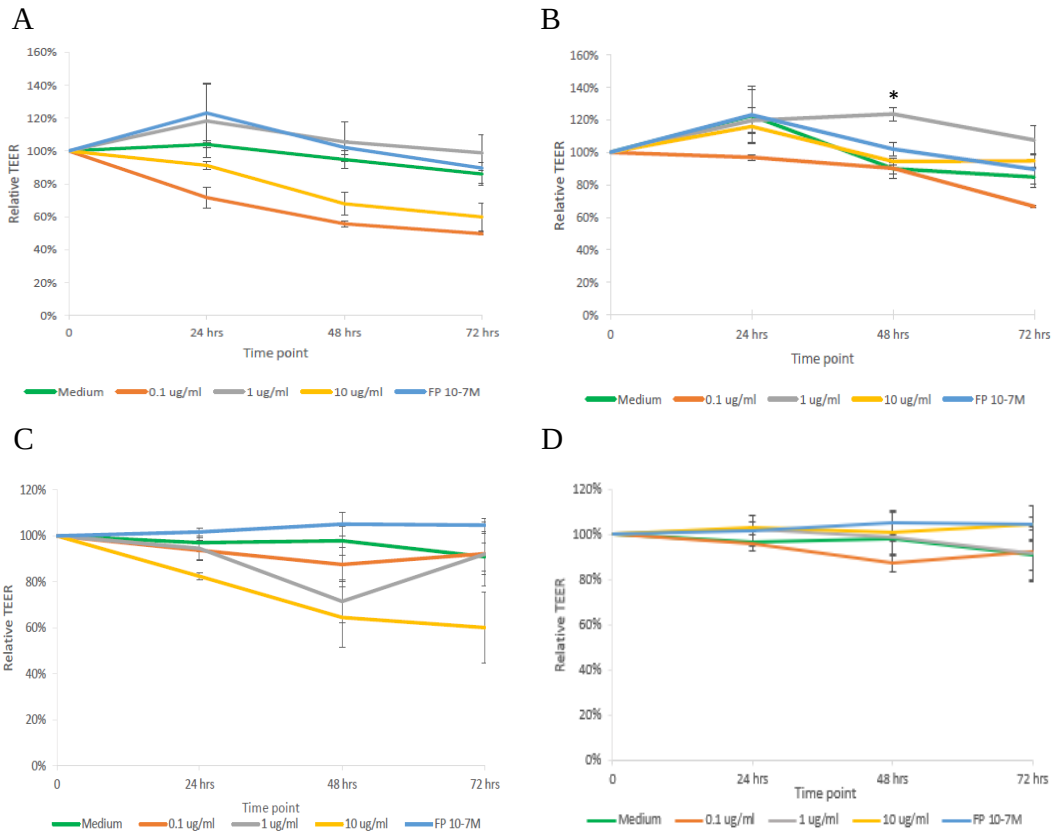

Fig. 1: Time course change in relative TEER values of pNECs from CRSwNP (A, B) and HCs (C, D) in presence and absence of different concentrations of doxycycline (A, C) and azithromycin (B, D) basolaterally (n=4). *$P < 0.05$.

Regarding doxycycline, we observed that adding concentrations of 0.1 or 10 ug/ml basolaterally to CRSwNP culture inserts caused decrease in TEER values over 72 hrs, in comparison to -ve control (only medium) (figure 1.A). However, adding a concentration of 1 ug/ml basolaterally causes increase in TEER over 72 hrs relative to medium control TEER values and even it had almost the same effect as +ve control (FP, 10^{-7} M) at time points 24 and 48 hrs. After 72 hrs, there was decrease in TEER values of FP treated cells but not for the 1 ug/ml treated cells; which remain higher than the medium control (figure 1.A). On the other hand, in the epithelial cells of heathy control subjects (figure1.C), all tested concentrations of azithromycin showed lower TEER values relative to the medium and FP controls after 24 and 48 hrs. However, after 72 hrs the TEER values of 0.1 and 1 ug/ml treated culture inserts had increased to the level of medium control.

Considering azithromycin effect on CRSwNP epithelial cells, adding 10 ug/ml basolaterally did not demonstrate remarkable effect relative to the medium control, although inserts with 0.1 ug/ml added basolaterally showed a decrease in TEER values relative to the medium control at 24 and 72 hrs time points but not at 48 hrs time point. When fluticasone propionate was added, no clear increase in TEER values was observed. However, 1 ug/ml azithromycin in basolateral medium resulted in significant increase in TEER values relative to medium control. With

healthy epithelium, no tested concentrations of azithromycin or ever the FP added remarkable effect to the TEER values when compared to medium control.

2. Effect of tested anti-inflammatory antibiotics on primary nasal epithelial cells apically:

The effect of anti-inflammatory antibiotics added apically to the pNECs predicts the potential effect of using these antibiotics locally for managing CRSwNP. Cells isolated from two CRSwNP patients (n=2) and two healthy control (HC) subjects (n=2) were included in this experiment.

For the apical investigations, concentrations higher than ones used for the basolateral experiment were used because normally the delivered dose for cells is higher in case of local treatment than of systemic intake. Either doxycycline or azithromycin in a concentration of 1, 10 or 100 ug/ml were added to the apical side of the pNECs of CRSsNP and HCs. Also, FP was used as a control in concentration of 10^{-6}M rather than 10^{-7}M The TEER vales were measured at the beginning of the experiment, 12 hrs, 24 hrs and 48 hrs later (Fig. 2).

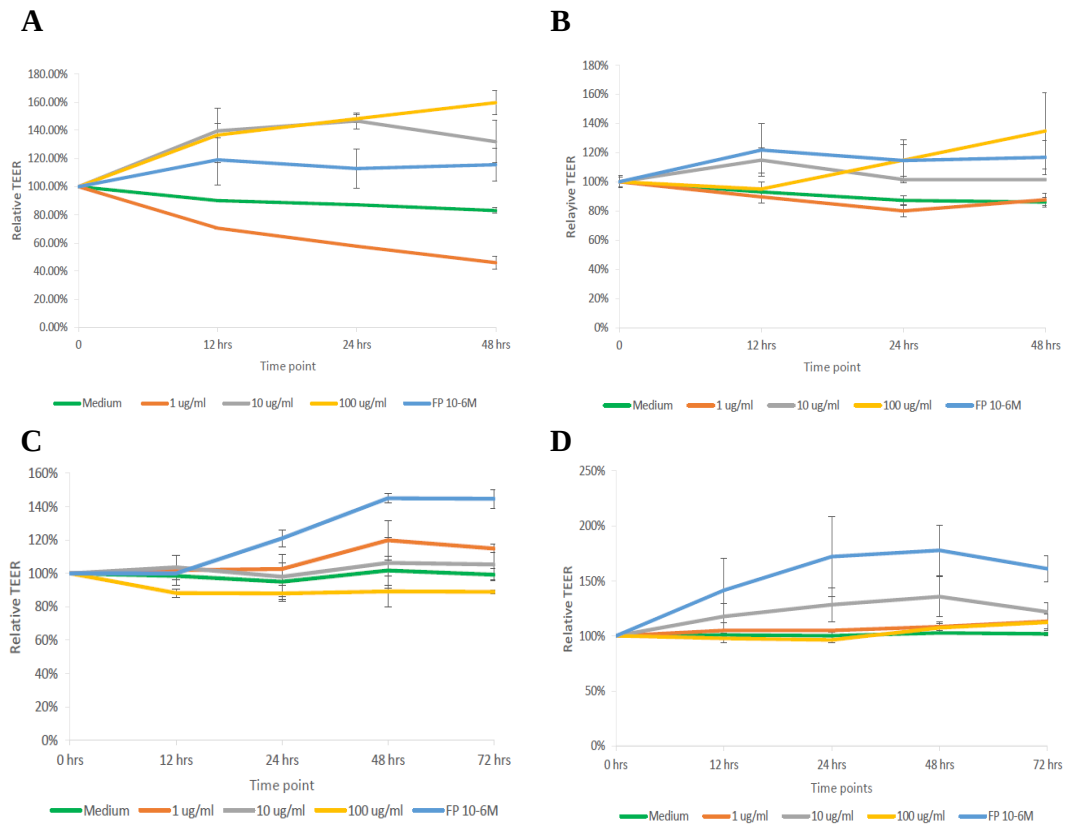

Fig. 2: Time course change in relative TEER values of pNECs from CRSwNP (A, B) and HCs (C, D) in presence and absence of different concentrations of doxycycline (A, C) and azithromycin (B, D) apically (n=2).

When doxycycline was apically applied to CRSwNP epithelial cells, increase in TEER values was observed with concentrations of 10 or 100 ug/ml even more than the level of FP, but there was decrease over time with 1 ug/ml of doxycycline, in comparison to medium control (Fig. 2.A). However, increase in TEER was observed for 1 ug/ml doxycycline with epithelial healthy cells over 48 hrs, but not for 10 or 100 ug/ml which had almost stable values over time relative to medium control (Fig. 2.C).

With regard to azithromycin effect on CRSwNP epithelial cells, concentrations of 10 and 100 ug/ml added apically had increasing effect on TEER vlaues relative to the medium control. For inserts with 1 ug/ml added apically, no added effect on TEER values was observed relative to the medium control. With healthy epithelium, medium control, inserts with 1 and with 100 ug/ml had almost the same values over time, but there was observed increase in values of inserts treated with either 10 ug/ml doxycycline or FP.

3. Effect of tested anti-inflammatory antibiotics on paracellular flux:

As doxycycline and azithromycin showed potential effect on increasing the epithelial barrier integrity (especially when they were applied apically), we then investigated change in transepithelial permeability over time, when cells where treated apically with azithromycin and doxycycline, by measuring the extent of FD4 passage across the epithelial monolayer using Calu-3 epithelial cell line. FD4 (2 mg/mL) was added apically to the ALI cultures together with the tested concentrations (1, 10 or 100 ug/ml) of either doxycycline or azithromycin and the FITC intensity of basolateral fluid was measured every 24 hours, FD4 concentration in basolateral fluid was then calculated (Fig. 3).

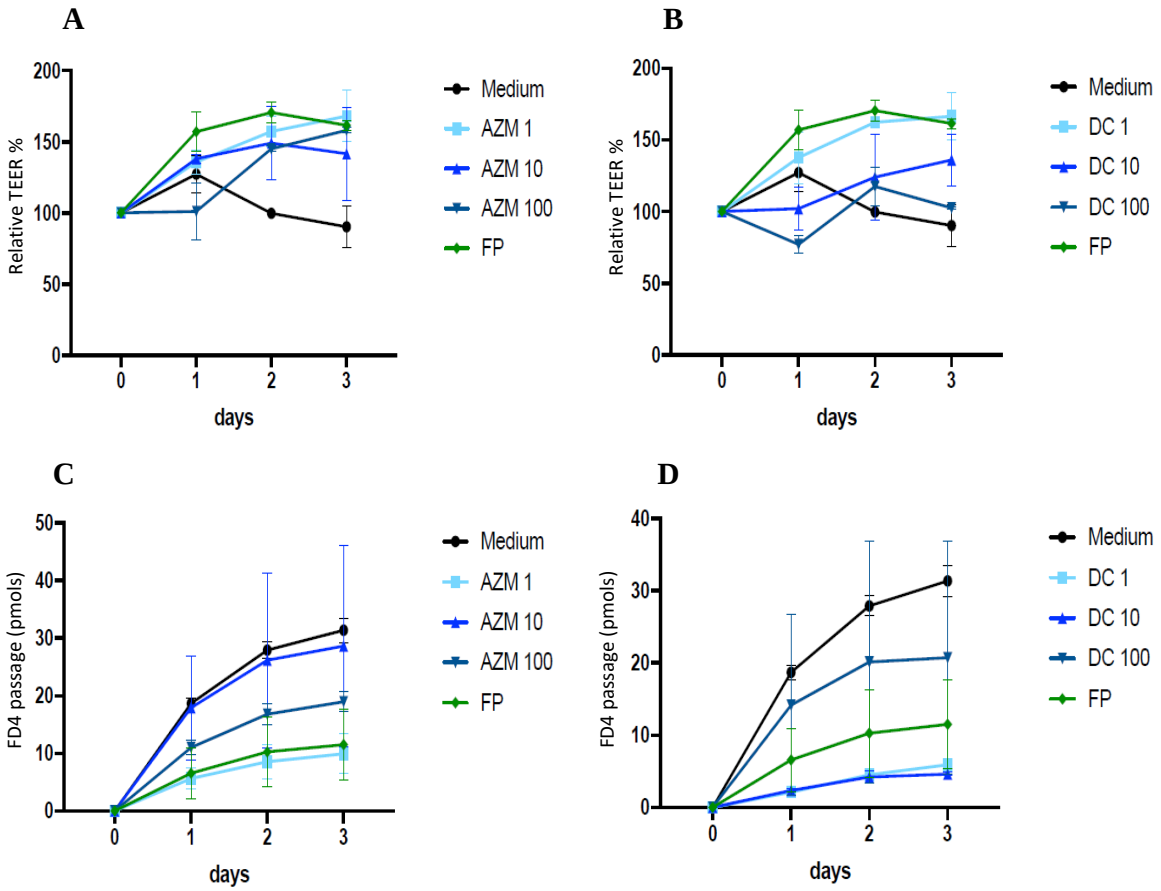

Fig. 3: Time course change in relative TEER values of Calu-3 epithelial cells in presence of different concentrations of azithromycin (A) and doxycycline (B), and FD4 permeability across monolayers of Calu-3 epithelial cells in presence of different concentrations of azithromycin (C) and doxycycline (D) (n=2).

For Calu-3 cells investigations, we used the same concentrations that were used for pNECs investigation in the apical model. Tested concentrations of drugs were added apically to Calu-3 cell inserts together with FD4. Concentrations of FD4 were assessed in the basolateral fluids at the beginning of the experiment and after 1, 2 and 3 days. TEER values also were measured at the same time points.

For azithromycin, time-dependent increase in TEER was observed for all the tested concentrations. Also, lower FD4 permeability than the medium control was observed for concentrations 1 and 100 ug/ml in a time-dependent manner. After 3 days, 1 ug/ml of azithromycin had the same effect on TEER values and FD4 permeability as FP.

Considering doxycycline, time-dependent increase in TEER was recorded for 1 and 10 ug/ml. For 100ug/ml, TEER values fluctuated between decrease after 24 hrs and increase in the following two days of the experiment. For the FD4 permeability, concentrations of 1 and 10 ug/ml showed increase in barrier integrity and hence decrease in FD4 permeability more than the effect of FP. 100 ug/ml doxycycline had also decrease in FD4 permeability than the medium control, but higher than the values obtained with FP treatment.

- ## Summary and conclusion:

The airway mucosa represents the first mechanical barrier against inhaled environmental particles, allergens and microbes and thus, increased permeability of the epithelial barrier can facilitate the penetration of harmful particles towards the submucosal regions with activation of the immune system as a consequence. Decreased epithelial barrier integrity was observed in different chronic airway diseases including chronic rhinosinusitis (CRS) [6]. CRS is characterized by an inflammation of the mucosa of both the nasal cavity and paranasal sinuses [7] which may lead to development of soft, painless, noncancerous growths on the lining of nasal passages or sinuses; called nasal polyps. The corresponding phenotype then is referred to as chronic rhinosinusitis with nasal polyps (CRSwNP) [7, 8]. Since impaired epithelial barrier function is a main hallmark in different airway diseases, restoring its integrity and function represent a promising treatment approach [8]. Two main classes of anti-inflammatory antibiotics are currently used in CRS: macrolides and tetracyclines [1]. However, a few studies investigated their beneficial effects on restoration of epithelial barrier integrity.

In our study, we used two models of applying anti-inflammatory antibiotics to pNECs of CRSwNP; basolateral model (which represent systemic intake of the drug) and apical model (which addresses local application of these tested drugs). Both models showed potential effect of the drugs in managing the epithelial integrity through affecting the TEER values of epithelial monolayers. Apical application of these drugs had more pronouncing effect than basolateral application. The effect was confirmed by testing the FD4 passage through the epithelial monolayers in presence and absence of the tested drugs in a Calu-3 epithelial cell line model. Most of the tested concentrations resulted in more integrity in epithelial monolayers leading to less FD4 passage across the cells. This observed effect was time dependent. All the results were compared to results of -ve controls (culture medium only) and +ve controls (FP), which were concurrently tested.

The same two models were also investigated with pNECs of healthy subjects. Most of the results of the tested antibiotics were in the same range of values as the -ve and/or the +ve controls. This may be due to the fact that healthy epithelial cells have no barrier dysfunction or

dysregulation, which then support our hypothesis that the tested antibiotics can help in restoring epithelial barrier integrity in defective monolayers.

In conclusion, the results of this study suggest a potential effect of macrolides (represented with azithromycin) and tetracyclines (represented with doxycycline) on restoring barrier integrity in CRSwNP patients having barrier dysfunction. However, the number of recruited pNECs in this study was low (due to lack of sufficient time). This is then suggests recruiting a greater number of subjects to have more reliable results.

- ## Future perspectives:

For better understanding of the beneficial effect of macrolides and tetracyclines on epithelial integrity in CRSwNP, more studies on primary nasal epithelium isolated directly from tissue/polyps of patients with CRSwNP should be performed. Since leaky epithelium and barrier dysfunction are predominant in CRS, it is therefore important to investigate the mechanisms of action of macrolides and tetracyclines on the epithelium and also more attentions should be given to potential effect on TJs proteins, which are controlling most of the epithelial integrity. A better understanding of these mechanisms will not only help to better treat CRS patients, but also patients with other comorbidities such as asthma, as the upper and lower airways are nowadays considered as one interconnected system.

- ## References:

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