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CICLO XXXIV

Functional and molecular analysis of Organic Cation Transporters (OCT/OCTN) in human airway epithelial cells

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Abbreviations

α- MT= Alpha-Methyltryptamine

ALI= Air-Liquid Interface

$ATB^{0,+}$/SLC6A14=Solute Carrier Family 6 Member 14

BSA= Bovine Serum Albumin

EBSS= Earle's Balanced Salt Solution

EMEM= Eagle's Minimum Essential Medium

FBS= Fetal Bovine Serum

HBSS= Hank's Balanced Salt Solution

ME= β-Mercaptoethanol

MPP^+ = ^{3}H1-Metil-4-Fenilpiridinio

OCT1/SLC22A1=Organic Cation Transporter 1/ Solute Carrier Family 22 Member 1

OCT2/SLC22A2=Organic Cation Transporter 2/ Solute Carrier Family 22 Member 2

OCT3/SLC22A3=Organic Cation Transporter 3/ Solute Carrier Family 22 Member 3

OCTN2/SLC22A5=Organic Cation/Carnitine Transporter 2/Solute Carrier Family 22 Member 5

PBS= Phosphate-Buffered Saline

PDVF= Polyvinylidene Difluoride

TBS= Tris-Buffered Saline

TEER= Transepithelial/Transendothelial Electrical Resistance

Abstract

Human transporters belonging to the solute carrier family 22 (SLC22) play a central role in physiology, pharmacology, and toxicology because of the broad spectrum of endogenous metabolites, drugs, and toxins that they can move across cell plasma membranes. The human SLC22 family includes Organic Cation Transporters (OCTs) that operate as electrogenic uniporters for organic cations and "Novel" Organic Cation Transporters (OCTNs) mediating Na^+-cotransport of selected zwitterions. The studies addressing OCTs/OCTNs in human airways were mostly performed in immortal or transformed cell lines; here, we studied organic cation transporters in primary models of human airways epithelial cells. To this purpose, EpiAirway™, a 3D organotypic in vitro model of human tracheal-bronchial cells, and EpiAlveolar™, a model of alveolar epithelium, were employed. To better reflect the proper in vivo-like conditions both cellular models were grown under Air Liquid Interface (ALI) condition. Calu-3 bronchial cell line was used in parallel for comparison, as a widely employed model in studies of drugs permeability. The activity of OCTs and OCTNs was evaluated by measuring the uptake of 1-methyl-4-phenylpyridinium (MPP^+) and L-carnitine, respectively, both at the apical and basolateral side of monolayers. Gene and protein expression of these transporters was determined by measuring the number of mRNA molecules through quantitative Polymerase Chain Reaction (qPCR) and Western Blot analysis. In addition the activity and function of $ATB^{0,+}$ Transporter (coded by SLC6A14), was evaluated since this transporter accepts also carnitine, beside neutral and cationic amino acids. The interaction of the transporters with drugs, such as bronchodilators, was also assessed.

Results obtained demonstrated that in EpiAirway™:

- Both OCT1 and OCT3 are expressed and functional only on the basolateral membrane
- OCTN2 transporter is expressed and functional at the basolateral side. This result was supported by the immunocytochemical analysis demonstrating a lateral distribution of the protein both in Calu-3 cell and in EpiAirway™
- $ATB^{0,+}$ is markedly expressed and functional only at the apical side. The protein was clearly present on this side of the membrane in immunocytochemical images
- The bronchodilators glycopyrrolate and tiotropium interact with $ATB^{0,+}$ on the apical membrane, while ipratropium with OCTN2 and OCTs on the basolateral membrane.
- Inflammatory stimuli, such as LPS or TNFα, cause an induction of the expression and activity of $ATB^{0,+}$.

The same analysis performed in EpiAlveolar™ reveal that EpiAirway™ and EpiAlveolar™ display close similarities in terms of transporters expression and interaction with drugs.
In conclusion, the identification of these transporters in these models of human epithelial airways can open new fields of investigation in the study of drug inhalation and pulmonary delivery.

Introduction

1.1 Respiratory System

The respiratory system includes the nose, nasal cavities, paranasal sinuses, pharynx, larynx, trachea, and smaller passageways that lead to efficient exchange of gases between the blood and the respiratory airspace. It can be divided into conducting airways and the respiratory region.

The conducting airways (1-2 m^2, a relatively small surface area) consist of the air-transmitting passages of the nose, pharynx, larynx, trachea, bronchi and bronchioles (Forbes, B. 2005). The epithelial lining of conducting airways comprises three major cell types: ciliated, secretory (mucous, goblet and serous) and basal cells. Gradual changes in the structure of the conducting airways occur as the diameter of the respiratory tubes becomes smaller. The epithelium changes from being pseudostratified and ciliated with goblet cells and submucosal glands interspersed to becoming a flattened squamous epithelium devoid of goblet cells or glands (Davies, D.E. 2014). Ciliated cells constitute the major cell population in the conducting airway epithelium, with their major function being the removal of inhaled particulate matter trapped in the mucus. Secretory cells correspond to 15-25% of cells epithelium and are present in several forms: mucous cells, also known as goblet cells, are the main producers of mucus; basal cells, the other major cells types in the airway epithelium, reside near the basement membrane, interspersed between the taller ciliated and secretory cells, not directly in contact with the airway lumen; basal cells are considered as the stem cells or progenitor cells for differentiated ciliated and secretory cells (Figure 1).

The respiratory region, where gas exchange takes place (80-120 m^2, a very large surface area), is composed of respiratory bronchioles, the alveolar ducts and the alveolar sacs. The alveolar epithelium is formed by two types of alveolar cells: AT-I, which usually occupy a very large area and facilitate the transport of gases; ATII, cuboidal alveolar cells that cover 3% of the alveolar surface area. In this area also alveolar macrophages are present (Steimeret, A. 2015). These different cells types have different functions: AT-I cells provide short diffusion path for gas exchanges, ions and protein transport, while AT-II cells produce and secrete the lung surfactants (phospholipids and proteins) that decrease the surface tension in the alveoli and prevent alveolar collapse. AT-I cells are unable to divide but it has been shown that AT-II cells have the ability to differentiate into AT-I, so as to allow the replacement and repair of the epithelium in case of lung injury (Meindl, C. 2015).

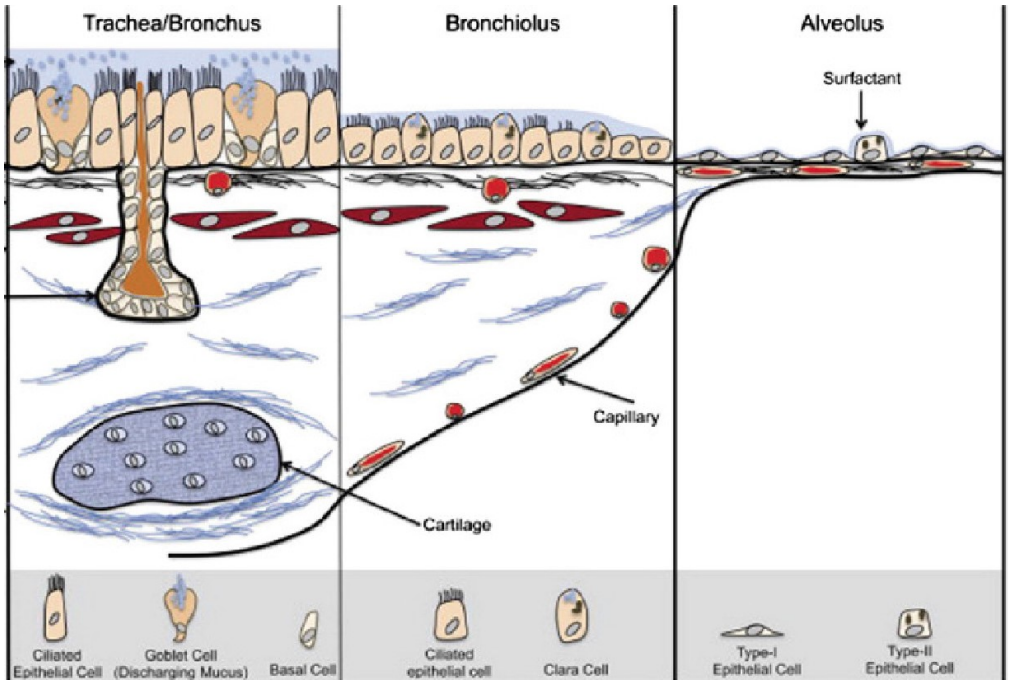

***Figure 1**. Different types of lung epithelial cells in the respiratory tract. Adapted from Klein, S.G. (2011)*

The administration of drugs through inhalation determines the action of some active substance across the upper respiratory tract, bronchi up to the alveoli. The inhalation route allows the exposure of the tissue to high drug concentrations, minimizing the systemic effects of the drug. The most common inhaled drugs are bronchodilators and anti-inflammatory agents used in the treatment of diseases such as asthma and chronic obstructive pulmonary disease (COPD). Many inhaled drugs are rapidly absorbed into the airways: due to their lipophilic chemical features (Patton, J.S. 2004) they reach specific receptors on the underlying smooth muscle, by crossing the lipid bilayer of cell membranes. However, most inhaled bronchodilators are positively charged at physiological pH; thus, e these organic cations require protein transporters for their translocation inside the cells to achieve the receptors.

Membrane transport proteins facilitate the movement of a specific substrate, either with or against its concentration gradient, and the conformational change is important in this transfer process (Vasiliou, V. 2009). There are specific transporters for the excretion and distribution of endogenous organic cations and for the absorption, elimination and distribution of drugs and toxins. Due to their essential role, especially in the absorption and excretion of pharmaceutical compounds, efforts have been made to identify new transporters and to determine detailed transport mechanisms and substrate preferences of

individual transporters. Two superfamilies of membrane transporters have been identified as relevant ones for these processes: ATP-binding Cassette (ABC) and solute carrier family (SLC) (Shin, N. 2015).

1.2 SLC transporters

The largest group of transporters are the Solute Link Carrier (SLC) proteins that include 55 gene families, having at least 362 putatively functional protein-coding genes (He, L., 2009). SLC transporters are often found in epithelial membranes and regulate the uptake and secretion of organic cations and other substrates (Koepsell, H. 2004).

The solute-carrier (SLC) superfamily comprehends passive transporters, symporters, and antiporters, as well as mitochondrial and vesicular transporters (see Figure 2).

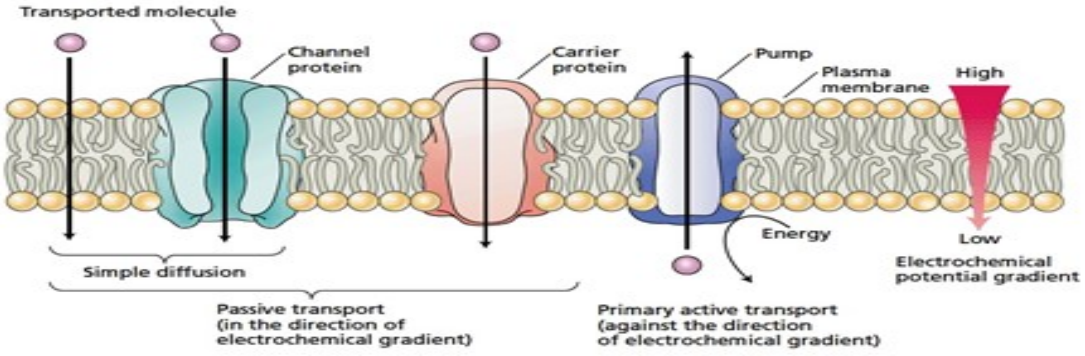

Figure 2. *Transport pathway through cell membrane and mechanism of transport. Figure adapted from Taiz L., Zeiger E., 2010*

SLC transporters are polyspecific and accept compounds with different sizes and molecular structures. These transporters can exhibit large variations in affinity and turnover for different compounds and can have specific physiological roles. They also transfer a wide range of drugs and toxins of different sizes and chemical formulations and are involved in the absorption and excretion of drugs in the liver, kidney and many other tissues (Koepsell, H. 2004).

Transporters involved in the transport of organic cations can be divided into various categories (Ciarimboli, G. 2008; Kouji, H., 2009):

- SLC22 family which includes electrogenic cation transporters OCT1-3 (SLC22A1-3), cation and carnitine transporters OCTN1 and OCTN2 (SLC22A4-5),
- Proton/cation antiporters of the MATE family (SLC47A1-2),
- Monoamine neurotransmitter transporters (SLC6 family),
- Cationic amino acid transporters (SLC7 family),
- Nucleoside transporters (SLC28A1-3 and SLC29A1-4)
- Choline transporters (SLC5A7 and SLC44A1-4)

1.3 The SLC22 family

The human SLC22 family includes Organic cation transporters (OCTs) that operate as electrogenic uniporters for organic cations, “Novel” organic cation transporters (OCTNs) mediating Na^+-cotransport of selected zwitterions, and Organic Anions Transporters (OATs), that physiologically work as organic anion exchangers (Cho, W. 2005). The first transporter of the SLC22 family in mammals, the organic cation transporter OCT1 (SLC22A1), was cloned in 1994 (Grundemann, D. 1994). In humans, genes coding for OCT1, OCT2 and OCT3 are localized within a cluster on chromosome 6.q26-7 (Koehler, M.R., 1997). These genes comprise 11 exons and 10 introns (Grundemann, D. 2000). OCTN1 and OCTN2 have been cloned from human and are localized in a cluster on chromosome 5q31 (Peltekova, V.D. 2004). Like most members of the SLC22 family, the organic cation transporters (OCTs and OCTN) have a predicted membrane topology that comprises 12 α-helical transmembrane domains (TMDs), an intracellular N-terminus, a large glycosylated extracellular loop between TMDs 1 and 2, a large intracellular loop with phosphorylation sites between TMDs 6 and 7 and, finally, an intracellular C-terminus (Figure 3).

These transporters play a central role in physiology, pharmacology, and toxicology because of the broad spectrum of endogenous metabolites, drugs, and toxins that they can move across cell plasma membranes (Ciarimboli, G. 2008, Nigam, S. K. 2018).

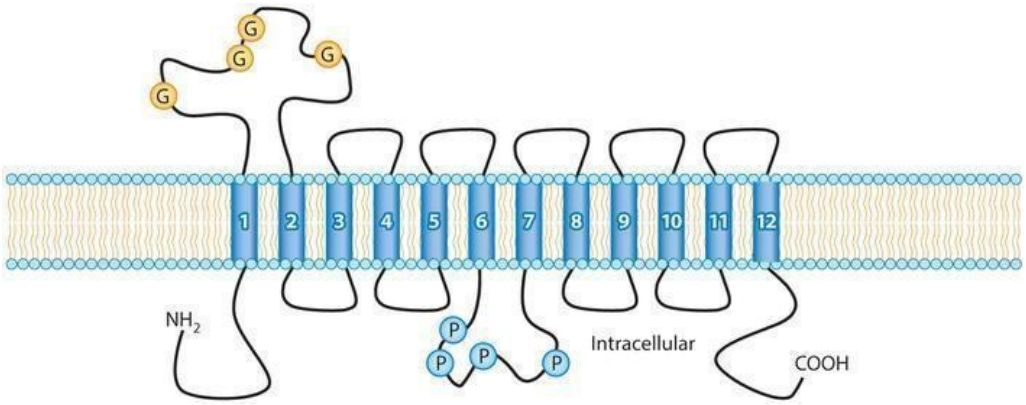

Figure 3. *SLC22 transporter. SLC22 transporters have 12 transmembrane domains. They contain a large glycosylated extracellular loop, located between the first and second TMD and a a large intracellular loop with phosphorylation sites between TMDs 6 and 7. These transporters are mostly expressed in epithelial tissues. Figure adapted from Nigam, K. 2018.*

1.3.1 Organic Cation Transporters (OCTs)

OCTs regulate the transport of small hydrophilic compounds, ranging in size from 60 to 350 Da, with at least one positively charged amino fraction at physiological pH, in both directions of plasma membrane. OCTs are frequently coupled to the obligatory exchangers multidrug and toxin extruders (MATE) belonging to the SLC47 family for the transepithelial secretion of organic cations (Shin, N. 2015). In kidney, OCTs operate the basolateral absorption of organic cations while MATEs are responsible for their apical efflux (Deeley, R. G. 2006). These transporters are present in many tissues such as the liver, kidney, heart, skeletal muscle, placenta, lung, brain, immune system (Nigam, S.K. 2015), and also in the entire gastrointestinal tract (Ortells, M.O. 1995). OCT1-3 carry endogenous compounds, such as monoamine neurotransmitters, carnitine derivatives and creatinine, as well as various drugs; model substrates for OCTs are 1-methyl-4-phenylpyridinium (MPP^+) and tetraethylammonium (TEA) (Cho, W. 2005, Koepsell, H. 2004).

SLC22A1/OCT1 is a hepatic uptake transporter, expressed on the sinusoidal membrane of hepatocytes and it plays a key role in the disposition and hepatic clearance of mostly cationic drugs. (Jonker, J. W. 2004) In addition to organic cations, hOCT1 has also been shown to mediate the transport of some anionic prostaglandins (Kimura, H. 2002), indicating that a positive charge is not an absolute prerequisite for OCT substrates. Recent findings revealed that OCT1 can mediate the uptake of drugs for treating various diseases such as cancers. The levels of OCT1 expression correlate with the responses towards

many drugs and functionally defective OCT1 leads to drug resistance. Recently, it has been proposed that OCT1 should be amongst the drug targets used for pharmacogenomic analyses (Brosseau, N. 2019).

SLC22A2/OCT2 transports cations and non-charged compounds in both directions across the plasma membrane, as was shown for hOCT1 (Nagel, G. 1997; Busch, A.E. 1998; Budiman, T. 2000). This transporter is most strongly expressed in the kidney and is also present in the small intestine and lung (Motohashi, H. 2004). OCT2 is also expressed in various regions of the brain; in particular, it is detected by in situ hybridization in the pyramidal cells of the cerebral cortex and hippocampus. This transporter might represent a "background" transporter for the removal of monoamine neurotransmitters that have escaped reuptake by high-affinity monoamine carriers, which are not members of the OCT family (Busch, A.E. 1998). Moreover, OCT2 is a renal uptake transporter that plays a key role in the disposition and renal clearance of drugs and endogenous compounds.

SLC22A3/OCT3 is an uptake transporter that plays a role in the pharmacokinetics and disposition of a variety of cationic drugs (Koepsell, 2004). OCT3 is involved in intestinal absorption and biliary excretion of drugs, and also plays an important role in the function of various physiological systems. In the SNC, OCT3 regulates the interstitial concentrations of monoamine neurotransmitters and cationic drugs and may thereby change neuronal activities and behavior (Baganz, N.L. 2008, Vialou, V. 2004). In humans, OCT3 and OCT1 mediate the first step in biliary excretion of most cationic drugs in the liver. OCT3 is also involved in renal excretion of epinephrine, histamine, and norepinephrine (Koepsell, H. 2004). The absorption of cationic drugs from the intestinal lumen is regulated by OCT3 and/or OCTN2 in the small intestine brush border membrane. In placenta and lung, OCT3 may also be responsible for the release of acetylcholine during non-neuronal cholinergic regulation (Koepsell, H. 2007). Genome-wide association studies have linked *SLC22A3* to the risk loci for coronary artery disease and prostate cancer (Tomlins, S.A. 2007, Trégouët, D.A. 2009). Mutations that impair the function of human OCT3 may reduce the hepatic excretion and the materno-fetal passage of OCT3 specific cationic drugs. (Chen, L. 2010).

Class	hOCT1	hOCT2	hOCT3	hOCTN1	hOCTN2
Metabolites					
Acetyl-L-carnitine					**8.5**
Betaine					(<500)
Choline	(16,700)[c]	**210 (381)**	(23,800)[c]	(231)	**(<2,000)**
Creatinine	(>20,000)	**(<2,000)**[c]	(15,700)[c]		
D-Carnitine				(<2,000)	**11, 98**
L-Carnitine	(12,400)[c]	(13,000)[c]	(5,590)[c]	**(24)**	**4.3, 4.8**
Guanidine	**(5,030)**[c]	**(2,300)**[c]	(1,300,6,200)[c]		
Thiamine					
Neurotransmitters					
Acetylcholine	**(580)**	**117 (149)**	(10,490)[c]		
Dopamine	(>20,000)[c]	**390–1,400**	1,200[c]		
Epinephrine	(>30,000)[c]	**400**	**240**		
Histamine	(>20,000)[c]	**940, 13,00**	**180, 220**		
Norepinephrine	(7,100)[c]	**1,500, 1,900**	**510–2,600**		
Serotonin	(>20,000)[c]	**80, 290 (310)**	(1,000)[c]		
Hormones					
Aldosterone					(<500)
Corticosterone	(7, 22)	(34)	(0.12, 0.29)		(<100)
Progesterone	(3.1)	(27)	(4.3)		
Prostaglandin E_2	0.66	0.03			
Prostaglandin $F_{2\alpha}$	0.48	0.33			
Testosterone	(10)[c]	(3)[c]	(44)[c]		
Miscellaneous					
Agmatine	**(24,000)**	**1,400 (3,251)**	**2,500**		
Ergothioneine[d]				**21**	
Stachydrine[d]				**270**	

***Table 1**. Endogenous Substrates and Inhibitors of Polyspecific Organic Cation Transporters. Adapted from Koepsell, H., 2004.*

1.3.2 Organic Cation and Zwitterions Transporters (OCTNs)

OCTNs present a very broad tissue expression pattern, they are expressed in epithelial cells, neurons, muscle cells, glial cells, enterocytes, renal and cancer cells (Inazu, M. 2003; Muller, J. 2005).

SLC22A4 encodes a 551 amino acid protein, known as OCTN1. OCTN1 is a widely expressed organic cation transporter and plays a role in L-carnitine tissue distribution and renal reabsorption. It acts as both a Na^+-dependent and Na^+-independent uptake transporter, or exchanger of organic cations, zwitterions and protons. It is involved in Crohn's disease, the renal secretion of gabapentin, and in the disposition of cationic respiratory drugs in the lung (Jong, N. N. 2011; Urban, T.J. 2008). OCTN1 specific substrate is ergothioneine, a mushroom metabolite (Grundemann, D. 2005).

OCTN2 is a high affinity, Na^+-dependent, pH-sensitive transporter, cloned from human kidney in 1998 (Tamai, I. 1998). 70% and 30% of the amino acids of hOCTN2 are identical to hOCTN1 and hOCT1, respectively.

Previous studies by our group demonstrated that SLC22A5/OCTN2 is expressed in human macrophages, where it has been identified as a novel target gene of the mTOR-STAT3 axis (Ingoglia, F. 2017). As far as human lung is concerned, OCTN2 is expressed in several respiratory epithelial models, such as A549, BEAS-2B, NCI-H441 and Calu-3 cells (Sakamoto, A. 2015; Salomon, J.J. 2012; Endter, S. 2009; Ingoglia, F. 2016). Although these models are usually employed as reference in studies of pulmonary drug absorption (Haghi, M. 2014), they all are immortalized or transformed cell lines, and criticisms exist as their biological functions can differ from those of primary differentiated human airway epithelial cells. Recently, various three-dimensional systems of human primary airway cells have been developed, reproducing respiratory epithelium *in vivo*. Among them, the EpiAirway™ and the EpiAlveolar™ system looks very promising and closely resembles epithelium in vivo due to its structure and culture conditions (Ingoglia, F. 2017, Barilli, A. 2021). Substrates of OCTN2 include quinidine, L-lysine, L-methionine (Ohashi, R. 1999) and L-carnitine. Particularly, L-carnitine is required for the transfer of activated long-chain fatty acids across the inner mitochondrial membrane for their degradation through β-oxidation (Reda, E. 2003). Over >99% of carnitine in the body is intracellular, and the organic cation transporter OCTN2 is the primary responsible for maintaining this tissue gradient (Shekhawat, P.S. 2013). OCTN2 operates the active absorption of carnitine in the small intestine and the secretion and reabsorption in renal proximal tubule. OCTN2 also mediates the transport of L-carnitine into adipocytes, cardiac myocytes, skeletal muscle cells, neurons, brain, lymphocytes, and across the blood-retinal barrier (Liang, Y. 2015). Therefore, this transporter is considered responsible for systemic carnitine deficiency, a recessive disorder of fatty acid oxidation leading to cardiomyopathy, hepatomegaly and cerebral dysfunction, among other symptoms (Tang, N.L., 1999).

The potential role of OCTN2 in the pathogenesis of Crohn's disease has been discussed but remains controversial (Silverberg, M.S. 2007, Talián, G. 2009). During inflammatory bowel disease and celiac disease, the expression of OCTN2 in the terminal ileum is decreased (Wojtal, K.A. 2009). Patients with ulcerative colitis and colonic inflammation may benefit by supplementation of L-carnitine or by increasing the expression of OCTN2 by treatment with PPARγ activators such as the antidiabetic rosiglitazone (D'Argenio, G. 2010, Fortin, G. 2009, Lewis, J.D. 2008).

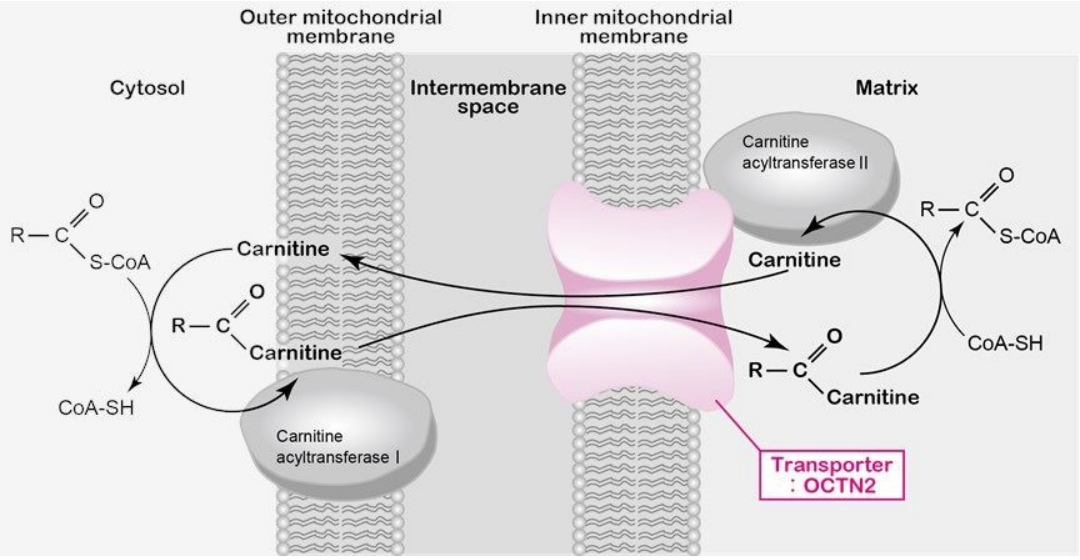

***Figure 4**: SLC22A5 transports carnitine into the mitochondrial matrix. SLC22A5 participates in the intestinal absorption process of carnitine.(www.creative-biolabs.com/slc22a5-membrane-protein-introduction)*

Recently, OCTN2 has been included in the list of transporters responsible for the interaction with drugs by the International Transporter Consortium (ITC) (Giacomini, K. M. 2010); its involvement in the transport of bronchodilators has been also suggested (Nakamura, T. 2010). OCTN2 transports some important respiratory medicines such as ipratropium and tiotropium, and due to its expression in the lung, it may influence the disposition and absorption of these drugs. Tiotropium bromide, the first Long-acting muscarinic antagonist (LAMA) available for COPD in clinical practice, is structurally related to ipratropium (Disse, B. 1999). Ipratropium has a short duration of action, with a half-life of 0.3 hours and requires four times daily dosing (QD), affecting adherence to therapy. In contrast, tiotropium has a long duration of action, allowing once-daily dosing (Panning, C.A. 2003).

1.4 Monoamine neurotransmitter transporters (SLC6 family)

The largest family of neurotransmitter transporters is the solute carrier 6 (SLC6) family, which operates a reuptake of released neurotransmitters by utilizing the electrochemical gradient of sodium. In humans, The SLC6 family includes neurotransmitters, amino acids, betaine, taurine, and creatine transporters. Transporters belonging to SLC6 family have 12 transmembrane helix and are composed by two domains: the larger scaffold domain that anchors the protein in the membrane, and the bundle domain that moves and rotates during the transport cycle, alternating the access to the substrate-binding site located midway through the membrane (Forrest, L.R. 2009). These transporters present two conformational states, whereby the binding site alternates between the intracellular side and extracellular side of the membrane.

Sequence similarity profiles and substrate specificities allow the division of the family into four subgroups (Kristensen, A.S. 2011):

1. The monoamine transporter (MAT),
2. The GABA transporters (GAT),
3. Amino acid (AA) subgroup I
4. Amino acid (AA) subgroup II

In particular amino acid (AA) subgroups include amino acid transporters such as Glycine transporters, the proline transporter PROT and the amino acid transporter B0,+ ($ATB^{0,+}$).

1.4.1 Amino acid transporter $B^{0,+}$ ($ATB^{0,+}$)

SLC6A14 encodes the plasma membrane amino acid transporter $B^{0,+}$ ($ATB^{0,+}$), which is responsible for the Na^+/Cl^--dependent influx of neutral and cationic amino acids. SLC6A14, located on chromosome X (Xq23), was cloned in 1999 from human mammary gland cDNA (Sloan, J.L. 1999) and contains 14 exons (each approximately 100-200 base pairs in length). It has 12 transmembrane domains with intracellular N and C terminals. This transporter has a low affinity (Km = 800 μM) for the substrates, but a high concentration capacity, being energized by transmembrane gradients as well as by the membrane potential (Nakanishi, T. 2001). This transporter is involved in carnitine absorption. $ATB^{0,+}$, under normal conditions, is most expressed in the lung and intestine (Hatanaka, T. 2004), where it is mainly involved in the absorption of nutrients. Among respiratory in vitro

models, only some airway epithelial cells, such as Calu-3 and NCI-H441, actually express $ATB^{0,+}$, while other cells, such as A549 and BEAS-2B, do not (Ingoglia, F. 2016).

Due to its wide substrate specificity and its concentrative ability, this transporter can be considered as a drug delivery system; in fact, many $ATB^{0,+}$ substrates are used as therapeutic agents such as nitric oxide synthase inhibitors (Rotoli, B.M., 2005). It also can transport antiviral drugs such as acyclovir and ganciclovir when they are covalently coupled to the side chain of anionic amino acids (Ganapathy, M.E. 2005).

1.5 Aim of the study

Due to their role in drug absorption great interest has been recently being paid to OCTs and OCTNs transporters. However, studies on this issue mainly focused on hepatocytes and proximal tubules of the kidney, while the physiological role of OCT/OCTN in the lung remains thus far mostly unclear, despite their role in the transport of inhaled drugs.

In this context, reference models for studies of pulmonary drug absorption are traditionally Calu-3 and A549 (Haghi, M. 2014); however, the biological functions of these transformed and immortalized cells may differ from those of primary differentiated human airway epithelial cells. Recently, three-dimensional human primary airway cell systems have been developed for reproducing the respiratory epithelium in vivo. Among these, the EpiAirway™, a 3D organotypic in vitro model of human tracheal-bronchial cells, and EpiAlveolar™, a model of alveolar epithelium, have been used in the present study. These cell models seem particularly promising and innovative since, grown under air-liquid interface (ALI) conditions, they closely resemble epithelium in vivo (Berube, K. 2010, Chemuturi, N.V. 2005). To date, however, little is known about the biophysical characteristics of this model and, more precisely, only minimal information is available on membrane transporters responsible for nutrient flux, such as amino acids, vitamins and other substances such as drugs through the plasma membrane. Therefore, the aim of this study is to characterize the expression and activity of OCTs/OCTNs transporters in EpiAirway™ and EpiAlveolar™, as well as to define their interaction with anticholinergic drugs such as tiotropium, ipratropium and glycopyrrolate in both cell models.

Materials and Methods

2.1 Cell culture and experimental treatments

Calu-3 cells (American Type Culture Collection, USA), obtained from a human lung adenocarcinoma and derived from serous cells of proximal bronchial airways, were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal serum (FBS), sodium pyruvate (1 mM) and 1% penicillin/streptomycin and routinely cultured under physiological conditions (37.5°C, 5% CO_2, 95% humidity). For the experiments, Calu-3 cell monolayers were grown at air-liquid interface (ALI). To this end, cells were seeded onto transwell polyester inserts (0.33 cm^2, 0.4 µm pore size; Falcon) at the density of 10^5 cells/insert; the apical medium was removed 24 hours after seeding, while basolateral medium was renewed every other day. The monolayers were differentiated under ALI condition over 8 or 21 days.

EpiAirway™ tissues (AIR-200-PE6.5), supplied by MatTek Lifesciences (Ashland, MA, USA), were used to recapitulate aspects of the *in vivo* microenvironment of the lung. EpiAirway™, indeed, are produced from primary human tracheal-bronchial epithelial cells that form a fully differentiated, pseudostratified columnar epithelium containing mucus-producing goblet cells, ciliated cells and basal cells. Upon arrival, tissue inserts, cultured on microporous membrane at the air-liquid interface (ALI), were transferred to 24-well plates containing 600µl of the AIR 200-M125 medium provided by the manufacturer, and equilibrated overnight at 37°C and 5% CO_2; the growth medium at the basolateral side was, then, renewed every other day, while apical washes for mucus removal were performed by employing the solution provided by the manufacturer.

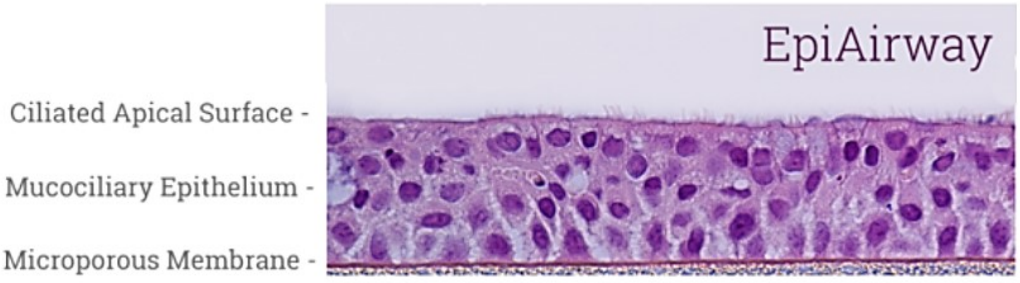

Figure 5. *Schematic illustration of EpiAirway™ system.*

EpiAlveolar™ (ALV-100-FT-PE12), purchased from MatTek Corporation (Ashland, MA) were also employed as an *in vitro* organotypic model of the human alveolar tissue, composed of primary human alveolar epithelial cells co-cultured with human pulmonary

fibroblasts and endothelial cells. EpiAlveolar™ were cultured on microporous membrane inserts at the air-liquid interface (ALI), according to the manufacturer's instructions.

***Figure 6**. Schematic illustration of EpiAlveolar™ systems consisting of primary alveolar epithelial cells, human fibroblast and endothelial cells.*

The integrity of polarized cell monolayers was ascertained before each experiment by measuring trans-epithelial electrical resistance (TEER) with an epithelial voltohm-meter (EVOM, World Precision Instruments); monolayer was considered “tight” for values > 500Ω/cm^2.

A549 cells are obtained from American Type Culture Collection (ATCC, USA) and cultured on plasticware in high glucose DMEM added with 10% fetal bovine serum.

2.2 RNA extraction and Reverse Transcription

For expression studies, cells were seeded onto a 24-well plate and total RNA was isolated with GeneJET RNA Purification Kit and quantified through measurement of the A_{260}/A_{280} ratio with NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific, Italy). RNA was, then, reverse transcribed with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). 11 µl with up to 1 µg of RNA for each sample were transferred into the tubes and additioned with 1 µl of random primers. The tubes were placed in the thermal cycler for a first incubation at the temperature of 65° for 5 minutes for primer annealing. The samples were then cooled to 4° and 8 µl of the mix containing the 1X reaction buffer, RevertAid Reverse Transcriptase (10U/µL), RiboLock RNase Inhibitor (1U/µL) and 0.5 mM dNTPs were added. The reverse transcription reaction was then continued through an incubation at 42° for 60 minutes, followed by a step at 70° for 5 minutes to inactivate the enzymes.

2.3 RT-qPCR analysis

RT-qPCR was performed on a StepOnePlus Real-Time PCR System (ThermoFisher Scientific) by employing specific forward/reverse primer pairs (Table 1) and SYBR™ Green Master Mix (ThermoFisher Scientific).

The amount of the genes of interest (GOI) was calculated relative to that of the reference gene (RPL15), either calculating the number of molecules for each GOI (see below) or employing the comparative CT method (ΔΔCT Method), when comparing control and treated cells (Rotoli B.M., 2018); under this latter condition, gene expression was shown as “fold increase” with respect to its levels in untreated control cells (=1).

2.4 Absolute quantification of mRNA expression

In order to perform an absolute quantification of the mRNAs for the above-mentioned transporters, we needed to preliminary create a standard sample for each genes of interest (GOI), as well as for a reference gene (ribosomal protein like 15, RPL15). Absolute quantification of GOI involves the construction of a standard curve with known dilutions of purified PCR products obtained with each primer pair. To this end, the PCR products were separated through electrophoresis on agarose gel; the single band corresponding to each GOI was purified by employing a commercial DNA gel extraction kit. The cDNA was, then, diluted to 1ng/µl and three serial dilutions were obtained from this first sample, covering so the expected range of expression within our samples. The number of molecules in each dilution was calculated according to the formula $x=N_A/amplicon_{MW}$ where x is the number of GOI molecules in 1 gram of cDNA, N_A is Avogadro number and $amplicon_{MW}$ is the molecular weight of the amplicon. PCR was, lastly, performed on these samples, with the primer pairs originally employed to create the standard sample for each gene of interest; StepOne Plus by Thermo Fisher Scientific was employed. Once reliable standard curves were obtained, mRNA expression analysis in any biological sample was performed with the usual protocol as described above and the number of molecules in each sample was expressed upon normalization for that of the reference gene.

2.5 Western blot analysis

To study the expression of transporter proteins, cell monolayers were washed with ice-cold phosphate-buffered saline (PBS), and then covered with Sample Buffer (62.5 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, 0.2 M dithiothreitol (DTT)). 20 µg of proteins were separated on SDS-PAGE (4-12% acrylamide) and electrophoretically transferred to PVDF membranes (Immobilione-P membrane, Merck). Membranes were incubated for 1 h at RT in blocking solution (Tris-Buffered Saline solution, TBS: 50 mM Tris-HCl pH 7.5, 150 mM NaCl) added with 5% non-fat dried milk, or 1% BSA and 1% casein. The incubation with the specific primary antibodies: anti-OCT1 (1:1000 Merck), anti-OCT3 (1:1000 Merck), anti-OCTN2 (1:1000 Merck) and anti-$ATB^{0,+}$ (1:1000 TermoFisher) were carried out overnight at 4°C. Blots were then exposed to horseradish peroxidase-conjugated anti-rabbit IgG (1: 20.000) for 1 h at RT. Western Blot images were captured with iBright FL1500 Imaging System (ThermoFisher Scientific) and analyzed with iBright Analysis Software. α-tubulin or vinculin, detected with a monoclonal antibody (1:2000; Merck), were employed as internal standard.

2.6 Uptake studies

OCTs and OCTNs activities were evaluated by measuring the uptake of the radiolabeled substrate [^{3}H]1-methyl-4-phenylpyridinium (MPP^+) and L-[^{3}H]carnitine respectively. Amino acid transport was measured both at the apical and the basolateral side of the monolayers. After two rapid washes in pre warmed transport buffer Earle's Balanced Salt Solution (EBSS) (containing, in mM, 117 NaCl, 1.8 $CaCl_2$, 5.3 KCl, 0.9 NaH_2PO_4, 0.8 $MgSO_4$, 5.5 glucose, 26 TRIS-HCl adjusted to pH 7.4), cells were incubated in fresh transport buffer containing each radiolabeled substrate (2 µCi/ml) for the times detailed in each experiment. For the determination of sodium-independent uptake, a modified EBSS in which NaCl was replaced with equimolar N-methylglucamine chloride was employed. Where indicated, the inhibitors or the drugs were added to the transport buffer at the designated concentrations. At the times indicated, transport buffer was removed and the experiment was terminated by two rapid washes in ice-cold urea (300 mM). The filter was then detached from the insert and the ethanol soluble pool was extracted from monolayers. Radioactivity in cell extracts was determined with MicroBeta2 liquid scintillation spectrometer (Perkin Elmer, Milano, MI, Italy).

The uptake was normalized for the protein content, determined using a modified Lowry procedure (Ingoglia F., 2015). The substrate uptake is expressed as pmol/mg of protein/min or nmol/ml intracellular water (see later).

2.7 Determination of cell volume

Cell volume of Calu-3 cells and EpiAirway™ was determined from the distribution space of [^{14}C]Urea. To this end, [^{14}C]Urea (0.5 mM; 2 µCi/ml) was added for 15 minutes at both the apical and basolateral side of polarized monolayers; the incubation was terminated by two rapid washes in ice-cold urea. Cell monolayers were extracted in ethanol and the radioactivity in cell extracts was determined as described above.

2.8 Confocal laser scanning microscopy (CLSM)

Calu-3 monolayers and EpiAirway™ tissues were washed twice in PBS, then fixed with ice-cold methanol for 7 min or with 3.7% paraformaldehyde for 15 min at room temperature, respectively. Cells were incubated with 0.2% Triton X-100 at room temperature for 20 min and then washed three times by PBS. After 1h in blocking solution (5% of BSA in PBS) at 37°C, cells were incubated overnight at 4°C with the polyclonal antibodies ATB$^{0,+}$ or OCTN2 (1:100, Merck), then washed and further incubated for 45 min with Alexa Fluor 488 secondary antibody (1:400, Abcam). Afterward, cells were incubated with propidium iodide solution (Cell Signaling, EuroClone) for 10 min at 37 °C, so as to stain nuclei; control filters were stained with propidium iodide in the absence of primary antibodies, so as to obtain only nuclei signal. After staining procedures, the permeable filters were detached from the culture inserts and mounted on microscope glass slides with fluorescence mounting medium (FluorSave Reagent, Calbiochem). Coverslips were analyzed with a Zeiss® 510 LSM Meta confocal microscope using a multi-track detection system and a 63x (NA 1.4) oil objective. Excitation at 488 nm and emission recorded through a 505-530 nm band pass barrier were used for the detection of the transporters; excitation at 543 nm and emission recorded through a 580-630 nm band pass barrier filter were adopted for cell nuclei. The two signals were rendered with scales of green and red for transporters and nuclei, respectively. Vertical sections were obtained with the function

Display - Cut (Expert Mode) of the LSM 510 confocal microscope software (Microscopy Systems, Hartford, CT).

2.9 Statistical analysis

The statistical analysis was performed using GraphPad Prism 6 XML (GraphPad Software, San Diego, CA, US). *p* values were calculated with a two tailed Student's t-test $p<0.05$ was considered significant.

2.10 Materials

Fetal bovine serum was purchased from EuroClone (Italy). Carnitine-L-[N-methyl-^{3}H]HCl (80 Ci/mmol), Mannitol, D-[1-^{14}C] (57.2 mCi/mmol), N-Methyl-4-phenylpyridinium acetate-[N-methyl-^{3}H] (MPP^{+}) 81.3Ci/mmol, and [^{14}C]-Urea, 55.42 mCi/mmol were obtained from Perkin Elmer (Italy). Merck (Italy) was the source of the antibodies and, unless otherwise specified, of all other chemicals.

Gene	Forward primer	Reverse primer
SLC22A1 *(NM_003057.3)*	TGTCACCGAAAAGCTGAGCC	TCCGTGAACCACAGGTACATC
SLC22A2T2 *(NM_003058.4)*	CATCGTCACCGCGTTTAACCTG	AGCCGATACTCATAGAGCCAAT
SLC22A3 *(NM_003058.4)*	AGGTATGGCAGGATCGTCATT	GCAGGAAGCGGAAGATCACA
SLC22A5 *(NM_001308122.1)*	TCCACCATTGTGACCGAG	ACCCACGAAGAACAAGGAGAT
SLC6A14 *(NM_007231.5)*	CTGCTTGGTTTTGTTTCTTCTTGGTC	GCAATTAAAATGCCCCATCCAGCAC
RPL15 *(NM_001253379.2)*	GCAGCCATCAGGTAAGCCAAG	AGCGGACCCTCAGAAGAAAGC

Table 2 *Sequences of the primer pairs employed for RT-qPCR analysis*

Results

Chapter I – OCTs in human airway epithelial cells

3.1.1 Time-dependent accumulation of 1-methyl-4-phenylpyridinium (MPP^+) in EpiAirway™ and Calu-3 monolayers

The functional activity of OCTs in EpiAirway™ was evaluated by measuring 1-methyl-4-phenylpyridinium (MPP^+) uptake both at apical and basolateral sides. An appropriate concentration of MPP^+ (50 μM) was employed so as to appreciate the contribution of both OCT1 and OCT3 transporters, taking into account the different Km of MPP^+ for these two transporters (Ingoglia, F. 2016). The uptake of has been measured at different times, up to 30 min, at both sides of EpiAirway™ (Figure 7). On the basolateral membrane, MPP^+ uptake progressively increased in a time-dependent manner, following a linear trend. After 30min, MPP^+ uptake was about 150 μM, which is three times higher than the substrate concentration employed, i.e., 50 μM. Conversely, MPP^+ influx at the apical side was very low at any experimental time, so we can exclude any significant accumulation of the substrate within the cells. The transport of MPP^+, measured at 30 min in the absence of sodium, was comparable to the uptake measured in the presence of the cation, thus confirming the absolute sodium-independence of OCTs transport. For comparison, time-course of MPP^+ uptake was performed in polarized Calu-3 cells grown under air–liquid interface conditions (Calu-3 ALI). The accumulation of the substrate was linear up to 30 min, comparable when measured at the apical and basolateral side of the layers and completely sodium-independent.

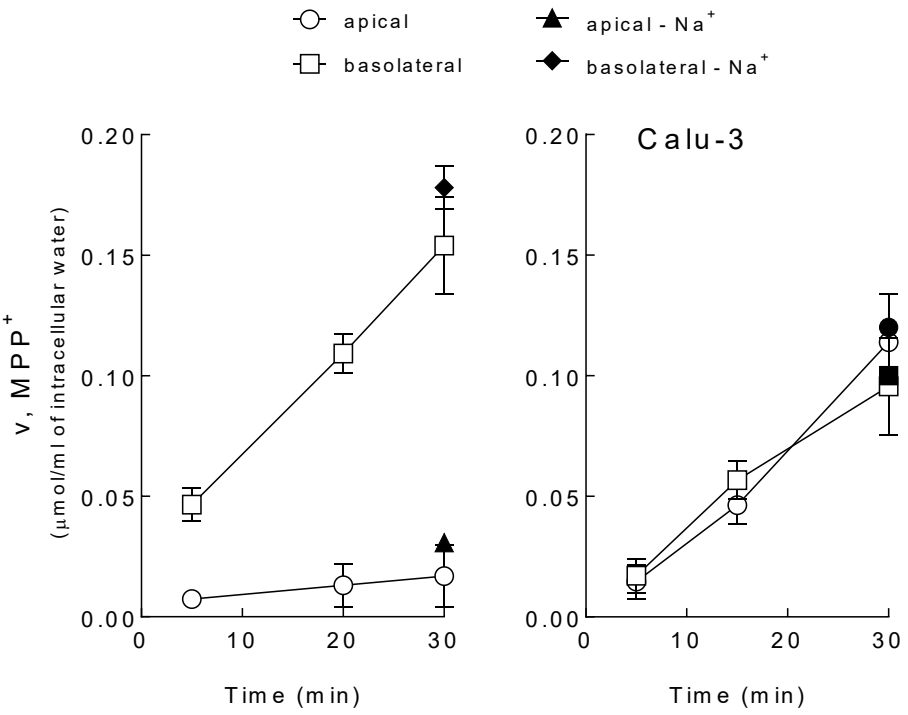

Figure 7. Time-dependent accumulation of MPP^+ in EpiAirway™ and Calu-3 cells. *Cells were washed with EBSS or Na^+-free EBSS (-Na^+) and incubated for the times indicated in the same buffer containing [^{3}H]MPP^+ (50 µM; 2 µCi/ml), either added to the apical or to the basolateral compartment, as indicated. The intracellular [MPP^+] was determined as described in section Material and Methods. Points represent the mean ± SEM of three independent determinations.*

3.1.2 Expression of OCTs in EpiAirway™ and Calu-3 cells

In order to identify the transporters responsible for MPP^+ transport in respiratory cells, we next evaluate the absolute quantitative expression of mRNA for OCTs in EpiAirway™ and Calu-3 cells. As far as EpiAirway™ are concerned, the number of molecules of SLC22A1/OCT1 and SLC22A3/OCT3 mRNA are quantitatively comparable. Calu-3, employed for comparison, expressed SLC22A1/OCT1 to the same extent as EpiAirway™ while SLC22A3/OCT3 was four time higher than in EpiAirway™. SLC22A2/OCT2 mRNA was undetectable. The presence of these transporters in both cells models is confirmed by protein bands corresponding to OCT1 and OCT3, obtained by Western blot analysis.

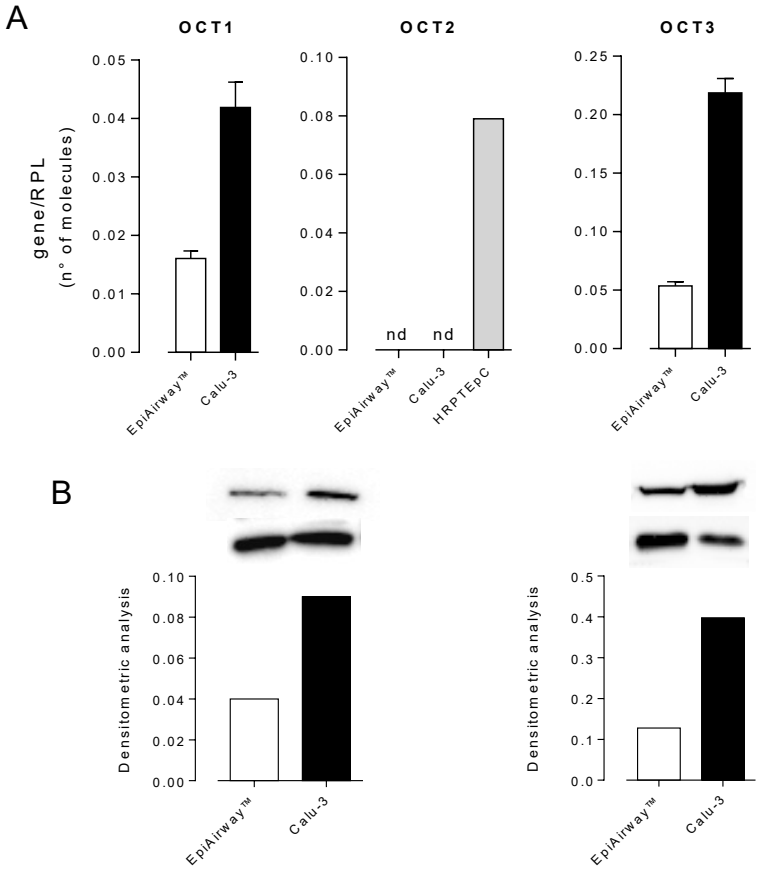

Figure 8. Expression of OCTs transporters in EpiAirway™ and Calu-3. *Panel A The absolute quantification of the mRNAs for SLC22A1/OCT1, SLC22A2/OCT2 and SLC22A3/OCT3 was performed as described in Material and Methods by means of qRT-PCR; the expression of the gene of interest is expressed as number of molecules, upon normalization for the reference gene (RPL15). Data are means ± SEM of four determinations. Panel B. The expression of OCT1 and OCT3 proteins was determined by Western Blot analysis, as described in Material and Methods. Lower panels present the results of the densitometric analysis of protein expression, normalized to that of α-tubulin. A representative Western blot is shown; the experiment was repeated two times with similar results.*

3.1.3 Characterization of MPP^+ uptake in EpiAirway™ and Calu-3 cells

The contribution of OCT1 and OCT3 to MPP^+ transport in EpiAirway™ was next addressed by measuring the uptake of the substrate in the presence of quinidine or corticosterone, which preferentially inhibit OCT1 (Nies A.T. 2011, Bi Y.A 2019) and OCT3 (Koepsell, H. 2007, Gasser, P.J. 2006), respectively. Calu-3 cells were used as a comparison. Results, presented in Figure 9 (Figure 9, panel A), demonstrate that both drugs significantly inhibited the uptake at basolateral side, thus pointing to the expression and activity of OCT1 and OCT3 on this side of cell cultures. In contrast, not significant effect of the inhibitors was observed at the apical side. These results confirm the absence of any relevant activity of the two transporters on the apical membrane. Instead, in Calu-3 cells the uptake of MPP^+ was significantly inhibited both at apical and basolateral side by corticosterone, while quinidine inhibited only the apical side. These results suggest that OCT3 is functional at both the basolateral and apical membranes in polarized Calu-3 cells, while a significant OCT1 activity is observed only at the apical side (Figure 9, panel B).

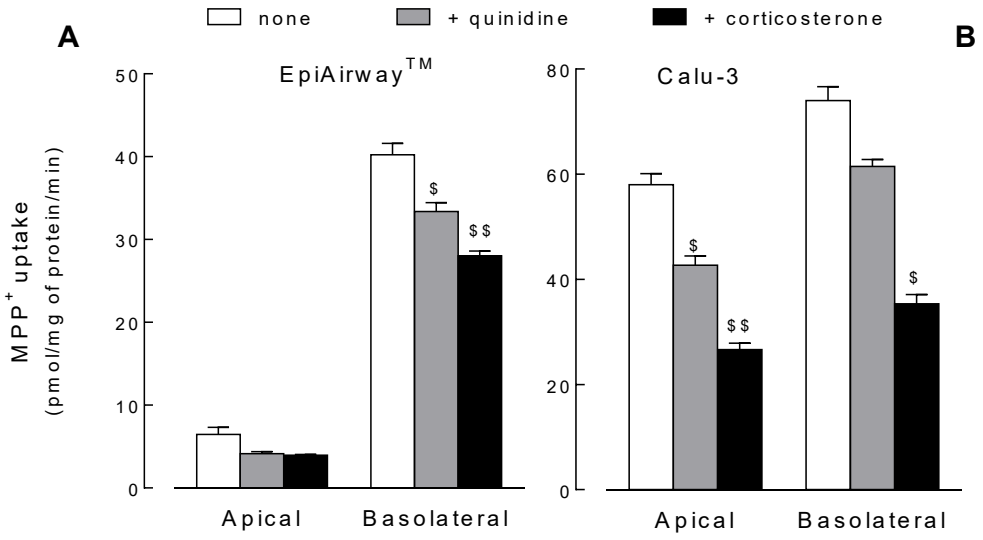

Figure 9. Characterization of MPP^+ uptake in EpiAirway™ and Calu-3 cells*. Monolayers of EpiAirway™ (panel A) and Calu-3 cells (panel B) were washed with EBSS and incubated for 5 min in the transport buffer containing [^{3}H]MPP^+ (50 μM; 2 μCi/ml), either added to the apical or to the basolateral compartment. Where indicated, quinidine (500 μM) or corticosterone (500 μM) were present during the transport assay. The intracellular [MPP^+] was determined as described in section Material and Methods. Bars represent the mean ± SEM of three independent determinations.* $^{\$}p< 0.05$; $^{\$\$}p< 0.01$ *vs none.*

3.1.4 Effect of bronchodilators on MPP^+ uptake in EpiAirway™ and Calu-3 cells

In light of the recognized role of OCT transporters in drug delivery (Braunlich, J. 2018, Hamelmann, E. 2018, Panduga, V. 2017, Wang, F. 2019), we investigated the effect of three bronchodilators (i.e. ipratropium, tiotropium and glycopyrrolate) on the absorption of MPP^+ and Calu-3 cells. Since EpiAirway™ have no transport activity at the apical membrane, uptake has been measured only at the basolateral side. As shown in Figure 10, ipratropium significantly reduced MPP^+ uptake in both monolayers, while the other two drugs were ineffective. This finding clearly denotes an involvement of OCT1 and OCT3 transporters in the transport of ipratropium, but not of glycopyrrolate nor tiotropium.

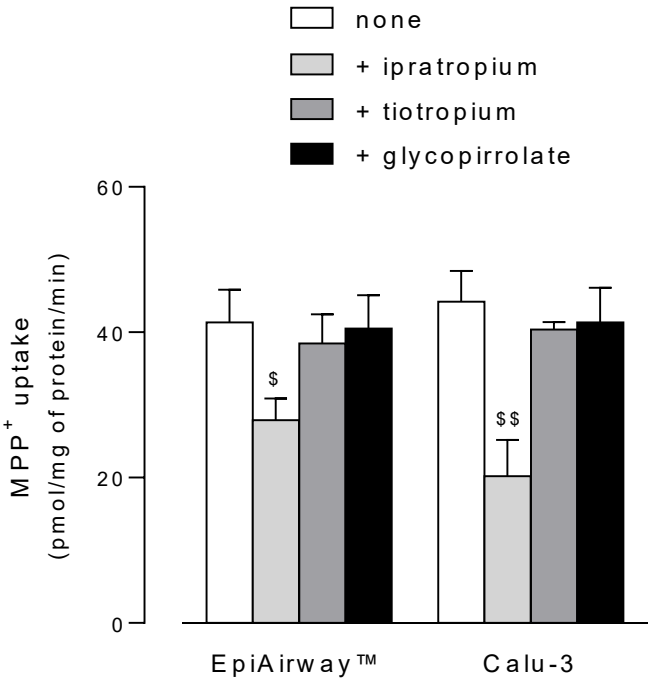

Figure 10. Effect of Bronchodilators on MPP^+ uptake in EpiAirway™ and Calu-3 cells. *Monolayers of EpiAirway™ and Calu-3 cells were washed with EBSS and incubated for 5 min, at the basolateral side, in EBSS containing [^{3}H]MPP^+ (50 μM; 2 μCi/ml) in the absence (none) or in the presence of 500 μM ipratropium, tiotropium, or glycopirrolate, as indicated. Bars represent the mean ± SEM of three independent determinations. $^{\$}p< 0.05$; $^{\$\$}p< 0.01$ vs none.*

3.1.5 Effect of inflammatory stimuli on the expression of SLC22A1 and SLC22A3 in EpiAirway™ and Calu-3 cells

Subsequently, we wondered if inflammatory stimuli could modify the expression of OCT transporters, making them potential targets for anti-inflammatory drugs. For this purpose, we studied the expression of SLC22A1/OCT1 and SLC22A/OCT3 in EpiAirway™ and Calu-3 cells incubated in the presence of inflammatory stimuli such as microbe-specific stimulus lipopolysaccharides (LPS) or tumor necrosis factor α (TNFα). The results obtained indicate that the proinflammatory conditions did not modify the gene expression of the two transporters in both cell models.

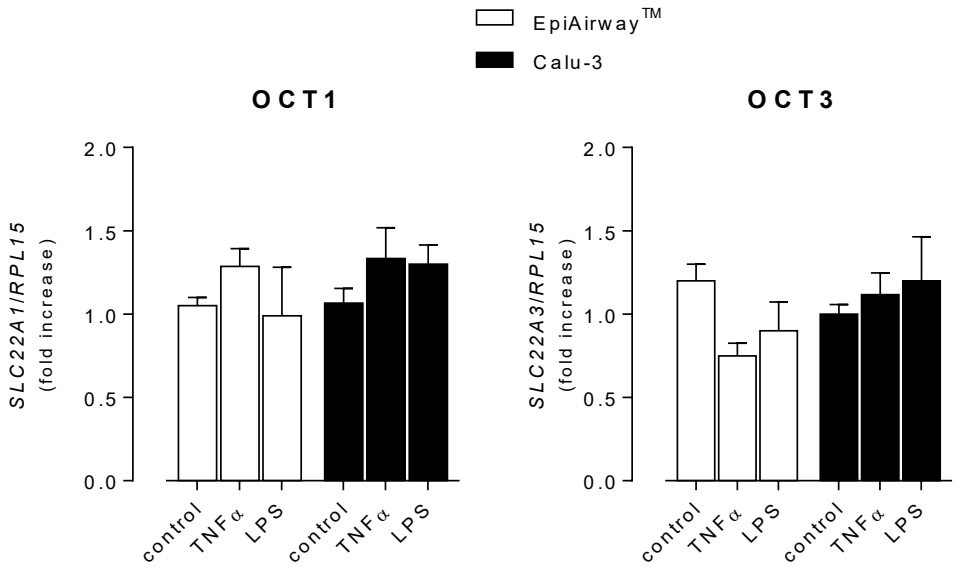

Figure 11. Effect of inflammatory stimuli on the expression of SLC22A1 and SLC22A3 in EpiAirway™ and Calu-3. *LPS (10 µg/ml) and TNFα (10 ng/ml) were added to the culture medium for 24 h. The expression of SLC22A1/OCT1 and SLC22A3/OCT3 was determined with qRT-PCR and shown after normalization for the expression of the reference gene (RPL15). Data are means ± SEM of three independent determinations.*

Chapter II – Carnitine and OCTNs in human airway epithelial cells

3.2.1 Characterization of carnitine uptake in EpiAirway™ e Calu-3 cells

In order to address the specific transporters involved in L-carnitine uptake in EpiAirway™ and Calu-3 cells, 30-min uptake of 1 μM carnitine was performed at both apical and basolateral side in the presence of specific transporters inhibitors. Previous studies performed on Calu-3 cells cultured on plasticware (Ingoglia, F. 2016) demonstrated that carnitine was transported by OCTN2 and $ATB^{0,+}$, a transporter responsible for the Na^+/Cl^- dependent influx of neutral and cationic amino acids.

In EpiAirway™, carnitine influx was totally sodium-dependent at both sides, being the transport almost completely suppressed in the absence of sodium. On the apical plasma membrane, L-carnitine uptake was almost completely inhibited by arginine, which compete with carnitine for $ATB^{0,+}$ transporter (Hatanaka, T. 2004), while betaine, substrate of OCTN2 (Bundey, R.A. 2003), was ineffective. Otherwise, at basolateral side, betaine markedly inhibited carnitine transport while arginine was completely ineffective. Overall, these findings indicate that L-carnitine transport in EpiAirway™ cell system is mediated by $ATB^{0,+}$ transporter on the apical and by OCTN2 in the basolateral side. As far as Calu-3 cells cultured at ALI condition for 21 days, carnitine uptake at the apical side was very low, not significantly inhibited by the presence of either arginine or betaine, and not affected by the absence of sodium. At the basolateral side, on the contrary, transport data were comparable with EpiAirway™ cells, being carnitine influx totally sodium-dependent and completely inhibited by betaine and not by arginine. In order to better address the differences observed between the two models, the same analysis was repeated in Calu-3 cells cultured for shorter time at ALI condition (8d): L-carnitine transport measured at the apical side was higher than in Calu-3 cultured for 21d and comparable to that of EpiAirway™, with the same inhibition pattern, i.e. none inhibition by betaine and a strong inhibition by arginine; similarly, also data obtained at the basolateral side overlapped with those of primary normal cells.

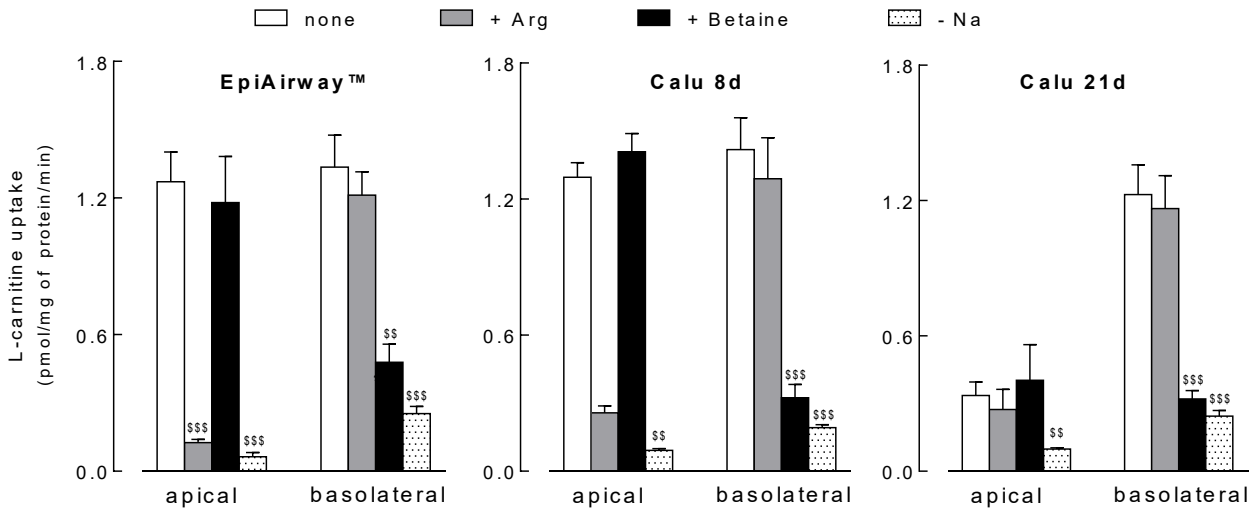

Figure 12. Characterization of carnitine uptake in EpiAirway™ and Calu-3 cells*. Monolayers of EpiAirway™ cells grown under air-liquid interface (ALI) conditions were washed with EBSS and incubated for 30 min, either at the apical or the basolateral compartment, in EBSS containing [^{3}H]carnitine (1 μM; 2 μCi/ml) in the absence (none) or in the presence of 2 mM arginine or 2 mM betaine, as indicated. For sodium-independent uptake (-Na), a Na+-free EBSS was employed (see Methods). Monolayers of Calu-3 cells grown under air-liquid interface (ALI) conditions for 21d (A) or 8d (B) were washed with EBSS and L-carnitine uptake was measured as described in Fig 1. Bars represent the mean ± SEM of four independent experiments. $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs none.*

3.2.2 Absolute quantification of SLC22A5/OCTN2 and SLC6A14/ATB$^{0,+}$ in EpiAirway™ and Calu-3 cells

Subsequently, we quantified the amount of mRNA for ATB$^{0,+}$ and OCTN2 carriers: the mRNA coding for SLC22A5/OCTN2 was similarly expressed in EpiAirway™ and in Calu-3, cultured for both 8 and 21 d; SLC6A14/ATB$^{0,+}$, on the contrary, was maximally detectable in EpiAirway™, expressed to a minor level in Calu-3 at 8d and much less abundant after 21 days of culture.

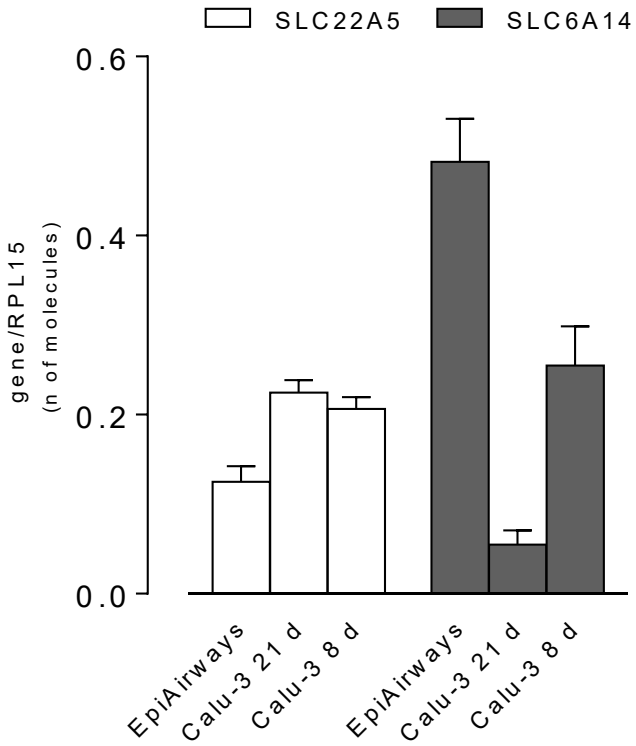

Figure 13. Absolute quantification of SLC22A5 and SLC6A14 in EpiAirway™ and Calu-3 cells*. The absolute quantification of the mRNAs for SLC22A5/OCTN2 and SLC6A14/ATB$^{0,+}$ was determined in EpiAirway™ and in Calu-3 cultured under ALI conditions for 8d or 21d, as indicated, by means of RTqPCR analysis. The expression of the gene of interest was shown after normalization for that of the reference gene (RPL15). Data are means ± SEM of five independent experiments. $^{\$}p<0.05$, $^{\$\$}p<0.01$.*

3.2.3 Immunolocalization of ATB^{0+} and OCTN2 transporters in EpiAirway™ and Calu

The expression of ATB$^{0,+}$ and OCTN2 was assessed at protein level by means of immunocytochemistry. This approach defined a clear-cut distribution of ATB$^{0,+}$ transporter on plasma membranes at the apical side of both EpiAirway™ (Figure 14, panel A) and Calu-3 monolayers cultured for 8d (Figure 14, panel B), with a more homogeneous staining in Calu-3 cells than in EpiAirway™; on the contrary, the protein was only barely detectable in Calu-3 after 21d of culture.

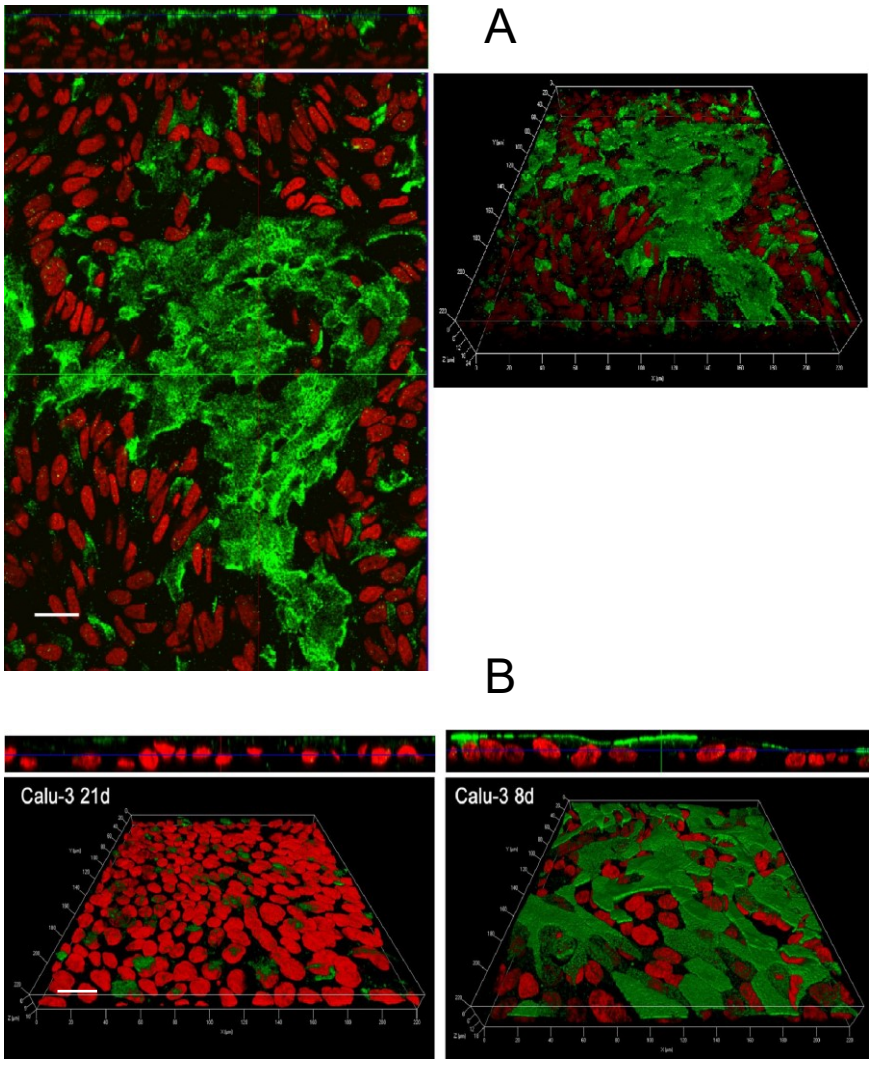

Figure 14. Immunolocalization of ATB$^{0,+}$ transporter in EpiAirway™ and Calu-3 cells*. Confocal laser scanning microscopy of EpiAirway™ and Calu-3 layers immunolabelled for ATB$^{0,+}$ transporter (green) is shown. Nuclei were stained with propidium iodide (red). Left: single XY scan acquired in correspondence of the apical membrane. Top: XZ section of the plane. Right: 3D reconstruction of zstacks confocal images; about 30 horizontal sections were acquired. Representative images from three independent experiments are shown. (Bar, 20 µm for EpiAirway and 10 µm for Calu-3). Bottom: 3D reconstruction of z-stacks confocal images; about 25 horizontal sections were acquired.*

As for OCTN2, the same analysis in Calu-3 cells both 8d and 21 revealed a continuous staining around cell borders on the lateral plasma membrane, confirming the basolateral localization of this transporter. In EpiAirway™, however, despite the positive staining the signal was not located precisely.

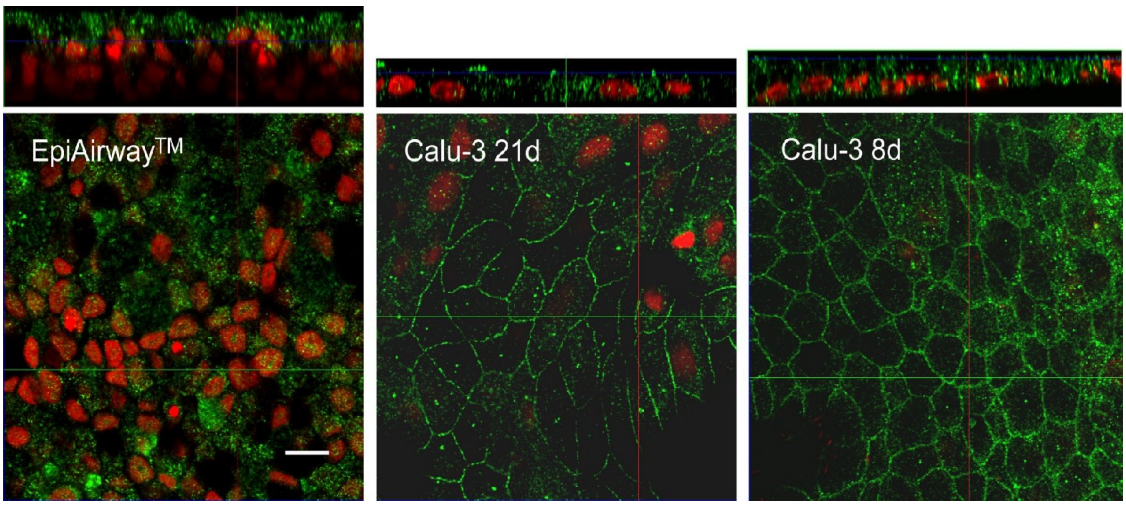

Figure 15. Immunolocalization of OCTN2 transporter in EpiAirway™ and Calu-3 cells*. Confocal laser scanning microscopy of monolayers immunolabelled for OCTN2 transporter (green) is shown. Nuclei were stained with propidium iodide (red). Single XY scans of EpiAirway™ and Calu-3 cultured under ALI conditions for 8d or 21d, as indicated, are shown; XZ sections of the planes are shown on the top. Representative images from three independent experiments are shown. Bar, 10 µm.*

Effect of bronchodilators on L-carnitine uptake in EpiAirway™ and Calu-3 cells

Furthermore we examined the role of L-carnitine transporters in drug delivery in the airways. The effect of three bronchodilators (ipratropium, tiotropium, and glycopyrrolate) was explored both in EpiAirway™ and in Calu-3 cells at 8d. Glycopyrrolate markedly reduced apical L-carnitine uptake in both EpiAirway™ (Figure 16, panel A) and Calu-3 (Figure 16, panel B); also tiotropium was significantly effective at the apical side of both models, although to a lower level, while ipratropium had no effect. Ipratropium, however, was the sole able to inhibit L-carnitine uptake at the basolateral side, where the other two drugs were completely ineffective**.** The results indicate that tiotropium and glycopyrrolate interact with $ATB^{0,+}$ at the apical side of membrane, while ipratropium is substrate of OCTN2.

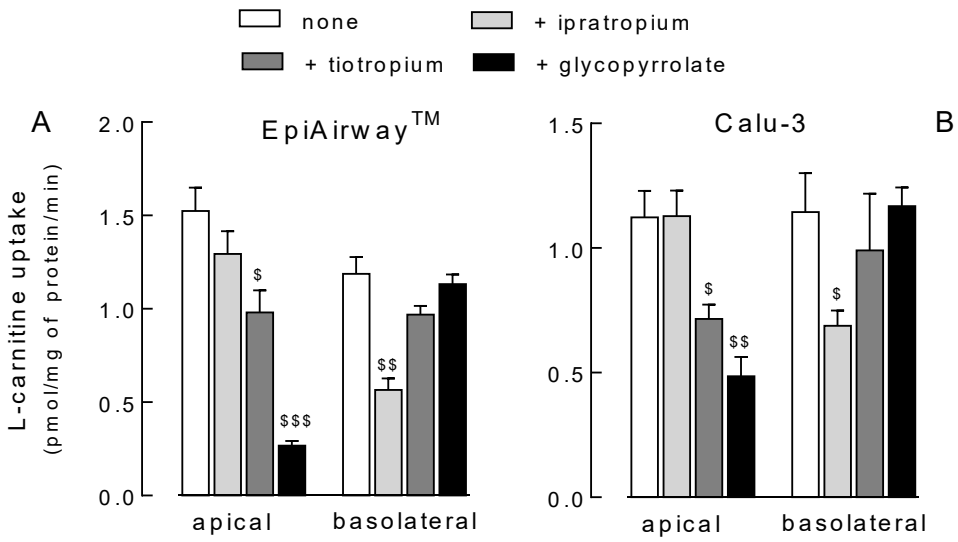

Figure 16. ***Effect of bronchodilators on L-carnitine uptake in EpiAirway™ and Calu-3.*** *Monolayers of EpiAirway™ (A) and Calu-3 cells grown under air-liquid interface (ALI) conditions for 8d (B) were washed with EBSS and incubated for 30 min, either at the apical or the basolateral compartment, in EBSS containing [^{3}H]carnitine (1 μM; 2 μCi/ml) in the absence (none) or in the presence of 200 μM ipratropium, tiotropium, or glycopyrrolate, as indicated. Bars represent the mean ± SEM of three (EpiAirway™) or four (Calu-3 cells) independent determinations. $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs none.*

3.2.4 Effect of inflammatory stimuli on the expression of OCTN2 and $ATB^{0,+}$ in EpiAirway™ and Calu-3 cells

Finally, we investigated the impact of inflammatory stimuli on the expression and function of carnitine transporters. To this end, the expression of $ATB^{0,+}$ and OCTN2 in both cells model has been evaluated under inflammatory conditions, i.e. in the presence of microbe-specific stimulus lipopolysaccharides (LPS) or the cytokine Tumor necrosis factor α (TNFα); the effect of the anti-inflammatory Interleukin 4 (IL-4) was also tested. Results showed that the expression of SLC22A5/OCTN2 was unaffected by any of the experimental conditions adopted in either cell model (Figure 17, panel A). Conversely, the exposure of cells to either LPS or TNFα markedly enhanced the expression of SLC6A14/$ATB^{0,+}$ in Calu-3 cells cultured for both 21d and 8d at ALI; a slight, although not significant increase was also observed in EpiAirway™ in the presence of LPS. In both cell models, IL-4 was ineffective, suggesting that the transporter is a target only of pro-inflammatory stimuli. Consistently, L-carnitine uptake (Figure 17, panel B) was significantly upregulated by LPS at the apical, but not at the basolateral side of Calu-3 cells at 8d, when $ATB^{0,+}$ reached the maximal expression.

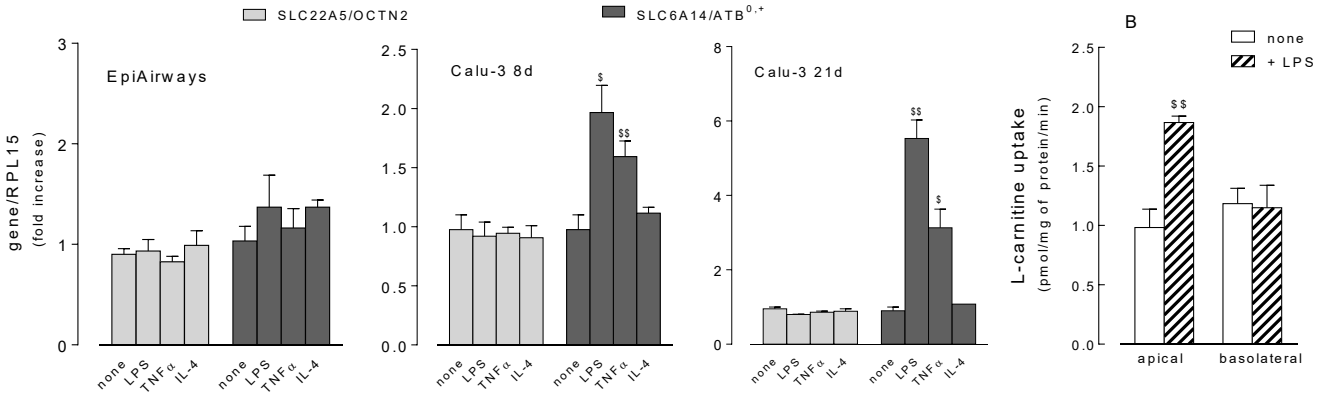

Figure 17. ***Effect of inflammatory stimuli on the expression of SLC22A5/OCTN2 and SLC6A14/ATB$^{0,+}$ in EpiAirway™ and Calu-3 cells cultured under ALI conditions for 8d or 21d.*** *LPS (10 µg/ml), TNFα (10 ng/ml) or IL-4 (10 ng/ml) were added to the culture medium for 24 h. The expression of SLC22A5/OCTN2 (A) and SLC6A14/ATB$^{0,+}$ (B) was determined by means of RT-qPCR analysis. Data are means ± SEM of four independent experiments. L-Carnitine uptake was measured in Calu-3 at 8d (C), as described in Fig 1; data are means ± SEM of three independent experiments. $^{\$}p<0.05$, $^{\$\$}p<0.01$ vs none.*

Chapter III – Characterization of OCTs/OCTNs in EpiAlveolar™

3.3.1 Activity and expression of L-Carnitine transporters in EpiAlveolar™

In order to evaluate OCTN activity in EpiAlveolar™, L-carnitine uptake was measured, at both the apical and the basolateral side of the monolayers, either in the absence or in the presence of sodium, and by employing specific inhibitors (Figure 18, panel A). On the apical membrane, the uptake L-carnitine was sodium-dependent and completely inhibited by α-methyltriptophane (α-MT), a specific substrate of $ATB^{0,+}$ transporter (Karunakaran, S. 2008); on the contrary, betaine, substrate of OCTN2 (Koepsell, H. 2007; Ingoglia, F. 2016), was completely ineffective. Betaine markedly inhibited L-carnitine transport at the basolateral side, where α-MT was, instead, completely ineffective. In light of these findings, we can conclude that L-carnitine transport in EpiAlveolar™ is mediated by the activity of $ATB^{0,+}$ and OCTN2 transporters, with the first operative at the apical and the latter at the basolateral side. The expression of these transporters, both at gene and protein levels, is shown in Panel B and C of Figure 18. EpiAirway™ and A549 cells were employed as comparison. *SLC6A14*/$ATB^{0,+}$ was expressed in EpiAlveolar™ at high level, even more pronounced than in EpiAirway™. Consistently a clearcut expression of the $ATB^{0,+}$ protein was observed in EpiAlveolar™. SLC22A5/OCTN2 was, instead, equally expressed both at mRNA and protein in EpiAlveolar™, EpiAirway™ and A549.

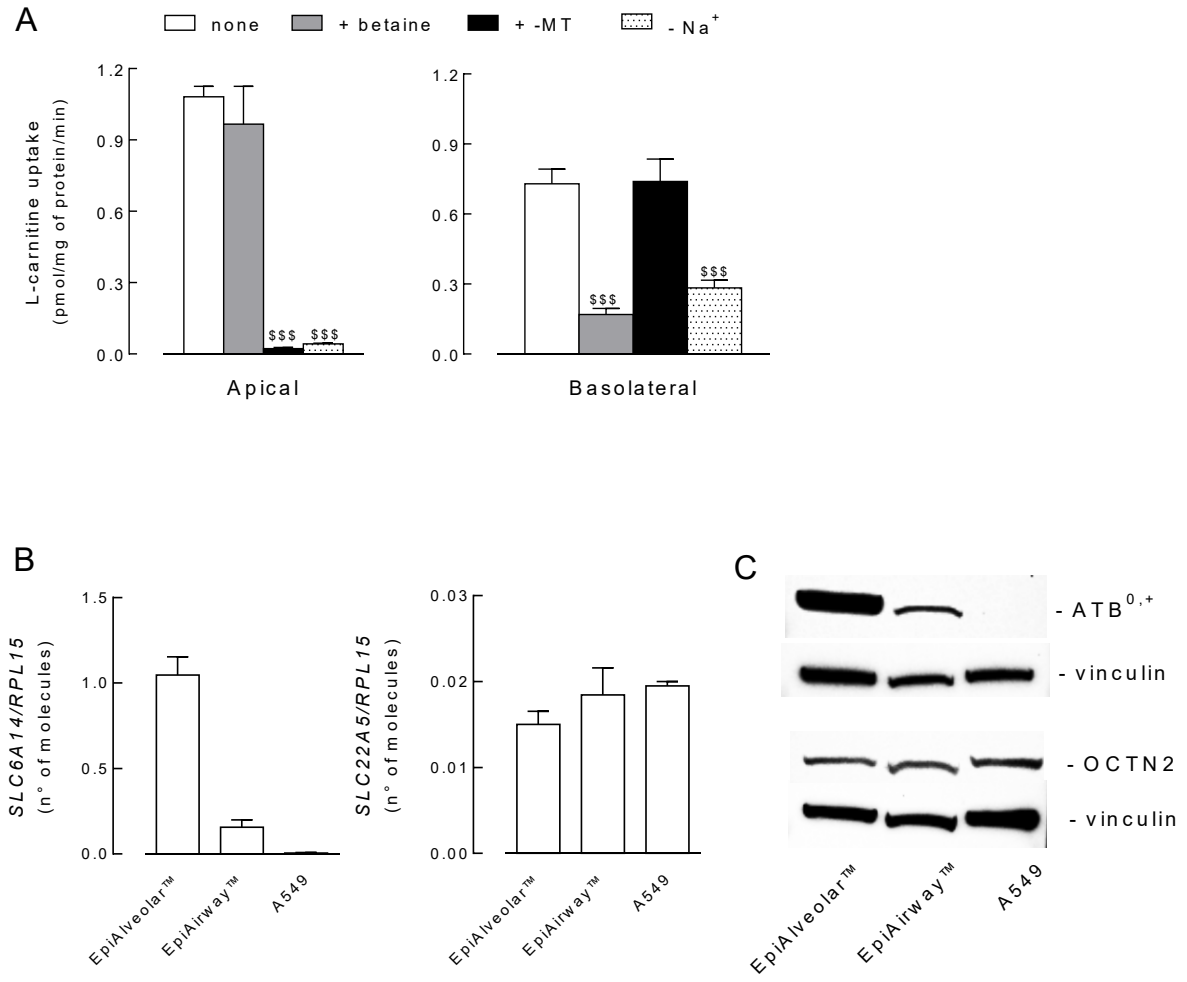

Figure 18 ***Carnitine uptake and expression of OCTN2 and ATB$^{0,+}$ transporters in EpiAlveolar™****. Panel A. Monolayers of EpiAlveolar™ cells grown under air-liquid interface (ALI) conditions were washed with EBSS and incubated for 30 min, either at the apical or the basolateral compartment, in EBSS containing [3H]carnitine (1 μM; 2 μCi/ml) in the absence (none) or in the presence of 2 mM α-methyl tryptophane (α-MT) or 2 mM betaine, as indicated. For sodium-independent uptake (-Na$^+$), a Na$^+$-free EBSS was employed (see Methods). Bars represent the mean ± SEM of four independent experiments. $^{\$\$\$}p < 0.001$, $^{\$\$}p < 0.01$ vs none. Panel B. mRNA levels were quantified by means of RT-qPCR analysis. The expression of the gene of interest was shown after normalization for that of the reference gene (RPL15) and presented as number of molecules. Data are means ± SEM of five independent experiments. Panel C. The expression of OCTN2 and ATB$^{0,+}$ proteins was determined by Western Blot analysis, as described in Material and Methods. A Representative Western blot is shown, the experiment was repeated two times with comparable results.*

3.3.2 Characterization of MPP^+ uptake and OCTs expression in EpiAlveolar™

The functional activity of OCTs in EpiAlveolar™ was evaluated by measuring MPP^+ uptake both at apical and basolateral sides (Figure 19, Panel A). The presence of atropine or corticosterone was employed to evaluate the contribution of OCT1, which is preferentially inhibited by atropine (Koepsell, H. 2007), and corticosterone as preferential inhibitor of OCT3 (Gasser, P.J. 2006; Koepsell, H. 2007) . MPP^+ uptake was greater at the basolateral than the apical side, where only corticosterone caused a modest but significant inhibition. On the basolateral side, instead, both corticosterone and atropine were effective in inhibiting MPP^+ influx, with higher inhibition by corticosterone (55%) in respect to atropine (35%), thus pointing to OCT1 and OCT3 operative on this side of cell cultures. The expression of *SLC22A1*/OCT1 and *SLC22A3*/OCT3 (Figure 19, Panel B and C) was detectable both at mRNA and protein level and comparable with those of EpiAirway™; *SLC22A2*/OCT2 was undetectable.

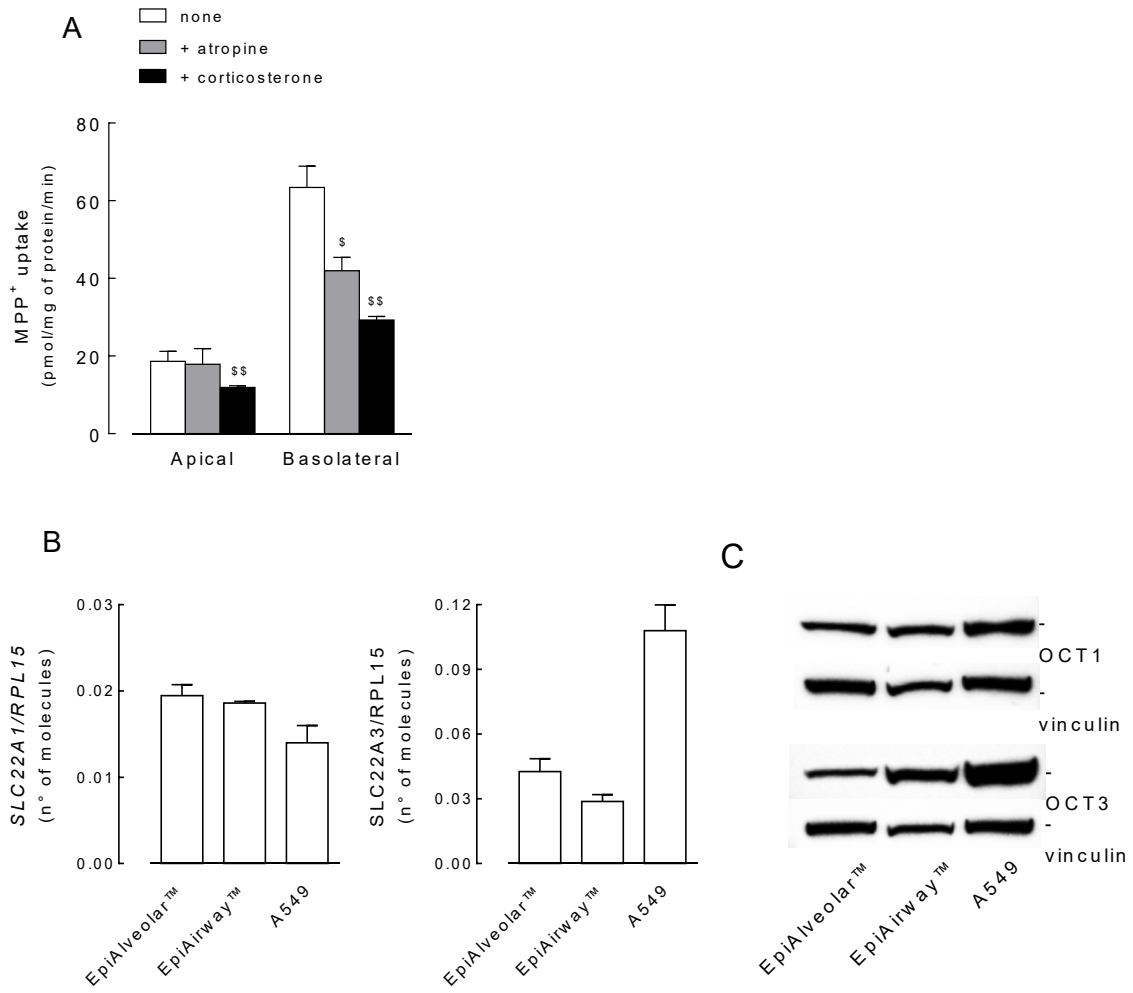

Figure 19: ***Characterization of MPP+ uptake and OCTs expression in EpiAlveolar™.*** *Panel A. Monolayers of EpiAlveolar™ were washed with EBSS and incubated for 5 min in the same buffer containing [^{3}H]MPP$^+$ (10 µM; 2 µCi/ml), either added to the apical or to the basolateral compartment. Where indicated, atropine (0.5 mM) or corticosterone (0.5 mM) were present during the transport assay. Bars represent the mean ± SEM of three independent determinations. $^{\$}p < 0.05$; $^{\$\$}p< 0.01$ vs none. Panel B. mRNA levels for SLC22A1/OCT1 and SLC22A3/OCT3 were determined by means of qRT-PCR and normalized for the expression of the reference gene (RPL15) (see Material and Methods). Data are means ± SEM of four determinations. Panel C. The expression of OCT1 and OCT3 proteins was determined by Western Blot analysis, as described in Material and Methods. A representative Western blot is shown; the experiment was repeated two times with similar results*

3.3.3 Role of OCTs/OCTNs in the uptake of ipratropium, tiotropium and glycopyrrolate in EpiAlveolar™

Lastly, in order to examine the role of OCTs and OCTNs transporters in drug delivery in alveolar epithelial cells, the effect of three bronchodilators (ipratropium, tiotropium, and glycopyrrolate) was investigated in EpiAlveolar™ both on carnitine and MPP^+ uptake. As shown in Figure 20, glycopyrrolate markedly reduced apical L-carnitine uptake; also, tiotropium was effective at the apical side, although to a lesser extent, while ipratropium had no effect. This latter, however, was the sole able to inhibit both L-carnitine and MPP^+ uptake at the basolateral side, where the other two drugs were, instead, completely ineffective. These results indicate that tiotropium and glycopyrrolate interact with $ATB^{0,+}$ at the apical side of alveolar epithelium, while ipratropium is substrate of both OCTs and OCTN2 transporters.

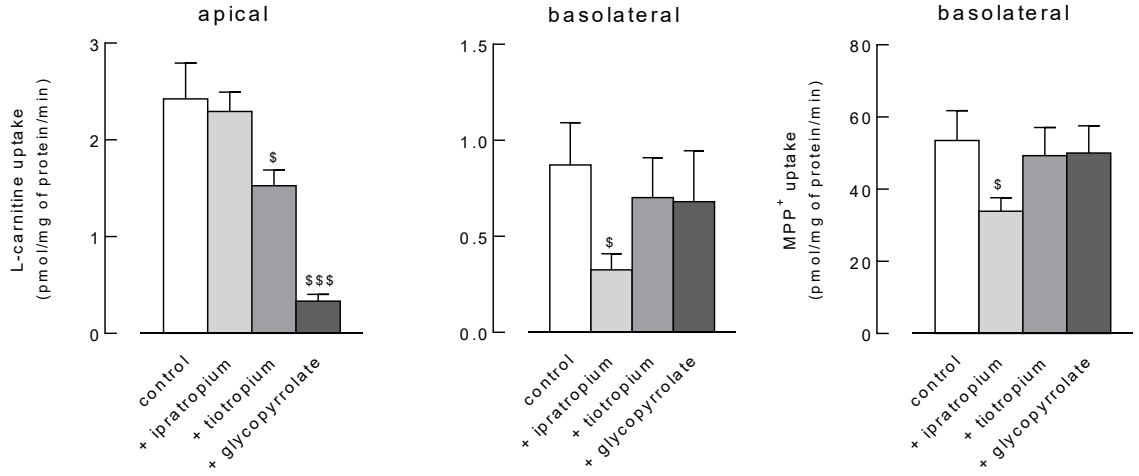

Figure 20 ***Effect of ipratropium, tiotropium and glycopyrrolate on L-carnitine and MPP+ uptake in EpiAlveolar™.*** *Monolayers of EpiAlveolar™ were washed with EBSS and incubated for 30 min, either at the apical or the basolateral compartment, in EBSS containing [3H] carnitine (1 μM; 2 μCi/ml) or only at the basolateral side for 5 min in EBSS containing [^{3}H]MPP^+ (10 μM; 2 μCi/ml), in the absence (none) or in the presence of 200 μM (for carnitine uptake) or 500 (for MPP^+ uptake) ipratropium, tiotropium, or glycopyrrolate, as indicated. Bars represent the mean ± SEM of three independent determinations. $^{\$}p < 0.05$, $^{\$\$\$}p<0.001$ vs none*

Discussion

The first and the second part of this project concerns the study of the expression and activity of OCTs/OCTNs transporters in EpiAirway™, an in vitro model of normal human airway epithelium. Most studies related to the expression of membrane transporters in human respiratory epithelium have been conducted in immortalized cell lines, such as BEAS-2B and Calu-3 cells; these cells, in particular, are considered a model in pulmonary drug uptake studies (Mathia, N.R. 2002). However, it is suspected that these models have different biological functions in respect to primary respiratory cells obtained ex vivo from tissues (Akhtar, J. 2010). Recently, three-dimensional cultures of primary airway cells, such as EpiAirway™ and EpiAlveolar™, have been developed that represent a promising and more realistic in vitro model for the human lung.

The SLC22 family contains 13 functionally characterized human plasma membrane proteins each with 12 predicted α-helical transmembrane domains. The family includes organic cation transporters (OCTs), organic anion transporters (OATs) and organic zwitterion/cation transporters (OCTNs) (Koepsell, H. 2013). Most transporters of the SLC22 family are polyspecific and mediate multiple structurally different substrates. The first subgroup comprises the OCT1, OCT2 and OCT3 transporter, encoded by SLC22A1-3, which transport organic cations and some neutral compounds. Results obtained demonstrate that EpiAirway™ exhibits OCTs transport activity at the basolateral side. In fact, OCT1 and OCT3 transporters exert an uptake of MPP^+, leading to a three-fold intracellular concentration of the substrate in 30 min at the basolateral membrane. These data are consistent with immunofluorescence studies performed in human bronchi showing OCT3 on the plasma membrane of basal cells and on the basolateral membrane of intermediate cells (Lips, K.S. 2005). In the same model, however, OCT1 transporter was present in the luminal membrane of ciliated epithelial cells. Basolateral localization of OCTs has also been observed in hepatocytes and proximal renal tubule (Koepsell, H. 2020), where the transcellular movement of organic cations has been shown to depend upon the combined action of electrogenic OCT-mediated uptake at the basolateral side and the apical efflux supported by the pump ABCB1/P-gp and MATEs (Koepsell, H. 2007). In our studies, however, the activity of the P-gp transporter was excluded in EpiAirways™ (Rotoli, B.M. 2020), while only a slight expression of MATE1 was detectable.

In addition, OCTN2 transporter, a polyspecific carrier primarily responsible for the uptake of L-carnitine, is present on the lateral plasma membrane in both EpiAirway™ and Calu-3 cells, as confirmed by immunocytochemical images and transport results. On the other

hand, a recent study had localized OCTN2 at apical side in epithelial bronchial cells of lung tissues of healthy and COPD patients and in Calu-3 cells (Berg, T. 2018). Our results exclude an also apical expression of OCTN2 on the basis of immunocytochemistry and the functional analysis. At the apical side of the EpiAirway™ cells we demonstrated a high activity of SLC6A14/$ATB^{0,+}$, a system responsible for the Na^+/Cl^- dependent influx of neutral and cationic amino acids. This transporter has a low affinity for carnitine, but a high concentrative capacity (Akhtar, J. 2010). Through absolute quantification, we showed that SLC6A14/$ATB^{0,+}$ is the most abundant transporter in EpiAirway™, being 10 times more expressed than SLC22A1/OCT1, SLC22A3/OCT3 and SLC22A5/OCTN2. The role of this transporter in the lung should be related to the effective clearance of proteins through the active reabsorption of amino acids, mainly in pathological conditions (Bosquillon, C. 2010). As far as Calu-3, this transporter is expressed and functional only in cells maintained at ALI for 8 days. Interestingly, SLC6A14 expression and carnitine uptake decrease in fully differentiated 21d monolayers, confirming the finding that the biological characteristics of Calu-3 cells can be modified by culture conditions (for example air or liquid interface and time in culture) (Kreft, M.E. 2015); therefore, we suggest to include the expression $ATB^{0,+}$ in the list of parameters that vary with the duration of the culture. In conclusion, our results indicate that in EpiAirway™ OCT1, OCT3 and OCTN2 are present and active on the basolateral membrane and not at the apical side, where the main carrier involved in the transport of organic cationic solutes is $ATB^{0,+}$.

The role of OCTs and OCTNs is well known in the absorption of cationic drugs in the intestine, and it is also recognized in the renal excretion of drugs (Koepsell, H. 2004; Pochini, 2013). These transporters regulate highly concentrative uptake of a broad category of substrates, including classic anticancer molecules, such as platinum derivatives, and newly developed targeted therapy drugs, such as imatinib and metformin (Gosens, R. 2018). In this context, the uptake is facilitated by the negative internal plasma membrane potential, which allows reaching intracellular concentrations higher than those external to the cells (Chien, H.C. 2016). A role of OCTs/OCTNs in drug delivery was also investigated in the airways, demonstrating that the OCT-mediated uptake of 4-(4-(diethylamino) styryl) -N-methylpyridinium iodide (ASP^+) at the apical level of the Calu-3 is inhibited by the bronchodilators formoterol, salbutamol, ipratropium and by the glucocorticoid budesonide (Panduga, V. 2017). OCTN2, in particular, is described to interact with inhaled drugs, such as muscarinic antagonists and β-adrenergic agonists cationic bronchodilators (Bosquillon, C. 2010); moreover, this transporter offers an efficient

mean to deliver drugs or drug-loaded nanoparticles conjugated to carnitine (Kou, L. 2018). Results about this issue are, however, controversial. A previous study performed in OCTN2-transfected HEK293 cells and in OCTN2-silenced BEAS-2B reports that tiotropium and ipratropium, anti-cholinergic drugs approved for treatment of asthma and COPD (Gosens, R. 2018), are taken up primarily by OCTN2 (Nakamura, T. 2010). On the contrary, a recent study in Calu-3 layers demonstrates that L-carnitine does not change ipratropium transepithelial transport, excluding an involvement of OCTN2 in the drug trafficking across the monolayer (Panduga, V. 2017).

Consistently, in this project, we demonstrate that the bronchodilator ipratropium interacts with OCTN2, OCT1 and OCT3 in both Calu-3 cells and EpiAirway™ at the basolateral side, while tiotropium and glycopyrrolate (other anti-cholinergic bronchodilator) inhibit $ATB^{0,+}$ activity at the apical side.

The capacity of $ATB^{0,+}$ to interact with drugs is very important if we consider the ability of inflammatory stimuli to modulate the expression of this transporter. Really, it has been recently suggested that $ATB^{0,+}$ typically localizes in places where the body interfaces with microbes, such as lung and colon, where it is involved in the reduction of available nutrients to bacteria; the transporter is constantly up-regulated in inflammatory states, such as ulcerative colitis, Crohn's disease and colon cancer (Broer, S. 2018). Furthermore, other studies have recently identified SLC6A14/$ATB^{0,+}$ as a genetic marker of lung disease severity in cystic fibrosis, providing a mechanism by which it regulates Pseudomonas aeruginosa attachment to human bronchial epithelial cells (Di Paola, M. 2017). In that study, Di Paola et al. demonstrated an enhancement of SLC6A14 expression by LPS in Calu-3 cells. In line with those observations, we here showed a marked induction of SLC6A14/$ATB^{0,+}$ upon exposure of polarized Calu-3 cells to inflammatory stimuli, i.e TNFα and LPS. In a recent paper we also demonstrate that flagellin induces an increase of SLC6A14 expression and activity (Barilli, A. 2021). On the contrary the anti-inflammatory IL-4 had no effect on SLC6A14 expression, so we can conclude that this transporter is a target of Th1 cytokines. These results were not evidenced in EpiAirway™, where the slight increase of SLC6A14 expression did not reach statistical significance; whether this discrepancy reflects the different structure of the layer, pseudostratified in EpiAirway™ and monolayer in Calu-3, or rather to the different cellular composition of the two tissues, remains to be determined. We also studied the same inflammatory stimuli on OCTN2 transporter and we did not show any modulation either on the gene expression or on the basolateral L-carnitine transport in EpiAirway™ or Calu-3 cells. These our data contradict

the findings by Mukherjee et al. (2017), showing that the exposure of polarized Calu-3 cells to LPS induces an impressive stimulation of OCTN2/SLC22A5. To this concern, however, a recent contribution (Li, D. 2019) demonstrates that the treatment with LPS even down-regulates OCTN2 expression and activity in alveolar A549 cells. Further studies will be required to define the effects of pro-inflammatory stimuli on OCTN2, as well as to investigate the role of L-carnitine transporters under inflammatory conditions.

In the third part of this study, we investigated the expression and the functional activity of Organic cation transporter in EpiAlveolar™ comparing them to EpiAirway™. EpiAlveolar™ is in vitro organotypic model of the human alveolar tissue, composed of primary human alveolar epithelial cells co-cultured with human pulmonary fibroblasts and endothelial cells. L-carnitine transport in EpiAlveolar™ is mediated by the activity of $ATB^{0,+}$ and OCTN2 transporters, with the first operative at the apical and the latter at the basolateral side. These results are in agreement with the previous observed activity and localization of $ATB^{0,+}$ transporter only at the apical side of the EpiAirway™ monolayer. SLC6A14/$ATB^{0,+}$, barely detectable in A549 cells, was expressed in EpiAlveolar™ at the highest level, both as mRNA and protein, even more than in EpiAirway™. The expression of this transporter is usually low under physiological conditions, while it is induced in malignant tumor progression and under inflammatory conditions (Broer, S. 2018; Sikder, M.O. 2017); a similar induction by inflammatory stimuli has been described also in airways epithelial cells in vitro. The massive expression of SLC6A14 at the apical side of normal respiratory cells is thus intriguing. SLC22A5/OCTN2 was, instead, equally expressed in EpiAlveolar™ and EpiAirway™. Our findings suggest that the alveolar and bronchial cells share similar properties as carnitine transport is concerned; in particular, for OCTN2, his activity is limited to the basolateral side and this is in consistent with the immunocytochemistry data we reported in EpiAirway™ and Calu-3 cells, where the protein localized around cell borders on the lateral membrane and was absent at the apical side. Subsequently, we evaluated the inhibitory effect of anticholinergic drugs (ipratropium, tiotropium, and glycopyrrolate) on carnitine and MPP^+ uptake in EpiAlveolar™. Results indicate that tiotropium and glycopyrrolate interact with $ATB^{0,+}$ at the apical side of alveolar epithelium, as in EpiAirway™, while ipratropium is substrate of OCTs as well as OCTN2. Since we demonstrate that some respiratory drugs can be substrate of these transporters, the definition of their pattern of expression and localization gain particular relevance in the field of pharmacokinetics; in particular, the differences among various cell models need to be considered when employing them for the screening of biopharmaceuticals molecules.

Conclusions

Despite the increasing relevance ascribed to OCTs/OCTNs in respiratory drug disposition, their regional expression and subcellular localization in the airways remain, thus far, elusive (Selo, M.A. 2020). In this study, we investigated the expression and activity of these transporters in EpiAlveolar™ and EpiAirway™, two model of human primary respiratory epithelia. We demonstrated that human alveolar and tracheal-bronchial epithelia share a similar pattern of expression and cellular localization of transporters, with OCTN2, OCT1 and OCT3 expressed on the basolateral membrane and $ATB^{0,+}$ at the apical side. In both models, bronchodilators drugs interact with these in different ways: in particular glycopyrrolate and tiotropium interact with $ATB^{0,+}$ and ipratropium with OCTN2 and OCTs.

From the data obtained in this study, it was possible to quantify the amount of mRNA for organic cation transporters in EpiAlveolar™ and EpiAirway™ comparing them with A549 and Calu-3 cells. mRNA coding for SLC22A1/OCT1 and SLC22A5/OCTN2 were similarly expressed in all cellular models studied. Instead, SLC22A3/OCT3 is less abundant in EpiAlveolar™ and EpiAirway™ in respect to A549 cells. SLC6A14/$ATB^{0,+}$, on the contrary, was maximally detectable in EpiAlveolar™, expressed to a minor level in EpiAirway™ and Calu-3 and barely detectable in A549 cells (Figure 21).

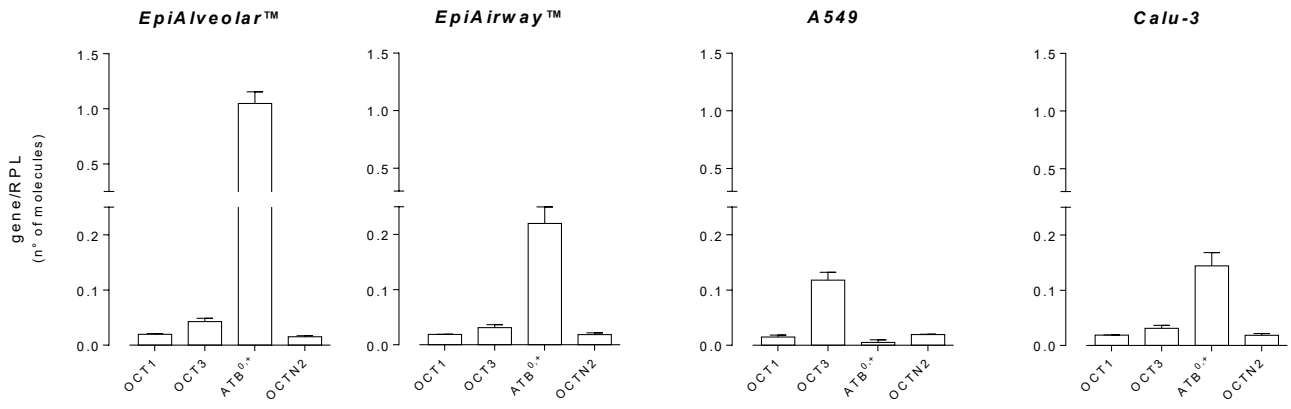

Figure 21 Absolute quantification of OCTs/OCTNs transporters in Human Airways Epithelial cells *The expression of the indicated genes was determined with RT-qPCR analysis as described in Material and Methods. Gene expression was calculated after normalization for the reference gene (RPL15) and presented as number of molecules; data are mean ±SEM of three independent determinations.*

These findings can open new fields of investigation on the mechanisms regulating the disposition of drugs through the epithelial layer, and, in this context, EpiAirway™ and Epialveolar™ provide a reliable tool for studies in the field of pulmonary drug delivery.

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