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Original Paper

Inhibition of Sphingolipid Synthesis as a Phenotype-Modifying Therapy in Cystic Fibrosis

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Key Words
Lipid metabolism • Ceramide • Proteinopathy • Autophagy • Cystic Fibrosis

Abstract
Background/Aims: Cystic Fibrosis (CF) is an inherited disease associated with a variety of mutations affecting the CFTR gene. A deletion of phenylalanine 508 (F508) affects more than 70% of patients and results in unfolded proteins accumulation, originating a proteinopathy responsible for inflammation, impaired trafficking, altered metabolism, cholesterol and lipids accumulation, impaired autophagy at the cellular level. Lung inflammation has been extensively related to the accumulation of the lipotoxin ceramide. We recently proved that inhibition of ceramide synthesis by Myriocin reduces inflammation and ameliorates the defence response against pathogens infection, which is downregulated in CF. Here, we aim at demonstrating the mechanisms of Myriocin therapeutic effects in Cystic Fibrosis broncho-epithelial cells. ***Methods:*** The effect of Myriocin treatment, on F508-CFTR bronchial epithelial cell line IB3-1 cells, was studied by evaluating the expression of key proteins and genes involved in autophagy and lipid metabolism, by western blotting and real time PCR. Moreover, the amount of glycerol-phospholipids, triglycerides, and cholesterols, sphingomyelins and ceramides were measured in treated and untreated cells by LC-MS. Finally, Sptlc1 was transiently silenced and the effect on ceramide content, autophagy and transcriptional activities was evaluated as above mentioned. ***Results:*** We demonstrate that Myriocin tightly regulates metabolic function and cell resilience to stress. Myriocin moves a transcriptional program that activates TFEB, major lipid metabolism and autophagy regulator, and FOXOs, central lipid metabolism and anti-inflammatory/anti-oxidant regulators. The activity of these transcriptional factors is associated with the induction of PPARs nuclear receptors activity, whose targets are genes involved in lipid

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transport compartmentalization and oxidation. Transient silencing of SPTCL1 recapitulates the effects induced by Myriocin. ***Conclusion:*** Cystic Fibrosis bronchial epithelia accumulate lipids, exacerbating inflammation. Myriocin administration: i) activates the transcriptions of genes involved in enhancing autophagy-mediated stress clearance; ii) reduces the content of several lipid species and, at the same time, iii) enhances mitochondrial lipid oxidation. Silencing the expression of Sptlc1 reproduces Myriocin induced autophagy and transcriptional activities, demonstrating that the inhibition of sphingolipid synthesis drives a transcriptional program aimed at addressing cell metabolism towards lipid oxidation and at exploiting autophagy mediated clearance of stress. We speculate that regulating sphingolipid *de novo* synthesis can relieve from chronic inflammation, improving energy supply and anti-oxidant responses, indicating an innovative therapeutic strategy for CF.

Introduction

Cystic fibrosis (CF) is a hereditary disease related to six different classes of mutations, affecting the chloride/carbonate channel CFTR [1]. CFTR dysfunction has a devastating effect primarily on the lungs and pancreas physiology and function. Although CF causes life threat because of pulmonary inflammation and infections, the improved therapeutic management of the last decades significantly increased life expectancy up to late adulthood. The disease, along with aging, is characterized by chronic inflammation and progressive manifestation and deterioration of comorbidities. Among comorbidities, dyslipidemia with high triglycerides and low LDL and cholesterol levels were identified in plasma of CF patients [2, 3], together with peripheral tissue fat accumulation [2, 4-8]. CF-related malabsorption of cholesterol is thought to enhance its synthesis, thus contributing to cholesterol accumulation, in the liver but also in other tissues [9, 10]. The most common CF mutations belong to class II and include the 508-phenylalanine deletion, which is encoded in 70% of mutant alleles in Caucasian patients. This mutation originates a proteinopathy due to ΔF508-CFTR unfolded protein accumulation, which saturates the clearance ability of the ER-associated degradation (ERAD), even when enhanced by the UPR system [11, 12]. The UPR modulates the expression of a variety of genes, that are involved in ER-related activities such as proteasome-lysosomal degradation, protein synthesis, sphingo- and glycerol-lipids and cholesterols metabolism, efficaciously adapting cell activities to a survival response to stress [13, 14]. Aggregates accrual, formed by misfolded mutant CFTR and a miscellaneous of sequestered proteins within, induces inflammation and oxidative stress, impairing proteins and lipids transport [1, 15]. Autophagy is a major supportive harm evoked under stress conditions and aimed to the degradation of unnecessary or un-wanted materials. Due to such aggregate-formation prone phenotype of ΔF508-CFTR expressing cells, autophagy is impaired by the segregation and degradation of key autophagic proteins, thus exacerbating the proteinopathy induced stress [12]. Intracellular organized lipid storages are a source for autophagosome membrane formation [16]. Consequently, an upregulated lipid synthesis or deregulated transport and metabolism was demonstrated to impair autophagy and to trigger ER stress [16-18]. TFEB is a master regulator of stress response insuring energy refueling via autophagy induction and lipid oxidation [19]. Autophagy sustains the clearance of proteins, lipids (lipophagy) [20] and infective pathogens (xenophagy) [21, 22]. A TFEB target is p62-sequestosome, a key component of autophagy vesicles that connects TFEB activation to lipophagy, in support of mitochondrial β-oxidation of fatty acids; interestingly, p62-sequestosome loss is also associated with diabetes and obesity [23]. Moreover, TFEB activates a set of genes involved in lipid mobilization and oxidation, mostly by the induction of the transcriptional activities of PPARs, their co-activator PGC-1α and FOXOs [20, 24].

FOXOs and PPARs transcriptional activity regulates lipid metabolism and inflammatory reaction. CF epithelial cells have reduced the level of FOXO1 [25], and CF models have a profound deficiency in the function of the lipid-activated PPAR-γ [26], possibly due to its sequestration into aggregates [27]. Overall, these observations suggest that protein

misfolding in the ER leads to global effects on lipid homeostasis [28]. Importantly, such cellular deregulation of lipid homeostasis is somewhat mirrored in CF patients by altered plasma lipid profile [3, 8, 9, 29-33]. Cholesterol increased synthesis was associated to ΔF508-CFTR expression, independently from CFTR channel function, but caused by late endosomal/lipid vesicle traffic impairment, in response to the proteinopathy stress. Moreover, the accumulation of esterified cholesterol in endosomal vesicles is accompanied by defects in the traffic of glycosphingolipids [7, 10, 34, 35]. These outcomes recall the Niemann-Pick disease type C (NPC) deficiency of cholesterol transport [36], responsible for membrane equilibrium alteration and impaired lipid trafficking [7]. The altered cholesterol transport and metabolism are sensed by the ER leading to the activation of SREBP (sterol regulatory element-binding protein) and endogenous cholesterol synthesis, in CFTR deficient cells [7] and mice [37]. Accordingly, CF patients present increased cholesterol in the lung and trachea sections [38]. Sphingomyelin, phosphatidylcholine, and cholesterol are the major components of cellular membranes. Their synthesis occurs at ER and it requires the coordinated activity of all the involved enzymes insuring that the modulation of one lipid class is related to the modulation of the other two classes [39], following the overall design of feeding the membranes [40-43]. Thus, triggering cholesterol synthesis may cause an increased synthesis of sphingolipids [14, 44]. Sphingolipid and glycerolipid metabolisms overlap at the enzymatic step where ceramide competes with diacylglycerol for the phosphocholine (deriving from CDP-choline). The ratio between the key enzymes of the two pathways, Serine Palmitoyl Transferase over Glycerol 3-phosphate Acyl Transferase, is significantly higher in the microsomal lung (and pancreas) than in most other adult rat tissues; accordingly, the percentage of sphingomyelin is higher in the total phospholipid content in these fractions [45, 46]. Sphingomyelin is formed, at Golgi or plasma membrane, by the addition of phosphocholine to ceramide. Ceramide is the core molecule of all sphingolipids metabolites, synthesized in the ER and translocated by vesicles or protein-mediated transport to the Golgi apparatus. Ceramide accumulation is involved in a variety of proteinopathies, that share with CF an inflammatory and ER stress condition, due to altered proteostasis, such as Retinitis Pigmentosa, Parkinson's, Alzheimer's, and Hungtinton's diseases [47, 48]. We and others previously demonstrated that ceramide content is pathologically increased in CF lungs and pulmonary epithelia, and that pharmacological impairment of ceramide accumulation reduces chronic inflammation and bacterial/fungal infections in CF [49-54]. Gulbins and his research group demonstrated that ceramide increases in CF lungs via enhanced sphingomyelin hydrolysis and they evaluated, in a clinical trial, the therapeutic effect of the sphingomyelinase inhibitor amitriptyline, a tricyclic antidepressant, which is already known for its action on lipid membranes and related signaling in neuropharmacology [49]. We proved, in different models, that *de novo* sphingolipid synthesis is an inflammation responsive pathway. It is enhanced by inflammatory mediators, both at transcriptional and at enzyme activity level and the accumulation of its metabolite ceramide potentiates inflammation in a vicious circle [52, 53, 55]. In this manuscript, we provide evidences that Myriocin (Myr), by inhibiting the first and rate-limiting reaction in the sphingolipid *de novo* synthesis pathway, is able to promote lipid oxidation and overall reduction and to induce autophagy, thus driving energy fueling and stress removal in ΔF508-CFTR bronchial epithelial cells.

Materials and Methods

Reagents and antibodies

The following materials were purchased: LHC Basal, LHC-8 w/o gentamicin culture media from Gibco (US); Fetal Bovine Serum and Minimum Essential Medium Earle's salt from EuroClone Life Science Italy; penicillin/streptomycin and RIPA buffer were purchased from Sigma-Aldrich; OA/BSA cell colture mix (Sigma); protease inhibitors cocktail (Roche); Quick Start™ Bradford Dye Reagent and Clarity™ Western ECL Blotting Substrates, iScript™ cDNA synthesis, retro-transcription kit (BioRad); BODIPY 493/503 (4, 4-difluoro-1, 3,5, 7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene), catalogue number D3922, and BODIPY™

558/568 C_{12} (4, 4-Difluoro-5-(2-Thienyl)-4-Bora-3a,4a-Diaza-*s*-Indacene-3-Dodecanoic Acid), catalogue number D3835, Prolong® Gold antifade reagent, were purchased from ThermoFisher Scientific, Molecular Probes™; NE-PER™ Nuclear and Cytoplasmic Extraction Reagents from ThermoFisher Scientific; ReliaPrep™ Miniprep RNA extraction System and GoTaq qPCR Master Mix (Promega); SYBR Green system (Qiagen); synthetic oligonucleotides from M-Medical Italy. The chemicals acetonitrile (ACN), 2-propanol (IPA), methanol, chloroform, formic acid (FA) and ammonium formate were purchased by Sigma-Aldrich (Milan, Italy). Cholesterol d7 (cod. 700041P), C15 ceramide d7 (cod. 860681P), 15:0-18:1-d7-phosphatidylcholine (cod. 791637C) and 16:0-18:0-16:0 d5 triglyceride (cod. 860902P), used as internal standards were purchased by Avanti Polar Lipids (Alabaster, AL). All aqueous solutions were prepared using purified water at a Milli-Q grade (Millipore, Milan, Italy). Primary antibodies: anti- PPAR-γ, Foxo1A and anti-Laminin A/C (ElabScience, US), SQSTM1/p62 (D1Q5S), anti β-actin (Sigma, US), anti-TFEB (ab2636, Abcam), anti-LC3 (Cell Signaling, US). The secondary antibodies were purchased from Jackson Laboratories (Bar Harbor, ME, US).

Cell lines and treatments

IB3-1 cells (named CF cells), an adeno-associated virus-transformed human bronchial epithelial cell line derived from a CF patient (ΔF508/W1282X) and provided by LGC Promochem (US), were grown in LHC-8 medium supplemented with 10% FBS, 1% penicillin/streptomycin at 37°C and 5% CO_2. Human lung bronchial epithelial cells 16HBE14o- (named healthy), originally developed by Dieter C. Gruenert, were provided by Luis J. Galietta, (Telethon Institute of Genetics and Medicine - TIGEM, Napoli) and cultured, as recommended, in Minimum Essential Medium (MEM) Earle's salt, supplemented with 10% FBS, 1% penicillin/streptomycin at 37°C and 5% CO_2. Myriocin (Myr) treatments were performed at a concentration of 50 μM, for the indicated time lengths, in 100 mm dishes plated at $1x10^5$ cells/each.

Protein extraction and western blotting

For transcriptional factors western blottings, nuclear and cytoplasmic extracts from cells were obtained with the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (ThermoFisher Scientific) according to the manufacturer's instructions. Total cell proteins were extracted from cells in RIPA buffer. The concentration of proteins in lysates was measured by Quick Start™ Bradford Dye Reagent (595 nm OD read). 50μg of proteins *per* sample were separated on SDS-PAGE gel and electro-blotted onto an either PVDF membrane for LC3 protein detection or nitrocellulose membrane for other protein targets. After washing in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and blocking with 5% non-fat dry milk for 1 hour at room temperature, membranes were probed overnight at 4°C with the primary antibodies. After three washes in TBS-T, the blot was incubated with the horseradish peroxidase-conjugated secondary antibodies. After the final washings, proteins were detected using an enhanced chemo-luminescent horseradish peroxidase substrate and the relative bands were captured and quantified by Alliance UVITEC Cambridge.

qRT-PCR

Cells were harvested, washed in PBS and total RNA was isolated from cell pellet with the ReliaPrep™ Miniprep RNA extraction System, according to the manufacturer's instructions. 1 μg of purified RNA was reverse transcribed and the obtained cDNA was stored at -20°C. The amplification of target genes was performed for the following targets: TFEB (*TFEB*), LAMP 2a, 2b and 2c (*LAMP2a,2b,2c*), PGC-1α (*PPARGC1A*), PPAR-α (*PPARA*), PPAR-γ (*PPARG*), FOXO 1a and 3a (*FOXO 1a, 3a*), FATP1 (*SLC27A1*), CPT-1a (*CPT1A*), CPT-1b (*CPT1B*), SCAD *(ACADS)*, MCAD (*ACADM*), LCAD (*ACADL*). Relative mRNA expression of target genes was normalized to the endogenous GAPDH control gene and represented as fold change *versus* control, calculated by the comparative CT method (ΔΔCT Method). All the primer sequences are reported in the Supplementary Table 1 (for all supplemental material see www.cellphysiolbiochem.com).

Lipidomic analysis

Lipids extraction from cell pellets was performed using a monophasic extraction method with water: chloroform: methanol (1:3:6 v/v/v) [56]. Then a small aliquot (2 μl) of the extracts was analysed by LC-HRMS (Shimadzu UPLC coupled with a Triple Tof 6600 Sciex). All samples were analysed in duplicate in positive mode with electrospray ionization for the identification and semi-quantification of sphingolipids, cholesterol and its esters, phospholipids and triacylglycerols. MS/MS spectra were acquired by data

dependent acquisition. Separation was achieved by an Acquity BEH C18 column 1.7 μm 2.1x50 mm (Waters, MA, USA) using as mobile phase A water/acetonitrile (60:40) and as mobile phase B 2-propanol/acetonitrile (90:10) both containing 10 mM ammonium formate and 0.1% of formic acid. The identification and semi-quantification were attained using MS-DIAL (ver. 3.82) [57]. Peak intensities of each lipid were normalized (Intensity norm.) by correcting for both total amount of protein (mg) measured by Bradford method and total-ion count. Results were presented as the sum of the normalized intensity for each lipid within a class. Absolute sphingolipid determination was achieved using a targeted analysis by a LC-MS/MS system (Dionex 3000 UltiMate coupled to a tandem mass spectrometer AB Sciex 3200 QTRAP) as already described [58].

Sptlc1 silencing by siRNA

CF cells were seeded in 6 wells plates and grown for 24 hours (70% confluency). For each well, 20pmol of Sptlc1 siRNA (or Scrambled sequence as negative control, Thermofisher) were diluted into 250 μl of Opti-MEM (w/o FCS); 5ul of Lipofectamine RNAiMAX (Thermofisher) was diluted into 250 μl of Opti-MEM LHC8 (w/o FCS); diluted oligomers and lipofectamine were gently mixed and incubated at RT for 15 minutes, then added to each cell colture well in a total volume of 2ml/well of serum free medium (LHC8). Cells were incubated at 37°C in a CO2 incubator for 5 hours, than fresh medium, containing 10% FCS, was replaced, either with or without 30 μM of oleic acid/BSA. Cells were additionally incubated up to 24-48 hours before collecting for analytical procedures.

Statistical analysis

All the experiments were performed in a minimum of 3 separate experiments. In some cases, the reported data derive from 5 independent experiments. Data are expressed as mean ± SD, calculated from experimental replicates. For western blotting analysis, the images are the most representative whereas the quantification of protein signals is calculated on the average of all the experiments performed. Data significance was evaluated by two-tailed Student T-test or ANOVA test followed by Bonferroni post test ($p<0.05$), as indicated in figure legends. Statistical analysis was performed by GraphPad Instat software (La Jolla, CA, USA) and graph illustrations generated by GraphPad Prism software (La Jolla, CA, USA).

Results

Myriocin induces autophagy in CF epithelial cells

Proteinopathies, in particular, ΔF508CFTR induced CF, are characterized by impaired cellular transports, ER stress, and deficiency in proteasomal and autophagic clearance of accumulated material. Therefore, we evaluated the effect of Myr treatment on autophagy induction in ΔF508CFTR and in a healthy broncho epithelial cell line as control. CF cells exhibit a reduced basal amount of lipidated form of LC3 (LC3II) in respect to healthy broncho epithelial cells (Fig. 1 A and B), as previously shown [12]. We demonstrated that Myr (50 μM) was effectively inducing the accumulation of the lipidated form of LC3 (LC3II) within 5 hours of treatment (Fig. 1A). In addition, we observed that Myr (50 μM) induced a significant reduction of p62-sequestosome in CF cells (Fig. 1B). These observations indicate that the treatment with Myr induced an increase in autophagic flux in CF cells.

Myriocin induces TFEB, PPARγ and FOXO1A nuclear translocation in CF epithelial cells

In order to understand why Myr is able to enhance autophagy, we investigated the activation of TFEB, a key inducer of autophagy and a regulator of energy homeostasis. TFEB nuclear migration and activation is significantly increased in CF cells treated with Myr (50 μM) *versus* untreated cells, already after 5 hours of treatment (Fig. 2A). By promoting autophagy, TFEB enhances lipid catabolism at the aim of conveying cell resources to energy production and to organize a survival stress response. To do so, TFEB promotes the activation of PPARs and FOXOs family of transcriptional factors involved in lipid homeostasis and inflammatory responses. We demonstrated that a slightly longer treatment with Myr (50 μM, 12 hours) stimulates nuclear translocation of PPAR-γ and FOXO1A (Fig. 2A, 2B).

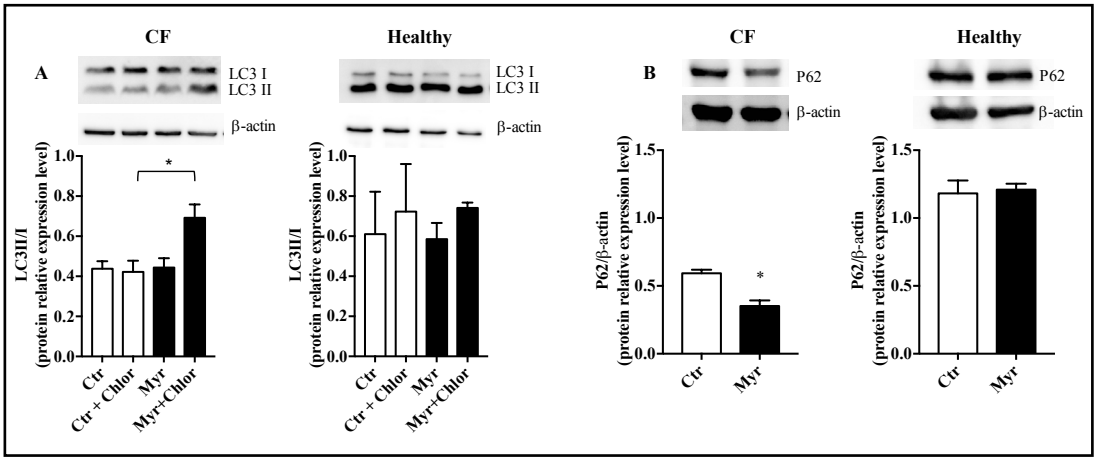

Fig. 1. Myriocin induces autophagy in CF cells. CF cells and healthy cells, treated with Myriocin for 12 hours. Detection of LC3 I and II protein expression by western blot in absence or in presence of 1 hour treatment with chloroquine and normalized on β-actin (A). Quantification of LC3II/I ratio obtained from triplicate samples and normalized onto β Actin and represented in the bars graph. Quantification of P62 protein expression by western blot analysis on whole lysates and normalized on β-actin (B); two-tailed unpaired Student's T-test.

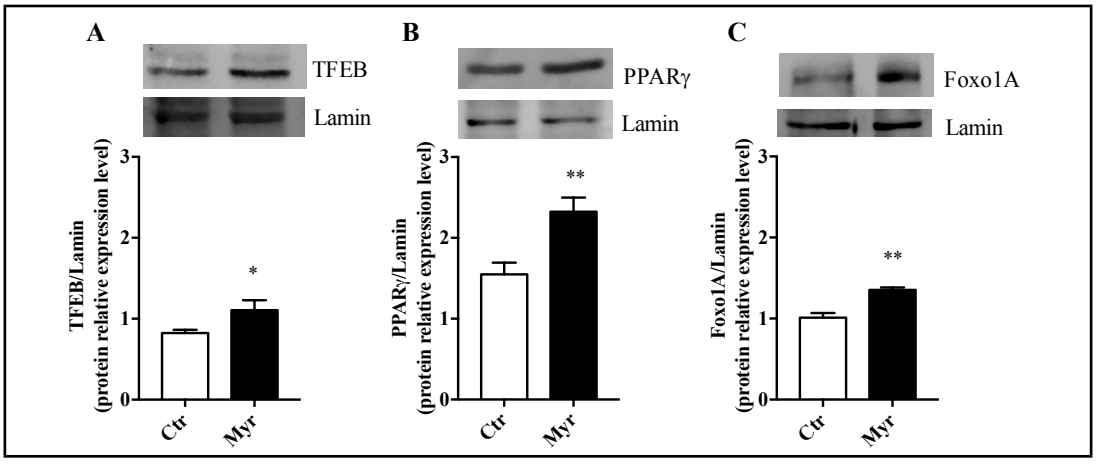

Fig. 2. Myriocin induces transcriptional factors activation. CF bronchoepithelial cells were treated or not with Myr (5 hours for TFEB evaluation and 12 hours for PPARγ; C, FOXO1A). Quantification of the transcriptional factor nuclear translocation by western blot on nuclear extracts and normalized on Lamin A/C: A, TFEB; B, PPARγ; C, FOXO1A. Protein signals were quantified by densitometry analysis and the normalized values reported in the graphs; two-tailed unpaired Student's T-test.

Myriocin activates a stress response transcriptional program

TFEB is a master regulator of the transcription of genes involved in autophagy and lysosome formation. First of all, the same TFEB expression resulted increased upon Myr treatment (50 μM, 12 hours; Fig. 3A) in CF cells *versus* healthy cells, indicating that initial activation of this critical regulatory factor is sustained, in the time, by the activation of a transcriptional program underlining TFEB induced effect. Next, we evaluated the expression of Myr nuclear translocation-activated transcriptional factors PPARγ and FOXO1A. We observed that their activation is sustained by their increased expression (Fig. 3B, 3E), together with the increased expression of PPAR-α, PGC1α and FOXO3A (Fig. 3C, 3D, 3F). Therefore, Myr drives the expression of a set of genes that are aimed at sustaining lipid catabolism and energy production inhibition. To note that Myr induced transcriptional activities is significantly regulated in CF cells but only to a minor extent in healthy cells.

Then, we evaluated the expression of TFEB targets, involved in sustain of autophagy flux, Lamp2a, Lamp2b, and Lamp2c. We observed that overnight (24 hours) treatment with Myr (50 μM) increased the expression of all these genes in CF cells *versus* healthy cells (Fig. 4A). In order to verify the effect of PPARs and FOXOs activation, we evaluated the expression of their target genes involved in lipid transport and catabolism. We proved that a 24 hours Myr treatment significantly increases the expression of the plasma membrane lipid transporter FATP1 (*Slcl27a1*), which mediates the uptake of long-chain FA, and the expression of the transporters Ctp1a and Cpt1b, which instead control the mitochondrial entry of FA for oxidation, as compared to untreated cells (Fig. 4B and 4C). In order to prove that fat entry and mobilization was finalized at oxidation and energy production, we evaluated the Myr-induced transcriptional response of enzymes involved in mitochondrial fatty acid oxidation (FAO). SCAD (*ACADS*), MCAD (*ACADM*) and LCAD (*ACADL*), which are the mitochondrial dehydrogenases primarily responsible for β-oxidation of small, medium and long chains of FAs respectively, are upregulated by 24 hours Myr treatment, reaching statistical significance for *ACADL* (Fig. 4C). To note that Myr significantly upregulated the transcription of the above mentioned genes in CF cells but only to a minor extent in healthy cells.

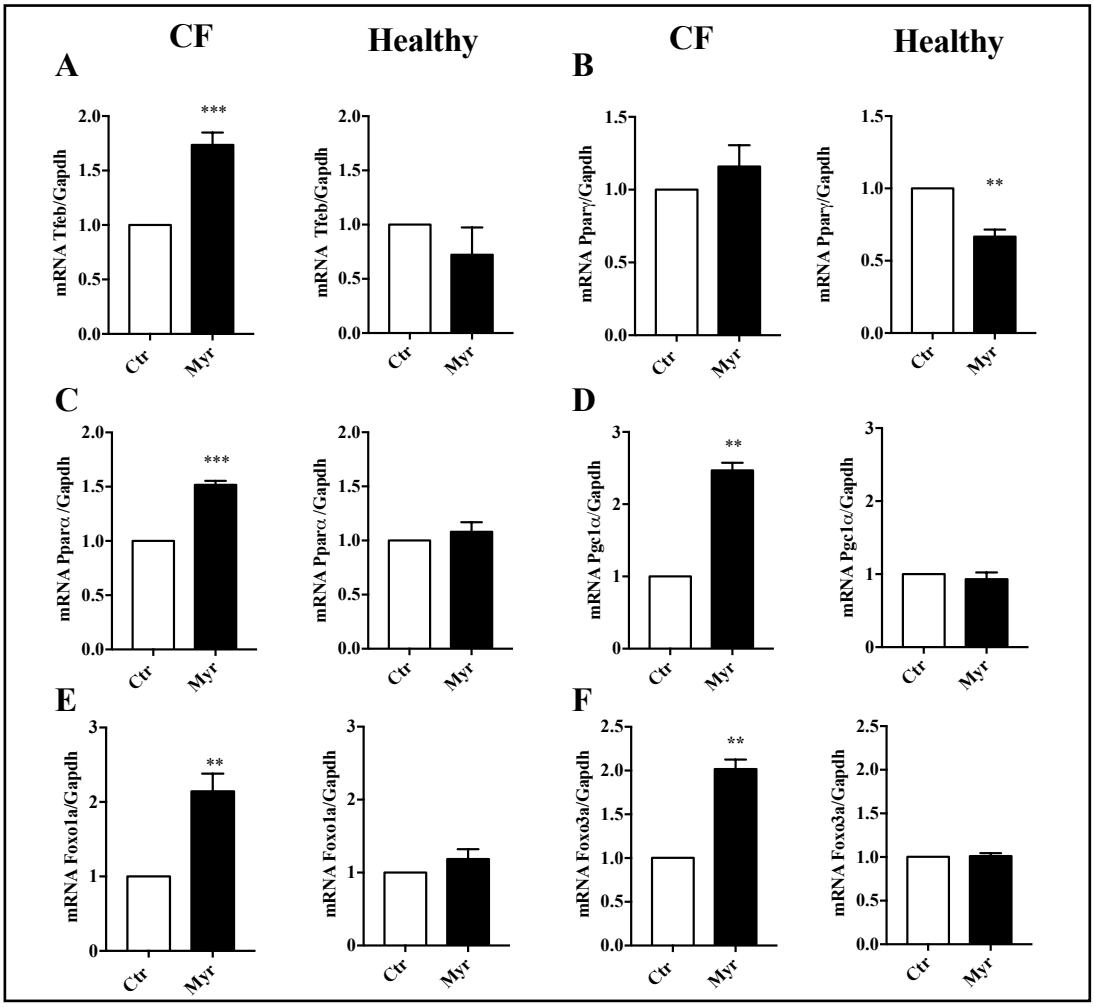

Fig. 3. Myriocin activates the transcription of TFEB-induced transcriptional factors. Quantification of the expression of genes encoding for transcriptional factors activated by TFEB: A, TFEB; B, Ppar-γ; C, Ppar-α;D, Pgc1α; E, Foxo1A; F, Foxo3A by qRT-PCR, in CF cells and healthy cells, treated and untreated with Myr (12 hours). All data are normalized on the housekeeping gene GAPDH and expressed as mean±SE (* $p<0.05$; ** $p<0.01$; *** $p<0.001$); two-tailed unpaired Student's T-test.

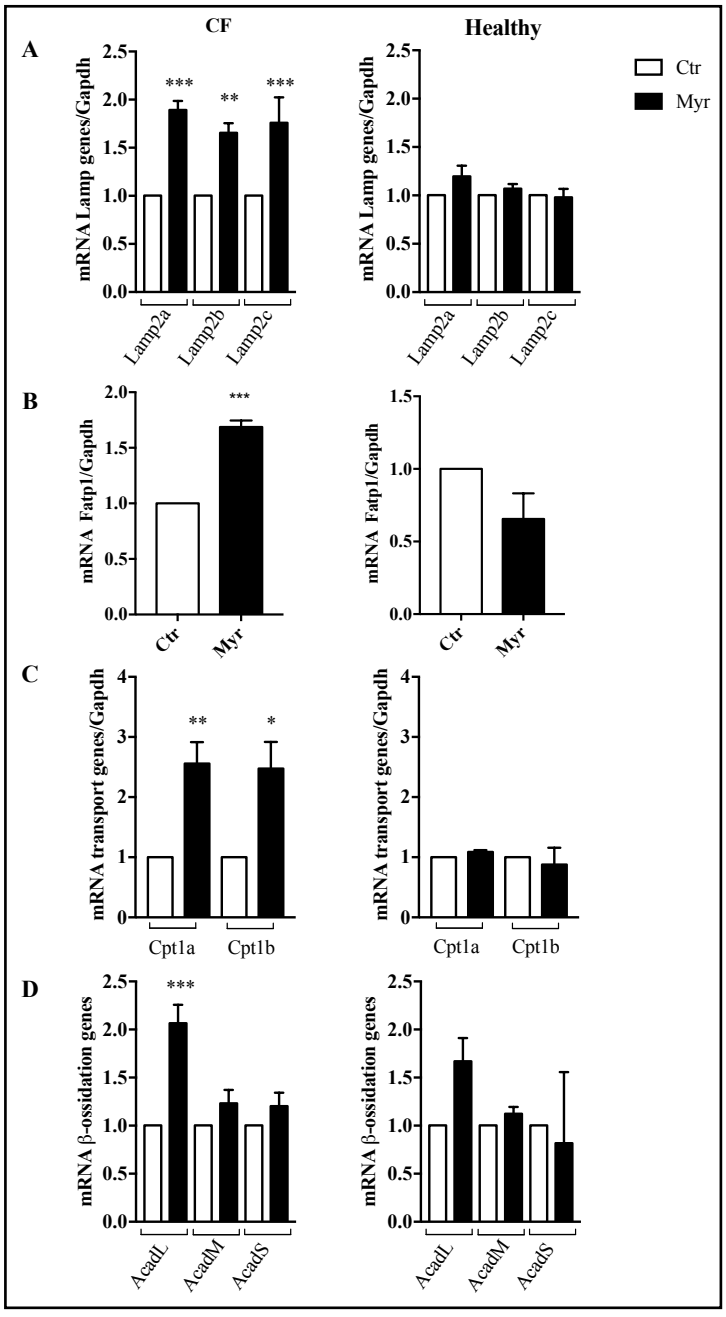

Fig. 4. Myriocin activates the transcription of TFEB-induced autophagy and lipid metabolism related genes in CF and healthy broncho epithelial cells. Quantification of the expression of genes involved in autophagy and cell lipid transport/metabolism: A, Lamp alternative splicing derived mRNA levels (Lamp2a, Lamp2b, Lamp2c);B, Fatp1; C, CPT1a, CPT1b and; D, AcadL, AcadM and AcadS by qRT-PCR in CF cells and healthy cells, treated and untreated with Myr (24 hours). All data are expressed as mean±SE (* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001); two-tailed unpaired Student's T-test for Fig. 4B and Anova followed by Bonferroni post-test for all other Figures.

Myriocin reduces lipid content in CF epithelial cells

Myr inhibits ceramide and related sphingolipids synthesis and, according to our results, it also promotes the expression of genes involved in lipid oxidation. We compared the amount of different lipid species in Myr treated healthy and CF cells, versus untreated ones. CF cells have a significantly higher content in sphingolipids (namely ceramides CER and sphingomyelins SM), glycerophospholipids (namely phosphatidylcholine PC and phosphatidylserine, PS), triacylglycerols (TAG) and cholesterol esters (CE) (Fig. 5 A, B, C, E, H, I). We observed that Myr not only reduces ceramide and sphingomyelin content (Fig. 5A-4C), but it also reduces glycerolipids (PC, PS and phosphatidylethanolamine, PE), Lyso-phosphatidylcholine (LPC), TAG, and cholesterol esters (CE), with a particular significance for the decrease of PC, PS, and CE (Fig. 5). Thus, the inhibition of sphingolipids biosynthesis causes an overall depletion of cellular lipids from different classes.

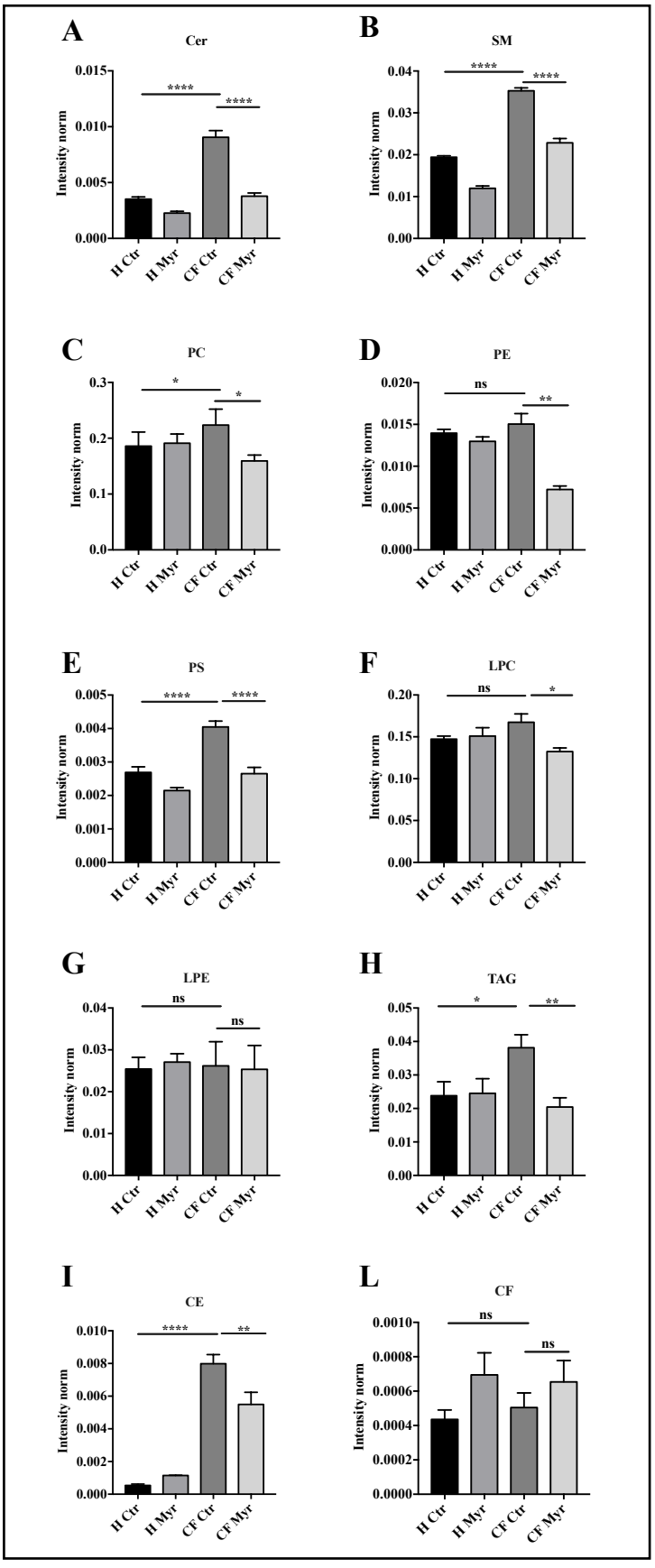

Fig. 5. LCMS measurement of different lipid species and Myriocin effects in CF cells and in healthy (H) broncho epithelial cells. The content of different lipid species was analyzed by LC-MS. CF cells lipid content is higher than healthy in all the observed species; 24 hours Myr reduced the content of all the evaluated lipid species in CF cells: A, Ceramide (Cer); B, Sphingomyelin (SM); C, phosphatidylcholine (PC); D, phosphatidylethanolamine (PE); E, phosphatidylserine (PS); F, Lysophosphatidylcholine (LPC); G, lysophosphatidylethanolamine (LPE); H, triacylglycerols (TE); I, cholesterol esters (CE); L, free cholesterols (CF). Data are expressed as mean ± SE (* $p<0.05$; ** $p<0.01$); Anova followed by Bonferroni post-test.

Reduced expression of SPT1 recapitulates Myr effects

To better prove that the control of sphingolipid synthesis is tightly linked to the overall cell metabolism and it can be used to switch on stress response and total lipid consume in CF model, we transiently silenced the expression of Sptlc1 gene, by specific targeting of its mRNA, siRNA, in CF cells. We observed a reduction of 83% gene expression within 24 hours, that was still stable at 48 hours (Fig. 6A), paralleled by a reduced protein expression of more than 50% (6B). After 48 hours, Cer and SM were significantly reduced, indicating the efficacy of gene expression silencing in counteracting sphingolipid *de novo* synthesis (Fig. 6C and D). In order to prove that Sptlc1 downregulation reproduces the effects of Myr treatment, we evaluated the induction of autophagy by LC3 lipidation analysis *via* western blotting. We observed that Sptlc1 silencing significantly enhances autophagic flux, either in the presence or in the absence of chloroquine for lysosomal activity inhibition (24 hours). Since nucleotide oligomers may activate autophagy, we added scrambled siRNA as a control, showing a slight and not significant increase of LC3II/LC3I ratio in respect to control (w/o scrambled siRNA) (Fig. 6E). Next, we compared the expression of the key genes activated by Myr in Sptlc1-silenced cells *versus* control. We investigated the expression of TFEB, master regulator of autophagy and energy metabolism, and we observed that 24 hours Sptlc1-silenced CF cells exhibit an increased expression of TFEB (Fig. 6F), and of one of its target genes involved in autophagic flux, LAMP2a (Fig. 6H). Similarly to Myr, Sptlc1 silencing increased the expression of PPARα (Fig. 6G), CPT1a (Fig. 6I), involved in fatty acids entry into mitochondria, and of ACADs (Fig. 6L), the dehydrogenases involved in fatty acids β oxidation (especially the enzyme which preferentially recognizes long chain fatty acids). From these data we conclude that the effects of Myr are entirely due to its inhibitory activity on SPT enzyme and that reducing the synthesis of sphingolipids triggers the TFEB mediated stress response of autophagy and lipid consume.

Discussion

We previously showed the therapeutic potential of Myr, a fungal-derived molecule known to be a specific inhibitor of the Serine Palmitoyl Transferase, rate-limiting enzyme of the sphingolipid synthesis. We proved that intra-trachea administration of this molecule reduces chronic inflammation and ameliorates endogenous rejection of bacterial and fungal infections in CF murine models [52, 53]. We also demonstrated that Myr corrects the defective ability of CF epithelial cells to kill internalized pathogens [53]. We here investigated the mechanisms underlying the therapeutic actions of Myr. CF proteinopathy is characterized by defective autophagy response to stress and altered lipid metabolism, as previously elucidated. In this manuscript, we demonstrate that Myr treatment of ΔF508CFTR broncho-epithelial cells is able to recover and stimulate autophagy, leading to LC3II accumulation and p62-sequestosome consume, thus counteracting the CF defective mechanism. In order to understand how an inhibitor of the sphingolipids' synthesis can be related to autophagy induction, we investigated the activation of TFEB, one of the master regulator of stress response. TFEB activation allows energy gain from the oxidation of lipid storage and the recycle of dispensable material via autophagy, inducing, at the same time, anti-oxidant response and sustaining mitochondrial activity [24, 59]. In doing so, TFEB stimulates the activation of other transcriptional factors such as PPARs and FOXOs, which regulate the lipid oxidation and the anti-inflammatory response [22, 24, 60, 61] We previously demonstrated that Myr induces TFEB transcriptional activation and its down stream pathway which is aimed at enhancing lipid oxidation and ATP production in myocardium [62]. We here observed that Myr promotes TFEB nuclear translocation and, subsequently, the nuclear translocation of PPAR-γ and Foxo1A, demonstrating that a transcriptional program is triggered by this molecule, aimed at enhancing cellular energy metabolism and reducing inflammation. In line with the activation of these transcriptional factors, we previously showed that Myr significantly reduces inflammatory cytokines transcription and release and favours the anti-

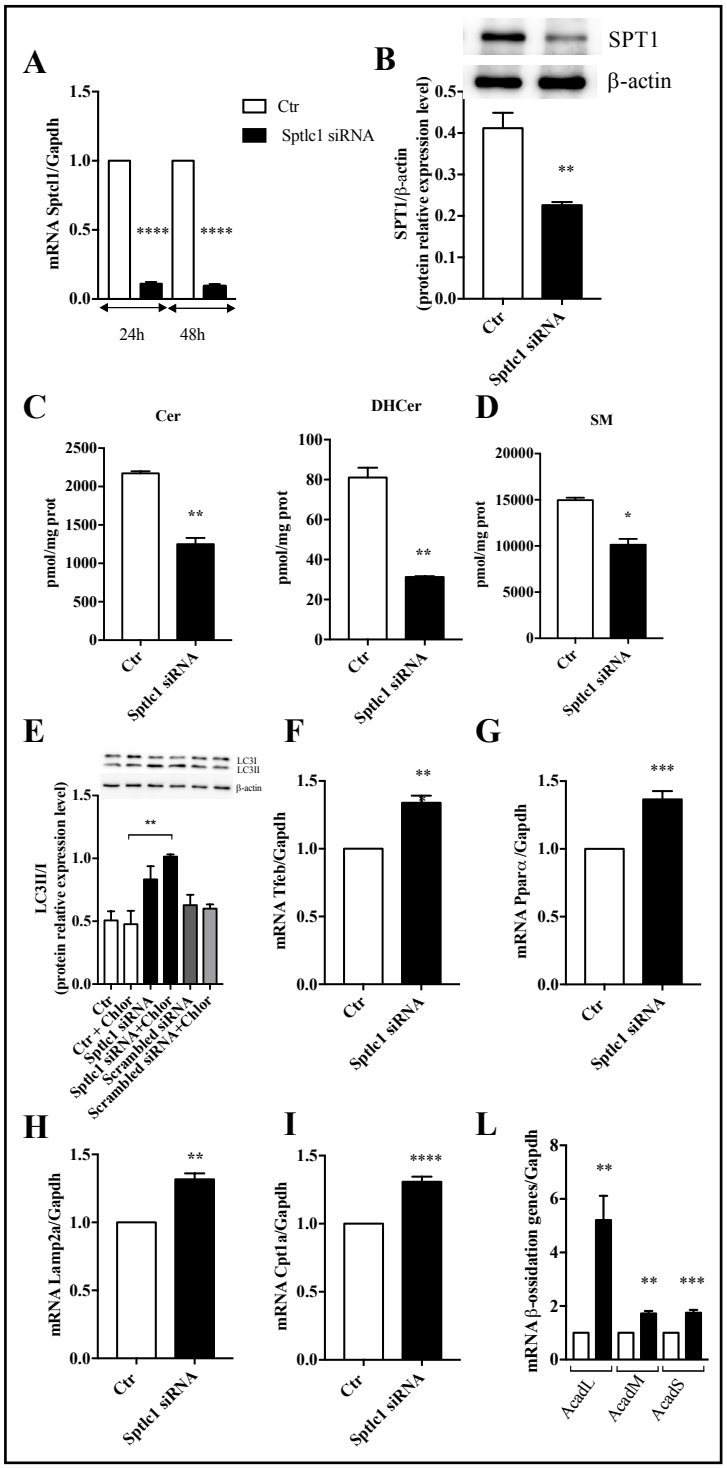

Fig. 6. Transient silencing of Sptlc1 expression recapitulates Myr effects in CF cells. A) Quantification of the expression of the gene encoding for Sptlc1 was obtained by qRT-PCR in CF cells treated with Sptlc1-directed siRNA for 24 and 48 hours. All data are normalized on the housekeeping gene GAPDH and expressed as mean±SE (* p<0.05; ** p<0.01; *** p<0.001); two-tailed unpaired Student's T-test. B). Detection of SPT1 protein expression by western blotting in cells treated with Sptlc1-directed siRNA for 24 hours. LCMS quantification of Ceramides (C) and Sphingomyelins (D) in cells treated with Sptlc1-directed siRNA for 48 hours. E) Detection of LC3 I and II protein expression by western blot, in absence or in presence of 1 hour treatment with chloroquine. Quantification of LC3II/I ratio obtained from triplicate samples of Sptlc1-siRNA or Scrambled-siRNa or vehicle was normalized onto β Actin and represented in the bars graph. Quantification by qRT-PCR of the expression of genes encoding for transcriptional factors activated by TFEB after 24 hours of Sptlc1 directed siRNA: F), TFEB; G), Ppar-α; quantification by qRT-PCR of the expression of genes encoding for TFEB- induced genes after 24 hours of Sptlc1 directed siRNA: H) Lamp2a; I) Cpt1a; L) AcadL, AcadM, AcadS. All data are normalized on the housekeeping gene GAPDH and expressed as mean±SE (* p<0.05; ** p<0.01; *** p<0.001); Anova followed by Bonferroni post-test for Fig. L and two-tailed unpaired Student's T-test for the other Figures.

oxidant response by inducing the HO-1 transcription in ΔF508CFTR CF epithelial cells [53]. We now demonstrate that the transcription of genes involved in lipid transport (cellular import and mitochondrial import of FA) and their mitochondrial oxidation (FA dehydrogenases), known to be stimulated by the TFEB/PPARs-axis, are significantly increased, indicating that, effectively, Myr enhances lipid consume. Consequently, we performed a lipidomic analysis of CF cells and healthy cells treated with Myr and observed an overall increased content of

glycerophospholipids, sphingolipids and cholesterols in CF cells *versus* healthy cells. To the best of our knowledge, this is the first report of increased lipids content in CF pulmonary epithelia and it is in line with reported cholesterol accumulation in peripheral tissues in CF patients. Lipids accumulation can derive from impaired membrane trafficking, and it is responsible for clogging the ER-Golgi network, exacerbating ROS and inflammation in CF. Lipids content was significantly reduced by Myr treatment in CF. The reduction is particularly evident for phosphatidylcholine and cholesterol esters. Moreover, lyso- glycerophospholipids, considered pro-inflammatory molecules [63, 64], were significantly reduced, indicating a decreased inflammation-driven metabolism of the lipid moiety. To note, healthy cells were poorly affected, indicating that Myr is acting on a CF specific defective mechanisms of lipid altered homeostasis.

Conclusion

Thus, lipids homeostasis is intrinsically deranged in ΔF508CFTR proteinopathy of CF, and lipid metabolism stands for an important therapeutic target that can be envisaged in sustain of pharmacological efficiency of correctors. Based on our results, we can envision that Myr treatment has the potential of partially restoring CF cellular dysmetabolism, releasing cells from the excessive stress which is deemed an important cause of correctors failure in CF patients. We can also speculate that this molecule may exert favourable systemic effects to reduce dyslipidemia occurring in CF patients. The limitation in this present study is the overall difference between a CF cell line and a patient's derived broncho epithelium. We are aware that molecular signaling might be affected by peculiarities of the specific cell line and this is the reason why we intend to investigate the mechanism of Myr induced autophagy and relief from proteinopathy stress, as well as lipid accumulation in CF primary cells. The comparison between primary broncho epithelial cells derived from CF patients and from control donors will furtherly shed light on lipid dysmetabolism in CF.

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Author Contributions

Mingione A. contributed to design and execute the experimental work, with analysis and interpretation; Bonezzi F., Piccoli M., Caretti A. contributed to data collection analysis and interpretation; Dei Cas M. and Paroni R.C. contributed by lipidomic analysis; Ghidoni R. contributed with critical revision of the manuscript; Anastasia L. contributed with data analysis and interpretation and with critical revision of the manuscript; Signorelli P. is the PI and contributed to design the work, its analysis and interpretation.

Disclosure Statement

All the authors of this manuscript have no conflicts of interest to declare.

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