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EFFECT OF SODIUM BUTYRATE ON TRANSCRIPTIONAL REGULATION OF CFTR GENE

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ABSTRACT

Cystic Fibrosis (CF) is an autosomal recessive disease caused by mutations in the *cystic fibrosis transmembrane regulator* (*CFTR*) gene, a member of the ATP-binding cassette (ABC) transporter superfamily. It acts in the apical part of epithelial cells as a plasma-membrane, cyclic AMP-activated chloride anion, bicarbonate anion and glutathione channel. *CFTR* is required for cell surface water-salt homeostasis and normal function of epithelia lining in the airways, intestinal tract, pancreatic ducts, salivary and sweat glands, liver and others. To date there are about 2000 mutations reported in *CFTR* gene that are generally classified in 6 different classes. The therapy of cystic fibrosis is still mainly simpthomatic but recently two novel classes of drugs were accepted for use in clinical practice even if in few patient with specific mutation called Ivafactor (potentiator of *CFTR* activity) and Lumafactor (corrector of *CFTR* activity). Although such therapies have improved the life expectancy of CFTR patients, they are still non resolutive in many cases and, moreover, are unable to work for all mutation's classes.

In order to identify novel molecules involved in *CFTR* transcriptional regulation, in addition to the correctors and potentiators that act at protein levels, we investigated the role of butyrate. Butyrate is a short-chain fatty acid and it is already in use in medical care. It's involved in a variety of cellular events but is still unknown if it could have a putative role, direct or indirect, in the expression and modulation of *CFTR*. Preliminary data from our group indicate that butyrate enhance the *CFTR* expression in some cellular lines. To define if this effect is due to a direct mechanism on the *CFTR* promoter activity, we have focused our attention to the promoter region of *CFTR* gene to identify potential butyrate responsive elements (BRE) and their putative binding protein factors. To this purpose, we have performed a luciferase assay to compare the transcriptional activity of 6 deletion mutants of *CFTR* promoter. All constructs have showed a similar increase of luciferase activity under butyrate treatment in 3 different cell types: A549, Caco2 and Panc1. These data suggest that the butyrate effect

on *CFTR* promoter activity is not mediated by a specific BRE element but most probably it is related to the Histone Deacetylase (HDAC) inhibitor activity of butyrate, and we confirmed this mechanism by treatment with Tricostatin (TSA), a specific histone deacetylase inhibitor, that demonstrated the same effect of butyrate on *CFTR* expression. Our study confirms that butyrate enhance *CFTR* expression. Such effect is not due to the interaction of butyrate with promoter responsive elements, but it depends on its effect on histone deacetylase inhibitor activity. Butyrate might be useful in patient with CF bearing mutations that permit a residual activity of the protein enhancing *CFTR* expression.

INTRODUCTION

1.1 Cystic Fibrosis

Cystic fibrosis (CF) was described as a clinical syndrome, for the first time, in 1938 and identified as a disease by Dorothy Andersen [1-4]. She described cystic fibrosis of the pancreas in 49 patients and the disorder was subsequently associated with autosomal recessive inheritance pattern in 1949 [1-4]. It manifests with exocrine pancreatic insufficiency, intestinal obstruction (meconium ileus), liver disease, increased sweat Cl- concentrations, male infertility, and airway disease (Figure 1). In CF patients, airway disease tipically leads to progressive lung dysfunction, responsible for over 95% of CF related morbidity and mortality (Figure 2).

CF is one of the most common mendelian life shortening disease in the world (Figure 3). The high incidence of CF carriers can be explained by a possible protective effect. Several works showed indeed that there is a heterozygote advantage for carriers of *CFTR* mutations. For example, Salmonella typhi, which is the etiologic agent of typhoid fever, was found to bind the *CFTR* protein. This binding mediates translocation of this pathogen into the gastrointestinal submucosa. Thus, decreased *CFTR* levels in the gastrointestinal epithelium of mice heterozygous for a *CFTR* mutation result in the resistance to typhoid fever [14]. It was also shown that mice carrying a *CFTR* mutation have an increased resistance to cholera toxin [15].

After the discovery of the abnormal Cl- transport in CF, the gene responsible for the disease was identified by Riordan, Tsui, Collins, and colleagues using linkage-based techniques and named cystic fibrosis transmembrane conductance regulator (*CFTR*) gene [7-8-9].

CF is a classic Mendelian autosomal recessive disorder. It is most common in populations with northern European ancestry where the predominant mutation is Phe508del (also known as

F508del) [12,13]. During the past six decades, median age of survival increased progressively, and now it is more than 40 years in developed countries [3,4]. The number of adults with cystic fibrosis will continue to increase with almost all deaths occurring in the adult population [5]. Indeed, in the past 5 years, in countries with well funded health-care systems, there were more adults than children with cystic fibrosis. In developed European countries, the number of adults with cystic fibrosis has been predicted to increase by around 70% by 2025 [6].

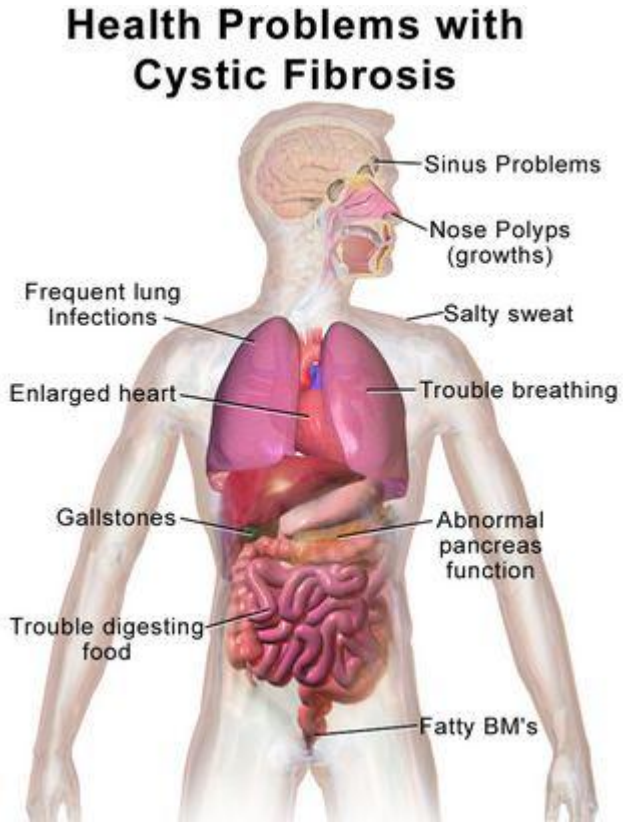


Figure 1. Health problems in patients with CF.

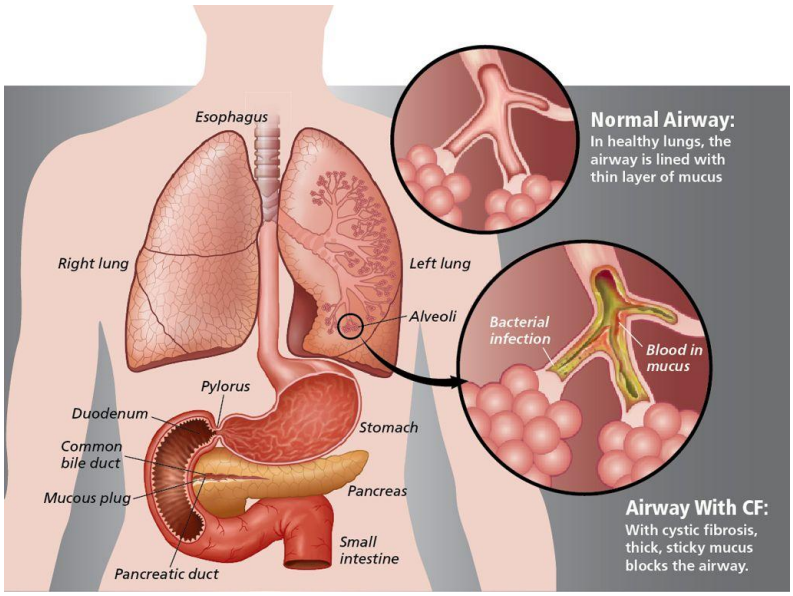


Figure 2. Representations of secretions disfunction in CF patients.

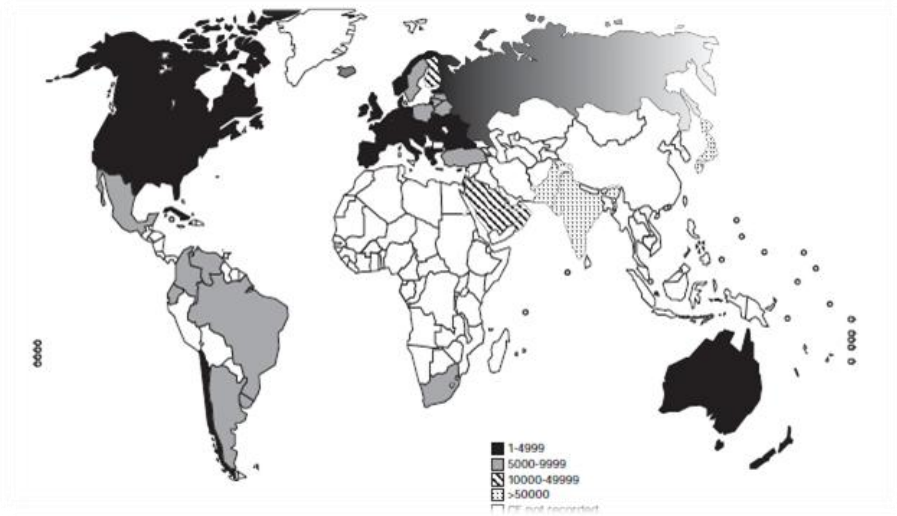


Figure 3. World incidence of CF disease (WHO data)

1.2 CFTR structure and function

The *CFTR* gene comprises 27 coding exons, spanning over 250 kb on chromosome 7q31.2, and the transcript is 6.5 kb. It encodes a chloride (Cl^-) channel in the apical membrane of exocrine epithelial cells [(10)] (Figure 4) that also acting as regulator of other transmembrane proteins. The *CFTR* protein, after its synthesis in the endoplasmic reticulum (ER), is glycosylated at Golgi level and transported to the apical plasma membrane [(12)]. *CFTR* protein is expressed in the trachea, lung, pancreas and several tissues of the reproductive system [(12,16,17)]. It is regulated by ATP hydrolysis and phosphorylation, and conducts Cl-, HCO3 -, SCN-, and other anions. [(18-22)]. The *CFTR* protein comprises 1,480 amino acids with a molecular weight of 170 kDa and five domains: two membrane-spanning domains (MSD1 and MSD2), each composed of six transmembrane segments (TM1 to TM12) that form the channel, two nucleotide-binding domains (NBD1 and NBD2), with ATP hydrolytic activity, and a regulatory domain (R), which contains numerous phosphorylation sites [(10,11)] (Figure 5). The R-region contains several Protein Kinase A (PKA) phophorylation sites highly conserved [(23,24)]. PKA is a cAMP-activated kinase, that phosphorylates CFTR when cellular levels of cAMP increase [(23)]. According to many Authors, the R-region blocks the NBDs from associating together keeping the channel in a closed conformation. Phosphorylation causes a structural change that removes the R-region from its steric-interfering position and allows NBD dimerization, triggering a much larger conformational change [(23, 25-27)]. Then, the binding of ATP initiate channel opening while channel closure is associated with ATP hydrolysis and release of ADP and inorganic phosphate [(28,29)].

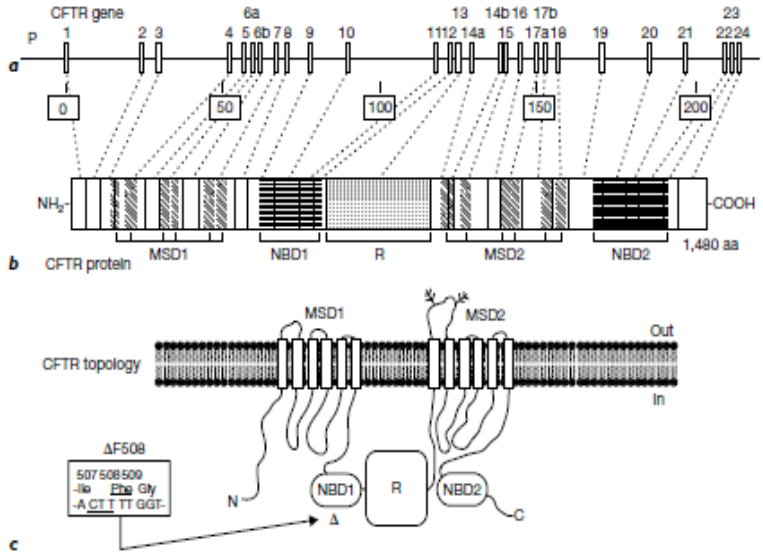


Figure 4. Schematic diagram of the CFTR gene. **a** Structure of the CFTR gene consisting of promoter region (P) and 27 exons. **b** CFTR polypeptide with predicted domains (highlighted). **c** topology of the CFTR protein relative to the cytoplasmic membrane and position of the most common mutation, _F508. Box: Deletion of 3 nucleotides, CTT (underlined), and subsequent loss of phenylalanine 508 (underlined).

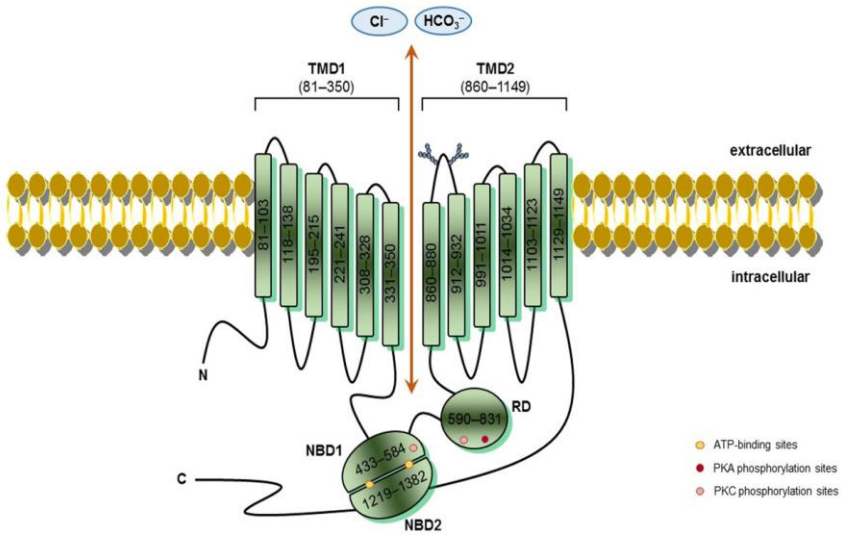


Figure 5. CFTR schematic structure – Cystic fibrosis transmembrane conductance regulator (CFTR) is a 1,480-amino acids protein inserted into the cell surface. CFTR possesses five domains: two transmembrane domains (TMD1/2), containing six hydrophobic alpha-helices, which cross the cell

surface lipid bilayer, and are joined by two intracellular loops and three extracellular loops, and with glycosylated residues linked in the extracellular loop 4 (N894, N900); two nucleotide-binding domains (NBD1/2) with highly conserved sequenced for ATP-binding, where occur hydrolysis; and one regulatory domain (RD) with multiple phosphorylation sites. CFTR channel open when protein kinase A (PKA) and protein kinase C (PKC) phosphorylate RD and ATPs bind to side chain charged amino acids in NBDs, thereby activating CFTR function. TMDs form the gate where occurs chloride conductance. The positions denoted into the boxes correspond to the first and last amino acid of each fragment and CFTR sequence was obtained in the Cystic Fibrosis Mutation Database (CFTR Modulators: Shedding Light on Precision Medicine for Cystic Fibrosis. M. Lopes-Pacheco. Front Pharmacol 2016).

In healthy subjects, *CFTR* is maintained in its correct apical plasma membrane location by the formation of a multiprotein complex made up of scaffold proteins (such as NHERF1) and signaling molecules (such as cAMP and protein kinases) guarantee its correct functioning [(12)].

In CF, a disorganized and dysfunctional airway epithelium brings an altered flux of ions and water into the lumen of bronchioles, consequent bacterial infections and influx of inflammatory cells (mainly neutrophils) into the airway lumen [(12)]. The lack/dysfunction of the *CFTR* protein causes the accumulation of sticky dehydrated mucus in various organs, such as lungs and pancreatic ducts. In the airways there is an abnormal ion and water flux consequent to decreased chloride secretion and hyperabsorption of sodium [(30)].

1.3 CFTR gene mutations and classes

The *CFTR* gene covers approximately 180,000 base pairs in the long arm of chromosome 7. The protein contains 1,480 amino acids. Up to date, more than 2000 *CFTR* mutations have been reported in patients with classic CF and CFTR-RD [(12, 28)]. Most mutations of the *CFTR* gene are missense alterations, but frameshifts, splicing, nonsense mutations, and inframe deletions and insertions have been described. [(32)]. About 15% of identified genetic variants are not associated with disease [(32)].

CFTR mutations are divided into six classes according to their effects on protein function (Figure 6).

Class I: *Defective Protein Synthesis*

Class I includes mutations which lead to the disruption of the CFTR protein synthesis. The mutations in this class include nonsense and frameshifts, which lead to the creation of premature termination codons. Genotype-phenotype studies revealed that CFTR premature termination codon mutations are associated with a severe form of the disease [(32)]. Common class I mutations are G542X (Britanny and Southern France), R1162X (Austria and Northern Italy), or W1282X (reaching 48% amongst Ashkenazi Jews) [(33)]

Class II: *Defective Protein Processing*

Class II mutations are associated with defective protein processing. Upon completion of the CFTR protein translation, the normal protein undergoes a series of processes in the endoplasmic reticulum (ER) and the Golgi apparatus. This includes glycosylation and folding that enable the protein trafficking to the apical cell membrane. Class II mutations cause impairment of this process, which leads to degradation of the abnormally processed protein. The major mutation, F508del, results in the synthesis of a CFTR protein that is unable to correctly fold into its appropriate tertiary conformation. Consequently, this protein is retained in the ER and abnormally degraded (99vs. 75% in normal proteins). [(32)]

Class III and IV: *Defective Protein Regulation and Altered Conductance*

Phosphorylation and dephosphorylation of the *CFTR* is considered the major pathway by which the chloride channel activity is physiologically regulated. Class III includes mutations that lead to the production of proteins (e.g. G551D and Y569D), which reach the plasma membrane; however, their regulation is defective and, thus, they cannot be activated by ATP or cAMP. Class IV mutations are associated with altered conductance (e.g. R347P, R117H and D1152H) thus, the rate of chloride transport is reduced. Thus, mutations in both class III and IV lead to a *CFTR* protein that can be produced, processed, transported and inserted into the apical membrane, but with a defective conductance.

Class V: *Reduced CFTR Level*

Class V mutations lead to the production of a normal protein, however at reduced levels. This class includes promoter mutations that reduce transcription and amino acid substitutions that cause inefficient protein maturation. Yet, most of the mutations are splicing mutations, which affect the normal splicing of the pre-mRNA and thus reduce the levels of correctly spliced mRNA, by partial exon skipping or inclusion of intronic sequences. These mutations account for 5% of *CFTR* mutations and can lead to variable levels of correctly spliced transcripts among different patients and among different organs of the same patient. [34,35] These levels were found to inversely correlate with the variable disease expression, such that lower levels of correctly spliced transcripts are associated with a severe disease, while higher levels are associated with milder disease. [36,37]

Class VI: *Reduced CFTR stability*

Class VI mutations decrease retention/anchoring at the cell surface, often associated with decreased protein stability at the plasma membrane. [38]

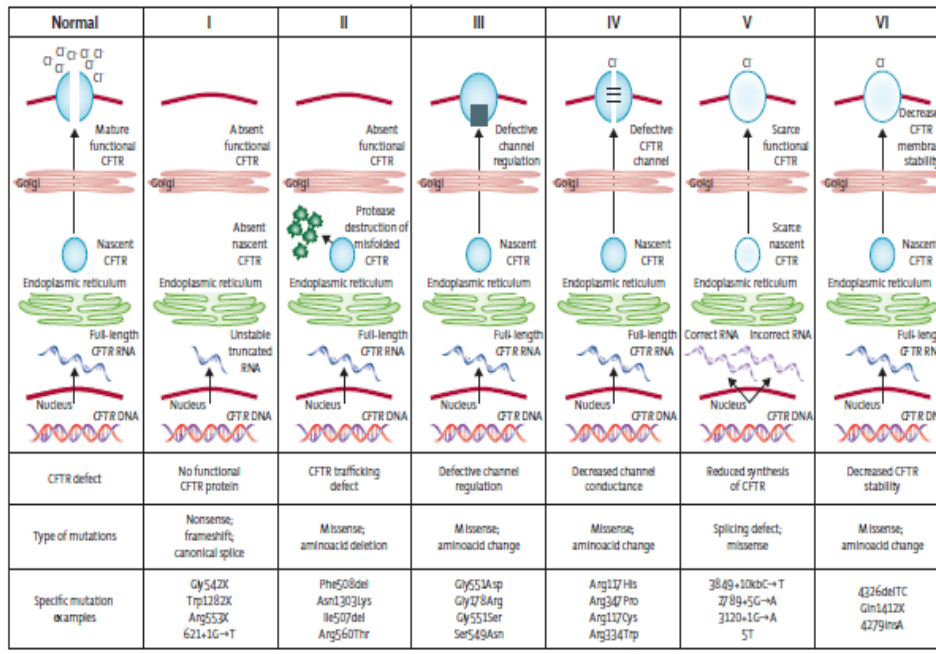


Figure 6. Classes of *CFTR* mutations

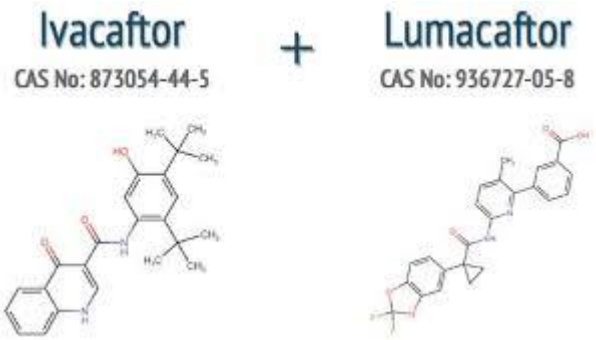
Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene can be divided into six classes. Class I mutations result in no protein production. Class II

mutations (including the most prevalent, Phe508del) cause retention of a misfolded protein at the endoplasmic reticulum, and subsequent degradation in the proteasome. Class III mutations affect channel regulation, impairing channel opening (eg, Gly551Asp). Class IV mutants show reduced conduction—ie, decreased flow of ions (eg, Arg117His). Class V mutations cause substantial reduction in mRNA or protein, or both, Class VI mutations cause substantial plasma membrane instability and include Phe508del when rescued by most correctors (rPhe508del).

1.4 CF mutation based therapy

The specific mutation, class and functional consequences are very important data to program treatments aimed to correct the basic molecular and cellular defects (i.e. mutation-specific therapies) [(39)]. In class I, for example, aminoglycoside antibiotics (e.g. gentamicin), can over-read the premature termination codons permitting the normal termination of the transcript. For Class II, chemical and molecular chaperones can avoid part of protein degradation in ER by promoting protein folding: the consequence is an increase in the protein presence in the cell surface. These compounds have been called correctors (e.g. VX-809, VX-661). In class III mutations, such as G551D, there is a class of molecules that have showed a therapeutic effect both *in vitro* and in clinical trials: these drugs, called potentiators (VX-770, ivafactor) work as activators of *CFTR* channel. In the class IV mutations, there is a reduction in the conductance that can be compensated by increasing the overall cell surface amount of these *CFTR* mutants with correctors, alternatively increasing the levels of channel activation (by potentiators). In class V mutations, there is the reduction of normal protein levels, often by affecting splicing and generating both aberrant and normal transcripts. So recent advances in the use of antisense oligonucleotide (AONs) make this promising approach a very powerful tool for the specific correction of misplicing. Meanwhile also potentiators and correctors may have some role.

Although the therapies have improved the life expectancy of CF patients, they are still non resolutive in many cases and, moreover, are unable to work for all mutation's classes.



1.5 Transcriptional regulation of CFTR

CFTR protein production is tightly regulated in the different tissues of human organism: it is mainly expressed in epithelial cells of respiratory and digestive systems, in the pancreatic duct epithelium and in the crypts of small intestine [40-43]. Low *CFTR* expression are described in many other tissue such as heart, brain, muscle [44].

The promoter of *CFTR* gene, like an housekeeping gene, is rich in CpG islands and has a lot of transcription start sites and multiple binding sites for putative transcription factors Sp1 and AP-1 [45,46]. Some authors showed that the inhibition of AP-1 binding site decrease *CFTR* transcription [46].

Promoters of constitutively expressed genes are often rich in CpG sites, generally not methylated, while tissue specific genes usually are poor in CpG sites, often not methylated [47]. There is an inverse correlation between CFTR promoter methylation and gene expression in cell lines [48].

The transcriptional regulation of *CFTR* also required a conserved sequence element CCAAT, named Y-box [49]. This Y-box element is crucial for the transcription regulation of *CFTR* gene mediated by transcription factor ATF-1 and human histone acetyl transferase protein GCN5 [49, 50].

Li et al. showed that levels of histone acetylation at the *CFTR* promoter are an indicator of *CFTR* transcription and that the effect of histone deacetylation by trichostatin A increases *CFTR* transcription [50].

1.6 Butyrate: mechanisms and effects

Among short chain fatty acid (SCFAs), butyrate has received a particular attention for its multiple beneficial effects, from the intestinal tract to the peripheral tissues. The butyrate is a fatty acid with numerous and diverse effects on cellular physiology [51]. It is a natural substance present in the gastrointestinal tract, in milk as well in the sweat and feaces of most mammals. Butyrate is naturally present in high concentration in the lumen of the large intestine: it is preferentially taken up by the colonic epithelium where is actively metabolized to produce energy [51].

Butyrate has multiple mechanism of action: many of these are directly correlate to its gene expression regulatory effects. In particular, butyrate is one of some molecules that have an important epigenetic influence known as histone deacetylase inhibitors (HDACi) [51].

Butyrate and phenylbutyrate are drugs already in use. One of their application is in neoplasia because for years have been shown to have HDACi activity. These SCFAs have HDACi activity at millimolar concentrations and have been described to have antiangiogenic and antimetastatic effects. Butyrate was found to repress angiogenesis, both in vitro and in vivo, and to reduce expression of proangiogenetic factors, including hypoxia inducible factors (HIF-1a) and vascular endothelial growth factor (VEGF) [53-55]. Butyrate also inhibits nuclear factor B (NFkB) activation, reduces the production of interferon gamma and the upregulation of peroxisome proliferator-activated receptor g (PPARg) by its inhibition of HDAC [51]. This confers to butyrate an anti-inflammatory effect that has been evaluated for its potential therapeutic capacity in the treatment of inflammatory bowel disease (IBD). Another important aspect of butyrate, actually under study, is its epigenetic regulation of the immune system, both innate and acquired. In particular, butyrate seems to play multiple anti-inflammatory activities by affecting immune cell migration, adhesion, cytokine expression and

modulating cellular processes such as proliferation, activation and apoptosis [56]. Finally, butyrate is used in some genetic diseases such as thalassemia or Congenital Chloridorrea. In thalassemia, butyrate showed the capacity to induce the synthesis of fetal hameoglobin (HbF) that is a therapeutic strategy for the treatment of beta thalassemia. Butyrate is also an inducer of SLC26A3 gene expression, whose mutations are involved in the pathogenesis of Congenital chloride diarrhea [51]. In conclusion butyrate is useful in the prevention and treatment of different chronic disorders such cancer, inflammatory disorders, cardiovascular diseases and immune-mediated chronic disorders [51].

1.7 Butyrate and CF

Butyrate and its derivatives have been investigated as potential molecules for the treatment of cystic fibrosis but their role in the disease and on *CFTR* regulation is still unknown. There are several sporadic studies that describe various aspects and effects of butyrate on *CFTR* gene expression, function or both [51, 55, 58, 61-65].

It is reported, for example, that butyrate can increase the protein trafficking pathway by manipulation of chaperone protein/*CFTR* interactions and then improve the *CFTR* activity in patient with class II mutations [51]. Some studies, in fact, demonstrated an increased production of mature *CFTR* and chloride transport at the cell surface [51]. The mechanism is still not fully understood but is probably due to an upregulation at the transcriptional level and modulation of protein folding step [51, 57]. Some data suggest that 4-Phenylbutyrate (4PBA) may improve *CFTR* trafficking by allowing a greater proportion of mutant *CFTR* to escape association with Hsc70 chaperone protein [58].

Singh et al, identified multiple putative butyrate-responsive cellular chaperones, protein processing enzymes and cell trafficking molecules associated with the amelioration of the chloride transport in cystic fibrosis bronchial epithelial cells

defect treating these cells with 4PBA and analyzing protein profiles by two-dimensional gel electrophoresis and mass spectrometry [59].

Others authors suggested that butyrate and the butyrate analog 4-phenylbutyrate may be therapeutic agents for treatment of CF in patients that carring the F508del CFTR mutation [61-63]. These molecules partially restored cAMP-activated Cl^- secretion in nasal, bronchial, and pancreatic epithelial cells expressing F508del CFTR by stimulating F508del CFTR gene expression and increasing the amount of F508del CFTR protein in the plasma membrane [61-63]. It was also demonstrated that butyrate increases apical membrane *CFTR* but reduces chloride secretion in MDCK cells: this inhibition of cAMP-stimulated Cl^- secretion across MDCK cells is in part caused by the reduction of the activity of the Na-KATPase [60].

One study quantifies the double effect of sodium butyrate and low temperature on *CFTR* protein expression at the cell surface of F508del cells demonstrating that these two interventions are synergistic regarding their ability to upregulate the expression of F508del *CFTR* [64].

Nguyen et al. showed that some short chain fatty acids can restore chloride secretion in cystic fibrosis [65]. In particular, they demonstrated that 2,2-dimethyl-butyrate and alpha-methylhydrocinnamic acid, exhibiting high oral bioavailability and sustained plasma levels, can correct the F508del *CFTR* trafficking defect [65].

2. AIM

In order to identify novel molecules involved in *CFTR* transcriptional regulation, we investigated a putative role of butyrate in the modulation of *CFTR* expression.

Butyrate is a short-chain fatty acid and it is already in use in medical care. It's reported to function as a modulator of *CFTR* and is involved in a variety of cellular events.

The aim of our project is to investigate the butyrate effects on *CFTR* expression by studying its molecular mechanism and in particular:

• evaluating the butyrate effect on promoter activity of *CFTR* gene in different cell lines

• looking for putative butyrate responsive element (BRE) in the promoter of *CFTR*

• defining its effect on deacethylation of *CFTR*

Given the role of butyrate in the regulation of *CFTR* gene expression, it is a candidate molecule to be used:

i) to enhance *CFTR* expression in patients with CF that bear mutation that permit some residual activity of the protein at membrane level

ii) in association with correctors and potentiators of *CFTR* protein

3. MATERIALS AND METHODS

3.1 Cell Culture

Cell lines were purchased from ATCC (Manassas, VA) and Lonza, SW. Human diploid fibroblast strain (IMR-90, ATCC number CCL-186), were grown in Eagle's Minimum Essential Medium (Gibco Invitrogen, Grand Island, NY). Normal human renal proximal tubule epithelial cells (RPTEC, Lonza, SW), were maintained in Renal epithelial cell basal medium (REBM Clonetics, USA), Human Bronchial Epithelial Cells (NHBE, Lonza, SW), in Bronchial Epithelial Cell Growth Medium (BEGM Clonetics, USA). Human colon primary cells were purchased by Celprogen, (CA) and seeding according to the manufacturer's. Cells were placed in CELL+T 25 flasks (Sarsdet) and maintained in culture with human colon cell culture complete growth serum free medium. Initial seeding density was 3000 viable cells/mm3 per flask.Medium was changed daily.

A549, Panc-1 and Caco2 cells were maintained in Dulbecco's modified Eagle's medium (Gibco Invitrogen) with 10% heat inactivated fetal bovine serum (HyClone), 2.5 mmol/L L-glutamine and 100 unit/ml Pen-Strep (Invitrogen). Cells were placed in Corning 100 x 20 mm Style Tissue Culture Dish in 5% (v/v) CO2 humidified atmosphere at 37°C. The medium was changed daily.

3.2 Real-Time PCR of CFTR

CFTR mRNA extraction and quantification

Total RNA is isolated from cells using TRIzol reagent (Invitrogen, Italy) according to the manufacturer protocol. RNA concentration and purity is determined with a NanoDrop ND-1000 spectrophotometer; reverse transcription is carried out on 1 μg of total RNA resuspended in DEPC-treated nano pure water using QuantiTect Rev Transcription Kit (Qiagen) using the protocol supplied by the manufacturer. To check levels of CFTR

transcripts in the cells, relative quantification by real-time PCR is performed in duplicates using LightCycler 480 Probes Master containing *CFTR* primers (Roche, Italy) and a TaqMan CFTR probe (ID. Assay 102716). Amplification is carried out with the LightCycler 480 Systems for Real-Time PCR (Roche) with a two-step PCR protocol (preincubation of 10 min at +95° C followed by 45 cycles of amplification: 95°C for 10 sec, 60°C for 25s, 72°C for 1s). mRNA quantification results are normalized using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene (Roche, ID. Assay 101128) as an endogenous control.

3.3 Treatment of cells with butyrate

Human primary cells were plated at a density of 4000 cells/cm^2 in CELL + T 25 flasks (Sarstedt Ltd, UK). The butyrate was added in fresh medium for 24 hours at sixth day, at different concentration. Cells were harvested for extraction of RNA and DNA. Control cells were incubated without the addition of butyrate.

3.4 Transfection and Luciferase Assay

Transfection of A549, CaCo2, Calu3 and Panc-1 cells with the 6 constructs (3kb, 2kb, 1kb, 0,5kb, 0,3kb and 0,1kb *CFTR* promoter cloned respectively into 6 different pGL3-basic luciferase vector) was performed with Attractene Transfection Reagent (Qiagen). Cells were seeded in 24-well plates and 24 hr after seeding the butyrate was added in fresh medium for 24 hours in half of transfected cells.

Twenty-four hours after treatment, cells were lysed and Renilla luciferase activities were determined using the Dual-Glo Luciferase Assay System (Promega Corporation). The relative reporter activity was obtained by normalization to the Renilla luciferase activity. Each experiment was done in triplicate and three independent experiments were performed for each construct tested.

3.5 Statistical Analysis

Comparisons of promoter activity between WT and variant CFTR promoter constructs from luciferase assays were conducted by using the unpaired t-test; data were considered statistically significant at $P < 0.001$; data are expressed as means ± StDv of three different assays.

4. RESULTS

4.1 Effect of butyrate on CFTR mRNA level

We tested the effect of butyrate on *CFTR* mRNA expression in different primary cells and in cell lines. In particular, all cells were exposed to standard or butyrate-containing medium, at different concentration, and the RNA was purified for Real-Time PCR to compare the levels of CFTR mRNA. As shown in Figure 7, CFTR mRNA expression in all cell types, except on Calu3 cell line, was significantly increased by butyrate treatment.

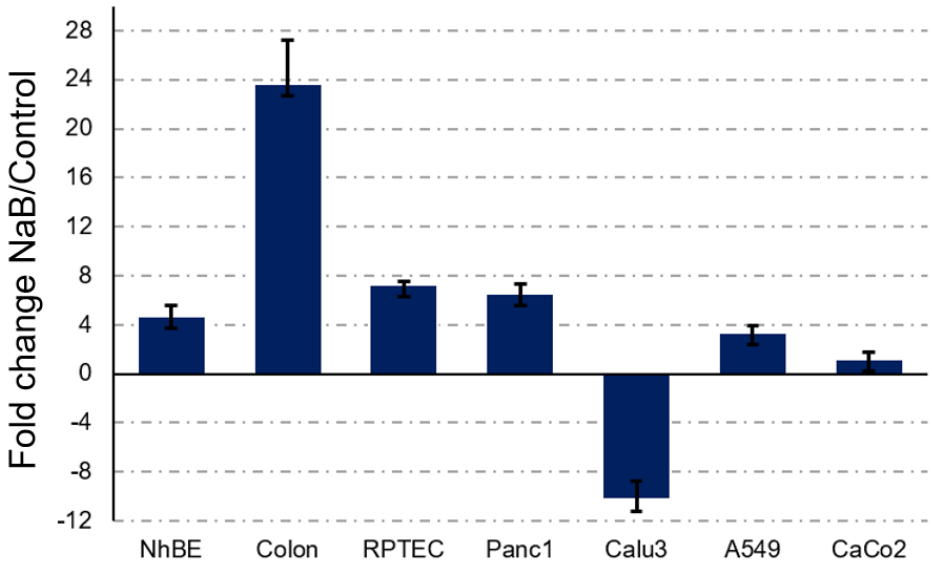


Figure 7. Effect of butyrate on the endogenous CFTR expression in human primary and cell lines. All cells were cultured in standard medium or butyrate-containing medium (5 mM, 24 h).

4.2 Promoter-reporter construct preparation

The human *CFTR* gene promoter constructs (pGL3b/CF-2910, pGL3b/CF-1899, pGL3b/CF-916, pGL3b/CF-410, pGL3b/CF-224, and pGL3b/CF-58) were made by PCR method using the primer pairs shown in table 1. Each promoter fragment

was amplified by PCR from human genomic DNA using primers with adapters containing Kpn I restriction enzyme sites in the forward primer and Xho I in the reverse primer.

CFPR150-Luc5`	GCAAAT**GGTACC**AAGGCGGGGGAAAGA
CFPR300-Luc5`	GCAAAT**GGTACC**TGGGAGTCAGAATC
CFPR500-Luc5`	GCAAAT**GGTACC**CTGACGCGAAGGAG
CFPR1000-Luc5`	GCAAAT**GGTACC**TGACTGAGACTAGG
CFPR2000-Luc5`	GCAAAT**GGTACC**TGTTTCACTCTTTCC
CFPR3000-Luc5`	GCAAAT**GGTACC**ACAGATTGTTTAGG
CFPRO-Luc3`	GATATT**CTCGAG**CAAGCTCCTAATGCC

Table 1. All primer used to amplify the CFTR promoter fragments. In red bold is shown the KpnI site and in blue bold the XhoI site.

Each PCR fragment was directionally cloned into the pGL3-basic vector and confirmed by sequencing Figure 8.

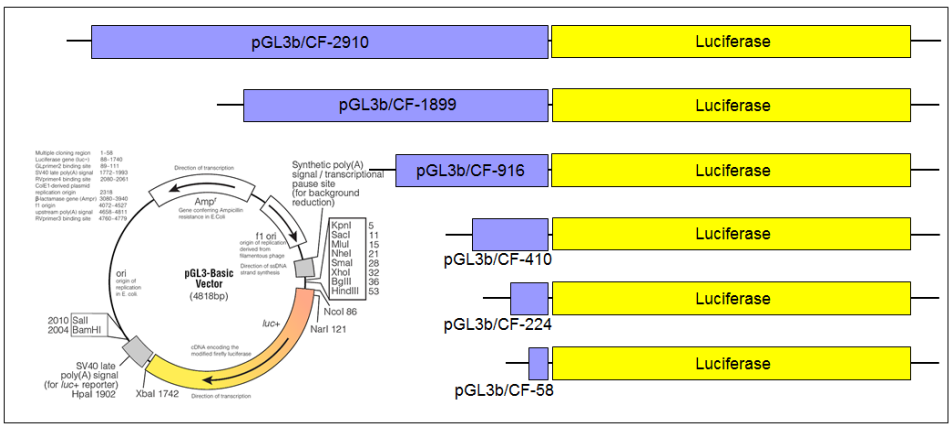


Figure 8. The figure is shown the six constructs with different fragments of CFTR promoter.

4.3 Effect of butyrate on human CFTR gene promoter activity.

To explore whether butyrate-mediated *CFTR* expression is due to increased gene transcription, we transfected different cell lines with our human *CFTR* gene promoter constructs followed by 24 h of treatment with 5 mM butyrate before analyzing promoter activity. In particular, we chose to test our constructs on Panc 1 and Calu3 cells. This is because *CFTR* expression, analyzed by qPCR assay, highlighted a different behavior in the two cell lines. In fact, the expression of *CFTR* increased in

Panc1 and greatly decreased in Calu3 cell line after treatment with sodium butyrate.

Unfortunately, although we had used several transfectants, from cationic liposomal formulation to electroporation methods, the Calu3 cell transfection efficiency did not have a good luciferase signal in contrast to Panc1 cells Figure 9. Surprisingly, no significant differences were observed between the different constructs.

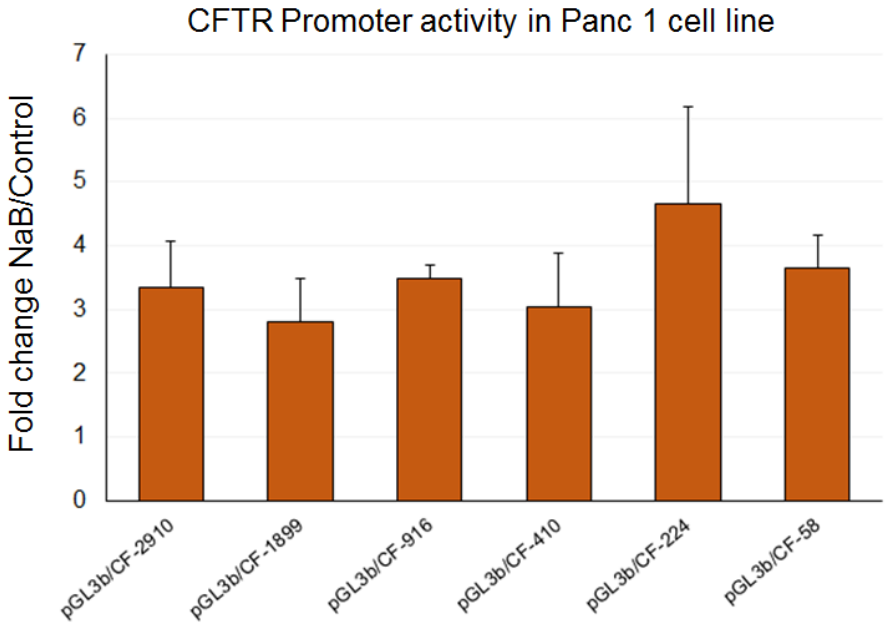


Figure 9. Effect of butyrate on human CFTR gene promoter activity. Cells were cotransfected with pRL-CMV and pGL3 basic (pGL3b) or human CFTR promoter constructs (pGL3b/CF-2910, pGL3b/CF-1899, pGL3b/CF-916, pGL3b/CF-410, pGL3b/CF-224, and pGL3b/CF-58). Butyrate was applied 24 h before measuring promoter activities and the degree of stimulation on CFTR promoter activity was considered as the ratio of relative activity in butyrate treated cells over relative activity in control cells. Results are means ± StDv from at least 3 different experiments.

4.4 Use of lentiviral particles for cell transduction.

In order to improve the Calu3 cell transfection efficiency, we decided to clone the expression cassette of the construct pGL3b/CF-2910, containing about 3kb of the CFTR promoter region and the luciferase coding region, in a lentiviral vector. To

transduce both Panc1 and Calu3 cells we used the supernatant of lentiviral, particles-producing 293T cells achieving good infection efficacy both in Panc1 and Calu3 cells.

As the use of lentiviral infections leads to the integration of the viral genome into the cell one, we used these cells, Panc1-CFTR-Luc and Calu3-CFTR-Luc, stably expressing the luciferase gene under the control of the CFTR promoter for our experiments with butyrate treatment. So, we treated Panc1-CFTR-Luc and Calu3-CFTR-Luc cells with butyrate for 24 hours before analyzing the luciferase activity. In Figure 10 is shown the luciferase activity from both cells after treatment with NaB in a dose dependent manner. The luciferase activity confirmed the endogenous CFTR expression in both cell lines.

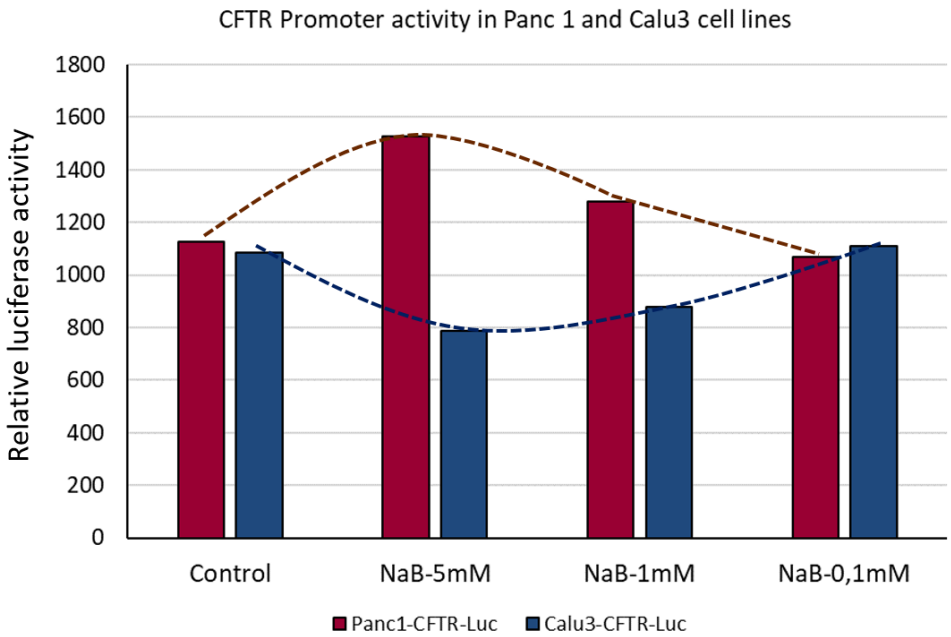


Figure 10. Luciferase activity in Panc1-CFTR-Luc and Calu3-CFTR-Luc cell lines. Contrary to Panc1 cells, 5 mM NaB treatment was able to suppress CFTR promoter activity in Calu3 cells by about 30%. Results are means ± StDv from at least 3 different experiments.

Phase contrast microscopic observations showed a dramatic decrease in the nucleus-cytoplasmic ratio in Panc1, wich seems to acquire a senescent-like morphology. Conversely, the N/C

ratio seems to increase to a lesser extent in Calu3 cells, which instead increase in Figure 11, suggesting a butyrate-mediated growth arrest of Panc1.

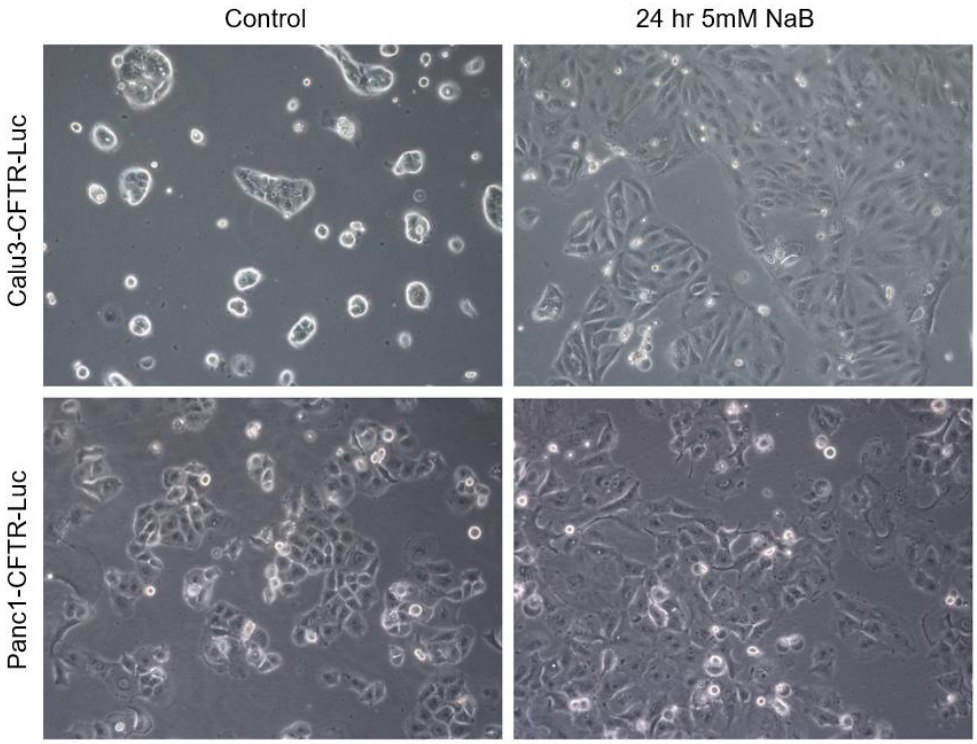


Figure 11. Treatment with 5 mM of sodium butyrate for 24 hr causes a large morphological and numerical change in Calu3 and Panc1 cells.

Indeed, several studies have reported the effect of butyrate in inhibiting cell growth [(63)]. So, we decided to analyze the expression of some genes involved in the cell cycle or in the process of senescence, a permanent condition of cell growth arrest, after 24 hr of butyrate treatment. Precisely, we have analyzed by qPCR the expression of cyclin A (CyclA), forkhead box-M1 (FOXM1), thymidylate synthase (TYMS), cyclin-selective ubiquitin carrier protein (UBE2C) and p21WAF1(p21) genes as marker of cell cycle arrest and interleukin-6 (IL-6) and β-galactosidase (GLB1) as marker of inflammation and senescence. In Figure 12 is shown the results of the qPCR analysis. After 24 hr of butyrate treatment we found a down-

regulation of Cycl A, FOXM1, UBE2C, and the up-regulation of p21 in Panc1 cell line according to a cell cycle arrest process. Conversely, in Calu3 cell line, we observed a

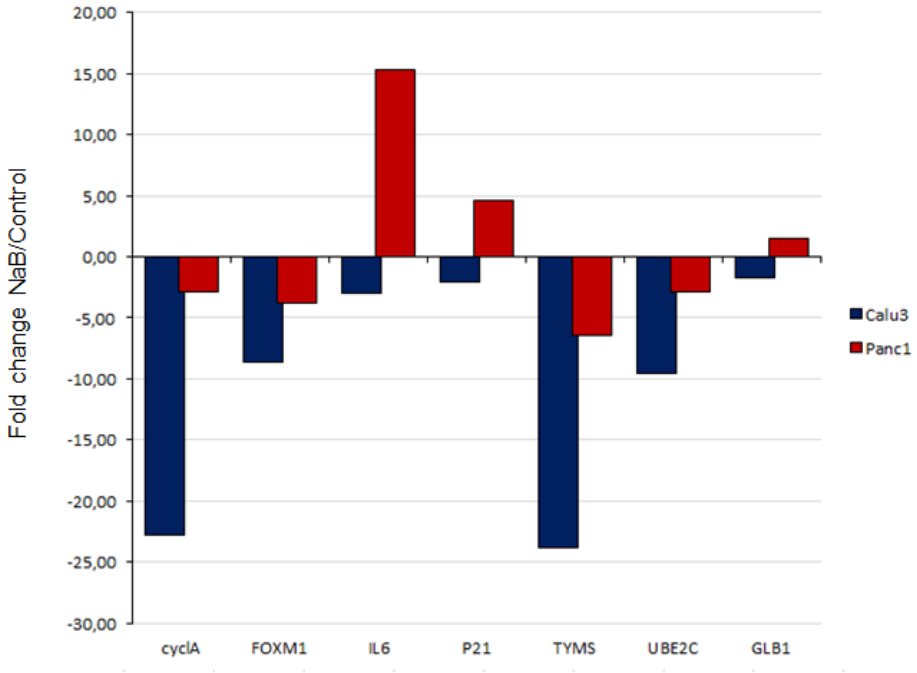
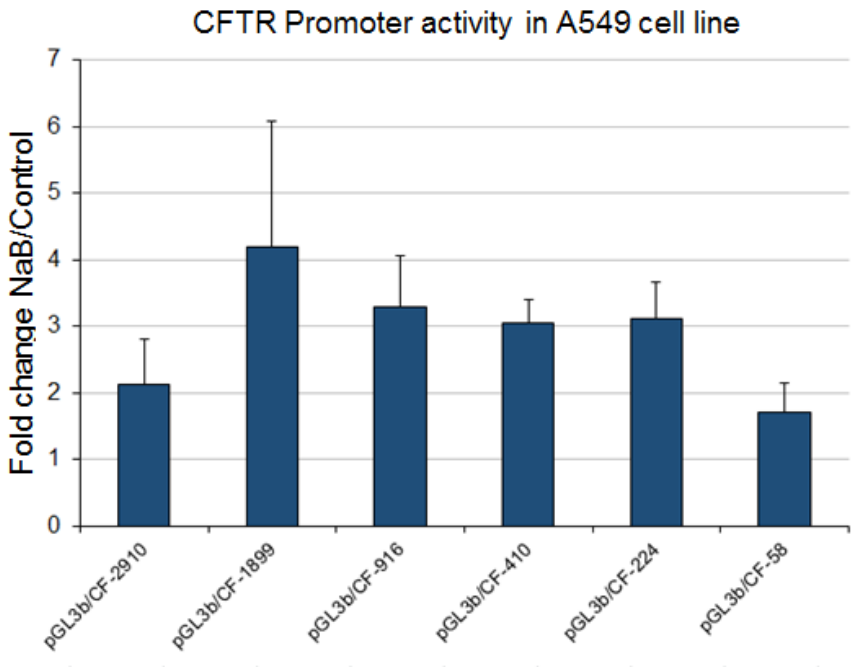


Figure 12. qPCR analysis of some cell cycle genes Effect of 24hr treatment with 5 mM sodium butyrate on endogenous CFTR expression in Panc1 and Calu3 cell lines.

down-regulation of CyclA, FOXM1, UBE2C, but not an up-regulation of p21 gene. Moreover, analyzing the IL-6 and GLB1 mRNA levels, we found both genes up-regulated in Panc1 cells and down-regulated in Calu3 cells

4.5 Effect of butyrate on human CFTR gene promoter activity in A549 and Caco2 cell lines.

To explore whether butyrate effect on CFTR gene promoter activity is cell line dependent, we transfected A549 and Caco2 cell lines with our human *CFTR* gene promoter constructs followed by 24 h of treatment with 5 mM butyrate before analyzing promoter activity.


CFTR Promoter activity in A549 cell line
Fold change NaB/Control
0
1
2
3
4
5
6
7
pGL3b/CF-2910
pGL3b/CF-1899
pGL3b/CF-916
pGL3b/CF-410
pGL3b/CF-224
pGL3b/CF-58

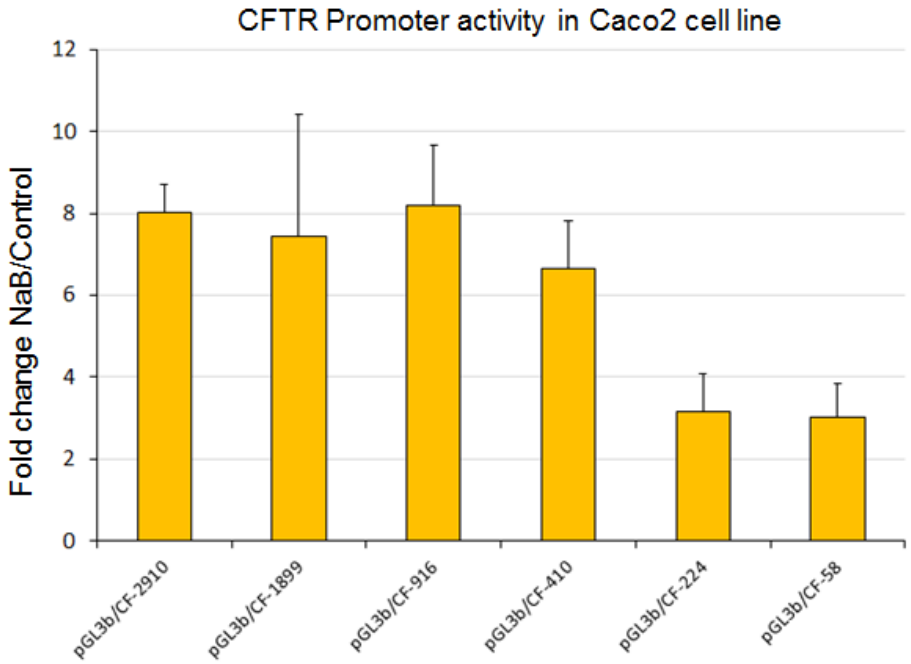

CFTR Promoter activity in Caco2 cell line
Fold change NaB/Control
0
2
4
6
8
10
12
pGL3b/CF-2910
pGL3b/CF-1899
pGL3b/CF-916
pGL3b/CF-410
pGL3b/CF-224
pGL3b/CF-58

Figure 13. Effect of butyrate on human CFTR gene promoter activity in A549 and Caco2 cell lines. Cells were cotransfected with pRL-CMV and pGL3 basic (pGL3b) or human CFTR promoter constructs (pGL3b/CF-2910, pGL3b/CF-1899, pGL3b/CF-916, pGL3b/CF-410, pGL3b/CF-224, and pGL3b/CF-58). Butyrate was applied 24 h before measuring promoter activities and the degree of stimulation on CFTR promoter activity was considered as the ratio of relative activity in butyrate treated cells over relative activity in control cells. Results are means ± StDv from at least 3 different experiments.

As shown in Figure 13, all reporter gene expression driven by human CFTR promoter were significantly induced in butyrate treated A549 and Caco2 cells. There was an induction of 2-8 fold in the promoter activity, without significant difference between the various constructs with different deletion mutants. This suggest that probably the effect of butyrate is due to its histone hyperacetylation action. So, to test this hypothesis, we treated the Caco2 cells, transfected with all set of constructs, with 1 μM trichostatin A (TSA) for 24 hr before the luciferase assay.

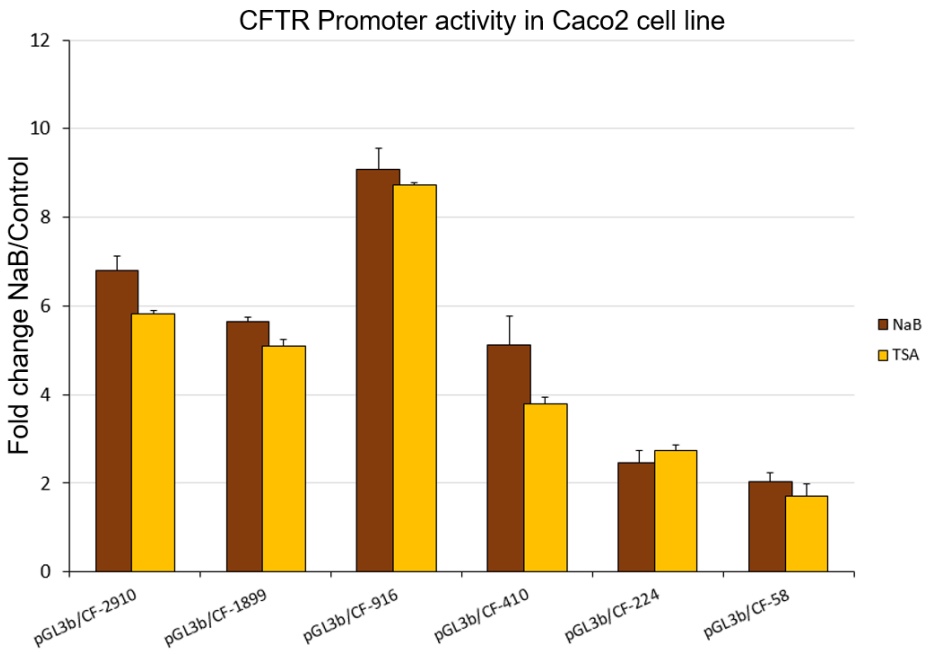


Figure 14. CFTR promoter activity in Caco2 cells after treatment with 1 μM of TSA or 5 mM of NaB for 24 hr before the luciferase assay.

As shown in Figure 14, each promoter activity was increased in a similar manner independently when treated with TSA or NaB, confirming the hypothesis that the effect of butyrate on CFTR promoter activity is due to its histone hyperacetylation action.

5. DISCUSSION

Cystic Fibrosis is one of the most important mendelian life shortening disease in the world and it is due to mutations in *CFTR* gene. Up to date, more than 2000 *CFTR* mutations have been reported and divided into six classes, according to their effects on protein function [12,31]. The specific mutation, class and functional consequences are very important informations because mutation based therapies become available in the last year. In particular, drugs known as potentiators and correctors are mainly used for the treatment of CF in patients with class II or III mutations [39]. Although the therapies have improved the life expectancy of *CFTR* patients, they are still not resolutive in many cases.

The aim of our work was to define the role of butyrate as a putative new drug for the therapy of CF by studying expression of *CFTR* gene. The expression of the *CFTR* gene is strictly tissue and cell specific. Of course, this tightly regulated expression is largely due to the promoter's activity [44]. The promoter of *CFTR* gene is rich in CpG island and has a lot of transcription start sites and multiple putative transcription factor protein binding sites [45,46]. Among these, we focused our attention on those that can be regulated by butyrate.

The butyrate is a fatty acid with numerous and diverse cellular effects with multiple mechanism of action: many of these are directly correlated to its important epigenetic function of histone deacetylase inhibitors (HDACi) [51]. Butyrate is reported to function as a potential useful molecule for the treatment of different diseases and it is already used in medicine. Several studies report that it has antiangiogenic, anti-metastatic, anti-inflammatory and immune-stimulations effects. Due to these capabilities butyrate finds application in treatment of some oncologic and autoinflammatory diseases [51,53,56]. Butyrate has also been proposed as a potential therapeutic drug in the therapy of some genetic disease such as Thalassemia or Congenital Chloridorrea and Cystic Fibrosis, because it had multiple effects both at genetic and epigenetic levels [51].

However, to date there are no studies showing whether butyrate has a direct or indirect action on the transcription promoter activity of the *CFTR* gene. Our project had investigated the butyrate effects on *CFTR* expression by studying its molecular mechanism.

We firstly showed that 24h of butyrate treatment causes a significant increase of *CFTR* in RPTEC, Colon and NHBe primary cell line and in Panc-1, A549 and Caco2 immortalized cells, while the *CFTR* mRNA level decrease in Calu3 cell line. In particular *CFTR* mRNA expression was increased by 2 or 4 fold up after butyrate treatment, except for Colon and Calu3 cells where the mRNA levels rise up than 20 fold and decrease down 8 fold respectively.

Once the butyrate-dependent activation of *CFTR* gene promoter was confirmed in primary and transformed cells, we constructed a series of *CFTR* promoter deletion mutants to try to locate butyrate responsive elements into promoter regions. The six constructs (pGL3b/CF-2910, pGL3b/CF-1899, pGL3b/CF-916, pGL3b/CF-410, pGL3b/CF-224, and pGL3b/CF-58) were transfected A549, Caco2 and Panc-1 cell lines, while lentiviral infection was used for Calu3 cells that resulted very difficult to transfect with. As expected, butyrate treatment increased *CFTR* promoter activity by up 2-4 fold in Panc1 and A549 and up 8 fold in Caco2 cells. However, smaller constructs (pGL3b/CF-224, and pGL3b/CF-58) have responded to butyrate treatment to a lesser extent as compared to the larger ones in Caco2 cells.

These experiments suggest that the effect of butyrate is due to its histone hyperacetylation activity rather than the presence of a specific butyrate responsive element in CFTR promoter region.

In fact, repeating the same experiment in Caco2 cells, treated this time with Trichostatin A, (TSA), a specific histone hyperacetylating agent, we note that the effect on the CFTR promoter is very similar to that of butyrate, confirming that the increase of the activity of the CFTR promoter is mainly due to histone hyperacetylation.

Concerning, the Calu3 cell line, the longer fragment of *CFTR* promoter (pGL3b/CF-2910) cloned into a lentiviral construct was down regulated by the butyrate treatment. Moreover, the observation of a dramatic decrease in the nucleus-cytoplasmic ratio in Panc1, similar to a senescent-like morphology (changes that were not observed in Calu3 cells) prompted us to analyze some genes involved in cell cycle arrest process. Indeed, several studies have reported the effect of butyrate in inhibiting cell growth [(66)]. The cell cycle is a set of events underlying cell proliferation. This complex machinery is characterized by cell cycle checkpoints that occur at the G1/S boundary, in S-phase, and during the G2/M-phases to monitor the fidelity with which genetic information is passed to future generations [(70)]. So, we decided to analyze the expression of cyclin A (CyclA), forkhead box-M1 (FOXM1), thymidylate synthase (TYMS), cyclin-selective ubiquitin carrier protein (UBE2C) and p21WAF1(p21) genes as marker of cell cycle arrest and interleukin-6 (IL-6) and β-galactosidase (GLB1) as marker of inflammation and senescence. Cyclin A can activate two different cyclin-dependent kinases (CDK1 and CDK2) and functions both in S phase and in mitosis. Its up-regulation could contribute to tumorigenesis (cell cycle proliferation) and its down-regulation is a symptom of cell cycle arrest (i.e. cellular senescence) [(68)]. FOXM1 (Forkhead box M1) is a proliferation-associated transcription factor, which regulates the expression of cell cycle genes essential for DNA replication and mitosis [(69)]. Thymidylate synthase (TYMS) is required for DNA replication and it plays an important role in the biosynthesis of the DNA-component thymidylate (dTTP). Its deregulation is an essential feature of cell cycle progression or arrest [(70)]. Cyclin-selective ubiquitin carrier protein (UBE2C) is involved in cell cycle progression because of its role in cyclin B destruction that is essential for exit from mitosis. Furthermore, UBE2C plays a key role in mitotic spindle checkpoint control [(71)]. The p21 (WAF1/CIP1) is a cell-cycle inhibitor directly controlled by p53 and/or p53-independent pathways. It is involved in maintaining the G1cell-cycle arrest when the checkpoint is triggered by

different stimuli such as by DNA damage, in a p53-dependent fashion or during cellular differentiation, by p53-independent pathways [72, 73]. After 24 hr of butyrate treatment we found a down-regulation of Cycl A, FOXM1, UBE2C, and the up-regulation of p21 in Panc1 cell line according to a cell cycle arrest process. Conversely, in Calu3 cell line, we observed a down-regulation of CyclA, FOXM1, UBE2C, but not an up-regulation of p21 gene. Moreover, analyzing the IL-6 and GLB1 mRNA levels, we found both genes up-regulated in Panc1 cells and down-regulated in Calu3 cells. Although partial, analysis of some cell cycle genes seems to indicate that 24 hr treatment with 5 mM of sodium butyrate causes cell cycle arrest in Panc1, and probably in other cell types analyzed where there is an increase in CFTR expression, and a partial proliferative effect, or no effect, in Calu3 cells.

6. CONCLUSION

In conclusion, we showed that the increased butyrate-dependent *CFTR* expression described in literature and confirmed by our study is mainly due to the histone hyperacetylating activity of butyrate and not as occurs for SLC26A3 gene, for the presence of butyrate responsive element in the *CFTR* promoter. Thus, these data suggest that butyrate, or butyrate derivatives, may be used as agents to increase expression of *CFTR* under certain clinical conditions.

Of course, this work represents the beginning of understanding a specific aspect of the transcriptional regulation of the *CFTR* gene that needs to be thoroughly investigated.

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