Targeting ENaC as a Molecular Suspect in Cystic Fibrosis

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Abstract: Cystic fibrosis (CF) is the most common life shortening autosomal inherited disorder, affecting 1 in 2500 newborns in the Caucasian population. In CF the lung pathology is associated with dehydration of the airways epithelial surface which in part results from Na⁺ hyperabsorption via the epithelial sodium channel (ENaC). The molecular mechanisms of this Na⁺ hyperabsorption and its correlation with the underlying genetic defect in the cystic fibrosis transmembrane conductance regulator (CFTR) are not fully understood. However, it is obvious that a reduced Cl⁻ secretion by CFTR and an enhanced Na⁺ absorption through ENaC lead to the so far incurable disease. Therefore, it could be indicated to pursue a double-tracked strategy in that way enabling Cl⁻ secretion by a reconstitution of the defect CFTR as well as blocking ENaC to prevent Na⁺ hyperabsorption. Since the cloning of CFTR great efforts have been done in delivery of CFTR for the correction of the reduced Cl⁻ secretion. Positive benefits for the inhibition of the CF related Na⁺ hyperabsorption offer technologies using small molecule inhibitors like ASOs or siRNA, which target translation and knockdown of ENaC, respectively. In this review we discuss possible CFTR/ENaC interactions in the context of CF, describe ENaC structure as well as some of the numerous attempts that were performed to prevent the Na⁺ hyperabsorption in CF related lung disease. Thus, we give a short summary of e.g. amiloride therapy approaches and focus on inventive blocking efforts using ASOs and siRNA.

Keywords: Amiloride, ASOs, cystic fibrosis, CFTR, ENaC, siRNA.

REVIEW ARTICLE

The cystic fibrosis transmembrane conductance regulator (CFTR) and the amiloride-sensitive epithelial sodium channel ENaC are located at the apical membrane of epithelial cells and maintain the first step in Na⁺ absorption and Cl⁻ secretion. This very fine tuned process is regulated by the Na⁺/K⁺-ATPase in the basolateral membrane. In case this carefully regulated system is disturbed by a dysfunction of one or two of these ion channels fatal consequences occur. The autosomal recessive genetic disease cystic fibrosis (CF) results from this kind of channelopathy. CF is caused by mutations in the CFTR gene which prevent proper functions of CFTR as a Cl⁻ channel and central regulatory protein. This results in an imbalance of Cl⁻ secretion and Na⁺ absorption and leads to transport defects in many different organs, but predominantly in the airways. There is a reduction of airway surface hydration which leads to an inefficient mucociliary clearance [1] following colonization by pathogens like Pseudomonas aeruginosa and Staphylococcus aureus which cause a permanent inflammation of the lung and thus airway obstruction (reviewed by Davis 2006, Cystic fibrosis since 1938). It was shown by many groups that besides this defect CFTR function the sodium transport mediated by ENaC is altered. Nearly twenty years ago it was already demonstrated that Na⁺ reabsorption was enhanced in CF airways [2, 3] followed by the idea that this Na⁺ hyperabsorption plays an important part in CF pathophysiology [4-7]. As it was shown that CF symptoms in the lung occur when one of the ENaC subunits is overexpressed in mice and this concept seems to be quite assuring [8]. This β-ENaC overexpressing mice was proposed to be the best model of CF disease, since all transgenic CF mice generated before did not provide a good model for the typical CF respiratory pathology (reviewed by [9]). Unfortunately, these β-ENaC overexpressing mice do not enable the possibility to study the dysfunctions caused by mutant CFTR [10], and an overexpression of CFTR failed to correct this CF-like lung disease [11].

Moreover, there is also no ideal cell line expressing all the defects of CF. Although there are some cell lines that express CFTR mutations such as the CFBE41o- cell line [12], yet these cells express only a low amount of functional ENaC measured as amiloride-sensitive transepithelial current, probably because these cells were generated from a very young child before the onset of CF symptoms [13]. Transfection of cells with mutant CFTR results in defects of the CFTR protein and absent Cl⁻ secretion, but to the best knowledge, Na⁺ hyperabsorption could never be detected in those transfected cells. Therefore, the only system that comes close to the CF situation is the primary cultured cells from CF patients and from non-CF patients that serve as controls [9, 14].

ARE THERE CFTR/ENaC INTERACTIONS?

There is still a controversial debate in how far CFTR and ENaC interact with each other and to which extent impaired Cl⁻ secretion is responsible for Na⁺ hyperabsorption.
Some groups used the oocytes of Xenopus laevis for a heterologous expression of ENaC from several tissues. Co-injection of CFTR-mRNA and mRNA coding for the α-, β- and γ-ENaC subunits leads to the functional co-expression of the particular proteins in the plasma membranes of the oocytes. Briel et al. [15] and Chabot et al. [16] showed that activation of CFTR by cAMP inhibited ENaC expressed in the same oocytes. Yet, it could be shown shortly after publication of the two papers that the apparent inhibition of ENaC current was artificial as a result of excessively large series resistance (R_s) that both groups failed to monitor during their experiments [17]. This large R_s led to considerable voltage-clamp errors and one of the groups supported this conclusion in an erratum [18]. Monitoring and compensating R_s and minimizing voltage-clamp errors during the measurements four groups showed independently that CFTR, whether activated by cAMP or not, had no inhibitory influence on ENaC [19, 20]. Furthermore, it has to be mentioned here that expression systems such as the Xenopus laevis oocytes are somewhat artificial when studying possible CFTR-ENaC interactions. In the recent years it has become even more clearer that there is no such ‘typical ENaC’. Indeed, expression and the modus operandi of ENaC are highly dynamic and vary depending on the physiological situation of the cell and the surrounding tissue. Especially the composition and the stoichiometry of the ENaC-subunits seem to be highly dependent on the specific tissue where ENaC is located. Depending on the specific physiological demands ENaC may vary form, function and regulation of the channel by other proteins such as proteases [21] second messengers (e.g. cAMP; [19]), ATP [22], kinases and ubiquitination [23], and many other intra- and extracellular factors. Furthermore, trafficking of ENaC into the apical plasma membrane and retrieving into intracellular pools are highly dynamic and subject to tight regulation. In the light of these findings putative CFTR-ENaC interactions should be only investigated in native tissue naturally expressing both proteins. Therefore, results obtained with Xenopus oocytes or any other artificial expression system cannot be transferred to the situation in the human respiratory tract or other organs. Even results from human kidney cells do not necessarily mirror the situation in any other organ. Yet, since these data are missing, the deficit is highlighted that ENaC stoichiometry and regulation in native tissues and especially in human airway epithelia is not well understood.

One model for CFTR-ENaC interactions postulated direct interactions between the two proteins yet all attempts to prove that hypothesis such as co-immunoprecipitation and the yeast-two-hybrid system failed [23]. A strong hint that CFTR and ENaC do not interact physically with each other at least in the respiratory tract and the oviduct came from data by Enuka et al. [24]. They showed that ENaC and CFTR are not located close to each other but are spatially separated from each other. They showed that ENaC molecules are uniformly distributed on the ciliary surface in the epithelia lining the bronchus in human lungs, while CFTR molecules are expressed at the apical membrane but not on cilia. Therefore, this spatial separation in human respiratory epithelia makes direct interactions between the two channel proteins impossible and contradicts the hypothesis that CFTR is down-regulating ENaC by direct protein-protein interactions.

Another possible way about how CFTR and ENaC interact with each other could be through non-direct interactions mediated by other proteins or other regulatory factors. It is one of the regulatory mechanisms that intra- or extracellular proteases activate ENaC by endoproteolytic cleavage [25]. One report showed that CFTR could act on proteases that activate ENaC by selective endoproteolytic cleavage [26]. The authors suggest that intact CFTR impedes the proteolytic stimulation of ENaC, while ΔF508 CFTR fails to protect ENaC from proteolytic cleavage and subsequent activation. The authors conclude that physical narrow co-localization of ENaC and CFTR could influence signaling cascades that regulate and control trafficking and transport patterns of ENaC in non-CF cells. Since ENaC and CFTR are spatially separated in human respiratory epithelia [24], this line of evidence does not seem to be quite convincing.

Yet, from all those data we cannot deduce a general model of CFTR-ENaC interactions.

In summary, there are certainly interactions between CFTR and ENaC of direct or indirect nature but we do not know currently the mechanism(s). There are still ongoing vivid discussions about that matter, however, no final solution has been obtained. A fact is that ENaC and its resultant Na^+ hyperabsorption contribute to the pathogenesis of CF, and the still unclear structure and stoichiometry of ENaC make it even more complicated to understand its exact influential impact.

**ENaC IS A HETEROMULTIMERIC PROTEIN - WHICH SUBUNIT SHOULD BE TARGETED?**

Initially, three subunits of ENaC were identified referred to as α, β and γ which form a heteromultimeric protein, with α as the pore-forming subunit and β and γ appeared to be the regulatory ones [27]. It could be shown that ENaC, which is a member of the ENaC/Degenerin ion channel family, is voltage-independent, highly Na^+-selective with a low single channel conductance and sensitive to the diuretic amiloride. Later on, these three subunits were detected in many different epithelial tissues and it was obvious that not all subunits were expressed at the same time in the same tissue [28, 29]. Therefore, it was assumed that the expression as well as the assembly is tissue specific. Still, stoichiometry is a matter of discussion but since Jasti et al. determined the crystal structure of chicken ASIC1 [30], a neuronal acid-sensing ENaC/DEG channel, it is proposed that ENaC could have a trimeric structure, containing 1α, 1β and 1γ subunit [31]. The situation turned more complicated when the fourth subunit, already identified and characterized as δ-ENaC subunit in 1995 [32] attracted more interest. This subunit is broadly expressed in tissue of non-epithelial and epithelial origin (for reviews refer to [33, 34]). This δ subunit can substitute the α subunit and may form homomeric channels as well as in combination with β and γ. Although delta shares 37% amino acid sequence identity with the alpha subunit, it differs in tissue distribution and channel properties. This subunit is predominantly expressed in tissues not directly related to Na^+ absorption as brain, testis, and ovary [32] but in respect to the CF associated Na^+ hyperabsorption in the airways which is quite interesting, that the subunit was detected in the lung [35] and in primary cultures of human na-
enal epithelia [36]. There, it could be shown by knocking out this subunit with specific siRNA that nearly 50% of amiloride-sensitive Na\(^+\) absorption contributed to δ-ENaC in functional Ussing chamber measurements. Nevertheless, α-ENaC remains to be the subunit that is targeted by several attempts to block ENaC expression in order to prevent Na\(^+\) hyperabsorption in CF.

**AMILORIDE AND ITS ANALOGS - STILL A POSSIBLE THERAPY?**

As described above the proper function of ENaC is impaired in cystic fibrosis airway epithelia causing a gain of function and Na\(^+\) hyperabsorption. Therefore, an attractive approach towards this aspect of cystic fibrosis would be to block Na\(^+\) hyperabsorption and the resulting dehydration and deficient mucociliary clearance via inhalation of potential non-toxic ENaC inhibitors, recently partly reviewed by [37].

ENaC is effectively blocked by the synthetic pyrazinoylguanidine amiloride and its synthetic analogs. The diuretic amiloride inhibits ENaC specifically at a concentration in the micromolar range and the inhibition is rapidly reversed after removal of amiloride from the bathing solution. Its high specificity for ENaC at micromolar doses without affecting other ion channels or transport proteins makes amiloride unique: there is no such highly specific blocker for other ion channels. Only in higher concentrations above 100 \(\mu\)M amiloride affects other transport systems such as the Na\(^+\)/H\(^+\) antiporter, the Na\(^+\)/Ca\(^{2+}\) exchanger and Na\(^+\)-cotransporters [38]. The carbonylguanidino substituents benzamil and phenamil have even higher specificity and affinity (IC\(_{50} \approx 10\) nM) for ENaC with potencies that are tenfold (benzamil) and seventeenfold (phenamil) higher as compared with amiloride [38].

In the 1980s, it has been shown that inhalation of a single dose of amiloride improved mucociliary and cough clearance in patients with cystic fibrosis [39]. The same group extended later their study to a three-week trial of nebulized amiloride twice daily [40]. Hofmann et al. amended the technique of amiloride delivery and let the patients inhale high doses (i.e. in the millimolar range) of aerosolized amiloride [41]. The authors showed that the inhalation of aerosolized amiloride had clear positive effects on the bioelectric parameters and sputum expectoration was improved in cystic fibrosis patients. However, the beneficial effects of amiloride for the patients lasted only around two hours and the improvements were only seen in patients younger than 15 years old [40]. Safety and efficacy of nebulized amiloride versus placebo inhalation was further demonstrated in a double-blind, placebo-controlled, cross-over pilot study with cystic fibrosis patients chronically colonized with *Pseudomonas aeruginosa* [42]. During the 6-month course of this pilot study amiloride normalized the electrolyte composition of airway secretions and improved mucociliary and cough clearance. Furthermore, viscosity of airway secretions was reduced and the loss of pulmonary function was slowed down during the course of the study. In a follow up, another randomized, double-blind, placebo-controlled, cross-over trial, however, revealed no therapeutic benefit of inhalation of nebulized amiloride [43]. Yet, another study contradicted these findings and demonstrated reduced mucus viscoelasticity and increased mucus clearance after nebulized amiloride inhalation as compared with placebo [44]. Interestingly, sputum water content did not improve during the 25-week, four times a day with amiloride therapy, arguing for a more complex mechanism for mucus improvement by amiloride. The positive outcome of these clinical trials was confirmed and extended by other studies that included additionally the high affinity amiloride analogs benzamil and phenamil [45, 46]. Benzamil even increased the beneficial effects to about seven hours [46]. At the moment, the interest in further investigating amiloride and its analogs as possible therapies for the treatment of cystic fibrosis lung disease seems to have fallen since no new data on clinical trials have been published. Furthermore, a recent survey covering four clinical trials with inhaled amiloride failed to demonstrate any beneficial effects by inhalation of sodium channel blockers [47].

Interesting insights into possible beneficial effects of amiloride came from an animal model. The above mentioned genetically modified mice that overexpress the ENaC β-subunit develop similar symptoms such as airway surface liquid depletion and chronic bronchitis as also seen in human cystic fibrosis lung disease [8]. Treatment of these animals with amiloride was completely ineffective when the mice had already developed respiratory disease symptoms. However, when the animals were treated with amiloride shortly after birth the treatment with the sodium channel blocker could not only prevent the development of chronic changes in the lung but also showed positive influence on the survival rate [48]. It will be interesting to see whether early amiloride treatment of cystic fibrosis patients before the onset of severe clinical symptoms could show more positive effects than those obtained in treatments of patients with fully developed disease. On the other hand, it might be possible that blocking the ion channel function of ENaC alone is not enough and it could be imaginable that a successful therapy must involve the reduction of ENaC protein level as recently suggested [14].

**ASOs AS EFFECTIVE ENaC BLOCKER IN CF ASSOCIATED LUNG DISEASE**

The ENaC hyperabsorption caused by CFTR dysfunction seems to play a certain role in the underlying CF pathogenesis and gives rise to identify global players for the down regulation of ENaC. Therefore, constitutive inactivation of ENaC could reveal an important role in designing strategies to inhibit ENaC function that may result in clinical benefit. Several compounds have been developed to inhibit ENaC (reviewed by [37]). Thus, small molecule drugs became more focused in disease treatment. Some years ago the inhibition of gene expression by the use of antisense oligonucleotides (ASOs) has become a valuable tool in many different studies and clinical trials. ASOs are short synthetic DNA molecules (15-18 bases), complementary to specific mRNA sequences and offer the ability to prevent the functional expression of the target protein. Depending on their sequence and chemical structure ASOs can generate gene silencing by e.g. degradation mediated by RNase H activity, by modulating splicing activity or by polyadenylatational site selection [49]. The first ASO based drug Formiviren® (Vitravene® by Isis Pharmaceuticals, Carlsbad, CA, USA) was approved almost 15 years ago [50]. The last years have succeeded in gaining a huge
interest in ASO based technologies and numbers of new ASO studies have been designed. By now ASO based therapeutics enter also the fields of e.g. inherited neurodegenerative diseases like Alzheimer’s disease (AD), Huntington disease (HD) or Amyotrophic lateral sclerosis (ALS) [51], and ASOs are also generated for the treatment of cardiovascular, metabolic and cancer related diseases. Two different ASO based drugs were recently approved by Isis Pharmaceuticals. KY-NAMRO® targets the apolipoprotein B (apoB) and results in a reduction of low density lipoprotein-cholesterol (LDL-C), apoB and total cholesterol (TC). The other approved drug, named Alicaforsen® inhibits the production of the intracellular adhesion protein ICAM-1 which is affected in different inflammatory disorders (http://www.isispharm.com/ Pipeline/index.html).

With regards to an ENaC inhibition for a CF therapeutic effect a precise design of potential ASOs is absolutely essential to achieve a sufficient ENaC down regulation. Since a complete inactivation of the three prominent ENaC subunits (α,β,γ) leads to early postnatal death, it turned out that the down regulation of the α-ENaC subunit is essential for the ENaC channel in lung fluid clearance [52].

Earlier investigations of the ENaC amiloride-sensitive transepithelial potential difference in α-ENaC knockout mice showed that the ENaC activity was completely prevented by the ASOs [53]. This demonstrates a potential benefit of ASOs directed against the α-ENaC subunit for the treatment in cystic fibrosis therapy by inhibition of the underlying Na⁺ hyperabsorption. Former investigations in rat alveolar type II cells also showed that ASOs against the α-ENaC decreased the lung epithelial cation-channel activity by reducing the channel density in the plasma membrane, whereas the inhibition of the β and γ subunits of ENaC showed no effects [54]. Moreover, recent studies clearly confirmed this specific down regulation of ENaC by ASOs directed against α-ENaC in therapeutically relevant human primary nasal epithelial cells [14]. Using Ussing chamber measurements it was shown that these ASOs effectively repress the amiloride-sensitive Na⁺ absorption mediated by ENaC in CF by about 75% and in non-CF tissues by about 66%. Furthermore, the used ASOs sustained this functional ENaC inhibition for more than 72 h, which is considered a long-term benefit in CF treatment by increasing the ASL hydration. More lately, the use of RNA interference (RNAi) appeared to be an alternative tool for the inhibition of gene expression and also entered the potential CF therapeutic treatment. RNAi is a natural mechanism of gene silencing using short double stranded siRNA and results in RNA-induced gene silencing which is comparable to the ASO based method. Since some concerns about the influence on the innate immune system have been raised [55], siRNA needs to be chemically modified to improve stability and reduce toxicity and immunogenicity. There are a numbers of studies and clinical trials in the field of RNAi therapeutics but nearly all are still in the pipeline [56, 57]. But previous approaches have already show a potential effect for the treatment of cystic fibrosis by the use of ENAC siRNA [58, 59]. In another broad-based study a selective siRNA could be identified, which is a potent negative regulator of ENaC on mRNA and on protein levels. Furthermore, this siRNA was able to functionally inhibit ENaC in human primary epithelial cells with a long lasting effect of nearly 72h. Positive immunogenicity screenings showed that the toll-like receptors (TLR3, TLR7 and TLR8) were not stimulated in primary cells and cell lines. Moreover, in vivo studies using a lipid nanoparticle formulation resulted in an inhibition of ENaC expression in mice lungs [60]. When referring to siRNA and RNAi mechanisms, there are several small molecule based technologies that could be mentioned for their potential benefit in CF therapeutics, although most of them target the correction of the mutated CFTR and the resulting defect in CF secretion. A more potent RNAi effect could also be achieved by the use of the precursor form, the short-hairpin RNA (shRNA), which could result in a long lasting suppression with a comparatively low rate of degradation. This vector based RNAi mechanism could be useful especially for rapidly dividing cells and could be promising in cancer therapies [61, 62].

Moreover, the use of another RNA type was already described related to CF therapy. It could be shown that the expression of different microRNAs (miRNAs) could influence the CFTR mRNA expression and that distinct miRNA concentrations are increased in CF epithelia [63]. miRNAs are small endogenous RNA molecules which regulate post-transcriptional gene expression and therefore became increasingly interesting as post-translational regulators. Therefore, mRNA modulation of miRNA by replacement of miRNA (premiRs) presents a new therapeutic approach in CF research [64]. Although toxicity and immunogenic aspects of all these RNA based approaches still raise safety concerns, a lot of these technologies are under positive evaluation so far. It seems very likely that small molecule inhibitors like ASOs or siRNA could reveal positive beneficial effects for the therapy of cystic fibrosis by inhibiting the Na⁺ hyperabsorption of ENaC. Nevertheless, the delivery of such nucleotides is still a hurdle to overcome. Different formulations and delivery techniques for a target uptake and distribution need to be validated for in vitro and in vivo applications. Although very small, as compared to e.g. plasmid DNA, these nucleotides have to pass different barriers for the treatment of CF lung disease. In vitro studies demonstrated that the use of lipid based formulations like cationic lipids or liposomes is a possible way to transfect the target cells under cell culture conditions by reaching quite good transfection efficiencies [14, 60, 65], but this could not be the method of choice. Since in the end the nucleotides need to be taken up into the airway epithelium, different more stable formulations like nanoparticle or exosome based systems must be validated for inhalation and nebulization to target intratracheal routes. The biocompatibility is a huge issue and a number of different colloidal nanocarriers, like polymers (biopolymers e.g. chitosan) or peptides were taken under extensive revision (reviewed in: [66]). The delivery of nanoparticles to the respiratory epithelium especially the lungs has shared a growing interest but for the success of a delivery system thorough biophysical and chemical analysis need to be carried out to address the problems that these kind of respiratory targets afford.

DOUBLE-TRACKED STRATEGY TO TARGET ENaC AND CFTR

Taken together ENaC is an attractive target and the described attempts are auspicious. Nevertheless, the underlying defect is caused by a mutation in the CFTR gene, and there-
fore it seems likely to restore its function and consequently Na⁺ hyperabsorption would not even arise. There have been numerous approaches to reconstitute CFTR function in general or to bring a functional protein into the cell using gene therapy techniques [67, 68]. In the case of the most common CFTR mutation (ΔF508) misfolding of the protein leads to its retention in the endoplasmatic reticulum (ER) and proteosomal degradation [69]. Consequently, the majority of CFTR molecules failed to reach the plasma membrane resulting in strongly reduced chloride conductance. Therefore, the restoration of the CFTR function is another possibility to circumvent the underlying CF defects. For this reason, several therapeutic approaches focus on so-called corrector compounds which aim to reconstitute the trafficking and insertion of the CFTR protein. In addition, the enhancement of the inserted CFTR activation is investigated by the use of potentiators [70]. In the last few years, our group was working on a new approach using mRNA instead of DNA to bring functional CFTR into the affected cells [71]. Thus, we transfected cells which express AF508 CFTR (CFBE41o- cells) or just a low amount of CFTR (primary cells of human nasal epithelium) with modified wtCFTR-mRNA. Amongst others we carried out functional measurements using Ussing chambers. In contrast to the non-transfected control cells, where no CFTR Cl⁻-conductance could be detected, the transfected cells showed an increased CFTR current, conductance and capacitance after CFTR activation by cAMP. Remarkably, CFTR currents in wtCFTR-mRNA transfected cells were approximately identical to cells natively expressing CFTR (16HBE14o-). This method has several advantages compared with a DNA based approach, e.g. the nuclear envelope, one of the major obstacles to non-viral transfection, could be circumvented and the risk of insertional mutagenesis might be avoided. Furthermore, the viral vector induced immunogenicity should be avoided but has to be elucidated in more detail in upcoming experiments. Nevertheless, we demonstrated a strategy for the delivery of CFTR-mRNA directly to epithelial cells. Meanwhile, it was successfully demonstrated that the CFTR function can be restored using this method, so far we could not determine any effect on ENaC after reconstitution of CFTR neither in the cell line nor in the human primary cells that were used (data not published). However, this can be due to the above mentioned matter of fact that the CFBE41o- cells do not show an adequate amiloride-sensitive current in general. Furthermore, the primary cultured human cells indeed showed a good amiloride-sensitive current but rarely Cl⁻ secretion [6, 14]. Therefore, the primary culture should be a better choice with regard to the question if the restored CFTR function influences ENaC. But the reason why this Na⁺ hyperabsorption appears in the pathogenesis of CF is still open. And, as it has been shown by others that the hyperabsorption develops within the clinical pattern of CF (reviewed by [72]) and therefore a long-time study is required to address the issue, if a CFTR reconstitution affects ENaC activity or expression. But here the circle around the first main theme can be closed again. There is no sufficient cell culture or animal model that resembles both ion channel abnormalities that can be found in CF lung disease.

Nevertheless, it should be an advantage to pursue a double-tracked strategy in that way enabling Cl⁻ secretion by a reconstitution of the defect CFTR as well as blocking ENaC to prevent Na⁺ hyperabsorption. Finally, the issue remains unsolved unless both methods are applied and proofed in CF patients and both approaches should be traced, maybe combined?

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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