Inhibition of Na\(^+\)/H\(^+\) exchanger isoform 3 improves gut fluidity and alkalinity in cystic fibrosis transmembrane conductance regulator-deficient and F508del mutant mice

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Background and Purpose: Constipation and intestinal obstructive episodes are major health problems in cystic fibrosis (CF) patients. Three FDA-approved drugs against constipation-prone irritable bowel syndrome were tested for their ability to increase luminal fluidity and alkalinity in cystic fibrosis transmembrane conductance regulator (CFTR) null (cftr\(^{-/-}\)) and F508del mutant (F508del\(^{mut/mut}\)) murine intestine.

Experimental Approach: Guanylate cyclase C agonist linaclotide, PGE\(_1\) analogue lubiprostone and intestine-specific NHE3 inhibitor tenapanor were perfused through a \(\sim\)3 cm jejunal, proximal or mid-distal colonic segment in anaesthetized cftr\(^{-/-}\), F508del\(^{mut/mut}\) and WT mice. Net fluid balance was determined gravimetrically and alkaline output by pH-stat back titration.

Key Results: Basal jejunal fluid absorptive rates were significantly higher and basal HCO\(_3^-\) output was significantly lower in cftr\(^{-/-}\) and F508del\(^{mut/mut}\) compared to WT mice. In cftr\(^{-/-}\) and F508del\(^{mut/mut}\) mice, all three drugs significantly inhibited the fluid absorptive rate and increased alkaline output in the jejunum and tenapanor and lubiprostone, but not linaclotide, in the colon. After tenapanor pre-incubation, linaclotide elicited a robust fluid secretory response in WT jejunum, while no further change in absorptive rates was observed in cftr\(^{-/-}\) and F508del\(^{mut/mut}\) jejunum, suggesting that the increase in gut fluidity and alkalinity by linaclotide in CF gut is mediated via NHE3 inhibition. Lubiprostone also inhibited fluid absorption in cftr\(^{-/-}\) and F508del\(^{mut/mut}\) jejunum via NHE3 inhibition but had a residual NHE3-independent effect.

Conclusion and Implications: Linaclotide, lubiprostone and tenapanor reduced fluid absorption and increased alkaline output in the CF gut. Their application may ameliorate constipation and reduce obstructive episodes in CF patients.
1 | INTRODUCTION

With markedly improved clinical outcomes for lung disease and therefore much longer life expectancy in cystic fibrosis (CF) patients, CF-related intestinal disease becomes an increasing burden for many patients. Malnutrition in CF patients has been attributed to pancreatic insufficiency, low duodenal and upper jejunal pH and has indeed been amenable to pancreatic enzyme substitution and gastric acid blockade. In contrast, the incidence of distal intestinal obstruction and chronic obstruction increases as patients reach adulthood and is particularly high in CF patients with severe phenotypes that have received a lung transplant (Abraham & Taylor, 2017; Houwen et al., 2010; Mavilia, 2019). A recent database search confirmed CF-related intestinal obstructive episodes in the CF gut. However, it has also been shown that NHE3 expression and/or function is down-regulated in CFTR-deficient small intestine (Gawenis et al., 2004). In the isolated small intestinal mucosa of CFTR-deficient mice, cAMP analogues did not inhibit $^{22}\text{Na}^+$ absorption (Clarke & Harline, 1996) and this was explained by a lack of CFTR-mediated cell shrinkage, with shrinkage being the NHE3-inhibiting function (Gawenis et al., 2003). However, we had previously observed an electroneutral cAMP-dependent increase in luminal alkalinization in CFTR-deficient colonic epithelium that was inhibited by the NHE3 inhibitor S1611 (Xiao, Li, et al., 2012). We therefore considered it important to test the ability of currently available drugs, with proven or suspected NHE3 inhibitory activity, to increase luminal fluidity and alkalinity in the small and large intestine of mice that express either no CFTR protein or the F508del mutant protein.

CFTR-deficient mice do not show major histopathological alterations in their lungs and pancreas, which is possibly related to the expression of alternative anion channels in those organs (Clarke et al., 1994). In contrast, they are strongly affected in the intestine and the majority of them die of intestinal obstructions during the weaning period, if not on lifelong treatment with osmotic laxatives (Clarke, Gawenis, Franklin, & Harline, 1996). Interestingly, they also show a marked survival benefit if the major intestinal sodium absorptive transporter, the NHE3 (Slc9a3) Na/H exchanger, is knocked out (Bradford, Sartor, Gawenis, Clarke, & Shull, 2009). This suggests that an inhibition of fluid absorption may also improve intestinal obstructive episodes in the CF patient. What is already known

- Constipation, inflammation and intestinal obstructive episodes are major health problems in cystic fibrosis patients.

What does this study adds

- This study investigates the ability of three FDA-approved drugs to increase gut fluidity and alkalinity.
- Experiments were carried out in mice expressing no CFTR protein or the F508del mutant protein.

What is the clinical significance

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CFTR (cystic fibrosis transmembrane conductance regulator) channel is strongly expressed in all segments of the mammalian intestine, with crypt predominance (Ameen, Alexis, & Salas, 2000; Strong, Boehm, & Collins, 1994). CFTR activation, followed by fluid and $\text{HCO}_3^-$ secretion will result in a “flushing” of the crypts and is necessary for proper mucus granule expulsion (Liu, Walker, Ootani, Strubberg, & Clarke, 2015), mucus release from the membrane (Schutte et al., 2014), and mucus expansion and hydration (Yang, Garcia, & Quinton, 2013). In CF patients and CF animal models, diluted and mucus filled cryptal lumina and signs of mucosal inflammation have been observed, which are considered to be major drivers of mucus hypersecretion and intestinal obstruction (Munck, 2014). The restoration of the flushing effect in the cryptal base will only be possible with new treatment strategies that target the basic defect (Burgener & Moss, 2018) or with an activation of crypt-expressed “alternative” anion channels (Li, Salomon, Sheppard, Mall, & Galietta, 2017). Whether these recently introduced therapies that target the basic defect have any effect on intestinal secretory function in patients with CF-related intestinal obstructive disease is unknown.

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NHE3 is inhibited by a variety of agonists that result in an increase in enterocyte cAMP, cGMP or Ca$^{2+}$ levels (Zachos, Tse, & Donowitz, 2005). Linaclotide binds to the guanylate cyclase C (GC-C) in the apical membrane of enterocytes and results in cGMP production, which stimulates enterocyte CFTR-mediated anion secretion and presumably inhibits NHE3-mediated salt absorption (Steinbrecher, 2014). Lubiprostone is a PGE$_1$ analogue and chloride channel activator and does not elicit a short circuit response in the intestine of CFTR null mice (Ambizas & Ginzburg, 2007; Bijvelds, Bot, Escher, & de Jonge, 2009, Figures S3 and S4). Tenapanor is an intestinal-selective NHE3 inhibitor (Spencer et al., 2014). All three agents result in low systemic exposure and have been approved for
the treatment of constipation-prone irritable bowel (IBS-C) syndrome. Despite an interest to apply these agents in the fight against CF-associated intestinal disease (De Lisle, Mueller, & Roach, 2010; McHugh et al., 2018; O’Brien, Anderson, & Stowe, 2011), the efficacy of these agents to increase gut fluidity and alkalinity in vivo is currently unknown. Therefore, small and large intestinal fluid movements and bicarbonate output were assessed in cftr−/−, F508delmut/mut and WT mice in vivo.

2 | METHODS

2.1 | Materials

All reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany) and Applichem (Darmstadt, Germany) unless mentioned otherwise. Linacotide and lubiprostone was from Cambridge Bioscience (Cambridge, UK). Tetrodotoxin was from Tocris Bioscience (Bristol, UK). Tenapanor was from Adooq Bioscience (Irvine, CA, USA).

2.2 | Animals

All experiments involving animals were approved by the Hannover Medical School Committee and an independent committee assembled by the local government. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020). The application and permission numbers are Az. 33.14-42502-04-14/1549 and Az 33.12-42502-04-19/3197 for breeding “stressed strains” and Az 33.9-42502-04-18/2829 for experimental procedures. Experiments were performed with wild type (WT) and CFTR null (cftr−/−) mice (FVB/N-CFTRtm1CAM) (Ratcliff et al., 1993), as well as with mice that were homozygous for the F508del mutation (F508delmut/mut) (FVB/N(B6)129P2-CFTRtm1Eu) (French et al., 1996). The strains had been bred for >10 generations on a congenic FVB/N background at Hannover Medical School. For each experimental animal, an age- and sex-matched littermate was raised under identical breeding conditions. Special breeding conditions for the mid-distal colonic segment (beginning of the distal half of the colon) from the base of crypt to the surface. Fifteen sections were prepared from the base of crypt to apex of villus in small intestine and for the mid-distal colon (beginning of the distal half of the colon) from the base of crypt to the surface. Fifteen sections were prepared from three mice from the intestine of cftr−/− and from the respective WT mice (three sections per mouse intestinal segment), and 15 sections from three mice from the intestine of F508delmut/mut and WT, respectively (five sections per mouse). Three to six villi and up to 10 crypts were measured and averaged for every section. Statistical evaluation was done with the Wilcoxon rank test.

2.3 | Histochemistry

Colonic tissues were harvested and fixed in 4% paraformaldehyde (PFA); 3 μl paraffin-embedded sections were stained for the different mucins with Alcian blue/periodic acid-Schiff stain (AB/PAS) previously described (Xiao, Juric, et al., 2012). For the determination of the crypt-villus or crypt length, the indicated crypt-villus length for the jejunum is from the base of crypt to apex of villus in small intestine and for the mid-distal colon (beginning of the distal half of the colon) from the base of crypt to the surface. Fifteen sections were prepared from five mice from the intestine of cftr−/− and from the respective WT mice (three sections per mouse intestinal segment), and 15 sections from three mice from the intestine of F508delmut/mut and WT, respectively (five sections per mouse). Three to six villi and up to 10 crypts were measured and averaged for every section. Statistical evaluation was done with the Wilcoxon rank test.

2.4 | Quantitative PCR

Gene expressions of the pro-inflammatory cytokines TNFα and IL-1β, as well as of NHE3, were analysed in different intestinal segments of cftr−/−, F508delmut/mut and the respective WT littermates by qPCR using β-actin and ribosomal protein S9 (Rps9) as reference genes. RNA extraction, cDNA transcription and qPCR analysis were performed as per manufacturer’s instructions. Briefly, the total RNA was extracted using RNeasy® Mini Kit (Qiagen GmbH) and the quality was assessed using QIAxcel RNA QC Kit v2.0 (Qiagen GmbH); 1 μg RNA was then reverse transcribed with the QuantiTect® Reverse Transcription Kit (Qiagen GmbH). cDNA was diluted 1:40 with DNase free water and 4 μl of the dilution was used as a template for PCR. Each reaction additionally contained 5 μl 2x qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems) and an appropriate amount of primers. (Table S1).

2.5 | In vivo fluid transport measurements by single pass perfusion of a jejunal, proximal-mid or mid-distal colonic segment

The in vivo fluid transport experiments were performed as previously described (Xia, Yu et al., 2014), with slight modifications: Mice were
anaesthetized with isoflurane (Forene, Abbott, Wiesbaden, Germany) via tracheal intubation and connected to a mechanical ventilator (MiniVent Type 845, Hugo Sachs Elektronik, March Hugstetten, Germany). Respiration rate and TV (tidal volume) depended on the weight of mice according to the recommendations from the “Operating Instructions for the Mouse Ventilator MiniVent Type 845.” The respiration rate ranged from 140 per min to 150 per min, and tidal volume (TV) ranged from 200 to 250μl, respectively, and was set according to the manufacturers’ suggestions according to the individual mouse weight. The anesthetized mice received metamizol (200mg/kg) in 30μl saline subcutaneously and were laid on a heating pad to maintain the body temperature at approx. 37.5 °C, which was monitored by a rectal thermistor probe (except experiments in mid-distal colon, because the anus was occupied by tube for collecting the outflow). The depth of anaesthesia was tested by probing the pedal withdrawal reflex. After the reflex disappeared, the concentration of isoflurane in the anaesthetic gas mixture was reduced from 5% to 2.5%

A catheter was placed in the left carotid artery to monitor BP and to allow continuous infusion of an isotonic sodium carbonate solution ([in mM] 200 Na+ and 100 CO32−) at the rate of 0.1ml10g−1 h−1 to maintain systemic acid base balance. Another catheter was placed in the left femoral vein for continuous infusion of saline solution at the rate of 0.2ml h−1 for compensation of the fluid loss during the procedure to ensure haematocrit stability throughout the experiment.

The abdomen was opened along the abdominal midline. For preparing the jejunal segment, the small intestine was ligated at a distance of 8 to 10 cm from the pylorus to prevent pancreatic juice and bile from entering the intestinal segment. For the influx, a small incision was made close to the ligature along the anti-mesentery side and a small polyethylene tube (inner diameter 1 mm) was inserted and secured by ligature for collecting the outflow. Another small incision was made along the anti-mesentery side at a distance of 3 cm from the influx, and a small polyethylene cannula (inner diameter 2 mm) was inserted and secured by ligature for collecting the outflow.

For preparing the proximal colonic segment, the first incision was made close to the cecum, and the second incision approx. 2 cm distally. For preparing the mid-distal colonic segment, the first incision was made in the middle of the colon (~ 4 cm from the cecum) and the second catheter for the outflow was advanced through the anus and secured intraperitoneally. Each intestinal segment had intact blood supply and was gently flushed before perfusion. 30 min was allowed for the stabilization of cardiovascular, respiratory and intestinal functions before the experiments were commenced. The intestinal segment was perfused with an unbuffered solution, pH titrated to 7.4, 37°C, consisting of NaCl 145.5mM, KCl 4.0mM, CaCl2 1.2mM, at a rate of 30 ml h−1. For selected experiments, a 5%CO2/95%O2-gassed, HCO3− buffered perfusate (NaCl 121mM; NaHCO3 24mM; KCl 4.0mM; CaCl2 1.2mM), or an O2-gassed, HEPES/Tris buffered perfusate (17mM HEPES/3mM Tris, 125mM NaCl, KCl 4.0 mM; CaCl2 1.2mM) was utilized. All effluents from the isolated intestinal segment were visually free of bile and blood throughout all experiments. The rate of fluid absorption was calculated according to the weight of the influx and the outflow (effluent). Thus

\[ R_1 = \frac{(W_1 - W_2)}{L \cdot T} \cdot \frac{T}{1} \cdot \frac{1}{T} \cdot \frac{1}{1} \],

where \( R_1 \): the rate of fluid absorption (μl cm−2 h−1), \( T \): time (h), \( W_1 \): the weight of influx (g), \( W_2 \): the weight of outflow (g), \( L \): the length of segment (cm), \( P \): density of fluid (1g cm−3). The rate of luminal alkalization was determined by the back titration of the effluents to pH 5.0 with 5mM HCl.

After each experiment and prior to killing, a sample of arterial blood was taken and used for blood gas analysis in a radiometer blood-gas analyser (Radiometer, Kopenhagen) and haematocrit measurement in a haematocrit centrifuge (Hematokrit 210, Hettich, Kirchhengern, Germany). The haematocrit was measured in a series of mice before and after the experiment, and subsequently in each mouse after the end of the experiment, before killing.

### 2.6 | Ussing chamber experiments

Because of considerable discrepancies in the literature regarding the mechanism of action and the dependency of the anion secretory effect of lubiprostone on CFTR expression (Mizumori, Akiba et al., 2009; Bijvelds, Bot et al., 2009; Musch, Wang et al., 2013; Cuppoletti, Chakrabarti, et al., 2014), we performed experiments in muscle-stripped and chemically denervated jejunal, proximal and distal colonic mucosa to study the concentration-response curve, the sidedness of its action, and its effect in cfr−/− and F508delmut/mut mucosa (suppl. Figures 3,4). The experiments were performed as recently described (Liu, Tan et al., 2020). Muscle-stripped jejunum, proximal, and distal colonic mucosa was mounted in Ussing chambers with an exposed area of 0.126 cm2. To ensure a purely epithelial action, the muscle layers harbouring the mesenteric plexus neurons were stripped and 10−6M tetrodotoxin was added to the serosal side to chemically denervate the tissue. The serosal solution contained (in mM) 108 NaCl, 25 NaHCO3, 3 KCl, 1.3 MgSO4, 2 CaCl2, 2.25 KH2PO4, 8.9 glucose and 10 sodium pyruvate, and was gassed with 95%O2/5%CO2 (pH 7.4). Tetrodotoxin (1μM) and indomethacin (3μM) were added serially. The mucosal solution contained (in mM) 146 NaCl, 5 KCl, 2 CaCl2, and was gassed with 100% O2. After basal parameters were measured for 30 minutes, the respective drugs, as indicated in the figure legends, were added to the mucosal or serosal solution. Bicarbonate secretion (JHCO3−) was continuously titrated for the subsequent minutes of the experiment. Transepithelial short circuit current was calculated as μEq.cm−2 tissue surface area. Measurements of JHCO3− were continuously recorded and averaged for 5-minutes periods and expressed as μmol.cm−2.h−1. The peak electrical response was averaged over 60 s and taken as Δshort circuit current response.

### 2.7 | Statistics

The study design included an a priori power analysis to determine the sample size for each experiment/subgroup. We used data from prior/similar experiments to determine the effect sizes and SDs. These were used in a power analysis with the software G*Power (Faul, Erdfelder, Lang, & Buchner, 2007). To determine the necessary sample sizes, we
assumed a type I error of $\alpha = 5\%$ and a statistical power of at least 80%. The Ko/WT and mut/WT pairs were allocated to the experimental groups by a different person than the experimenter and this person also re-genotyped the mice after killing and allocated the genotype to the mouse number. A fully blinded experiment is not possible, because the experienced experimenter has a high chance of correctly guessing the genotype based on the different phenotypes of Ko and WT mice. Sample sizes were calculated as biological replicates and were allocated equally to the experimental groups. Studies were designed to generate groups of equal size; however, due to removal of individual data sets if the experiment had not met the predefined experimental quality standard (i.e., pre-PCR RNA quality check not satisfactory for isolated samples, arterial pressure drop during intestinal perfusion experiment, electrical resistance in Ussing-chamber experiments not stable throughout the experiment, death of mouse during the operative procedure), slightly uneven numbers could occur. Data are presented as means ± SEM, with the number of experiments (WT and KO pairs) given in parenthesis. For the comparison of rates between genetically altered and respective WT littermates, the rate in the time period before the addition of the drug and the rate in the time period of maximal alteration after drug addition were selected (marked with brackets) for each animal and these differences were compared to the equally treated group of WT littermates using the paired Student's $t$-test. In contrast, the fluid absorptive and $\text{HCO}_3^-$ secretory rates for all mice of each genotype are averaged for each time point and each bar indicates the mean and SEM for that time point. Significant differences are indicated with a star (NS, not significant; $^*P < 0.05$). For comparison of basal rates between different intestinal segments within one genotype, the one-way ANOVA for multiple comparisons was used ($^#P < 0.05$), with the Tukey’s post hoc analysis only if the $F$ value of the ANOVA reached significance. Homoscedasticity for ANOVA groups were tested with the Brown–Forsythe test in

![Image](https://via.placeholder.com/150)

**FIGURE 1** mRNA expression proinflammatory cytokines and of Na$^+$/H$^+$ exchanger NHE3, in cftr$^{−/−}$, F508del$^{mut/mut}$ and the respective WT intestine. (a, b) TNF$\alpha$ and IL-1$\beta$ mRNA expression was not different between cftr$^{−/−}$, F508del$^{mut/mut}$ and the respective WT intestinal segments. The proximal and distal colon of TNF$\Delta^{−/−}$ mice, which displays mild inflammation (Juric et al., 2013; Xiao, Juric, et al., 2012) was used as positive control tissue. (c) No difference in NHE3 mRNA expression in the different segments of cftr$^{−/−}$, F508del$^{mut/mut}$ and the respective WT intestine. Each value is shown individually and the bars represent mean and SEM. $N = 6$ (cftr$^{+/+}$ and cftr$^{−/−}$), $n = 5$ (F508del$^{mut/mut}$ and WT)
GraphPad Prism 7. For the evaluation of the crypt-villus length measurements, the Wilcoxon test was used. A minimum number of 5 was included in each group. The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos, et al., 2019; Alexander, Mathie, et al., 2019).

3 | RESULTS

3.1 | No evidence for intrinsic inflammation and NHE3 down-regulation in cftr−/− and F508delmut/mut intestine

Since the aim was to study the regulation of fluid and electrolyte movement in the absence or dysfunction of CFTR, unaffected by secondary alterations of the mucosa, optimized breeding conditions had previously been developed. A group of WT and cftr−/− littersmates, as well as WT and F508delmut/mut littersmates, respectively, were killed and expression of mucosal proinflammatory cytokines and of NHE3 mRNA was studied (Figure 1a–f). Distal colonic mucosa from TNFΔκB mice, which display their maximal inflammation in the distal ileum and show very mild distal colonic inflammation (Kontoyiannis, Pasparakis, Pizarro, Cominelli, & Kollias, 1999) was chosen as a control. TNFα and IL-1β mRNA expression was low in all studied intestinal segments and not different between WT and ko/mutant mucosa (Figure 1a,b). Likewise, Cd3 mRNA expression, a T-cell marker, was not elevated (Figure S2). The NHE3 mRNA expression was not different between WT and ko/mut mouse (Figures 1c and S2). A difference in the goblet cell phenotype, with slightly larger thecae, as well as villus and crypt elongation was observed in cftr−/− and to a lesser degree in F508delmut/mut intestine (Figure 2).

3.2 | Higher fluid absorptive and lower HCO3− output rates in cftr−/− and F508delmut/mut intestine in vivo

Basal fluid absorptive rates in WT mice were relatively similar in the different segments, whereas the basal HCO3− output rates were
significantly different between each intestinal segment (Table 1). During perfusion with unbuffered saline, the basal fluid absorptive rates were significantly higher in the jejunum and proximal colon but not in the distal colon in both cftr\(^{-/-}\) and F508del\(^{mut/mut}\) compared to WT mice. The HCO\(_3^{-}\) output rates were significantly lower in all studied segment of the cftr\(^{-/-}\) and F508del\(^{mut/mut}\) than in the respective WT mice. The basal fluid absorptive rates and HCO\(_3^{-}\) output rates were not significantly different between F508del\(^{mut/mut}\) and cftr\(^{-/-}\) intestine.

TABLE 1 Basal fluid absorptive and HCO\(_3^{-}\) output rates in cftr\(^{-/-}\), F508del\(^{mut/mut}\) and the respective WT jejunum, proximal and mid-distal colon

|                  | Jejunum | Proximal colon | Mid-distal colon |
|------------------|---------|----------------|-----------------|
|                  | FA      | \(J_{\text{HCO}_3^-}\) | FA              | \(J_{\text{HCO}_3^-}\) | FA              | \(J_{\text{HCO}_3^-}\) |
| cftr\(^{+/+}\)   | 118.5 ± 4.04 | 3.56 ± 0.26   | 234.4 ± 3.82\(^{\text{b,ns}}\) | 2.66 ± 0.25\(^{\text{b,#}}\) | 98.8 ± 4.84\(^{\text{b,#}}\) | 7.84 ± 0.27\(^{\text{b,#}}\) |
| cftr\(^{-/-}\)   | 144.1 ± 5.81\(^{a,**}\) | 1.66 ± 0.20\(^{a,**}\) | 158.5 ± 5.12\(^{a,**}\) | 1.73 ± 0.25\(^{a,**}\) | 106.4 ± 3.48\(^{a,**}\) | 5.68 ± 0.30\(^{a,**}\) |
| F508del\(^{ wt/ wt}\) | 116.0 ± 4.59 | 3.75 ± 0.30   | 118.3 ± 3.47\(^{b,ns}\) | 2.73 ± 0.25\(^{b,ns}\) | 89.8 ± 4.42\(^{b,\#}\) | 7.45 ± 0.47\(^{b,\#}\) |
| F508del\(^{ mut/ mut}\) | 139.8 ± 4.92\(^{a,**}\) | 1.88 ± 0.18\(^{a,**}\) | 139.0 ± 3.68\(^{a,**}\) | 1.77 ± 0.14\(^{a,**}\) | 97.6 ± 3.13\(^{a,NS}\) | 5.74 ± 0.53\(^{a,\#}\) |

Note: The table shows the basal fluid absorptive rates (left columns) and HCO\(_3^{-}\) output rates (right columns) in the jejunum, proximal, and mid-distal colon of cftr\(^{-/-}\), F508del\(^{mut/mut}\) and the respective WT intestine. Data are presented as mean ± SEM. \(N = 23\) (jejunum), \(n = 10\) (proximal colon), and \(n = 15\) (mid-distal colon).

\(^{a}\)Comparison of WT versus ko/Mut \(*P < 0.05\), NS: no significance.

\(^{b}\)Comparison between the different segments, \(# P < 0.05\), ns: no significance.
3.3 | Linaclotide reduces fluid absorptive rates and increases \( \text{HCO}_3^- \) output in \( \text{cftr}^{-/-} \) and \( F508\text{del}^{\text{mut/mut}} \) jejunum but not in the colon

Linaclotide, \( 10^{-7} \) M, applied luminally had been shown to elicit a robust short circuit current response in murine jejunal (Liu et al., 2020) and rat proximal colonic (Ahsan et al., 2017) isolated mucosa. In this study, it also stimulated a short circuit current response in isolated jejunal, proximal and distal colonic mucosa that was absent in \( \text{cftr}^{-/-} \), minimal in \( F508\text{del}^{\text{mut/mut}} \) and robust in WT jejunal, proximal colonic and lower in mid-distal colonic mucosa (Figure S3). This concentration is considered to be comparable to that presumably reached in the human intestinal lumen after taking the prescribed dose of 290 \( \mu \)g for constipation-prone irritable bowel syndrome and therefore was initially chosen but later increased to \( 5 \times 10^{-7} \) M for colonic perfusion. The luminal perfusion with \( 10^{-7} \) M (jejenum) and \( 5 \times 10^{-7} \) M (colon) linaclotide resulted in a significant decrease in jejunal, proximal colonic and mid-distal colonic fluid absorptive rates (Figures 3a–c and 4a–c), accompanied by a rise in luminal \( \text{HCO}_3^- \) output rates (Figures 3d–f and 4d–f) in WT mice. It also resulted in a decrease of fluid absorptive and increase in \( \text{HCO}_3^- \) output in the jejunum of both \( \text{cftr}^{-/-} \) (Figure 3a,d) and \( F508\text{del}^{\text{mut/mut}} \) (Figure 4a,d) mice. The total change of fluid absorptive rate in \( \text{cftr}^{-/-} \) as well as \( F508\text{del}^{\text{mut/mut}} \) jejunum reached ~25% of that seen in the respective WT jejunum (Figure 4a, FA, right panels). Surprisingly, the same effect was not seen in the colon, where a complete inhibition of fluid absorption and an increase in \( \text{HCO}_3^- \) output was observed in WT, but no significant change in both \( \text{cftr}^{-/-} \) and \( F508\text{del}^{\text{mut/mut}} \) mice was found, even after increasing the linaclotide concentration to \( 5 \times 10^{-7} \) M (Figures 3 and 4b,c,e,f).

**FIGURE 4** Effect of linaclotide on fluid absorptive and \( \text{HCO}_3^- \) output rates in \( F508\text{del}^{\text{mut/mut}} \) and WT jejunum and colon. (a–c, left panels) Time course of fluid absorptive rates in the 30 min before and after linaclotide (\( 10^{-7} \) M for jejunum, \( 5 \times 10^{-7} \) M for colon) addition (—Lina) to the luminal perfusate in the \( F508\text{del}^{\text{mut/mut}} \) and WT jejunum (a, left panel), the proximal colon (b, left panel) and the mid-distal colon (c, left panel). (a–c, right panels) Maximal linaclotide-induced decrease of fluid absorptive rates (\( \Delta F_A \)) in WT (left dots) and \( F508\text{del}^{\text{mut/mut}} \) (right dots) jejunum (a, right panel), proximal colon (b, right panel) and mid-distal colon (c, right panel). The percentage number after the brace indicates the percentage reduction of linaclotide-induced \( \Delta F_A \) in \( F508\text{del}^{\text{mut/mut}} \) versus WT intestine. (d–f, left panels) Time course of \( \text{HCO}_3^- \) output rates (\( J_{\text{HCO}_3^-} \)) in the 30 min before and after linaclotide (\( 10^{-7} \) M for jejunum, \( 5 \times 10^{-7} \) M for colon) addition (—Lina) to the luminal perfusate in the \( F508\text{del}^{\text{mut/mut}} \) and WT jejunum (d, left panel), the proximal colon (e, left panel) and the mid-distal colon (f, left panel). (d–f, right panels) Maximal linaclotide-induced increase in \( \text{HCO}_3^- \) output rate (\( \Delta J_{\text{HCO}_3^-} \)) in WT (left dots) and \( F508\text{del}^{\text{mut/mut}} \) (right dots) jejunum (d, right panel), proximal colon (e, right panel) and mid-distal colon (f, right panel). The percentage number after the brace indicates the percentage reduction of linaclotide-induced \( \Delta J_{\text{HCO}_3^-} \) in \( F508\text{del}^{\text{mut/mut}} \) versus WT intestine. \( N = 6 \) (jejunum), \( n = 5 \) (proximal and mid-distal colon). *\( P < 0.05 \). NS, no significance.
3.4 | Lubiprostone reduces fluid absorptive rates and increases HCO$_3^-$ output in WT, cftr$^{-/-}$ and $F508del^{mut/mut}$ jejunum and mid-distal colon

Because of conflicting data regarding the molecular mechanisms of lubiprostone action, a series of experiments were performed in isolated jejunal, proximal and distal colonic mucosa to verify the importance of CFTR channel expression for lubiprostone action on short circuit current response and to compare its effect when calculated jejunal, proximal and distal colonic mucosa to verify the importance of CFTR channel expression for lubiprostone action, a series of experiments were performed in iso-
dition in WT jejunum and a significant inhibition of fluid absorption in cftr$^{-/-}$ and $F508del^{mut/mut}$ jejunum, which reached ~50% of the fluid loss seen in WT jejunum (Figures 5a and 6a). The HCO$_3^-$ output was significantly stimulated by 10$^{-6}$ M lubiprostone in WT and to a lesser degree (~50% of the WT HCO$_3^-$ secretory response) in cftr$^{-/-}$ and $F508del^{mut/mut}$ jejunum (Figures 5c and 6c). In the mid-distal colon, 10$^{-6}$ M lubiprostone significantly inhibited fluid absorption in WT as well as in cftr$^{-/-}$ and $F508del^{mut/mut}$ (also ~50% of the WT response; Figures 5b and 6b). HCO$_3^-$ output was significantly increased in WT and to a lesser extent in cftr$^{-/-}$ and $F508del^{mut/mut}$ colon (Figures 5d and 6d). The proximal colon was not tested due to shortage of mice.

3.5 | Tenapanor results in reduction of fluid absorptive rates and increase in HCO$_3^-$ output in WT, cftr$^{-/-}$ and $F508del^{mut/mut}$ jejunum and mid-distal colon

The selective NHE3 inhibitor tenapanor (10$^{-5}$ M) significantly reduced jejunal, proximal and mid-distal fluid absorption in cftr$^{-/-}$ and $F508del^{mut/mut}$, as well as the respective WT mice (Figures 7a–c and 8a–c, left panels). No significant differences were seen in the percentage of inhibition after 30 min of tenapanor exposure (Figures 7a–c and 8a–c, right panels), although the time course of the inhibition was slower in cftr$^{-/-}$ as well as in $F508del^{mut/mut}$ than in WT mice (Figures 7a–c and 8a–c, left panels). Tenapanor (10$^{-5}$ M) also caused a similar increase in HCO$_3^-$ output rates in wt and $F508del^{mut/mut}$ as in WT mice (Figures 7d–f and 8d–f). While the absolute inhibition of fluid absorption was relatively similar in the three genotypes and different intestinal segments, the increase in HCO$_3^-$ output, while similar between the genotypes, had the previously observed segment-specific differences (Table 1).

3.6 | NHE3-dependency of the linaclotide- and lubiprostone-induced inhibition of jejunal fluid absorption in cftr$^{-/-}$ and $F508del^{mut/mut}$ mice

It has been shown that heat-stable Escherichia coli enterotoxin activates neural pathways in vivo, resulting in the stimulation of multiple

**FIGURE 5** Effect of lubiprostone on fluid absorptive and HCO$_3^-$ output rates in cftr$^{-/-}$ and WT jejunum and colon. (a and b, left panels) Time course of fluid absorptive rates in the 30 min before and after 10$^{-6}$ M lubiprostone addition (Lubi) to the luminal perfusate in the cftr$^{-/-}$ and WT jejunum (a, left panel) and the mid-distal colon (b, left panel). (a and b, right panels) Maximal lubiprostone-induced decrease of fluid absorptive rates ($\Delta$FA) in WT (left dots) and cftr$^{-/-}$ (right dots) jejunum (a, right panel) and mid-distal colon (b, right panel). The percentage number after the brace indicates the percentage reduction of lubiprostone-induced $\Delta$FA in cftr$^{-/-}$ versus WT intestine. (c and d, left panels) Time course of HCO$_3^-$ output rates ($J_{HCO3}$) in the 30 min before and after 10$^{-6}$ M lubiprostone addition (Lubi) to the luminal perfusate in the cftr$^{-/-}$ and WT jejunum (c, left panel) and mid-distal colon (d, left panel). (c and d, right panels) Maximal lubiprostone-induced increase in HCO$_3^-$ output rate ($\Delta J_{HCO3}$) in WT (left dots) and cftr$^{-/-}$ (right dots) jejunum (c, right panel) and mid-distal colon (d, right panel). The percentage number after the brace indicates the percentage reduction of lubiprostone-induced $\Delta J_{HCO3}$ in cftr$^{-/-}$ versus WT intestine. $n = 5$ for each group, *P < 0.05.
receptors and signalling pathways (Eklund, Jodal, & Lundgren, 1985; Harville & Dreyfus, 1995; Mourad & Nassar, 2000; Rolfe & Levin, 1994). For lubiprostone, multiple targets have been described because due to a compulsory breeding interruption the age of the mice in the two groups was very different (see figure legend). While the addition of lubiprostone to WT jejunum resulted in an increase in both fluid and HCO₃⁻ secretory rate, it did not cause an increase in HCO₃⁻ output in cfr⁻ ⁻ or in F508delmut/mut jejunum.

3.7 Difference in basal fluid absorptive rate between WT and ko/Mut, as well as the response to tenapanor and linaclotide, is dependent on the luminal pH and luminal CO₂

At first glance, the higher fluid absorptive rate in ko/mut jejunum (Table 1) might be explained by a lack of CFTR-mediated secretion. However, we had not observed this difference in previous experiments in which buffered solutions had been perfused (Liu et al., 2020). During luminal perfusion of the jejunum with unbuffered solution the pH decreased in WT mice and significantly more so in ko/mut mice (Table 2). We wondered whether the higher fluid absorptive rates in ko/mut mice may be related to the different luminal pH profiles during perfusion of unbuffered saline in cfr⁻⁻ or F508delmut/mut jejunum. To test this hypothesis, the jejunum of WT mice was first perfused with a HEPES/Tris buffered perfusate of pH 7.4 for 45 min and then switched to a buffered solution of either pH 6.4, 6.8 and 8.0 (Figure 10a–c). The highest fluid absorptive rates were observed at a luminal pH of 6.8, while pH 6.4 or 8.0 were inhibitory.
We also considered it necessary to perform an experiment with a CO₂/HCO₃⁻ buffered perfusate, because luminal CO₂ has been shown to stimulate fluid absorption presumably by stimulating an apical Na⁺/H⁺ exchange process (Turnberg, Fordtran, Carter, & Rector, 1970). We therefore perfused the jejunum of cftr⁻/⁻ and WT mice with a HEPES-buffered pH of 7.4 (Figure 10d), or with a CO₂/HCO₃⁻-buffered solution of pH 7.4 (Figure 10e). The difference in basal fluid absorptive rates between cftr⁻/⁻ and WT jejunum was abolished in both conditions but for different reasons. In the case of perfusion with the HEPES-buffer, the fluid absorptive rate of the cftr⁻/⁻ jejunum was lowered to that of the WT jejunum (Figure 10d). In the case of perfusion with a CO₂/HCO₃⁻-buffered solution with the same pH, both cftr⁻/⁻ and WT displayed significantly higher and similar, fluid absorptive rates (Figure 10e). The effect of tenapanor and linaclotide was also tested in either condition. In CO₂/HCO₃⁻-buffered solution fluid absorption was inhibited faster and to a stronger extent by 10⁻⁵ M tenapanor (Figure 10e). The same phenomenon was observed in F508delmut/mut jejunum (Figure 10f). The jejunal fluid absorptive rate is therefore strongly stimulated by luminal CO₂, in part via NHE3 activation. As seen with unbuffered luminal perfusates (Figures 3 and 4) the subsequent response to linaclotide was robust in WT jejunum but absent in cftr⁻/⁻ as well as F508delmut/mut jejunum (Figure 10d-f).

4 | DISCUSSION

This study explores the ability of three recently FDA-approved drugs for treatment of constipation-prone irritable bowel syndrome, to improve gut fluidity and alkalinity in the cftr⁻/⁻ and F508delmut/mut murine intestine and identifies the mechanisms of action of these drugs in the CF gut. All three drugs were able to decrease fluid absorption in the CF intestine but with significant differences regarding segmental efficacy or mode of action.

In order to get FDA-approval for the treatment of a benign disease such as constipation-prone irritable bowel syndrome, strict safety criteria have to be fulfilled. Safety is also essential for CF patients, because treatment should be started early and given...
continuously to prevent secondary and possibly irreversible alterations due to chronic intestinal inflammation. Therefore, these tested drugs are highly interesting candidates for the treatment of CF patients.

Mice with no or mutant CFTR expression display an intestinal phenotype early in life. A high percentage of them die of intestinal obstruction, with only little survival advantage of the F508del/mut/mut in comparison to the cftr−/− mice (Colledge et al., 1995; Ratcliff et al., 1992; Ratcliff et al., 1993). It was also shown that chronic PEG-based oral laxative application reduces the incidence of intestinal obstructions and prolongs survival in cftr−/− mice (Clarke, Gawenis, Franklin, & Harline, 1996). More recent studies demonstrated that this beneficial effect was due to the prevention of early pathological alterations in the murine CF gut, such as cryptal obstructions by mucus, Paneth cell demise, small bowel overgrowth and intestinal inflammation (De Lisle, Roach, & Jansson, 2007). This strategy of daily treatment with PEG-containing saline laxatives is also employed in the prevention of severe obstipation and intestinal obstructive episodes in CF patients. However, it is intolerable to the majority of patients for long-term use because of its bad taste.

A period of optimizations of laxative treatment and type of chow had preceded the present study. The aim was to breed cftr−/− and F508del/mut/mut mice free of intestinal inflammation, which causes a plethora of secondary alterations associated with the CF intestinal phenotype (De Lisle & Borowitz, 2013; Dorsey & Gonska, 2017). In the mouse cohort of the present study, proinflammatory cytokine expression was not increased in the small and large intestine of cftr−/− and F508del/mut/mut mice compared to WT littermates (Figure 1a,b) and NHE3 mRNA expression was not down-regulated (Figure 1c). Crypt architecture was not distorted by mucus obstruction (Figure 2a,b).
b), but the abnormally large goblet cells and the villus and crypt elongation (Figure 2c,d), which are probably intrinsic features of CFTR-deficient gut (Liu, Walker, Ootani, Strubberg, & Clarke, 2015; Strubberg et al., 2017), were also observed.

Linaclotide binds to the heat-stable Escherichia coli toxin/guanylin receptor GC-C in the apical membrane of intestinal epithelial cell (Bryant et al., 2010). Upon binding, cGMP production is stimulated. This activates the cGMP-dependent kinase II (PKG2), resulting in the activation of the CFTR channel (Ahsan et al., 2017; Pfeifer et al., 1996) and the inhibition of the Na"/H" exchanger NHE3 (Cha et al., 2005). In one study, 50 μg·kg"⁻¹ linaclotide had been applied to cftr"⁻/⁻" and F508del mutant mice by gavage and the effects on small intestinal transit time were assessed (McHugh et al., 2018). The authors concluded that the action of linaclotide in increasing small

FIGURE 9  Sequential application of tenapanor followed by linaclotide or lubiprostone reveals the effect mediated via NHE3 inhibition. (a, c) Application of 10⁻⁷ M linaclotide to the luminal perfusate that had already contained 10⁻⁵ M tenapanor for the preceding 30 min resulted in a robust secretory response to linaclotide in WT, but no further change in fluid dynamics in cftr"⁻/⁻" (a) and F508mut/mut jejunum (c). (b, d) The same was observed for HCO₃⁻ output. (e, g) Application of 10⁻⁶ M lubiprostone after 10⁻⁵ M tenapanor pre-incubation induced a strong further secretory response in WT and also a further reduction of fluid absorption from that seen in WT in the cftr"⁻/⁻" (e) and F508mut/mut jejunum (f). Interestingly, a further increase in HCO₃⁻ output was only observed in WT jejunum (f, h). Due to a compulsory breeding interruption, these F508delmut/mut and WT mice were 30–40 weeks per age, which may be the reasons for their strong fluid secretory response in comparison to the cftr"⁻/⁻" and WT mice, which were born when breeding was restarted. N = 5 for each group, *P < 0.05. NS, no significance.
Intestinal transit time is via NHE3 inhibition. Based on our calculation of the daily dose of 290 μg linaclotide for patient use (approx. 4 μg kg⁻¹) and a volume of 1–2 L (conservative estimation) of gastric and small intestinal juice to dissolve it, patients will likely experience not more than 1–2 × 10⁻⁷ M linaclotide concentration in their upper small bowel. The effect of luminally applied 10⁻⁷ M linaclotide on the

**TABLE 2**  pH of outflow in cftr⁻/⁻, F508delmut/mut, and the respective WT jejunum and mid-distal colon

|                  | Jejunum | Proximal colon | Mid-distal colon |
|------------------|---------|----------------|-----------------|
| cftr⁺/⁺         | 7.071 ± 0.001 | 6.908 ± 0.017¹ ²  | 7.263 ± 0.011 ² ³ |
| cftr⁻/⁻         | 6.783 ± 0.027³ ⁴ | 6.728 ± 0.012³ ⁴ | 7.151 ± 0.015³ ⁴ |
| F508delwt/wt     | 7.095 ± 0.001 | 6.904 ± 0.016³ ⁴ | 7.265 ± 0.011³ ⁴ |
| F508delmut/mut   | 6.731 ± 0.025³ ⁴ | 6.733 ± 0.013³ ⁴ | 7.113 ± 0.016³ ⁴ |

Note: The table shows the basal fluid absorptive rates (left columns) and HCO₃⁻ output rates (right columns) in the jejunum, proximal, and mid-distal colon of cftr⁻/⁻, F508delmut/mut, and the respective WT intestine. Data are presented as mean ± SEM. N = 23 (jejunum), n = 10 (proximal colon), and n = 15 (mid-distal colon).

¹Comparison of WT vs ko/mutant. *P < 0.05.

**FIGURE 10**  Basal jejunal fluid absorptive rates are dependent on luminal pH and luminal CO₂. (a–c) Change of the pH in a HEPES-buffered luminal perfusate significantly alters jejunal fluid absorptive rate in WT mice, with a pH-optimum of 6.8. Adjustment of perfusate pH to 7.4 in HEPES-buffer (d) or in CO₂/HCO₃⁻ buffer (e, f) results in similar basal fluid absorptive rates in cftr⁻/⁻ (d, e) and F508delmut/mut (f) versus WT jejunum. They are ~70% higher in CO₂/HCO₃⁻ buffer than HEPES-buffer perfusate at the same pH value of 7.4. Both the speed and magnitude of inhibition of fluid absorptive rate with tenapanor is higher in CO₂/HCO₃⁻ buffer. However, the response to linaclotide is similar, that is, completely inhibited by tenapanor in cftr⁻/⁻ and F508delmut/mut jejunum. N = 5 for each group. *P < 0.05. NS, no significance.
short circuit current response was therefore tested in isolated jejunal, proximal and distal colonic mucosa of the three genotypes in vitro (Figure S3) and subsequently on jejunal, proximal and distal colonic fluid movements and HCO$_3^-$ output in vivo. A reduction of jejunal fluid absorption and an increase in bicarbonate secretion were found in cftr$^{-/-}$ and F508del jejenum that was approx. 25% that in WT mice. Surprisingly, linaclotide did not significantly inhibit fluid absorption in the cftr$^{-/-}$ and F508delmut/mut proximal and mid-distal colon, even in at the higher concentration of $5 \times 10^{-7}$M, which elicited a strong secretory response in WT mice. Possible reasons are a thicker and less permeable mucus layer and an altered GC-C expression in cftr$^{-/-}$ and F508del colen. A significant reduction or even complete loss of the biological effect of luminaly applied toxins by the presence of the adherent mucus gel was demonstrated in rat duodenum (Flemstrom et al., 1999). Ilka et al. recently demonstrated that the expression of Gucy2c mRNA in the murine colon is strongest in the base of the crypts, as compared to a villous expression in the small intestine (Ilka et al., 2016). It is thus possible that linaclotide does not reach its receptor because of the viscous mucus in the CF colon. In a recent study in human volunteers, a dose of 870 μg did not result in a biological effect in the colon, unless a PEG-containing oral laxative solution was used to cleanse the bowel prior to linaclotide ingestion (Weinberg et al., 2017). Since it is unclear whether an exclusive action in the small bowel is sufficient to relieve CF-associated constipation, a galenic formulation of linaclotide that ensures colonic release seems necessary before clinical trials are started in chronically constipated CF patients. This would indeed be desirable, because linaclotide is generally well-tolerated, with diarrhoea being the most frequent side effect, which would likely not be an issue in CF patients (Black et al., 2018).

Lubiprostone is a PGE$_2$ analogue that has already been tested in CF patients in a small published pilot trial, with benefit in some patients (O’Brien, Anderson, & Stowe, 2011). In our study, $10^{-6}$ M lubiprostone significantly inhibited fluid absorptive rates and increased HCO$_3^-$ output in both the small and large intestine of cftr$^{-/-}$ and F508delmut/mut, reaching ~50% of the effect observed in the jejenum and colon of WT mice. This is in line with previous findings that showed an inhibition of ileal fluid absorption in CF patients by luminaly applied PGE$_2$ analogue misoprostol (Coates et al., 2004). While this result at first glance makes lubiprostone a very promising candidate for the treatment of CF-associated constipation and obstructive episodes, the concentrations that have been used in isolated mucosa and in animal studies, including ours, in the study by Coates et al. with misoprostol in humans, are high when compared with the 8 and 26 μg bid dosing of lubiprostone recommended for oral intake in constipation-prone irritable bowel syndrome. It is therefore not clear whether the beneficial action of lubiprostone in constipation-prone irritable bowel syndrome patients is predominantly mediated by its action on intestinal fluid transport. Lubiprostone has originally been characterized as a stimulant of ClC-2-mediated intestinal fluid secretion (Ambizas & Ginzburg, 2007), but this concept was later questioned. Reviews by Akiba and Kaunitz (2012) and Jin and Bliklagsler (2015) discuss the controversial experimental results (Jin & Bliklagsler, 2015). Meanwhile, lubiprostone has been reported to target multiple cellular structures, including muscle (Chan & Mashimo, 2013), pacemaker cells of Cajal (Jiao et al., 2014), goblet cells (De Lisle, 2012; Jakab, Collaco, & Ameen, 2012) and possibly nerves (Bassil et al., 2008). This broad range of target structures may explain side effects of lubiprostone such as nausea, which may limit its applicability in higher concentrations for increasing gut fluidity and alkalinity in the CF gut.

Tenapanor, a selective NHE3-inhibitor whose action is limited to the intestine because of very low systemic absorption (Spencer et al., 2014), received FDA approval in September 2019 for the treatment of constipation-prone irritable bowel syndrome (Markham, 2019). Dose-finding experiments, using the identical method used in the present study, in anaesthetized C57B/6 mice had shown that $10^{-5}$ M tenapanor inhibited jejunal fluid absorption reaching a plateau that was even somewhat lower than the fluid absorptive rate in NHE3-deficient mice, which may have undergone a degree of compensation (Xia et al., 2014). Therefore, we can assume full NHE3 inhibition with this concentration. Tenapanor inhibited fluid absorptive rate and increased HCO$_3^-$ output both in the jejenum and colon to a similar percentage in cftr$^{-/-}$, F508delmut/mut and WT mice, although the time course of inhibition was slower in the former. This difference in the onset of action was abolished when a CO$_2$/HCO$_3^-$-buffered perfusate was applied, which stimulates NHE3 activity (Turnberg, Fordtran, Carter, & Rector, 1970). Dose-ranging studies with tenapanor have been performed in healthy volunteers (Rosenbaum, Yan, & Jacobs, 2018) and doses of 50 mg twice daily were chosen for phase III trials for constipation-prone irritable bowel syndrome (Chey, Lembo, & Rosenbaum, 2020). One capsule achieves the concentration of $10^{-5}$ M, as used in our perfusates, in ~5 L of gastrojejunal fluid. Our chosen concentration is therefore likely to be in the range of the currently prescribed therapeutic drug concentration.

An issue of concern for the CF patient population is a potential interference of tenapanor with the absorption of nutrients that are taken up via a proton-coupled transport system such as the dipeptide transporter PEPT1 (SLC15A1) or the proton-coupled amino acid transporter PAT1 (SLC3A1) or the proton-coupled folate and heme transporter PCFT (SLC46A1), which depend on the action of NHE3 to restore intracellular pH$_3$-homeostasis (Thwaites & Anderson, 2007). This has been studied for PEPT1 but with a concentration of 2 x 15 mg, which is low compared to the recommended dose and found to not cause a significant decrease in the maximal serum concentration of the PEPT1 substrate cefadroxil (Johansson et al., 2017). Nevertheless, nutrient/micronutrient absorption should be closely monitored in a future tenapanor clinical trial in CF patients with severe constipation and/or prior DIOS episodes.

In order to evaluate the mechanism of the inhibitory action of $10^{-7}$ M linaclotide and $10^{-4}$ M lubiprostone in the CF gut, we tested the effect of either drug after tenapanor preincubation. In contrast to linaclotide, lubiprostone resulted in a further decrease in fluid absorption in F508delmut/mut mice, albeit significantly less than without tenapanor preincubation (Figure 10). This does not reflect
merely a further inhibition of NHE activity, because this would be associated with an increase in HCO$_3^-$ output. As mentioned above, lubiprostone shares with other prostaglandins the broad range of target structures.

In some laboratories, the F508del mutant mouse models showed better survival rate than that described for CFTR null mice, which was related to low levels of functional CFTR protein in the brush border membrane of intestinal epithelium and appeared to be dependent on the genetic background (van Doorninck et al., 1995; Wilke et al., 2011). This is the mouse strain and background that we also use and we also observe a residual short circuit current response to linaclotide and lubiprostone in isolated small and large intestinal mucosa of and F508del$^{mut/mut}$ mice, which is not seen in cftr$^{-/-}$ mucosa (Figure S3). It is therefore interesting that the changes in fluid absorptive and HCO$_3^-$ output rates did not differ in a major way in cftr$^{-/-}$ and F508del$^{mut/mut}$ intestinal segments, suggesting that the short circuit current response in isolated mucosa in vitro may not be a good readout of fluid secretory response in vivo.

As a potential predictor of treatment success of the tested drugs in CF-associated constipation and obstructive episodes, our study has several limitations: (1) The method assesses the direct effect on fluid movements and HCO$_3^-$ output in defined intestinal segments but ignores the indirect effects that links fluid secretion to intestinal motility via the enteric nervous system; (2) While the expression of CFTR and NHE3 has been studied in both murine and human intestine and is comparably distributed in the intestinal segments under investigation, differences in receptor or transporter affinity for the studied drugs may exist between mice and humans and (3), drug-related side effects are not recognized. Nevertheless, they yield valuable information about the drugs' effects on intestinal fluid and acid/base transport in vivo.

In summary, this study assessed fluid absorptive and HCO$_3^-$ output rates in anaesthetized and tighty acid/base and BP-controlled cftr$^{-/-}$, F508del$^{mut/mut}$ and WT mice in response to three recently FDA-approved drugs developed for the treatment of constipation-prone irritable bowel syndrome. Each of these substances has the term action of these drugs on the prevention of obstructive episodes and the reversibility of inflammatory alterations and dysbiosis in CF animal models and in patients.

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AUTHOR CONTRIBUTIONS
Q.T., G.d.S., X.T., X.R. and U.S. performed experiments, acquired and analysed data. G.d.S., D.R. and U.S. planned and supervised the mouse breeding. S.R.T. helped with statistical calculations. Q.T. and U.S. drafted the figures and wrote the manuscript.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR
This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT
Data are available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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