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CHAPTER

4

Ivacaftor restores FGF19 regulated bile acid homeostasis in cystic fibrosis patients with an S1251N or a G551D mutation

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Abstract

Objective: Disruption of the enterohepatic circulation of bile acids (BAs) is part of the gastrointestinal phenotype of cystic fibrosis (CF). Ivacaftor (VX-770), a cystic fibrosis transmembrane conductance regulator (CFTR) potentiator, improves pulmonary function in CF patients with class III gating mutations. We studied the effect of ivacaftor on the enterohepatic circulation by assessing markers of BA homeostasis and their changes in CF patients.

Methods: In CF patients with an S1251N mutation (N=16; age 9-35 years TICTAC-2 study/NTR4873) or a G551D mutation (N=101; age 10-24 years; GOAL study/ NCT01521338) we analyzed plasma fibroblast growth factor 19 (FGF19) and 7 α -hydroxy-4-cholesten-3-one (C4) levels, surrogate markers for intestinal BA absorption and hepatic synthesis, respectively, before and after treatment with ivacaftor.

Results: At baseline, median FGF19 was lower (52% and 53%, $P < 0.001$) and median C4 higher (350% and 364%, $P < 0.001$), respectively, for the S1251N and G551D mutation patient groups compared to healthy controls. Treatment with ivacaftor significantly increased FGF19 and reduced C4 levels towards normalization in both cohorts but this did not correlate with CFTR function in other organs, as measured by sweat chloride levels or pulmonary function.

Conclusions: We demonstrate that patients with CFTR gating mutations display interruption of the enterohepatic circulation of BAs reflected by lower FGF19 and elevated C4 levels. Treatment with ivacaftor partially restored this disruption of BA homeostasis. The improvement did not correlate with established outcome measures of CF, suggesting involvement of modulating factors of CFTR correction in different organs.

Introduction

Cystic fibrosis (CF) is the most common autosomal recessive disorder in the Caucasian population and is characterized by the production of abnormally thick, viscous mucus in the lungs, digestive system and various other tissues (1,2). The defect is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) gene that encodes a chloride channel protein (3). The diagnosis of CF is confirmed by an increased sweat chloride concentration which is considered a surrogate for the CFTR protein chloride channel function and/or by genetic testing. CF patients primarily suffer from progressive pulmonary disease as determined by a reduced forced expiratory volume in 1 second (FEV₁). Additionally, they frequently display exocrine pancreatic insufficiency, poor growth and weight gain resulting in a low body mass index (BMI) and intestinal complications like e.g. distal intestinal obstruction syndrome (1).

Gastrointestinal problems are common among CF patients. Increased fecal bile acid (BA) loss is an intrinsic part of the gastrointestinal phenotype of CF, found consistently in both CF patients and CF animal models (4,5). Compared to healthy controls, CF patients have an up to three-fold increase in fecal BA loss. This fecal BA loss is independent of CF-related exocrine pancreatic insufficiency and intestinal fat malabsorption (4,6). The higher fecal BA excretion indicates a partial interruption of the enterohepatic circulation of BA and is associated with a compensatory increase of *de novo* hepatic BA synthesis (6). Adequate BA homeostasis is essential to hepatic, intestinal and metabolic function (7). CF patients regularly display complications such as colonic dysbiosis and small intestinal bacterial overgrowth (SIBO) (8). The disruption in BA homeostasis may contribute to these complications and drugs affecting BA homeostasis could potentially benefit CF patients (9).

The enterohepatic circulation comprises hepatobiliary secretion and intestinal reabsorption of BA and is controlled via a negative feedback system (10). After uptake in the distal small intestine, BAs bind to the Farnesoid X receptor (FXR) which is localized in the enterocytes and induces the expression and secretion of fibroblast growth factor 19 (FGF19) into the portal circulation (11). In the liver, FGF19 binds to the FGF receptor 4 (FGFR4), leading to suppression of the cholesterol 7 α -hydroxylase (CYP7A1) gene, encoding the rate-limiting enzyme in BA synthesis. The protein activity of CYP7A1 can be inferred from the concentration of the BA intermediate, 7 α -hydroxy-4-cholesten-3-one (C4), that is used as a surrogate marker for BA synthesis (12). Clinically, altered plasma levels

of FGF19 and C4 have been used as markers for disrupted BA metabolism. For example patients with primary bile acid diarrhea (BAD) display BA malabsorption and increased BA synthesis, resulting in decreased plasma FGF19 levels, and elevated plasma C4 levels (13-16). Based on the CF phenotype of BA malabsorption, it is expected that FGF19 levels are low and C4 levels are high in CF patients, however, this has never been documented.

Ivacaftor, a drug developed for the treatment of CF patients, potentiates CFTR channel activity and thereby enhances chloride transport in patients with class III gating mutations, such as the S1251N and the G551D CFTR missense mutations (17,18). In The Netherlands, the S1251N mutation is the most common class III mutation with an occurrence of 1.2% in 2016 (19). The G551D mutation is present in ~4% of all CF patients in the United States and is the third most common CFTR mutation worldwide (20). In randomized placebo-controlled trials, it has been shown that ivacaftor improves sweat chloride levels, pulmonary function (FEV₁) and BMI in G551D and S1251N patients (21-23). Recently, a study also showed the efficacy of ivacaftor in the intestine where it improved the small intestinal pH after gastric emptying by increasing bicarbonate secretion (24).

The TICTAC-2 clinical trial was an open label observational study that assessed the effects of ivacaftor in a small Dutch cohort of patients with the S1251N mutation. In this study, fecal and plasma samples were obtained before and eight weeks after treatment with ivacaftor.

The multicenter clinical G551D observational (GOAL) study was a six months follow-up, phase 4 study exploring the therapeutic effect of ivacaftor in patients with a G551D mutation. The GOAL study confirmed, in these specific CF patients, the positive treatment effects observed earlier in the original clinical trials for ivacaftor on sweat chloride, FEV₁ and BMI (25). Within the GOAL study, blood samples were taken and stored before and at six months after the start of the treatment.

We aimed to evaluate the effects of CFTR modulation on the gastrointestinal tract. Specifically, we investigated whether ivacaftor treatment induces a recovery of the enterohepatic circulation of BAs in CF patients as reflected by the effects on FGF19 and C4 plasma levels.

Methods

Subjects

For the S1251N mutation study, samples were obtained before and 2 months after treatment with ivacaftor. A total of 16 patients (age 9-35 years) were recruited. All patients had an S1251N mutation at one allele. At the other allele 12 patients had a deltaF508 mutation, 2 patients a R117H mutation, 1 patient an 1717-IGA mutation and 1 an A455E mutation. 13 (81%) patients were reported to be exocrine pancreatic insufficient. Plasma samples before and after ivacaftor treatment were obtained for 14 participants. From 10 patients, random fecal samples were also obtained before and at 2 months after starting treatment with ivacaftor. Clinical data including age, BMI, FEV₁ scores, sweat chloride levels, comorbidity and medication use was gathered for all patients. The ethics committees of the University Medical Center Utrecht and 3 other Medical Centers (Erasmus MC, Rotterdam; AMC Amsterdam; HAGA The Hague) approved this study, and informed consent was obtained from all participating subjects.

Plasma samples from 101 CF patients (age 10-24 years) before and after six months of ivacaftor treatment were obtained from the G551D observational (GOAL) study (clinicaltrials.gov number, NCT01521338) (25). The protocol was reviewed and approved by the GOAL study review board. Patients enrolled in this study were six years and older, had a G551D gating mutation on at least one allele and no prior history of ivacaftor use. Patients using UDCA were excluded from the GOAL cohort. Available BMI, FEV₁ scores, and sweat chloride levels before and after ivacaftor treatment were obtained from the GOAL study database. Additional ethical approval was obtained from the medical ethical committee of the University Medical Center Groningen. Two patients were excluded based on a baseline sweat chloride level of ≤ 39 mEq/L, which indicated a sweat chloride level below the upper limit of normal (26). The final data analysis was performed on the data of the remaining 99 patients.

Control group

The control group consisted of non-fasted plasma samples of 120 healthy subjects, aged 18-50 years and with a BMI between 18.5-30, from the Dutch multidimensional cohort study and biobank LifeLines (27).

Plasma levels of FGF19 and C4

Plasma samples were stored at -20°C. FGF19 levels were determined using a quantitative sandwich enzyme-linked immunosorbent assay (Human FGF-19 Quantikine ELISA kit, cat no. DF1900; R&D systems). Plasma C4 levels were determined using high-performance liquid chromatography-tandem mass spectrometry (XLC-MS/MS) (28). All the samples were assayed in duplicate.

Fecal and plasma bile acids

Fecal samples were weighed, freeze-dried and mechanically homogenized. Samples were then solubilized in 1ml of methanol/0.1N sodium hydroxide (3:1) at 80 °C for 2 hrs. As an internal standard 15 nmol of 5 β -Cholanic acid 7 α ,12 α diol was added. Next, bile acids were extracted using Sep Pak C-18 columns (Mallinckrodt Baker, Deventer, The Netherlands) and eluted with 75% methanol followed evaporation at 65C under a stream of nitrogen. Dried samples were methylated with methanol/acetyl chloride for 25 minutes at 55 °C. Samples were dried under a stream of nitrogen and trimethylsilylated with a mixture of pyridine, N,O-Bis (trimethylsilyl) trifluoroacetamide and trimethylchlorosilane. The bile acid samples were then diluted in 150 μ l of heptane and analyzed by GC (Agilent 6890, Amstelveen, the Netherlands) using a CPSil 19 capillary column (25m \times 0.25mm \times 0.2 μ m) (Chrompack, Middelburg, The Netherlands) The total amount of bile acids was calculated as the sum of the individually quantified bile acids.

Plasma bile acid profiles were measured using liquid chromatography-mass spectrometry (LC-MS). For the analysis 25 μ l of plasma was used to which a 250 μ l internal standard solution was added. Samples were centrifuged at 15800 x g and the supernatant poured into a clean glass tube. The fluid was evaporated under nitrogen at 40C. Before measuring samples were reconstituted in 200 μ l 50% methanol in water, vortexed and centrifuged for 3 minutes at 1800 x g. The supernatant was transferred into a 0.2 μ m spin-filter and centrifuged at 2000 x g for 10 minutes. After filtering, the samples were transferred into LC-MS vials and analyzed (10 μ l injection volume).

For the quantitative determination of bile acids we used a Nexera X2 Ultra High Performance Liquid Chromatography system (SHIMADZU, Kyoto, Japan), coupled to a SCIEX QTRAP 4500 MD triple quadrupole mass spectrometer (SCIEX, Framingham, MA, USA) (UHPLC-MS/MS). The LC-MS/MS system is controlled by Analyst MD 1.6.2 software.

Bile acids were separated with a ACQUITY UPLC BEH C18 Column (1,7 μm x 2,1 x 100 mm) equipped with a ACQUITY UPLC BEH C18 VanGuard Pre-Column (1,7 μm x 2,1 x 5 mm), (Waters, Milford, MA, USA). Separation was achieved in 28 minutes using 10 mM ammonium acetate in 20% acetonitrile (mobile phase A) and 10 mM ammonium acetate in 80% acetonitrile (mobile phase B), total flow rate: 0.4 ml/min.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 22 (International Business Machines, Armonk, New York). FGF19, C4 and fecal BA data were not normally distributed and are displayed as Tukey plots where boxes represent the median with interquartile range (IQR) and whiskers extend to the largest value or 1.5 times the IQR if the largest value extends that. A Mann-Whitney U test was performed to compare CF patients versus controls. For the statistical analysis of treatment effect a Wilcoxon Signed Rank test was performed. Correlation analysis was performed only on the GOAL data as the sample size of the Tic-Tac study was considered too small for a reliable correlation analysis. For the relationship between FGF19 and C4 measurements a Spearman's rank coefficient was used as data were not normally distributed. However, all changes in parameters were normally distributed (determined by Q-Q plot) and therefore these correlations were analyzed using a Pearson R correlation coefficient. $P < 0.05$ was considered significant. All authors had access to the study data and have reviewed and approved the final manuscript.

Results

S1251N mutation cohort

Ivacaftor improves sweat chloride level, BMI and FEV₁ scores in CF patients with an S1251N mutation

Table 1 shows the patient characteristics of the control subjects and CF patients with an S1251N mutation before and after 2 months of ivacaftor treatment. Of the 14 CF patients analyzed for FGF19 and C4 data, 10 were male. In the control group 60 of the 120 subjects were male. In line with improved CFTR function, the median sweat chloride level significantly decreased after ivacaftor treatment. Median FEV₁ and BMI significantly increased, showing clinical efficacy of ivacaftor treatment.

The median age of the controls was 33 years which was significantly higher than in the CF group (16 years) at baseline ($P < 0.001$). Controls had a median BMI of 24.4 kg/m² which was significantly higher than in CF patients at baseline (19.0 kg/m², $P < 0.001$) and after ivacaftor treatment (20.1 kg/m², $P < 0.001$).

	Tic-Tac study			
	controls	Before ivacaftor	After Ivacaftor	p-value
n	120	14	14	
Age	33 (25-41)	16 (9-35)	+ 2 months	
BMI	24.4 (22.5-26.6)	19.0 (16.3-22.8)	20.1 (17.2 -23.6)	0.017
Sweat chloride (mEq/L)		72.0 (63.0-109.0)	20.5 (18.5-33.0)	<0.001
FEV1 (% of predicted)		73 (66-95)	92 (92-102)	<0.001

Table 1. Basic characteristics of controls and CF patients before and after ivacaftor treatment in the Tic-Tac study cohort. Data displayed as median with interquartile range (IQR) between brackets. *P*-values displayed represent the differences before and after ivacaftor treatment; Wilcoxon signed rank test.

Ivacaftor improves bile acid homeostasis in CF patients with an S1251N mutation

Fig. 1 shows the changes in plasma FGF19 and C4 levels and fecal BA concentrations at 2 months after ivacaftor treatment. As a surrogate marker for BA absorption plasma FGF19 was measured. **Fig. 1A** shows the individual changes in FGF19 level of all patients with an S1251N mutation. One patient had diagnosed cystic fibrosis liver disease and received ursodeoxycholic acid treatment. However, as the measured plasma parameters were similar to untreated patients, these

results were included in the final analysis. No fecal samples were obtained for this patient. Median FGF19 level increased significantly from 45 to 113 pg/ml (increase in 12 of 14 patients, **Fig. 1B**, $P = 0.003$). In accordance with the BA malabsorption phenotype of CF, median FGF19 levels in CF patients at baseline were significantly lower as compared to healthy controls (45 vs. 94 pg/ml, $P < 0.001$). After 8 weeks of ivacaftor treatment median FGF19 levels were similar between CF patients and controls (113 vs. 94 pg/ml, $P = 0.91$). These results suggest that ivacaftor treatment induces an increased BA reabsorption in CF patients with an S1251N mutation .

FGF19 inhibits hepatic BA synthesis. We, therefore, determined plasma C4 levels as a surrogate marker for BA synthesis. **Fig. 1C** shows individual changes in plasma C4 levels. All except two patients showed a decrease in C4 level after 2 months of ivacaftor treatment. These two patients showed an increase of 6 and 4 ng/ml. The latter subject was the same patient that showed the highest decrease in FGF19 level (-54 pg/ml). The other subject showed despite the slight increase in C4 also an increase in FGF19 level (+83 pg/ml). In **Fig. 1D** the median change in plasma C4 level is shown. Median plasma C4 levels decreased significantly from 49 to 24 ng/ml ($P = 0.001$) suggesting a decrease in hepatic BA synthesis. While median C4 levels improved towards normality, values at baseline and after ivacaftor treatment were still significantly higher than control values (49 vs 14 ng/ml; $P < 0.001$ and 24 ng/ml; $P = 0.001$; respectively).

Fig. 1E shows individual changes in fecal BA concentrations. Random fecal samples before and after ivacaftor treatment were available for 10 patients. **Fig. 1F** shows the median change in fecal BA concentrations. Median fecal BA decreased after Ivacaftor treatment from 8.8 to 6.8 mg/g but the difference did not reach statistical significance ($P = 0.06$). Fecal and plasma BA profiles were similar before and after treatment (**table S1, S2**).

Together these results show that 2 months of ivacaftor treatment restores the enterohepatic circulation of BAs in CF patients with an S1251N mutation towards normality.

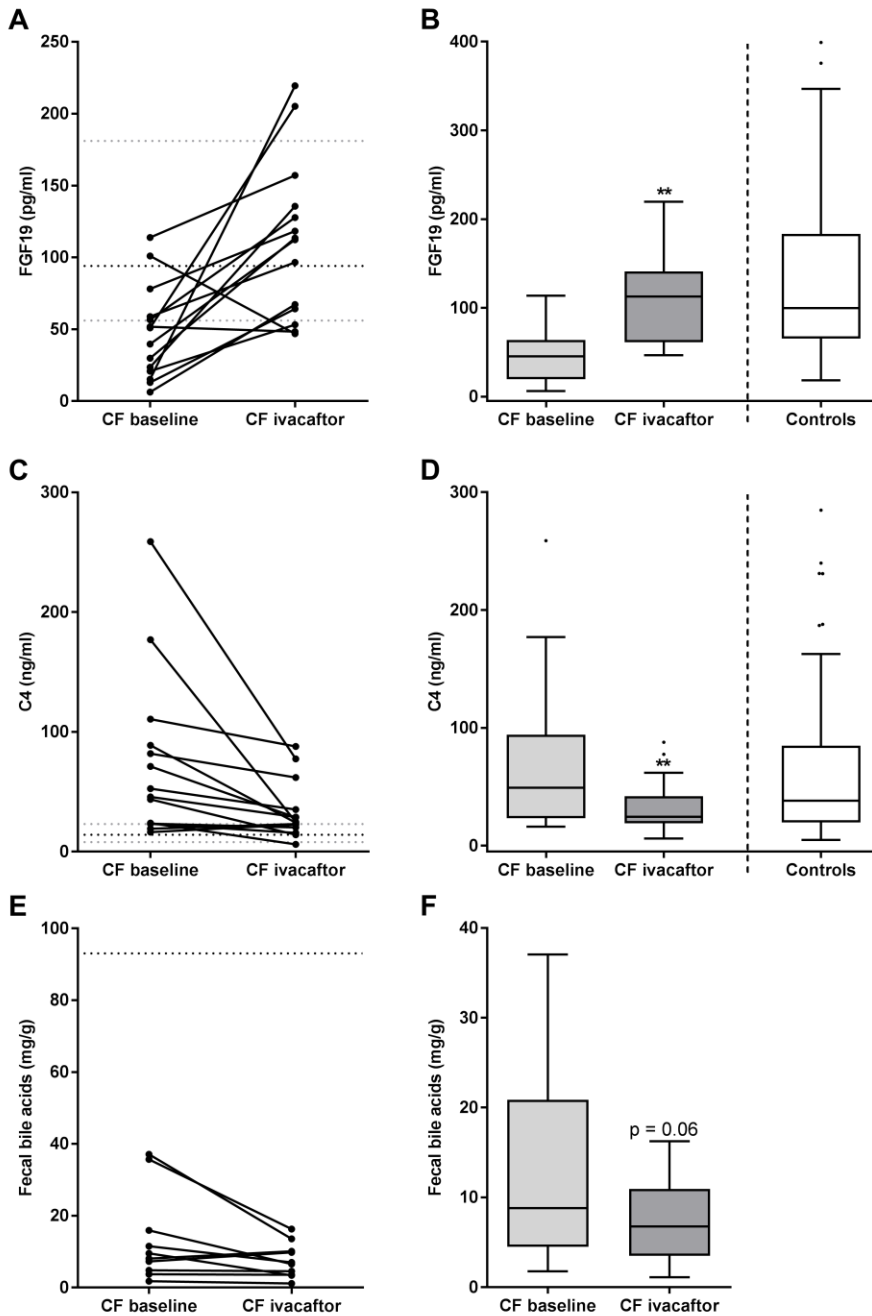


Figure 1. Improvement of bile acid metabolism markers after ivacaftor treatment in the Tic-Tac study cohort. (A) change in plasma FGF19 of individual patients, **(B)** change in plasma FGF19 on group level n = 14; **(C)** change in plasma 7 α -hydroxy-4-cholesten-3-one (C4) of individual patients, **(D)** change in plasma C4 on group level n = 14 , dotted lines represent median in black and interquartile

range (IQR) in dark grey of healthy controls; **(E)** change in fecal bile acid (BA) concentrations of individual patients, **(F)** change in fecal BA concentrations on group level (n=10). **p<0,01; Wilcoxon signed rank test. Data expressed as Tukey plots, outliers represented as separate dots.

G551D mutation cohort

Ivacaftor improves sweat chloride level, BMI and FEV₁ scores in CF patients with a G551D mutation

Table 2 shows the characteristics of the control patients and of the patients with a G551D mutation before and 6 months after treatment with ivacaftor. Improvements in sweat chloride level, FEV₁ and BMI were published before by *Rowe et al.* (29). Also in the GOAL cohort, median age was significantly lower compared to the control group (16 vs. 33 years, *P* < 0.001). BMI was also significantly lower before (20.4 vs. 24.4 kg/m², *P* < 0.001) and after ivacaftor treatment (21.0 vs. 24.4 kg/m², *P* < 0.001) compared to the control group.

Of the 99 patients included, 49% was male. More detailed baseline characteristics and the effect of ivacaftor on the improvement in BMI, FEV₁ and sweat chloride level of this cohort have recently been published as part of the first GOAL study report (25).



	controls	GOAL-study		p-value
		Before ivacaftor	After Ivacaftor	
n	130	99	99	
Age	33 (25-41)	16 (10-24)	+ 6 months	
BMI	24.4 (22.5-26.6)	20.4 (17.2-23.2)	21.0 (18.4-24.1)	<0.001
Sweat chloride (mEq/L)		104.8 (99.9-109.6)	43.0 (29.0-58.3)	<0.001
FEV1 (% of predicted)		93.4 (73.4-104.8)	99.6 (81.0-110.3)	<0.001

Table 2. Basic characteristics of controls and CF patients before and after ivacaftor treatment in the GOAL study cohort. Data displayed as median with interquartile range (IQR) between brackets. *P*-values displayed represent the differences before and after ivacaftor treatment; Wilcoxon signed rank test.

Ivacaftor improves plasma FGF19 and C4 levels towards healthy control values in CF patients with a G551D mutation

Fig. 2A shows the FGF19 levels of CF patients with a G551D mutation at baseline and after 6 months of ivacaftor treatment. At baseline median FGF19 levels were significantly lower in CF patients as compared to healthy controls (44

vs. 94 pg/ml; $P < 0.001$). After 6 months of ivacaftor treatment median FGF19 levels significantly increased from 44 to 77 pg/ml ($P < 0.001$). An increase was observed in 73% of the ivacaftor treated patients. Ivacaftor treatment normalized FGF19 levels to healthy control values (77 vs. 94 pg/ml, $P = 0.11$), similar as found in patients with an S1251N mutation.

Fig. 2B shows the median levels of plasma C4 before and after 6 months of ivacaftor treatment. At baseline median C4 levels, representing compensatory hepatic BA synthesis, were significantly higher in CF patients with a G551D mutation as compared to healthy controls (51 vs. 14 ng/ml, $P < 0.001$). In accordance with the observed increase in FGF19 level, C4 levels decreased in 70% of patients and median C4 levels decreased from 51 to 30 ng/ml ($P < 0.001$). While median C4 levels improved towards normality after ivacaftor treatment, they still remained significantly higher than median levels of healthy control value (30 vs 14 ng/ml, $P < 0.001$). While the increase in FGF19 level was significantly correlated with a decrease in C4 level ($r = -0.43$, $P < 0.0001$), the decrease in C4 can only partially be explained by the increase in FGF19 ($r^2 = 0.18$). **Fig. 2C** shows the inverse correlation between C4 and FGF19 levels.

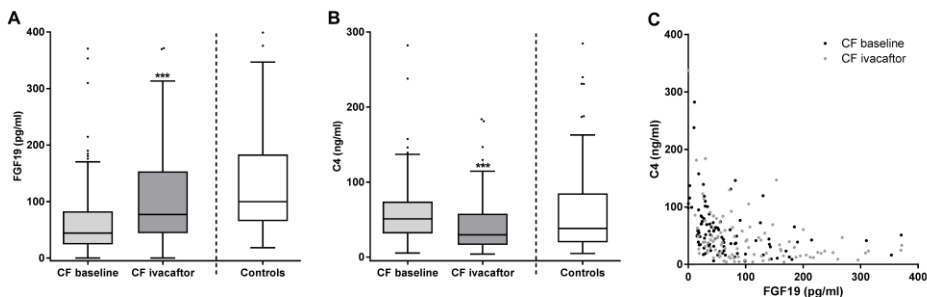


Figure 2. Effect of ivacaftor treatment on bile acid metabolism markers in the GOAL study cohort. (A) change in FGF19 on group level and **(B)** change in C4 on group level ($n=99$). *** $P < 0.001$; Wilcoxon signed rank test. Data expressed as Tukey plots, outliers represented as separate dots, dotted lines represent median in black and interquartile range (IQR) in dark grey of healthy controls; **(C)** C4 and FGF19 levels are similarly inversely correlated before (Spearman $r = -0.50$, $P < 0.001$) and after (Spearman $r = -0.42$, $P < 0.001$) ivacaftor treatment, $n = 99$;

Changes in FGF19 and C4 do not correlate with effects of ivacaftor on sweat chloride concentrations and FEV₁

An elevated sweat chloride level (**Table 1 and 2**) is considered a surrogate marker for a decreased *in vivo* CFTR chloride channel function.²⁴ We, therefore, correlated the changes in FGF19 and C4 of patients with a G551D mutation to the

ivacaftor-induced decrease in sweat chloride levels (**Fig. 3A-B**). There was no significant correlation between the change in either FGF19 or C4 and the improvement in sweat chloride levels after ivacaftor treatment ($r = -0.14$, $P = 0.19$ and $r = 0.11$, $P = 0.28$, respectively).

To determine whether the difference in BA homeostasis upon ivacaftor treatment was related to improvement in clinical outcome parameters of CF, we correlated FGF19 and C4 level changes to ivacaftor induced changes in FEV₁ (**Fig. 3C-D**). No significant correlation was found between either FGF19 ($r = 0.05$, $P = 0.63$) or C4 ($r = 0.02$, $P = 0.85$) and FEV₁. Similarly, no significant correlation was found between FGF19 or C4 and BMI (**Fig. S1**).

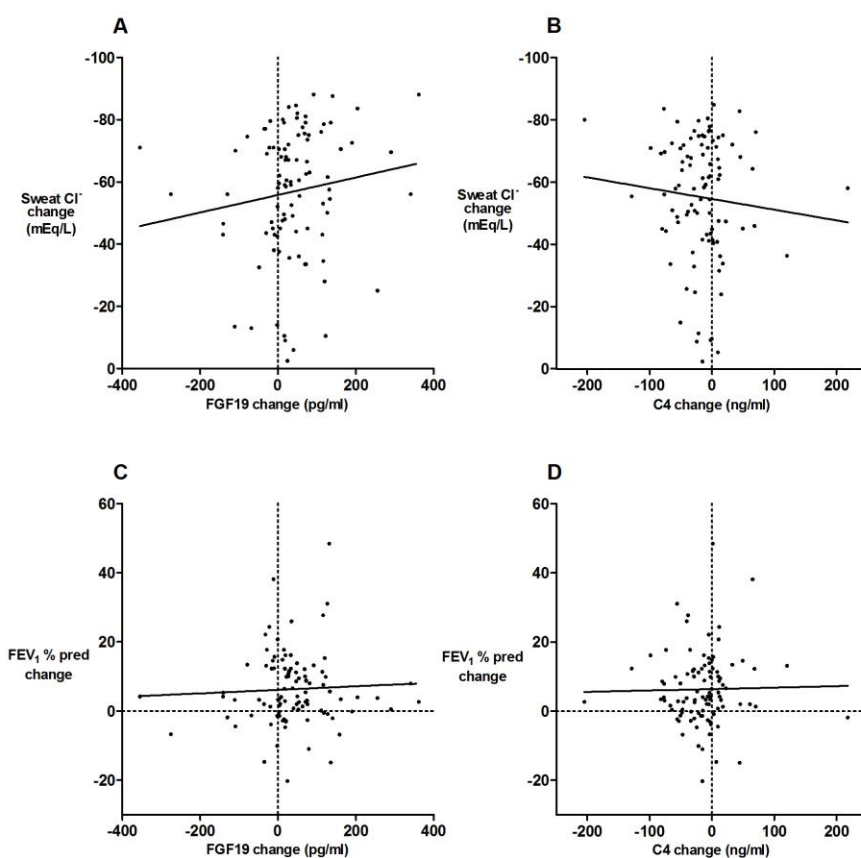


Figure 3. Correlation between FGF19 and C4 and sweat chloride and FEV₁. (A) an increase in FGF19 (Pearson $r = -0.14$, $P = 0.19$) and (B) decrease in C4 (Pearson $r = 0.11$, $P = 0.28$) are not significantly correlated with a greater decrease in sweat chloride level $n = 96$; (C) an increase in FGF19 (Pearson $r = 0.05$, $P = 0.63$) and (D) decrease in C4 (Pearson $r = 0.02$, $P = 0.85$) are not significantly correlated with an increase in FEV₁ % of predicted, $n = 97$

Discussion

In this study, we demonstrate, in two independent studies, that the enterohepatic feedback regulation of BAs is impaired in CF patients with a class III CFTR gating mutation as reflected by decreased plasma FGF19 and increased plasma C4 levels compared to control values. Our data also demonstrate that ivacaftor treatment restored these markers towards normalization.

To establish baseline disturbances in FGF19 and C4 we compared CF values to healthy controls. We found that FGF19 levels were lower and C4 levels higher in both CF patients with an S1251N or a G551D mutation, in line with a disruption of BA homeostasis. Unfortunately, we could not obtain control samples of age and BMI matched patients. However, aside from a surge of FGF19 in very early life (2-12 months of age) to our knowledge, there is no correlation with FGF19 and C4 and age (30). Additionally, the median C4 level in our control group was 14 ng/ml which corresponds to healthy control values reported in the literature, ranging from 9 to 19 ng/ml (31). To confirm that age did not play a role in our study we performed a sub-analysis of FGF19 and C4 in different age groups and did not find a significant difference (data not shown). As for BMI, it has been shown that overweight and obesity correlate with lower plasma FGF19 levels (32). As the median BMI in our control group was 24.4, control FGF19 values could represent a slight underestimation compared to a lower BMI controlled group. However, we found no correlation between BMI and FGF19 levels in our control group nor in CF patients (data not shown). Additionally, BMI values in children under 18 are dependent on age and could, therefore, represent an underestimation compared to similar values in adults (33).

Even though ivacaftor treatment significantly improved median FGF19 and C4 levels, there was a large variation between patients. We have identified several factors which may contribute to the variation. First, plasma FGF19 and C4 levels both express a diurnal rhythm and respond to food intake (10). Postprandial FGF19 levels are known to rise about 2-fold within 1-3 hours after having a meal and are also differentially affected by carbohydrates, lipids, and proteins (34). Especially for C4 diurnal variation is pronounced with two peaks of a 2 to 4 fold increase during the day (35). For the GOAL and TICTAC-2 study patients were not fasted and the time at which blood samples were taken was not standardized. This likely added more variation to the measured parameters and could account for the small portion of patients for which FGF19 and C4 changed in the opposite

direction. However, on group level, we observed a similar change in FGF19 and C4 in two independent cohorts supporting our conclusion that parameters of bile acid metabolism improve on ivacaftor treatment in CF patients with specific mutations. However it remains possible that the observed beneficial effects would be more pronounced if obtained after fasting and under more standardized conditions.

These results elicit speculation on the exact mechanism underlying the effects of ivacaftor on the enterohepatic circulation of BAs. There could be a direct effect of potentiated CFTR on ileal BA uptake or on intestinal FXR activation. CFTR has previously been implicated in modulating the expression and in enhancing the function of the apical sodium bile acid transporter (ASBT), the main BA uptake transporter in the distal small intestine (5). Alternatively, the effect of ivacaftor could be indirect, for example on intestinal factors that could modulate BA metabolism such as BA absorption, inflammation or microbial composition. Absorption might also be increased by an alteration in the intestinal luminal mucus layer resulting in higher permeability for BAs towards the apical membrane of the enterocyte. Ivacaftor could ameliorate intestinal inflammation or dysbiosis, common features of the CF gastrointestinal phenotype (9,36,37).

The relevance of an improved enterohepatic BA circulation is not limited to possible improvement of nutrient (particularly fat) absorption. Recent studies suggest an important role of BA homeostasis in various metabolic processes (38). In addition, FGF19 has hormone-like properties and is implicated to beneficially affect glucose homeostasis, metabolic syndrome, and liver regeneration (39,40). Since CF patients are prone to develop metabolic complications, an improved bile acid homeostasis could potentially ameliorate these disorders (41,42).

The improvements in plasma FGF19 and C4 levels did not correlate with the improvement in sweat chloride levels. This suggests that the ivacaftor-induced improvement in CFTR function is quantitatively different *in vivo* between various organ systems (e.g. sweat gland and GI system) (43). However, a recent study in CF patients with a G551D mutation found that there is a high rate of inter- and intra-subject variability in sweat chloride levels which could also account for some of the discrepancy (44). Another factor that could contribute to the difference is that ivacaftor is taken orally and results in a higher bioavailability in the intestine compared to other organs. The effect could, therefore, be more potent in the

intestine. A recent study supports this idea by showing that the combination of lumacaftor/ivacaftor treatment in CF patients with a Phe508del mutation showed greater improvements *in vivo* intestinal CFTR function (assessed by intestinal current measurement) compared to respiratory epithelial CFTR function (measured by nasal potential difference) (45). In this study there was also no correlation between these parameters and other CF-related outcomes (FEV₁, sweat chloride and BMI) supporting the notion of heterogeneity in the individual responses to CFTR correction therapies. Additionally, CFTR potentiation in the intestine could affect various other GI factors that are disturbed in CF and influence BA metabolism directly or indirectly. These include effects on intestinal pH, microbiota, small intestinal bacterial overgrowth (SIBO) and inflammation. Ivacaftor has also been shown to affect other ATP-binding cassette (ABC) transporters such as ABCB4 (MDR3), which mediates phosphatidylcholine secretion into the bile (46,47). BMI in CF is mainly dependent on pancreatic insufficiency and the pulmonary condition (48). Changes in BMI were not correlated to the changes in BA homeostasis. However, BMI is not only a representation of nutritional status or the functionality of the GI tract but is also related to whole body metabolism (49). FEV₁ is a reflection of pulmonary function and dependent on many variables including pulmonary infection, inflammation, smoking, and exercise. Also, when baseline FEV₁ level is low, there is usually some irreversible damage present making treatment induced improvement more tedious. It is known that there is a relatively large variation in the FEV₁ improvement of individual patients treated with ivacaftor. The large inter-individual variation might corroborate the correlation between FEV₁ and parameters of BA homeostasis.

These observations indicate that the effect of CFTR modulation, potentially, varies in different organ systems, e.g. in the pulmonary system vs. the gastrointestinal tract. Different individual outcome parameters such as FEV₁, BMI and sweat chloride concentration, may differ in the dependency on direct CFTR protein function. There could also be a variation in the involvement of gene and environmental protein function modifiers in the various organ systems. Based on these considerations, we conclude that it is sensible to use an extended panel of available outcomes measures in different organ systems to access the overall therapeutic effects of CFTR modulators (50).

In conclusion, our data show that FGF19 and C4 provide information as parameters on the disruption BA homeostasis in CF and their improvement upon

CFTR modulation therapy. Since FGF19 and C4 can be measured in plasma, they are relatively easy to obtain and can be used even in young children and neonates with still preserved growth and lung function (51). However, additional studies are indicated to investigate the relationship between FGF19 and C4 and parameters of intestinal and liver function. Additionally, research is needed to validate FGF19 and C4 as potential biomarkers for clinical trials in CF.

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Supplementary tables and figures

	Fecal bile acid profile (Tic-Tac study)		
	Before ivacaftor	After Ivacaftor	p-value
n	10	10	
Deoxycholic acid	26.1 (7.7-56.5)	34.3 (11.4-47.7)	0.77
Cholic acid	18.6 (6.2-32.0)	12.9 (1.0-29.9)	0.49
Ursodeoxycholic acid	6.5 (5.4-20.3)	9.3 (4.0-20.6)	1.00
Chenodeoxycholic acid	13.2 (5.3-20.0)	11.7 (5.1-23.8)	0.92
Lithocholic acid	10.9 (4.8-22.0)	15.8 (3.2-34.3)	0.43
Iso-lithocholic acid	1.4 (0.0-2.7)	2.4 (0.0-7.3)	0.49
Total primary	35.9 (11.5-50.0)	23.5 (5.4-53.5)	0.56
Total secondary	64.1 (50.0-88.5)	76.5 (46.5-94.7)	0.56

Table S1. Profile of individual BA species in percentage before and 2 months after treatment with ivacaftor in S1251N patients. Data displayed as median (IQR). *P*-values displayed represent the differences before and after ivacaftor treatment; Wilcoxon signed rank test.

	Plasma bile acid profile (Tic-Tac study)		
	Before ivacaftor	After Ivacaftor	p-value
n	14	14	
Total bile acid concentration (μM)	3.4 (1.9-6.7)	4.1 (3.0-5.8)	0.33
All deoxycholic acid (%)	4.2 (0.2-11.7)	13.5 (0.4-27.8)	0.24
All Cholic acid (%)	11.0 (8.1-15.8)	9.7 (5.3-12.9)	0.19
All Ursodeoxycholic acid (%)	18.0 (13.4-21.6)	13.1 (10.3-27.7)	0.86
All Chenodeoxycholic acid (%)	58.4 (44.6-69.3)	50.8 (38.4-65.8)	0.12
All Lithocholic acid (%)	0.0 (0.0-0.0)	0.0 (0.0-0.1)	n.d.
Total unconjugated (%)	26.6 (9.8-40.9)	19.4 (8.4-30.6)	0.63
Total glycine conjugated (%)	70.8 (58.5-77.9)	74.3 (65.2-82.8)	0.71
Total taurine conjugated (%)	1.8 (0.7-2.7)	4.6 (2.2-8.7)	0.05
Total primary (%)	73.9 (60.8-84.0)	61.9 (46.8-72.4)	0.09
Total secondary (%)	26.1 (16.0-39.2)	38.1 (27.6-53.2)	0.09

Table S2. Concentration and profile of individual BA species in plasma before and 2 months after treatment with ivacaftor in S1251N patients. Data displayed as median (IQR). *P*-values displayed represent the differences before and after ivacaftor treatment; Wilcoxon signed rank test.

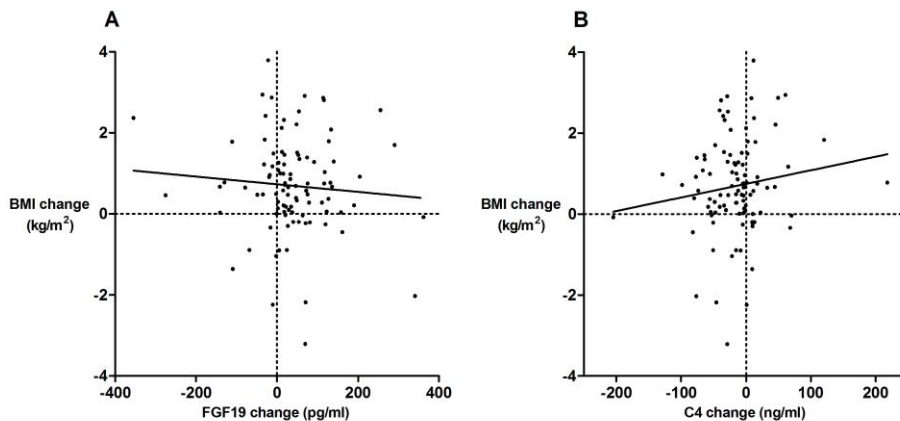


Figure S1. Correlation between changes in FGF19 and C4 and BMI. (A) correlation between an increase in FGF19 (Pearson $r = -0.08$, $P = 0.42$) and (B) decrease in C4 (Pearson $r = 0.14$, $P = 0.17$) with an increase in BMI, $n=99$.

