No indications for altered essential fatty acid metabolism in two murine models for cystic fibrosis

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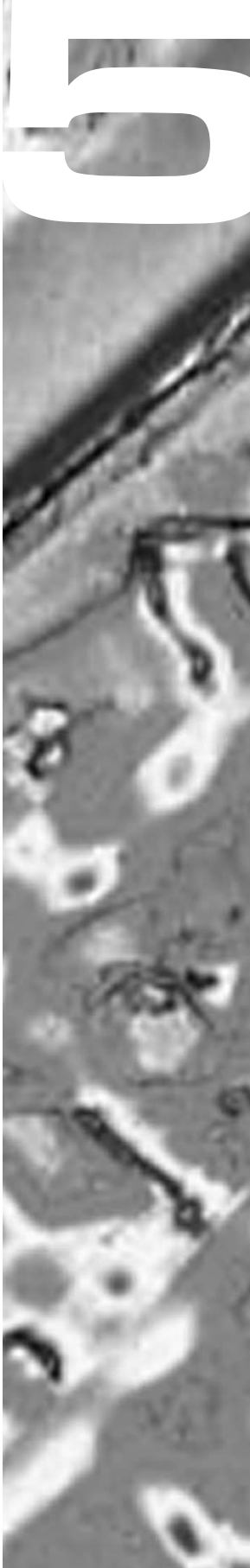

ABSTRACT

Background: A deficiency of essential fatty acids (EFA) is frequently described in cystic fibrosis (CF), but whether this is a primary consequence of altered EFA metabolism or a secondary phenomenon, is unclear. It was suggested that defective long-chain polyunsaturated fatty acid (LCPUFA) synthesis contributes to CF phenotype. To establish whether CFTR dysfunction affects LCPUFA synthesis, we quantified EFA metabolism in *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice.

Methods: Effects of intestinal phenotype, diet, age and genetic background on EFA status were evaluated in *cftr* $^{-/-CAM}$ mice, dF508/dF508 mice and littermate controls. EFA metabolism was measured by ^{13}C stable isotope methodology *in vivo*. EFA status was determined by gas chromatography in tissues of *cftr* $^{-/-CAM}$ mice, dF508/dF508 mice, littermate controls and C57Bl/6 wildtypes, fed chow or liquid diet.

Results: After enteral administration of ^{13}C-EFA, arachidonic acid (AA) and docosahexaenoic acid (DHA) were equally ^{13}C-enriched in *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice, indicating similar EFA elongation/desaturation rates. LA, ALA, AA and DHA concentrations were equal in pancreas, lung and jejunum of chow-fed *cftr* $^{-/-CAM}$ and dF508/dF508 mice and controls. LCPUFA levels were also equal in liquid diet-weaned *cftr* $^{-/-CAM}$ mice and littermate controls, but consistently higher than in age- and diet-matched C57Bl/6 wildtypes.

Conclusions: *Cftr* $^{-/-CAM}$ mice adequately absorb and metabolize EFA, indicating that CFTR dysfunction does not impair LCPUFA synthesis. A membrane EFA imbalance is not inextricably linked to CF genotype. EFA status in murine CF models is strongly determined by genetic background.

INTRODUCTION

A deficiency of essential fatty acids (EFA) or their long-chain polyunsaturated metabolites (LCPUFA) has frequently been reported in CF patients[1-4] and has formerly been attributed to fat malabsorption due to pancreatic insufficiency. Current high-fat, hypercaloric nutritional strategies and improved pancreas enzyme replacement therapies can usually maintain patients in optimal nutritional status, thus normalizing EFA status in many CF patients[5]. Yet, several reports still indicate the occurrence of EFA deficiency in CF[6-8]. Although some authors have suggested that residual fat malabsorption and increased EFA turnover in CF may compromise EFA status[9; 10], the exact pathophysiology of EFA deficiency in CF patients has not been elucidated.
A direct link between CFTR dysfunction and EFA metabolism has been postulated by Gilljam *et al.*[11], and Bhura-Bandali *et al*.[12] described impaired EFA incorporation into phospholipids in human pancreatic CF cells. In *cftr* $^{-/-UNC}$ mice, Freedman *et al.*[13] reported a profound membrane fatty acid imbalance, characterized by increased concentrations of arachidonic acid (AA) and decreased concentrations of docosahexaenoic acid (DHA) in membrane phospholipids of organs typically affected in CF, such as pancreas, lung and intestine. Oral supplementation with DHA, but not with its precursor ALA, corrected this lipid imbalance and was reported to reverse certain pathological features of the disease. These studies suggested that CFTR exerts control over LCPUFA synthesis from EFA, and that impaired EFA processing primarily contributes to CF pathology[13; 14]. However, it has not been elucidated whether perturbed EFA status in CF is a primary result of CFTR malfunction or secondary to fat malabsorption or increased turnover.

Several CF mouse models have been developed in the past decade, including total null mice with no detectable CFTR production[15-17] as well as mice with the delta F508 mutation (dF508/dF508 mice), which have low-level residual CFTR activity[18]. Similar to CF patients, CF mouse models display significant phenotypic variability, particularly concerning the severity of gastrointestinal symptoms such as intestinal obstruction and fat malabsorption.
To assess the effect of CFTR on LCPUFA synthesis, we quantified conversion of EFA into LCPUFA *in vivo* in *cftr* $^{-/-CAM}$ mice and littermate controls. In addition, we analyzed fecal fatty acid excretion and membrane fatty acid composition in tissues of *cftr* $^{-/-CAM}$ mice[17] and homozygous dF508 mice[18], and of their respective littermate controls. These particular CF models have been demonstrated to differ in intestinal phenotype, with fat malabsorption present in *cftr* $^{-/-CAM}$ mice but absent in dF508/dF508 mice[19].

Furthermore, we determined the effects of age, diet and genetic background on EFA status in these mouse models and in C57Bl/6 wildtype mice. Our results indicate that *cftr* $^{-/-CAM}$ mice adequately absorb, elongate and desaturate intragastrically administered EFA, and that a membrane fatty acid imbalance in CF-affected tissues is not inherent to CF genotype in mouse models with and without fat malabsorption. Rather, EFA status in CF mice is strongly determined by genetic background, diet and age.

METHODS

Animals

C57Bl/6/129 *cftr* $^{-/-tm1CAM}$ mice and *cftr* $^{+/+tm1CAM}$ littermates[17], homozygous dF508 mice and sex-matched littermate controls (N/N) of FVB/129 background[18] and wildtype C57Bl/6 mice were accommodated at the breeding colony at the Erasmus Medical Center, Rotterdam, the Netherlands. Southern blotting of tail-clip DNA was performed to verify the genotype of individual animals[20]. Mice were housed in a light-controlled (lights on 6 AM to 6 PM) and temperature-controlled (21°C) facility and were allowed tap water and standard laboratory chow (Hope Farms BV Woerden, the Netherlands) or liquid diet (Peptamen) *ad libitum* from the time of weaning. The Ethical Committee for Animal Experiments in Rotterdam approved of the experimental protocols.

Experimental diets

The standard laboratory chow contained 6 weight% fat and 14 energy% fat, and had the following fatty acid composition: 18.2 mol% palmitic acid (C16:0), 7.0 mol% stearic acid (C18:0), 25.8 mol% oleic acid (C18:1n-9), 39.1 mol% linoleic acid (C18:2n-6), 3.5 mol% alpha-linolenic acid (C18:3n-3), 0.3 mol% arachidonic acid (C20:4n-6) and 0.05 mol% docosahexaenoic acid (C22:6n-3) (Hope Farms BV, Woerden, the Netherlands). The Peptamen liquid diet (Nestle Clinical Nutrition, Brussels, Belgium) contained 3.7 g fat/100 ml (33 energy%) and had the following fatty acid composition: 16.4 mol% palmitic acid (C16:0), 6.7 mol% stearic acid (C18:0), 22.2 mol% oleic acid (C18:1n-9), 43.6 mol% linoleic acid (C18:2n-6), 4.6 mol% alpha-linolenic acid (C18:3n-3), 0.1 mol% arachidonic acid (C20:4n-6) and 0.08 mol% docosahexaenoic acid (C22:6n-3).

Experimental procedures

Cftr $^{-/-CAM}$ mice and *cftr* $^{+/+CAM}$ littermates (n=5-6 per group) were fed standard laboratory chow from weaning. At 3 months of age, mice were anesthetized with isoflurane

and a baseline blood sample was obtained by tail bleeding. Subsequently, a 100 ml lipid bolus containing uniformly labeled ^{13}C-LA and ^{13}C-ALA was slowly administered by intragastric gavage, for determination of *in vivo* conversion of EFA into LCPUFA and partitioning to different organs. The lipid bolus was composed of olive oil mixed with U-^{13}C-LA (0.40 mg) and U-^{13}C-ALA (0.40 mg) (Martek Biosciences Corporation, Columbia, MD, USA). U-^{13}C-LA and U-^{13}C-ALA were 99% ^{13}C-enriched, with a chemical purity exceeding 97%. At 24 hours after bolus administration, a large blood sample was obtained by cardiac puncture and pancreas, liver, lungs and intestine were removed and stored at -80°C until further analysis. Intestine and lungs were flushed with ice-cold 0.9% (w/w) NaCl before storage. Blood was collected in heparinized vials and plasma and erythrocytes were separated by centrifugation. Erythrocyte membrane lipids were hydrolyzed and methylated for fatty acid analysis the same day[21] to prevent fatty acid oxidation, and plasma was stored at -80°C.

To establish the effect of fat malabsorption, diet, age and genetic background on body EFA status, homozygous dF508 mice of FVB/129 background, sex-matched N/N littermates and *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice (n=5-6 per group) were fed standard laboratory chow from weaning. At the age of three months, mice were anesthetized with isoflurane and sacrificed by means of cardiac puncture. Lung, pancreas and jejunum were removed and samples of each were immediately stored at -80°C for fatty acid and protein analysis. Fecal fatty acid excretion was quantified by gas-chromatographic analysis of feces aliquots obtained after a 72h fat balance.

A separate group of *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice and C57Bl/6 wildtypes (n=6 per group) were weaned at 23 days of age, and subsequently put on Peptamen liquid diet *ad libitum* for 7 days. At postnatal day 30, mice were anaesthetized and blood, pancreas, lung, jejunum, ileum, and liver samples were obtained. Jejunum, ileum and lungs were flushed with ice-cold saline and ileal mucosa was separated from submucosal layers by scraping with a glass microscope slide on an ice-cooled glass plate. Pancreatic cell suspensions were prepared by mechanical dissociation and addition of collagenase as described by Bruzzone *et al.*[22]. Lung tissue was flushed with Krebs-Henseleit buffer (KHB), pH 7.4, containing 0.5% BSA to rinse off contaminating blood. Lung tissue was then finely cut and suspended in 10 ml of oxygenated KHB containing 1000 units of collagenase, 2000 units of DNAse and 0.5 units of thermolysin, and incubated for 30 min at 37°C. The lung cell suspension was then sedimented through KHB containing 4% BSA and washed once in KHB. All organ samples were stored at -80°C until further analysis.

Analytical techniques

^{13}C enrichment analysis

Gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS; DeltaPlusXL, Thermo Finnigan, Bremen, Germany) was used to measure ^{13}C enrichment of LA and ALA and their metabolites. The GC-C-IRMS was equipped with a 50mx0.22mm BPX70 capillary column and the injector temperature was set at 275°C with splitless injection. The gas chromatograph oven was programmed from an initial temperature of 50°C to a final temperature of 250°C in 3 steps (50°C, held 1 min isotherm; 50-100°C, ramp 7°C/min; 100-225°C, ramp 10°C/min; 225-250°C, ramp 25°C/min, held 10 min). Helium was used as a carrier gas with a constant flow rate of 0.5 ml per minute. $^{12}CO_2^+$ and $^{13}CO_2^+$ ions were measured at m/z 44 and 45. Correction for ^{17}O was achieved by measurement of ^{18}O abundance at m/z 46.

Fatty acid analysis

Fatty acid profiles were determined by hydrolyzing, methylating and extracting total plasma lipids and erythrocyte membrane lipids as described by Muskiet *et al.*[21]. For fatty acid analysis of liver, intestine, pancreas and lung tissue, samples were mechanically homogenized in 0.9% NaCl and lipids were extracted from aliquots of tissue homogenate as described by Bligh and Dyer[23]. The lipid extract was partly methylated *in toto* for GC analysis, and partly fractionated into phospholipids, cholesterol esters, triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids using thin-layer chromatography (TLC) (20x20 cm, Silica gel 60 F254, Merck) with hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as solvent. TLC plates were dried and colored by iodine, and PL and TG spots were scraped. Of these scrapings, fatty acid methyl esters were prepared as mentioned above. To account for losses during lipid extraction, heptadecanoic acid (C17:0) was added to all samples as internal standard prior to Bligh & Dyer procedures. BHT was added as antioxidant. Aliquots of chow diet and feces were freeze-dried and homogenized, after which lipids were hydrolyzed, methylated and extracted for fatty acid analysis. Similarly, fatty acid composition of Peptamen liquid diet was determined after dissolution in chloroform/methanol (2/1 v/v).

Fatty acid methyl esters were separated and quantified by gas liquid chromatography (GLC) on a Hewlett Packard gas chromatograph model 6890, with a 50mx0.2mm Ultra 1 capillary column (Hewlett Packard, Palo Alto, CA) and a FID detector as described previously[24]. We verified purity of AA and DHA peaks as separated by GLC, using a gas chromatography-mass spectrometer (GC-MS; Finnigan MAT SSQ7000), alternately equipped with a 50mx0.2mm Ultra 1 capillary

column or a 50mx0.22mm BPX70 capillary column (SGE, Weiterstadt, Germany). Both methylesters and pentafluorbenzylbromide (PFB-Br) derivatives of tissue fatty acids were analyzed, no indications for impurity of AA or DHA peaks were detected.

Protein analysis

Total protein contents of tissue homogenates were determined with Folin phenol reagent as described by Lowry *et al.*[25] Standard Pierce BSA was used as reference.

Calculations

^{13}C abundance was expressed as $d^{13}C$-PDB value, i.e. the difference between the sample value and baseline compared to Pee Dee Belemnite limestone. $d^{13}C$-PDB values were converted to atom% ^{13}C values. Enrichment (atom% excess) was calculated by subtracting baseline ^{13}C abundance from all enriched values.

Relative concentrations (mol%) of individual fatty acids in plasma, erythrocytes, liver, intestine, pancreas and lung were calculated by summation of all fatty acid peak areas and subsequent expression of areas of individual fatty acids as a percentage of this amount. Fatty acid contents were quantified by relating the areas of their chromatogram peaks to that of the internal standard heptadecanoic acid (C17:0).

Statistics

All results are presented as means ± S.D. for the number of animals indicated. Data were statistically analyzed using Student's t-test or, for comparison of more than two groups, ANOVA-test with post-hoc Bonferroni correction. Statistical significance of differences between means was accepted at $p<0.05$. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL).

RESULTS

In vivo conversion of ^{13}C-labeled EFA into LCPUFA

LCPUFA in specific tissues originate either from the diet or from endogenous synthesis by elongation and desaturation of EFA. As CFTR dysfunction has been postulated to affect EFA tissue incorporation or rate of metabolism[12; 13; 26; 27], we quantified *in vivo* the appearance in different organs of ingested ^{13}C-labeled EFA, and their conversion into LCPUFA. At 24 hours after intragastric administration of ^{13}C-LA and ^{13}C-ALA, ^{13}C enrichment of LA and ALA could be demonstrated in all analyzed tissues of *cftr* $^{-/-CAM}$ mice and *cftr* $^{+/+CAM}$ controls. ^{13}C-LA and ^{13}C-ALA concentrations were not significantly different between *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice (Figure 1).

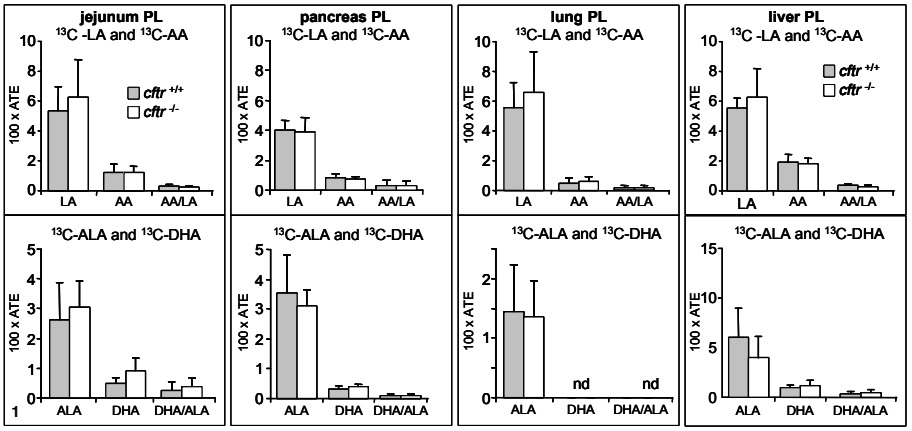

Figure 1: ^{13}C enrichment of linoleic acid (LA, C18:2n-6), arachidonic acid (AA, C20:4n-6), alpha-linolenic acid (ALA, C18:3n-3) and docosahexaenoic acid (DHA, C22:6n-3) in phospholipids of pancreas, lung, intestine and liver of *cftr*$^{+/+}$ mice (grey bars) and *cftr*$^{-/-}$ mice (white bars) at 24h after intragastric administration of ^{13}C-LA and ^{13}C-ALA. Individual fatty acid ^{13}C enrichment was calculated from the difference between the sample value and baseline compared to Pee Dee Belemnite limestone (d^{13}C-PDB), and is expressed as 100 x atom % excess. Data represent means ± S.D. of 6 mice per group. No significant differences in ^{13}C enrichment were detected between *cftr*$^{+/+}$ and *cftr*$^{-/-}$ mice, indicating normal conversion of EFA into LCPUFA.

Similarly, ^{13}C-AA levels did not significantly differ between *cftr* $^{-/-CAM}$ mice and littermate controls. ^{13}C enrichment of DHA was below detection limit in liver triglycerides (not shown) and in lung PL, but ^{13}C-DHA in jejunum, pancreas and liver phospholipids was similar in *cftr* $^{-/-CAM}$ mice and controls. The ratios between ^{13}C-LA and ^{13}C-AA and between ^{13}C-ALA and ^{13}C-DHA in liver, pancreas, lung, and intestine of *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice were highly comparable, suggesting adequate rates of LA and ALA elongation and desaturation in this CF mouse model.

Fatty acid composition of feces and tissue homogenates

To determine the effects of intestinal phenotype on EFA status, we analyzed fatty acid composition of feces and of CF-affected organs in CF mouse models with and without fat malabsorption. Figure 2 shows the daily fecal excretion of the main dietary fatty acids in dF508/dF508 and *cftr* $^{-/-CAM}$ mice and their respective controls. Fecal fatty acid excretion was similar in homozygous dF508 mice and controls, in contrast to *cftr* $^{-/-CAM}$ mice which secreted significantly more fatty acids in feces than littermate controls, confirming the presence of fat malabsorption in this CF mouse model. Malabsorption of saturated fatty acids was slightly more pronounced than that of unsaturated fatty acids.

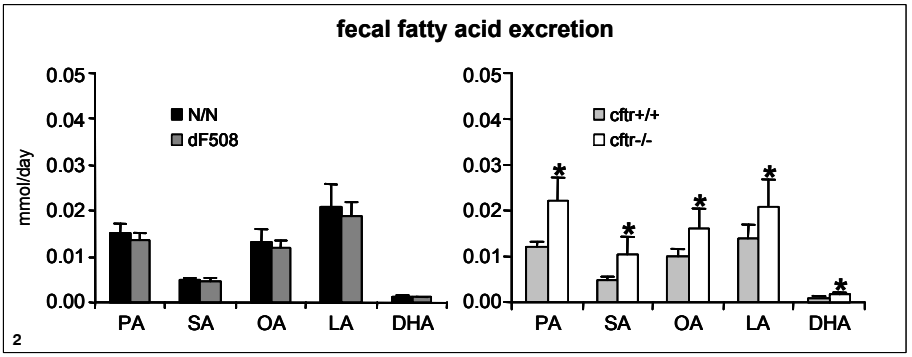

Figure 2: Relative concentrations of palmitic acid (PA, C16:0), stearic acid (SA, C18:0), oleic acid (OA, C18:1n-9), linoleic acid (LA, C18:2n-6) and docosahexaenoic acid (DHA, C22:6n-3) in fecal lipid extracts of homozygous dF508 mice and *cftr*$^{-/-}$ mice and their respective littermate controls. Fecal fat excretion was quantified by means of a 72h fecal fat balance. Individual fatty acid concentrations are expressed as mmol of fatty acid excreted per day. Data represent means ± S.D. of 5-6 mice per group. No significant differences were detected for any of the fatty acids between homozygous dF508 mice and controls, but daily fecal fatty acid excretion was significantly higher in *cftr*$^{-/-}$ mice than in *cftr*$^{+/+}$ controls ($p<0.05$).

Figure 3 shows LA, AA, ALA and DHA concentrations in tissue homogenates of pancreas, lung and jejunum in homozygous dF508 mice and *cftr* $^{-/-CAM}$ mice compared to their respective littermate controls. No significant differences were observed in relative concentrations of either these EFA and LCPUFA, nor of saturated and non-essential fatty acids (data not shown).

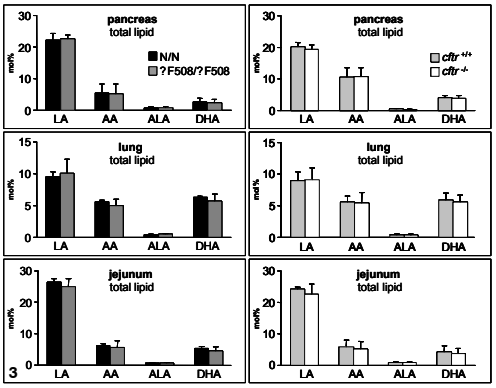

Figure 3: Relative concentrations of linoleic acid (LA, C18:2n-6), arachidonic acid (AA, C20:4n-6), alpha-linolenic acid (ALA, C18:3n-3) and docosahexaenoic acid (DHA, C22:6n-3) in total lipid extracts of homozygous dF508 mice and *cftr*$^{-/-}$ mice and their littermate controls. Fatty acid concentrations are expressed as mol% of total fatty acids. Data represent means±S.D. of 5-6 mice per group. No significant differences were detected for any fatty acid between dF508 or *cftr*$^{-/-}$ mice and littermate controls.

The fatty acid composition of the total lipid fraction depends on the fatty acid composition of the various lipid classes present (i.e., triglycerides, diglycerides, monoglycerides, phospholipids, cholesterol esters, free fatty acids), and the proportions of these lipid classes may vary considerably between organs. Since phospholipids (PL) may be a more adequate indicator for determination of EFA status, we specifically analyzed fatty acid composition of tissue PL fractions of CF-affected organs. Figure 4a shows relative fatty acid concentrations in the PL fraction of pancreas, lung and

jejunum of the two CF mouse models and their respective controls. DHA concentrations in pancreas PL were 25% lower in dF508/dF508 mice compared to N/N controls ($p<0.05$) and in lung PL, there was a small significant increase of AA in dF508/dF508 compared to N/N mice (5.3±0.7 vs. 4.3±0.5, respectively; $p<0.05$). In jejunum PL of dF508/dF508 mice, however, AA was 37% decreased (3.1±1.3 vs. 5.0±0.7, $p<0.05$) and ALA was 15% increased (0.48±0.04 vs. 0.41±0.04, $p<0.01$) compared to N/N controls. Similar differences were not observed in tissues of *cftr* $^{-/-CAM}$ mice, in which LA, AA, ALA or DHA concentrations were consistently similar compared to *cftr* $^{+/+CAM}$ littermates.

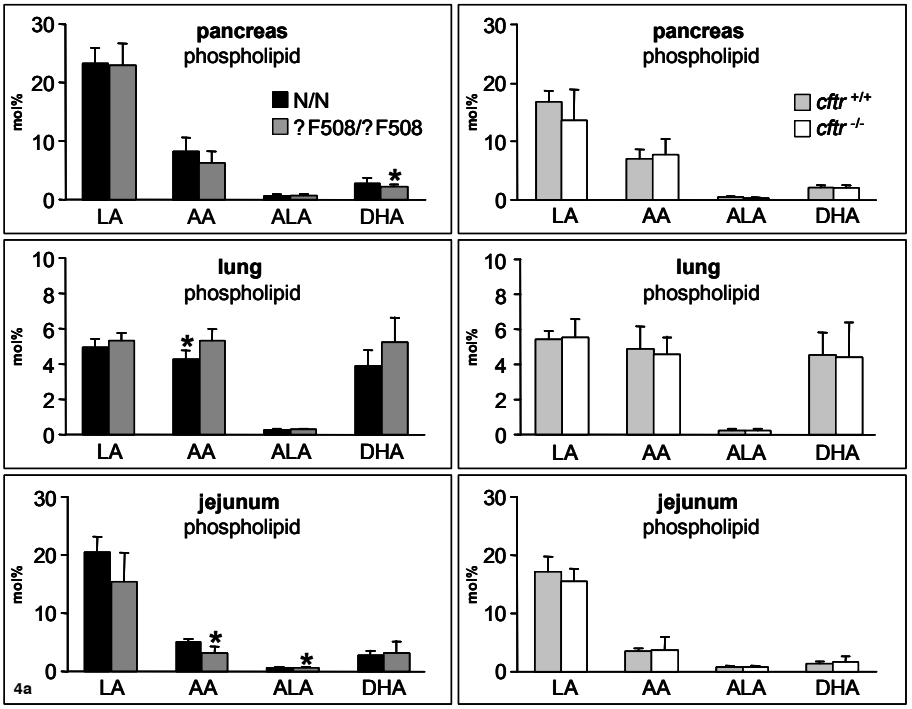

Figure 4a: Relative concentrations of linoleic acid (LA, C18:2n-6), arachidonic acid (AA, C20:4n-6), alpha-linolenic acid (ALA, C18:3n-3) and docosahexaenoic acid (DHA, C22:6n-3) in purified PL extracts of homozygous dF508 mice and cftr $^{-/-}$ mice and their littermate controls. Fatty acid concentrations are expressed as mol% of total fatty acids. Data represent means±S.D. of 5-6 mice per group, *$p<0.05$.

The severity of CF phenotype has been implicated in the EFA status of CF patients[7]. Body weight is an important clinical parameter related to severity of CF symptoms[28], exemplified by a consistently lower weight of *cftr* $^{-/-CAM}$ mice compared to littermate controls (26.5±4.4 g vs. 30.7±3.8 g, respectively, $p<0.05$). In contrast, homozygous dF508 mice, displaying normal fat absorption as a consequence of milder gastro-intestinal pathology, show normal weight gain (22.4±1.9 g vs. 23.0±1.4 g, NS).

To investigate the possible influence of nutritional status on EFA levels, EFA molar percentages were related to bodyweight for each individual mouse (Figure 4b). Neither in *cftr* $^{-/-CAM}$ nor in dF508/dF508 mice or their corresponding controls, significant correlations between relative EFA or LCPUFA concentrations and body weight could be identified in pancreas (Figure 4b), lung or intestinal PL (data not shown).

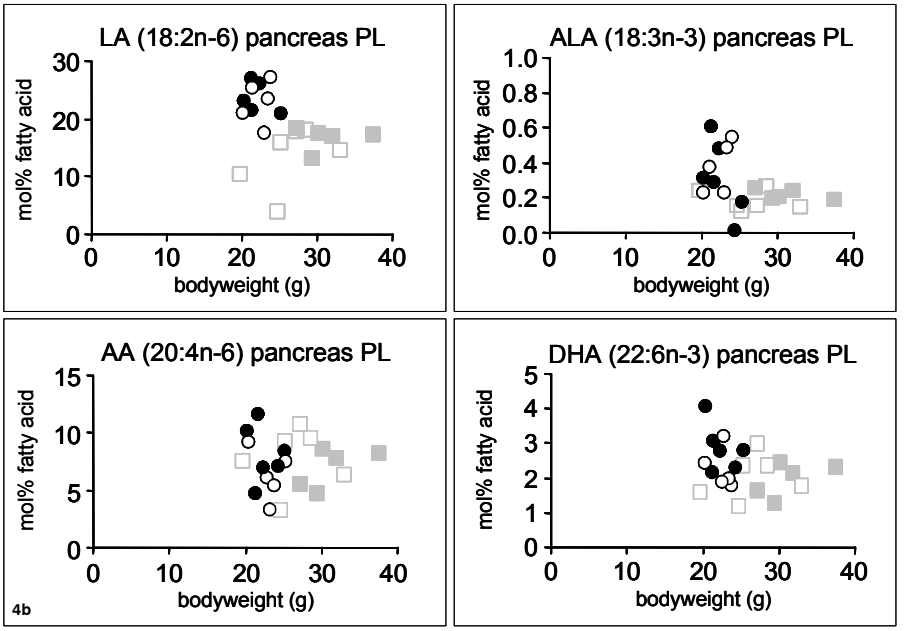

Figure 4b: Relative concentrations (mol%) of linoleic acid (LA, C18:2n-6), arachidonic acid (AA, C20:4n-6), alpha-linolenic acid (ALA, C18:3n-3) and docosahexaenoic acid (DHA, C22:6n-3) in pancreas PL related to bodyweight of homozygous dF508 mice (open circles) and *cftr*$^{-/-}$ mice (open squares) and littermate controls (N/N, closed circles; *cftr*$^{+/+}$, closed squares). No correlations were detected between body weight and fatty acid concentrations. Data represent means±S.D. of 5-6 mice per group.

In addition to relative fatty acid concentrations, absolute concentrations in tissues were determined and expressed per mg protein. Figure 4c shows that absolute LA, ALA, AA and DHA contents were similar in pancreas tissue of the two CF mouse models compared to controls. Similarly, absolute fatty acid concentrations in lung or jejunum were not significantly different between CF and control mice (not shown).

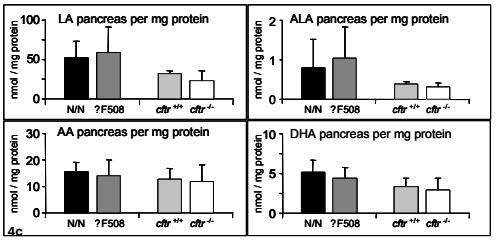

Figure 4c: Absolute concentrations (nmol) of LA, AA, ALA and DHA acid in pancreas PL per mg pancreas protein for homozygous dF508 mice and *cftr*$^{-/-}$ mice and littermate controls. No significant differences in absolute fatty acid concentrations per mg protein were detected between homozygous dF508 mice and *cftr*$^{-/-}$ mice and their littermate controls. Data represent means±S.D. of 5-6 mice per group.

Membrane EFA concentrations appeared unaffected in the presently used CF mouse models compared to littermate controls. Yet, in 1-month-old *cftr* $^{-/-UNC}$ mice weaned on a liquid diet (Peptamen), a profound membrane lipid imbalance was reported[13]. This suggests that dietary composition, caloric intake and/or age affect EFA status. To test this hypothesis, fatty acid profiles were determined in tissues of 1-month-old *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice, after weaning on Peptamen liquid diet for 7 days. Furthermore, to assess the role of genetic background, EFA profiles were determined in tissues of age-matched, liquid diet-weaned C57Bl/6 wildtype mice.

Analysis of fatty acid composition of chow pellets and Peptamen revealed relatively small differences in EFA and LCPUFA contents, with Peptamen containing less AA and more DHA than standard chow (Figure 5). Although Peptamen is frequently used in CF mouse models to prevent intestinal obstruction and to improve nutritional status, 1-month-old *cftr* $^{-/-CAM}$ mice weaned on Peptamen still had a significantly lower body mass than their *cftr* $^{+/+CAM}$ littermates (11.4±2.2g vs. 13.6±1.1g, $p<0.05$).

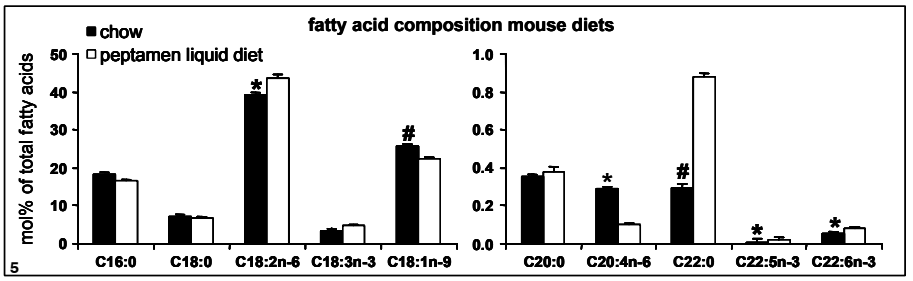

Figure 5: Fatty acid composition of standard laboratory chow and Peptamen elemental liquid diet. Data represent means ± S.D. of triple aliquot analyses of each diet. *$p<0.05$ for linoleic acid (C18:2n-6), arachidonic acid (C20:4n-6), eicosapentaenoic acid (C22:5n-3) and docosahexaenoic acid (C22:6n-3) and #$p<0.001$ for oleic acid (C18:1n-9) and behenic acid (C22:0).

PUFA concentrations in pancreas, lung and jejunum of 1-month-old, Peptamen-weaned *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice significantly differed from those of adult chow-fed *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice (Figure 6a; age effect). When compared at the same age, however, LA, ALA, AA and DHA concentrations in pancreas, lung, intestine and plasma PL were not significantly different between *cftr* $^{-/-CAM}$ mice and littermates, neither at the age of 1 month after Peptamen-weaning (Figure 6b), nor at adult age during chow-feeding. Yet, AA and DHA concentrations were consistently higher than in age- and diet-matched C57Bl/6 wildtype mice for all tissues studied ($p<0.01$). Similarly, LA concentrations in pancreas, lung and jejunum PL of *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice were significantly higher than in C57Bl/6 wildtype controls, but not significantly different between *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice. ALA levels were low in all

tissues analyzed, and although there was a tendency for lower ALA values in wildtype C57Bl/6 mice compared to *cftr* $^{-/-CAM}$ mice, this only reached significance in plasma and ileum ($p<0.05$ each). Similarly, fatty acid analyses of erythrocyte- and ileum phospholipids revealed consistently different fatty acid concentrations in C57Bl/6 mice when compared to *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice (data not shown). Other LCPUFA of the n-3 and n-6 series (C20:5n-3, C22:5n-3, C22:4n-6) also only differed between C57Bl/6 mice on one hand, and *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice on the other hand, and not between *cftr* $^{-/-CAM}$ mice and *cftr* $^{+/+CAM}$ littermates (not shown).

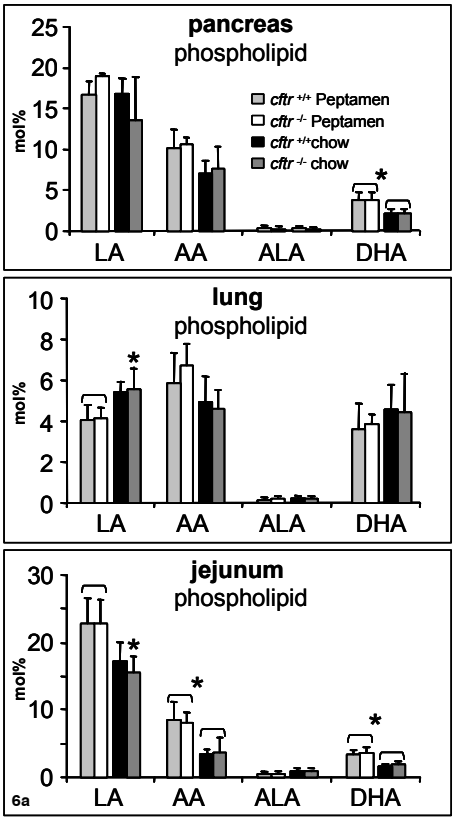

Figure 6a: Relative concentrations of linoleic acid (LA, C18:2n-6), arachidonic acid (AA, C20:4n-6), alpha-linolenic acid (ALA, C18:3n-3) and docosahexaenoic acid (DHA, C22:6n-3) in purified PL extracts of pancreas, lung and intestine of 1-month-old Peptamen-fed *cftr*$^{+/+}$ mice (light grey bars) and *cftr*$^{-/-}$ mice (white bars), adult chow-fed *cftr*$^{+/+}$ mice (black bars) and *cftr*$^{-/-}$ mice (dark grey bars). Individual fatty acid concentrations are expressed as mol% of total fatty acids. Data represent means±S.D. of 6 mice per group. *$p<0.05$ for DHA of pancreas and jejunum PL, for LA of lung and jejunum PL and for AA of jejunum PL from Peptamen-fed *cftr*$^{+/+}$ and *cftr*$^{-/-}$ mice compared to adult chow-fed *cftr*$^{+/+}$ and *cftr*$^{-/-}$ mice. No significant differences were detected between *cftr*$^{+/+}$ and *cftr*$^{-/-}$ littermates.

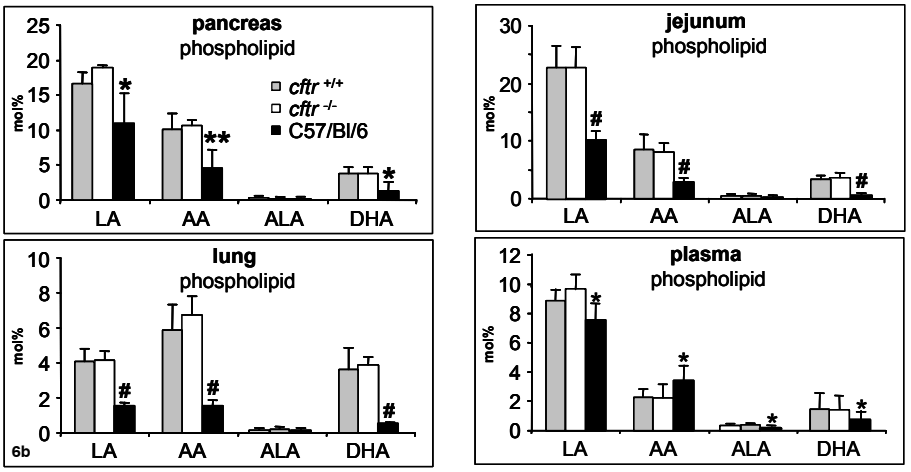

Figure 6b: Relative concentrations of LA (C18:2n-6), AA (C20:4n-6), ALA (C18:3n-3) and DHA (C22:6n-3) in purified PL extracts of pancreas, lung and intestine of *cftr*$^{+/+}$ mice (grey bars), *cftr*$^{-/-}$ mice (white bars) and wildtype C57/Bl/6/129 mice (black bars). All mice were weaned on Peptamen liquid diet from post-natal day 23 and fatty acid analyses were performed at post-natal day 30. Fatty acid concentrations are expressed as mol% of total fatty acids. Data represent means±SD of 6 mice per group. *p<0.05 for LA and DHA and **p<0.005 for AA in pancreas PL of wildtype C57/Bl/6/129 mice vs. *cftr*$^{+/+}$ and *cftr*$^{-/-}$ mice. #p<0.001 for LA, AA and DHA in lung and intestinal PL of C57/Bl/6/129 mice vs.to *cftr*$^{+/+}$ and *cftr*$^{-/-}$ mice. No significant differences were detected between *cftr*$^{+/+}$ and *cftr*$^{-/-}$ mice.

DISCUSSION

We aimed to establish whether perturbed EFA metabolism and altered membrane EFA composition in CF-affected organs are inextricably linked to CF. Our present study in two murine models for CF shows no disturbance in EFA metabolism nor in membrane fatty acid composition, indicating that a membrane EFA imbalance is not an intrinsic characteristic of CF genotype in mice. By inference, our data indicate that the altered EFA compositions reported in CF are a secondary phenomenon, possibly related to inflammation or malnutrition.

Freedman *et al*.[13] reported markedly increased membrane AA- and decreased DHA-concentrations in CF-affected organs of a subset of *cftr* $^{-/-UNC}$ mice compared to non-littermate C57Bl/6 controls. Oral supplementation with DHA, but not with its precursor ALA, corrected this membrane EFA imbalance and was reported to alleviate certain phenotypic manifestations of the disease. The authors suggested a causative relation between impaired capacity for conversion of EFA into LCPUFA and CF symptoms. Using ^{13}C-labeled EFA, we quantified rates of EFA elongation, desaturation and tissue incorporation *in vivo* in *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice. After intragastric administration of ^{13}C-EFA, levels of ^{13}C-AA and ^{13}C-DHA in jejunum, pancreas

and liver PL were equal in *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice, indicating that in this CF mouse model, EFA elongation and desaturation is unaffected and that impaired LCPUFA synthesis is no inextricable feature of CF phenotype.
The phenotypic manifestations of CF are highly variable in patients as well as in different murine models for CF. We analyzed EFA status in two CF mouse models: homozygous dF508 mice with the dF508 exon 10 insertional mutation[18], expressing a mild phenotype without fat malabsorption, and *cftr* $^{-/-CAM}$ (University of Cambridge) mice, in which exon 10 replacement results in complete absence of CFTR activity and a severe gastrointestinal phenotype, including fat malabsorption[17]. For comparison, we used sex-matched littermates as controls. Quantification of fecal fatty acid excretion demonstrated that *cftr* $^{-/-CAM}$ mice indeed malabsorbed dietary fatty acids. Yet, in neither of the two murine CF models we found indications for major membrane EFA alterations in CF-affected organs as compared to littermate controls. The slight and inconsistent alterations of AA levels that we did measure in lung and jejunum, and the marginally decreased DHA levels in pancreas, were found only in dF508/dF508 mice and not in *cftr* $^{-/-CAM}$ mice, despite the fact that the more severe phenotype of *cftr* $^{-/-CAM}$ mice would be expected to correlate with a higher incidence of membrane lipid imbalances[29]. Only when *cftr* $^{-/-CAM}$ and *cftr* $^{+/+CAM}$ mice were compared to wildtype controls of different (C57Bl/6) genetic background, pronounced differences in membrane fatty acid composition became apparent.

The discrepancies between our observations and those of Freedman *et al*. are unlikely to be explained by differences in preparative steps prior to GC injection. The discrepancies could however be related to the age difference between mice in our study (3 months) and in Freedman's study (1 month)[13]. In contrast to the chow-fed adult mice used by us, 1-month-old liquid-diet weaned *cftr* $^{-/-UNC}$ mice displayed a profound lipid imbalance in CF-affected tissues, compared to C57Bl/6 mice. Theoretically, a conditional essentiality of dietary LCPUFA during early life may result in transiently low LCPUFA levels in young mice, which may resolve when EFA metabolizing capacity reaches maturity at adult age. Young *cftr* $^{-/-}$ mice might be more vulnerable than wildtype controls for such a transient deficiency of LCPUFA due to impaired fat absorption in CF. However, comparison of membrane fatty acids of 1-month-old, liquid diet-fed *cftr* $^{+/+CAM}$ and *cftr* $^{-/-CAM}$ mice with those of 3-month-old, chow-fed mice indicated that the former actually had higher relative levels of EFA and LCPUFA. Similar to the 3-month-old mice, no differences in fatty acid composition were detected between 1-month-old *cftr* $^{-/-CAM}$ mice and *cftr* $^{+/+CAM}$ littermates, suggesting that differences in fatty acid levels between 1- and 3-month-old mice is more likely related to the different diets, or to an age-dependent effect unrelated to CFTR malfunction.

The different diets fed to *cftr* $^{-/-UNC}$ mice and *cftr* $^{-/-CAM}$ mice could theoretically account for the inconsistency regarding EFA levels in these two models. Both *cftr* $^{-/-CAM}$ mice and *cftr* $^{-/-UNC}$ mice display a severe phenotype characterized by fat malabsorption, goblet cell hyperplasia and failure to thrive, although *cftr* $^{-/-UNC}$ mice are more severely affected. When weaned on a chow-based diet, mortality due to intestinal obstruction is considerable in *cftr* $^{-/-UNC}$ mice during the first weeks of life. Weaning on a complete elemental liquid diet, such as Peptamen, significantly improves survival rates, but CF mice fed Peptamen remain considerably smaller when compared to normal littermates. To meet daily caloric needs, adult mice have to consume up to 15 ml of Peptamen per day[28], and lower intake may result in malnutrition. Striking similarities have been described between Peptamen-fed *cftr* $^{-/-UNC}$ mice and a malnourished CF mouse model regarding pulmonary cytokine profiles[30], suggesting that malnutrition secondary to liquid diet feeding may contribute to symptoms in Peptamen-fed CF mice[29]. Relative EFA concentrations differ only slightly between chow and Peptamen, with Peptamen containing relatively less AA and more DHA than solid chow. *Cftr* $^{-/-UNC}$ mice fed Peptamen, however, had high levels of AA and low levels of DHA, which makes the fatty acid composition of the liquid diet an unlikely contributor to the observed membrane EFA imbalance in these mice. Yet, quantitative absorption studies would be required to fully exclude differences in net enteral uptake of EFA from chow or from Peptamen.

The discrepancy between our results and those of Freedman *et al.* may also be due to variations inherent to the use of different mouse models for CF. To date, over 10 different murine CF models have been characterized, which can be categorized into mutants in which CFTR expression is simply disrupted (i.e., *cftr* $^{-/-1HGU}$, *cftr* $^{-/-HSC}$, *cftr* $^{-/-BAY}$, *cftr* $^{-/-UNC}$ and *cftr* $^{-/-CAM}$ mice[15-17; 31; 32]) and mutants that model specific clinical mutations such as the dF508 mutation in *cftr* $^{-/-EUR}$ and *cftr* $^{-/-1KTH}$ mice[18; 33]. Within the group with CFTR gene disruption, the potential to produce CFTR mRNA ranges from no detectable CFTR mRNA in absolute null mice (*cftr* $^{-/-CAM}$, *cftr* $^{-/-CAM}$, *cftr* $^{-/-HSC}$) to mutants in which up to 10% of CFTR mRNA production is retained (*cftr* $^{-/-1HGU}$). Generally, mice with lowest residual CFTR activity display the most severe phenotype, but phenotypic differences can also result from the different genetic backgrounds onto which CFTR mutations have been introduced. The UNC mutation has been crossed into three different strains, i.e., C57Bl/6/129, B6D2/129 and BALB/C/129 mice, while the CAM mutation has been outcrossed to a C57Bl/6/129 population. Whereas we used sex-matched littermates as controls for *cftr* $^{-/-CAM}$ mice to evaluate EFA status, Freedman *et al*. used non-littermate, wildtype C57Bl/6 mice. Our present data indicate that genetic background and age have an overriding effect

on EFA status in general and on DHA and AA levels in particular, so any meaningful comparisons of EFA status between CF mice and controls should take these confounding factors into account.

In addition to the specific type of CFTR mutation and to environmental influences, phenotypic variability between CF patients and mouse models is thought to be related to independently segregating disease-modifying genes. Proteins encoded by genes other than the CFTR gene may partially substitute for mutant CFTR, and individual variability in levels of tissue expression and functional activity for these other proteins may explain the inter-individual phenotypic differences between patients or mice with identical CFTR mutations[34; 35]. Several candidate modifier genes have been postulated to account for the wide spectrum of lung disease severity in patients homozygous for the dF508 mutation[36]. Rozmahel *et al*. demonstrated in mice the presence of a CFTR-independent locus that modulated severity of gastrointestinal disease[32], and Zielenski *et al*. identified a similar modifier gene for meconium ileus on human chromosome 19[37]. Similarly, the expression of liver disease has been described to be modulated by independently inherited modifier genes. This again underlines the prerequisite of using littermate controls in murine models for CF.

Our findings of normal membrane fatty acid composition in two CF mouse models correspond to results described by Dombrowsky *et al.*, who found normal levels of DHA and even decreased levels of AA in phospholipid species of standard diet-fed adult *cftr* $^{-/-1HGU}$ mice[38]. As in our study, the differences in EFA levels were very small, and inconsistent between phospholipid classes. The fact that HGU mice have 10% residual CFTR mRNA makes conclusions regarding the role of CFTR in EFA metabolism difficult, yet, both Dombrowsky's and our results underline the variability in membrane PL composition between different CF mouse models. Strandvik *et al*. described essential fatty acid deficiency in plasma phospholipids of CF patients[7], but differences were small and AA levels were normal in all patients. The most pronounced EFA alterations were found in patients with severe mutations (i.e., dF508 and 394delTT), and although no correlations were reported with other genotypes, a relation with fat malabsorption cannot be excluded.

In summary, from *in vivo* analyses of LCPUFA synthesis in a mouse model for CF, we conclude that impaired LCPUFA synthesis or imbalanced membrane fatty acid composition are no inextricable features of CF phenotype. Fat malabsorption does not have a strong effect on EFA status in CF mice. Extrapolating these conclusions to CF patients may implicate that sufficient oral EFA intake could effectively prevent

compromised EFA status in CF. For studying essential fatty acid metabolism in murine CF models and inferring observations to the human condition, meticulous verification of mouse background strains and the use of littermate controls is of crucial importance.

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