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

Anniek Werner,* Marloes E. J. Bongers,* Marcel J. Bijvelds,† Hugo R. de Jonge,† and Henkjan J. Verkade[1,*]

Department of Pediatrics,* Center for Liver, Digestive and Metabolic Diseases, Academic Hospital Groningen, The Netherlands; and Department of Biochemistry,† Erasmus Medical Center, Rotterdam, The Netherlands

Abstract **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 the CF phenotype. To establish whether cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction affects LCPUFA synthesis, we quantified EFA metabolism in *cftr*$^{-/-CAM}$ and *cftr*$^{+/+CAM}$ mice. Effects of intestinal phenotype, diet, age, and genetic background on EFA status were evaluated in *cftr*$^{-/-CAM}$ mice, ΔF508/ΔF508 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, ΔF508/ΔF508 mice, littermate controls, and C57Bl/6 wild types fed chow or liquid diet. 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 ΔF508/ΔF508 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 wild types. We conclude that *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 the CF genotype. EFA status in murine CF models is strongly determined by genetic background.**—Werner, A., M. E. J. Bongers, M. J. Bijvelds, H. R. de Jonge, and H. J. Verkade. **No indications for altered essential fatty acid metabolism in two murine models for cystic fibrosis. *J. Lipid Res.* 2004.** 45: **2277–2286.**

Supplementary key words arachidonic acid • cystic fibrosis transmembrane conductance regulator • docosahexaenoic acid • essential fatty acid deficiency • α-linolenic acid • linoleic acid • modifier genes

A deficiency of essential fatty acids (EFA) or their long-chain polyunsaturated metabolites (LCPUFA) has frequently been reported in cystic fibrosis (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). Nonetheless, 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 cystic fibrosis transmembrane conductance regulator (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 (PL) 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 PL 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), and mice with the ΔF508 mu-

Abbreviations: AA, arachidonic acid, 20:4n-6; ALA, α-linolenic acid, 18:3n-3; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DHA, docosahexaenoic acid, 22:6n-3; EFA, essential fatty acid; KHB, Krebs-Henseleit buffer; LA, linoleic acid, 18:2n-6; LCPUFA, long-chain polyunsaturated fatty acid; PL, phospholipids.

[1] To whom correspondence should be addressed.
e-mail: h.j.verkade@med.rug.nl

Manuscript received 21 June 2004 and in revised form 16 September 2004.
Published, JLR Papers in Press, October 1, 2004.
DOI 10.1194/jlr.M400238-JLR200

tation, with 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 (University of Cambridge) (17) and homozygous ΔF508 mice (18), as well as of their respective littermate controls. These particular CF models have been shown to differ in intestinal phenotype, with fat malabsorption present in *cftr*$^{-/-CAM}$ mice but absent in ΔF508/ΔF508 mice (19). Furthermore, we determined the effects of age, diet, and genetic background on EFA status in these mouse models and in C57Bl/6 wild-type 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 the 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 ΔF508 mice and sex-matched littermate controls (N/N) of FVB/129 background (18) and wild-type 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 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% α-linolenic acid, 0.3 mol% arachidonic acid, and 0.05 mol% docosahexaenoic acid (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% α-linolenic acid, 0.1 mol% arachidonic acid, and 0.08 mol% docosahexaenoic acid.

Experimental procedures

The 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 μl 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, Columbia, MD). [U-^{13}C]LA and [U-^{13}C]ALA were 99% ^{13}C-enriched, with a chemical purity exceeding 97%. At 24 h after bolus administration, a large blood sample was obtained by cardiac puncture and pancreas, liver, lungs and intestine were removed and immediately stored at −80°C until further analysis. Intestine and lungs were flushed with ice-cold 0.9% (w/v) NaCl solution 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 ΔF508 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 3 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 72 h fat balance.

A separate group of *cftr*$^{-/-CAM}$ and *cftr*$^{+/+CAM}$ mice and C57Bl/6 wild types (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 anesthetized 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 onto 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 1,000 units of collagenase, 2,000 units of DNase, and 0.5 units of thermolysin and was 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 50 m × 0.22 mm 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. The $^{12}CO_2^+$ and $^{13}CO_2^+$ ions were measured at m/z 44 and 45, respectively. 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 solution 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 PL, cholesterol esters, triacylglycerols, diacylglycerols, monoacylglycerols, and free fatty acids using TLC (20 × 20 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 triacylglycerol spots were scraped. Of these scrapings, fatty acid methyl esters were prepared as mentioned above. To account for losses during lipid extraction, heptadecaenoic acid (C17:0) was added to all samples as internal standard prior to Bligh and Dyer procedures (23). Butylated hydroxytoluene 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, equipped with a 50 m × 0.2 mm Ultra 1 capillary column (Hewlett Packard, Palo Alto, CA) and a flame ionization detector as described previously (24). We verified the purity of AA and DHA peaks as separated by GLC, using a gas chromatography-mass spectrometer (GC-MS; Finnigan MAT SSQ7000), equipped with either a 50 m × 0.2 mm Ultra 1 capillary column or a 50 m × 0.22 mm BPX70 capillary column (SGE, Weiterstadt, Germany). Both methyl esters and pentafluorobenzyl bromide (PFB-Br) derivatives of tissue fatty acids were analyzed, but no indications for impurity of AA or DHA peaks could be 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

Abundance of ^{13}C was expressed as $\delta^{13}C_{PDB}$ value (i.e., the difference between the sample value and baseline compared with Pee Dee belemnite limestone). The $\delta^{13}C_{PDB}$ 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 heptadecaenoic acid (C17:0).

Statistics

All results are presented as means ± SD 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]EFA into LCPUFA

LCPUFA in specific tissues originate either from the diet or from endogenous synthesis by elongation and desaturation of EFA. Because 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 h 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 (**Fig. 1**). 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 triacyclglycerols (data not shown) and in lung PL, but [^{13}C]DHA in jejunum, pancreas, and liver PL was similar in *cftr*$^{-/-CAM}$ mice and controls. The ratios between [^{13}C]LA and [^{13}C]AA and between [^{13}C]LA 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 ΔF508/ΔF508 and *cftr*$^{-/-CAM}$ mice and their respective controls. Fecal fatty acid excretion was similar in homozygous ΔF508 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 3 shows LA, AA, ALA, and DHA concentrations in tissue homogenates of pancreas, lung, and jejunum in homozygous ΔF508 mice and *cftr*$^{-/-CAM}$ mice compared with their respective littermate controls. No significant differences were observed in relative concentrations of these EFA and LCPUFA, nor of saturated and nonessential fatty acids (data not shown).

The fatty acid composition of the total lipid fraction depends on the fatty acid composition of the various lipid classes present (i.e., triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, cholesterol esters, free fatty acids), and the proportions of these lipid classes may vary considerably between organs. Since 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 ΔF508/ΔF508 mice than in N/N controls ($P < 0.05$); in lung PL, there was a small significant increase of AA in ΔF508/ΔF508 compared with N/N mice (5.3 ± 0.7 vs. 4.3 ± 0.5, respectively; $P < 0.05$). In jejunum PL of ΔF508/ΔF508 mice, however, AA was 37%

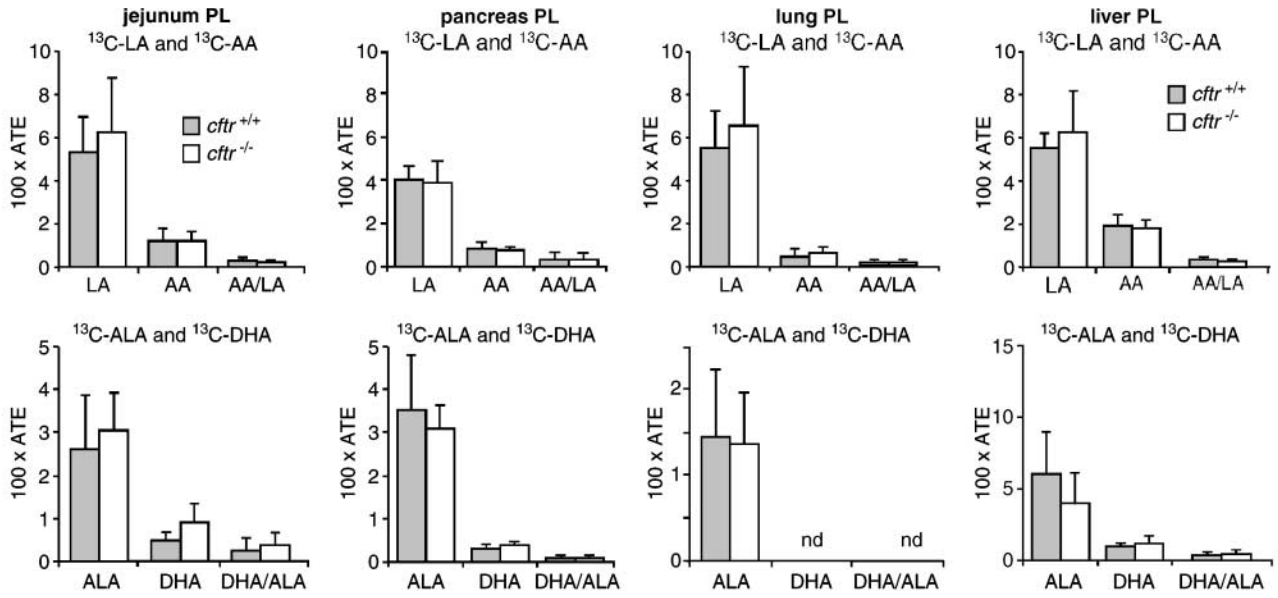


Fig. 1. ^{13}C enrichment of linoleic acid (LA, 18:2n-6), arachidonic acid (AA, 20:4n-6), α-linolenic acid (ALA, 18:3n-3), and docosahexaenoic acid (DHA, 22:6n-3) in phospholipids (PL) of pancreas, lung, intestine, and liver of *cftr*$^{+/+}$ mice (gray bars) and *cftr*$^{-/-}$ mice (white bars) at 24 h after intragastric administration of [^{13}C]LA and [^{13}C]LA. Individual fatty acid ^{13}C enrichment was calculated from the difference between the sample value and baseline compared with Pee Dee Belemnite limestone ($\delta^{13}C_{PDB}$), and is expressed as 100 × atom % excess. Data represent means ± SD of six mice per group. No significant differences in ^{13}C enrichment were detected between *cftr*$^{+/+}$ and *cftr*$^{-/-}$ mice, indicating normal conversion of essential FAs (EFA) into long-chain PUFAs (LCPUFA).

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 with 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 to those of *cftr*$^{+/+CAM}$ littermates.

The severity of the 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 with littermate controls (26.5 ± 4.4 g vs. 30.7 ± 3.8 g, respectively, $P < 0.05$). In contrast, homozygous ΔF508 mice, displaying normal fat absorption as a consequence of milder gastrointestinal 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 body weight for each individual mouse (Fig. 4B). Neither in *cftr*$^{-/-CAM}$ nor in ΔF508/ΔF508 mice or their corresponding controls could a significant correlation between relative EFA or LCPUFA concentrations and body weight be identified in pancreas (Fig. 4B), lung, or intestinal PL (data not shown).

In addition to relative fatty acid concentrations, absolute concentrations in the different tissues were determined and expressed per milligram protein. Figure 4C shows that absolute LA, ALA, AA, and DHA contents were similar in pancreas tissue of the two CF mouse models and

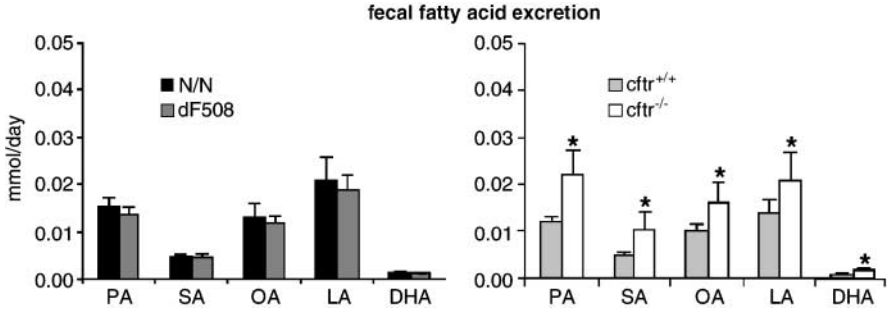


Fig. 2. Relative concentrations of palmitic acid (PA, 16:0), stearic acid (SA, 18:0), oleic acid (OA, 18:1n-9), linoleic acid (LA, 18:2n-6), and docosahexaenoic acid (DHA, 22:6n-3) in fecal lipid extracts of homozygous ΔF508 mice and *cftr*$^{-/-}$ mice and their respective littermate controls. Fecal fat excretion was quantified by means of a 72 h fecal fat balance. Individual fatty acid concentrations are expressed as mmol of fatty acid excreted per day. Data represent means ± SD of 5–6 mice per group. No significant differences were detected for any of the fatty acids between homozygous ΔF508 mice and controls, but daily fecal fatty acid excretion was significantly higher in *cftr*$^{-/-}$ mice than in *cftr*$^{+/+}$ controls (* $P < 0.05$).

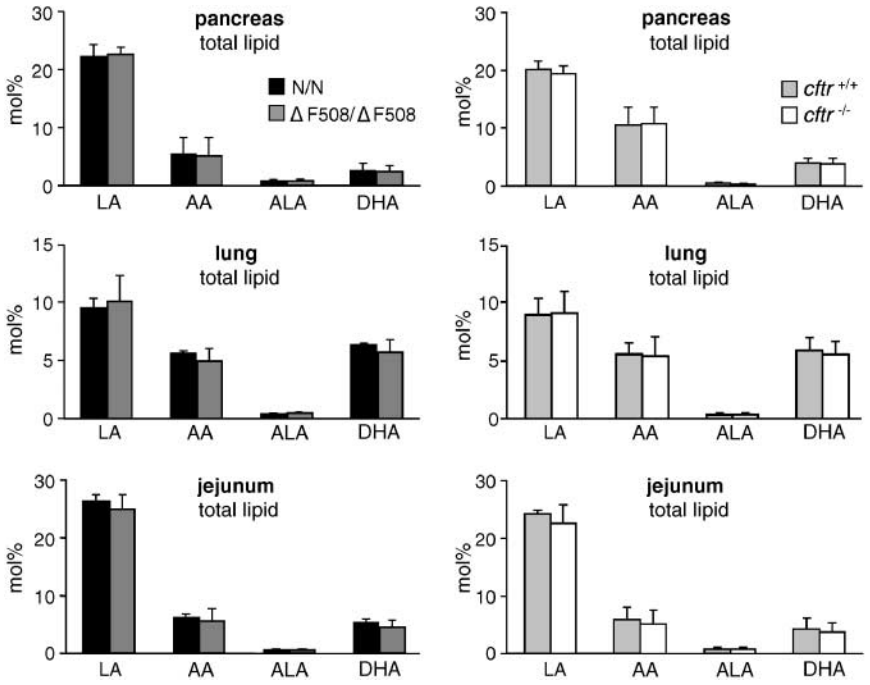


Fig. 3. Relative concentrations of linoleic acid (LA, 18:2n-6), arachidonic acid (AA, 20:4n-6), α-linolenic acid (ALA, 18:3n-3), and docosahexaenoic acid (DHA, 22:6n-3) in total lipid extracts of homozygous ΔF508 mice and $cftr^{-/-}$ mice and their respective littermate controls. Individual fatty acid concentrations are expressed as mol% of total fatty acids. Data represent means ± SD of 5–6 mice per group. No significant differences were detected for any of the fatty acids between ΔF508 or $cftr^{-/-}$ mice and their littermate controls.

their controls. Similarly, absolute fatty acid concentrations in lung or jejunum were not significantly different between CF and control mice (data not shown).

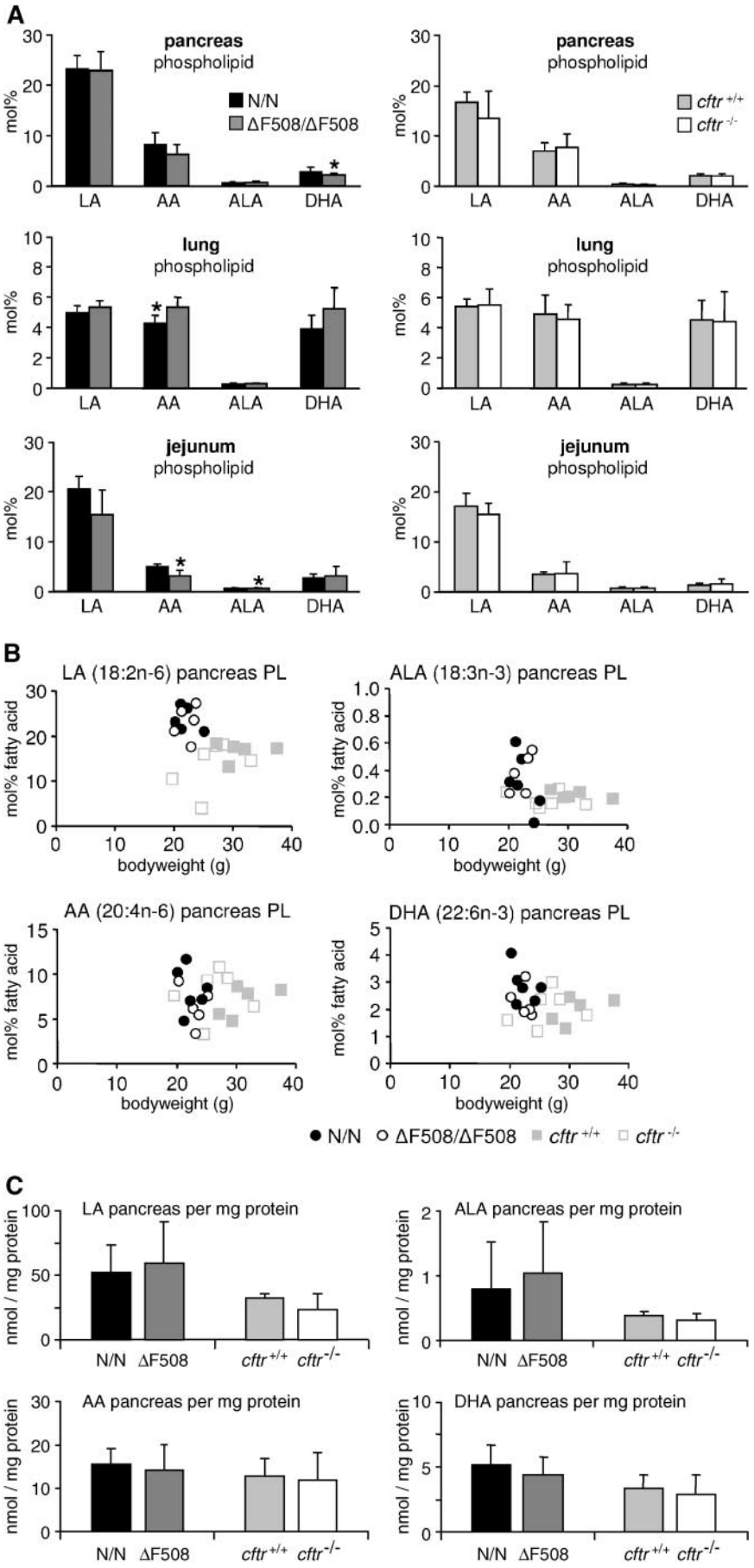
Membrane EFA concentrations appeared unaffected in the presently used CF mouse models compared with littermate controls. However, in 1-month-old $cftr^{-/-UNC}$ mice weaned on a liquid diet (Peptamen), a profound membrane lipid imbalance was reported (13). This suggests that dietary composition, caloric 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 wild-type 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 (**Fig. 5**). Although Peptamen is used frequently 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 ± 2.2 g vs. 13.6 ± 1.1 g, $P < 0.05$).

The 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 (**Fig. 6A**; age effect). 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 littermate controls, neither at the age of 1 month after Peptamen weaning (Fig. 6B), nor at adult age during chow-feeding. However, AA and DHA concentrations were consistently higher than in age- and diet-matched C57Bl/6 wild-type 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 wild-type 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 wild-type C57Bl/6 mice compared with $cftr^{-/-CAM}$ mice, this reached significance only in plasma and ileum ($P < 0.05$ each). Similarly, fatty acid analyses of erythrocyte and ileum PL revealed consistently different fatty acid concentrations in C57Bl/6 mice, compared with $cftr^{-/-CAM}$ and $cftr^{+/+CAM}$ mice (data not shown). Other PUFA of the n-3 and n-6 series (i.e., 20:5n-3, 22:5n-3, 22:4n-6) also differed only between C57Bl/6 mice and $cftr^{-/-CAM}$ and $cftr^{+/+CAM}$ mice, and not between $cftr^{-/-CAM}$ mice and $cftr^{+/+CAM}$ littermates (data not shown).

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 either



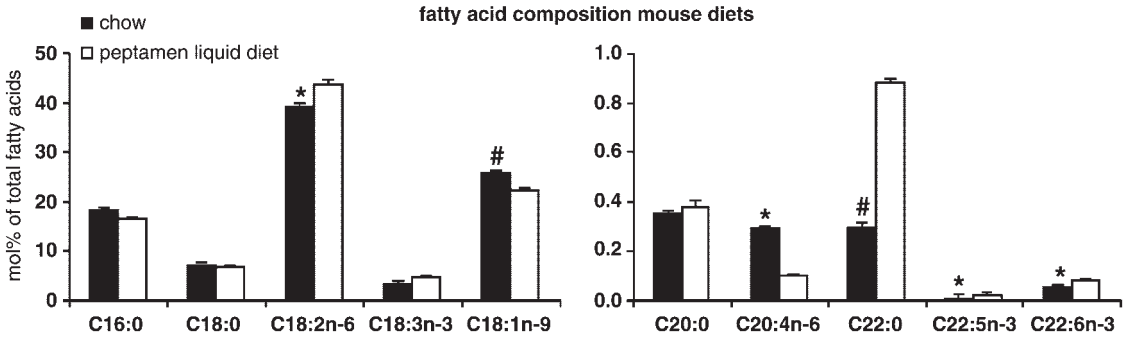


Fig. 5. Fatty acid composition of standard laboratory chow and Peptamen elemental liquid diet. Data represent means ± SD of triple aliquot analyses of each diet. *$P < 0.05$ for linoleic acid (C18:2n-6), arachidonic aid (C20:4n-6), eicosapentaenoic acid (C22:5n-3), and docosahexaenoic acid (C22:6n-3)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

EFA metabolism or membrane fatty acid composition, indicating that a membrane EFA imbalance is not an intrinsic characteristic of the 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 with nonlittermate 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]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 not an inextricable feature of the CF phenotype.

The phenotypic manifestations of CF are highly variable in both patients and different murine models for CF. We analyzed EFA status in two CF mouse models: homozygous ΔF508 mice with the ΔF508 exon 10 insertional mutation (18), expressing a mild phenotype without fat malabsorption, and $cftr^{-/-CAM}$ 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 showed that $cftr^{-/-CAM}$ mice indeed malabsorbed dietary fatty acids. In neither of the two murine CF models, however, did we find indications for major membrane EFA alterations in CF-affected organs, compared with 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 ΔF508/ΔF508 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 with wild-type controls of different (C57Bl/6) genetic background did pronounced differences in membrane fatty acid composition become apparent.

The discrepancies between our observations and those of Freedman et al. (13) 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 their study (1 month). In contrast to the chow-fed adult mice that we used, their 1-month-old liquid-diet weaned $cftr^{-/-UNC}$ mice displayed a profound lipid imbalance in CF-affected tissues, compared with C57Bl/6 mice. Theoretically, a

Fig. 4. A: Relative concentrations of linoleic acid (LA, 18:2n-6), arachidonic acid (AA, 20:4n-6), α-linolenic acid (ALA, 18:3n-3), and docosahexaenoic acid (DHA, 22:6n-3) in purified PL extracts of homozygous ΔF508 mice and $cftr^{-/-}$ mice and their respective littermate controls. Individual fatty acid concentrations are expressed as mol% of total fatty acids. Data represent means ± SD of 5–6 mice per group, *$P < 0.05$. B: Relative concentrations (mol%) of linoleic acid (LA, 18:2n-6), arachidonic acid (AA, 20:4n-6), α-linolenic acid (ALA, 18:3n-3), and docosahexaenoic acid (DHA, 22:6n-3) in pancreas PL related to body weight of homozygous ΔF508 mice (open circles) and $cftr^{-/-}$ mice (open squares) and their respective littermate controls (N/N, closed circles; $cftr^{+/+}$, closed squares). No correlations were detected between body weight and individual fatty acid concentrations. Data represent means ± SD of 5–6 mice per group. C: Absolute concentrations (nmol) of linoleic acid (LA, 18:2n-6), arachidonic acid (AA, 20:4n-6), α-linolenic acid (ALA, 18:3n-3), and docosahexaenoic acid (DHA, 22:6n-3) in pancreas PL expressed per milligram pancreas protein for homozygous ΔF508 mice and $cftr^{-/-}$ mice and their respective littermate controls. Individual fatty acid concentrations were quantified by relating the areas of their chromatogram peaks to that of the internal standard heptadecaenoic acid (C17:0). No significant differences in absolute fatty acid concentrations per milligram protein were detected between homozygous ΔF508 mice and $cftr^{-/-}$ mice and their respective littermate controls. Data represent means ± SD of 5–6 mice per group.

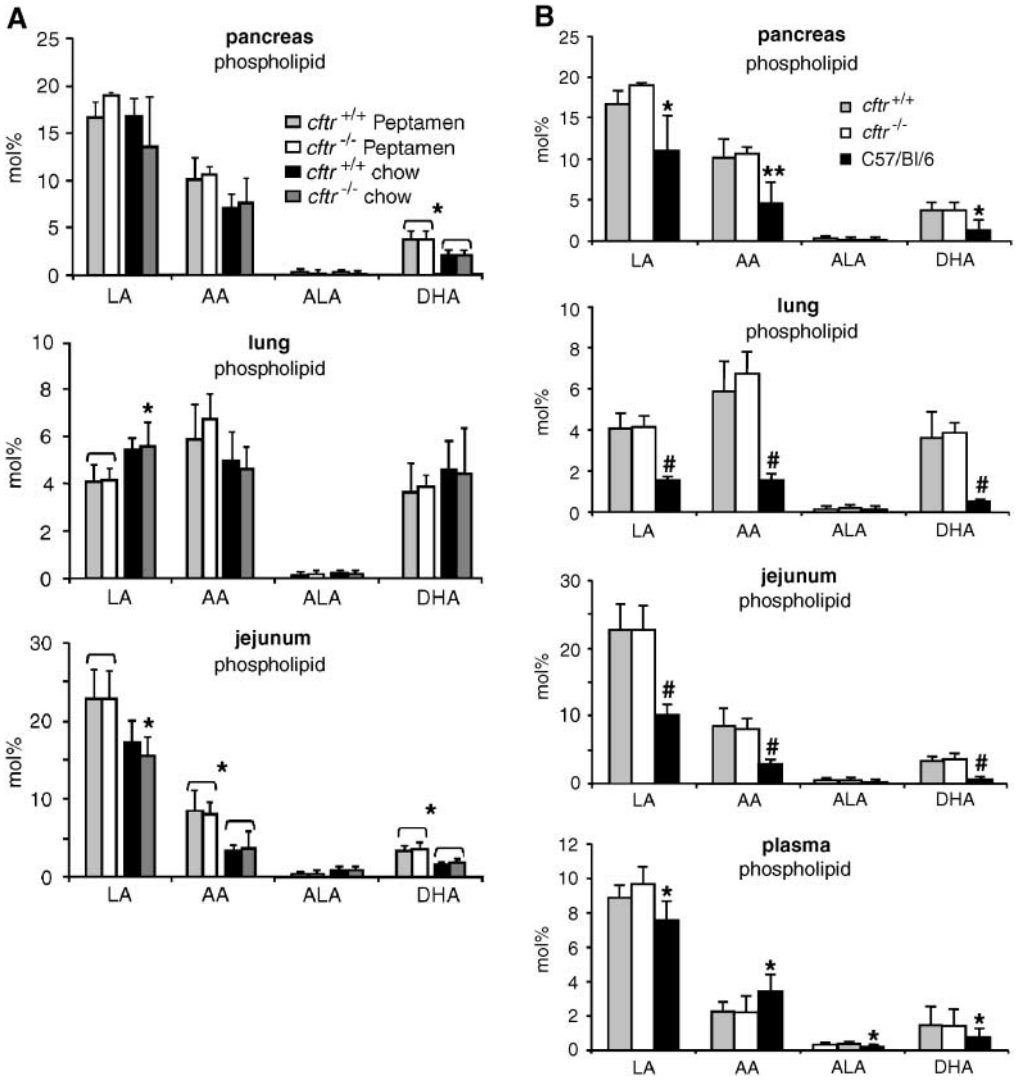


Fig. 6. A: Relative concentrations of linoleic acid (LA, 18:2n-6), arachidonic acid (AA, 20:4n-6), α-linolenic acid (ALA, 18:3n-3), and docosahexaenoic acid (DHA, 22:6n-3) in purified PL extracts of pancreas, lung, and intestine of 1-month-old Peptamen-fed $cftr^{+/+}$ mice (light gray bars) and $cftr^{-/-}$ mice (white bars), adult chow-fed $cftr^{+/+}$ mice (black bars) and $cftr^{-/-}$ mice (dark gray bars). Individual fatty acid concentrations are expressed as mol% of total fatty acids. Data represent means ± SD 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 with adult chow-fed $cftr^{+/+}$ and $cftr^{-/-}$ mice. No significant differences in fatty acid concentrations were detected between $cftr^{+/+}$ and $cftr^{-/-}$ littermates. B: Relative concentrations of linoleic acid (LA, 18:2n-6), arachidonic acid (AA, 20:4n-6), α-linolenic acid (ALA, 18:3n-3), and docosahexaenoic acid (DHA, 22:6n-3) in purified PL extracts of pancreas, lung, and intestine of $cftr^{+/+}$ mice (gray bars), $cftr^{-/-}$ mice (white bars) and wild-type C57/Bl/6/129 mice (black bars). All mice were weaned on Peptamen liquid diet from postnatal day 23 and fatty acid analyses were performed at postnatal day 30. Individual 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 wild-type C57/Bl/6/129 mice compared with $cftr^{+/+}$ and $cftr^{-/-}$ mice. # $P < 0.001$ for LA, AA and DHA in lung and intestinal PL of C57/Bl/6/129 mice compared with $cftr^{+/+}$ and $cftr^{-/-}$ mice. No significant differences in fatty acid concentrations were detected between $cftr^{+/+}$ and $cftr^{-/-}$ mice.

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 wild-type 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 c*ftr*$^{-/-CAM}$ mice could theoretically account for the inconsistency regarding EFA levels in these two models. Both c*ftr*$^{-/-CAM}$ 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 than with 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. However, 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. (13) 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; these 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 ΔF508 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*$^{-/-UNC}$, *cftr*$^{-/-CAM}$, *cftr*$^{-/-HSC}$) to mutants in which up to 10% of CFTR mRNA production is retained (c*ftr*$^{-/-1HGU}$). Generally, mice with lowest residual CFTR activity display the most severe phenotype, but phenotypic differences can also result from the different genetic backgrounds into 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), and the CAM mutation has been outcrossed to a C57Bl/6/129 population. Whereas we used sex-matched littermates as controls for c*ftr*$^{-/-CAM}$ mice to evaluate EFA status, Freedman et al. (13) used nonlittermate, wild-type 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 interindividual 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 ΔF508 mutation (36). Rozmahel et al. (32) demonstrated in mice the presence of a CFTR-independent locus that modulated severity of gastrointestinal disease, and Zielenski et al. (37) identified a similar modifier gene for meconium ileus on human chromosome 19. Similarly, the expression of liver disease has been described to be modulated by independently inherited modifier genes. This again underscores 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. (38), who found normal levels of DHA and even decreased levels of AA in PL species of standard diet-fed adult *cftr*$^{-/-1HGU}$ mice. As in our study, the differences in EFA levels were very small, and inconsistent between PL classes. The fact that HGU mice have 10% residual CFTR mRNA makes conclusions regarding the role of CFTR in EFA metabolism difficult; nonetheless, both Dombrowsky's and our results underscore the variability in membrane PL composition between different CF mouse models. Strandvik et al. (7) described essential fatty acid deficiency in plasma PL of CF patients, 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., ΔF508 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 not inextricable features of the CF phenotype. Fat malabsorption does not have a strong effect on EFA status in CF mice. Extrapolating these conclusions to CF patients could imply that sufficient oral EFA intake may 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 crucial.

The authors would like to thank Rick Havinga, Henk Elzinga, and Ingrid Martini for their technical expertise and assistance

in the experiments described in this article and Dr. Frans Stellaard for his help with the stable isotope studies. This study was supported by the Netherlands Organization for Scientific Research (NWO Grant 90462210).

REFERENCES

1. Lloyd-Still, J. D., S. B. Johnson, and R. T. Holman. 1981. Essential fatty acid status in CF and the effects of safflower oil supplementation. *Am. J. Clin. Nutr.* **34:** 1–7.
2. Biggemann, B., Laryea, M. D., Schuster, A., Griese, M., Reinhardt, D., and Bremer, H. J. 1988. Status of plasma and erythrocyte fatty acids and vitamin A and E in young children with CF. *Scand. J. Gastroenterol. Suppl.* **143:** 135–41.
3. Strandvik, B. 1989. Relation between essential fatty acid metabolism and gastrointestinal symptoms in CF. *Acta Paediatr. Scand. Suppl.* **363:** 58–65.
4. Lepage, G., E. Levy, N. Ronco, L. Smith, N. Galeano, and C. C. Roy. 1989. Direct transesterification of plasma fatty acids for the diagnosis of essential fatty acid deficiency in cystic fibrosis. *J. Lipid Res.* **30:** 1483–1490.
5. Parsons, H. G., E. V. O'Loughlin, D. Forbes, D. Cooper, and D. G. Gall. 1988. Supplemental calories improve EFA deficiency in cystic fibrosis patients. *Pediatr. Res.* **24:** 353–356.
6. Roulet, M., P. Frascarolo, I. Rappaz, and M. Pilet. 1997. Essential fatty acid deficiency in well nourished young CF patients. *Eur. J. Pediatr.* **156:** 952–956.
7. Strandvik, B., E. Gronowitz, F. Enlund, T. Martinsson, and J. Wahlstrom. 2001. Essential fatty acid deficiency in relation to genotype in patients with cystic fibrosis. *J. Pediatr.* **139:** 650–655.
8. Benabdeslam, H., I. Garcia, G. Bellon, R. Gilly, and A. Revol. 1998. Biochemical assessment of the nutritional status of cystic fibrosis patients treated with pancreatic enzyme extracts. *Am. J. Clin. Nutr.* **67:** 912–918.
9. Portal, B. C., M. J. Richard, H. S. Faure, A. J. Hadjian, and A. E. Favier. 1995. Altered antioxidant status and increased lipid peroxidation in children with cystic fibrosis. *Am. J. Clin. Nutr.* **61:** 843–847.
10. Rogiers, V., I. Dab, Y. Michotte, A. Vercruysse, R. Crokaert, and H. L. Vis. 1984. Abnormal fatty acid turnover in the phospholipids of the red blood cell membranes of cystic fibrosis patients. *Pediatr. Res.* **18:** 704–709.
11. Gilljam, H., B. Strandvik, A. Ellin, and L. G. Wiman. 1986. Increased mole fraction of arachidonic acid in bronchial phospholipids in patients with cystic fibrosis. *Scand. J. Clin. Lab. Invest.* **46:** 511–518.
12. Bhura-Bandali, F. N., M. Suh, S. F. Man, and M. T. Clandinin. 2000. The dF508 mutation in the cystic fibrosis transmembrane conductance regulator alters control of essential fatty acid utilization in epithelial cells. *J. Nutr.* **130:** 2870–2875.
13. Freedman, S. D., M. H. Katz, E. M. Parker, M. Laposata, M. Y. Urman, and J. G. Alvarez. 1999. A membrane lipid imbalance plays a role in the phenotypic expression of cystic fibrosis in $cftr^{-/-}$ mice. *Proc. Natl. Acad. Sci. USA.* **96:** 13995–14000.
14. Freedman, S. D., P. G. Blanco, M. M. Zaman, J. C. Shea, M. Ollero, I. K. Hopper, D. A. Weed, A. Gelrud, M. M. Regan, M. Laposata, J. G. Alvarez, and B. P. O'Sullivan. 2004. Association of cystic fibrosis with abnormalities in fatty acid metabolism. *N. Engl. J. Med.* **350:** 560–569.
15. Snouwaert, J. N., K. K. Brigman, A. M. Latour, N. N. Malouf, R. C. Boucher, O. Smithies, and B. H. Koller. 1992. An animal model for cystic fibrosis made by gene targeting. *Science.* **257:** 1083–1088.
16. O'Neal, W. K., P. Hasty, P. B. McCray, Jr., B. Casey, J. Rivera-Perez, M. J. Welsh, A. L. Beaudet, and A. Bradley. 1993. A severe phenotype in mice with a duplication of exon 3 in the cystic fibrosis locus. *Hum. Mol. Genet.* **2:** 1561–1569.
17. Ratcliff, R., M. J. Evans, A. W. Cuthbert, L. J. MacVinish, D. Foster, J. R. Anderson, and W. H. Colledge. 1993. Production of a severe cystic fibrosis mutation in mice by gene targeting. *Nat. Genet.* **4:** 35–41.
18. van Doorninck, J. H., P. J. French, E. Verbeek, R. H. Peters, H. Morreau, J. Bijman, and B. J. Scholte. 1995. A mouse model for the cystic fibrosis delta F508 mutation. *EMBO J.* **14:** 4403–4411.
19. Bijvelds, M., C. Hulzebos, H. Bronsveld, R. Havinga, F. Stellaard, M. Sinaasappel, H. De Jonge, and H. J. Verkade. 2003. Digestive Disease Week and the 104th Annual Meeting of the American Gastroenterological Association. May 17–22, 2003. Orlando, FL. Fat absorption and enterohepatic circulation of bile salts in cystic fibrosis mice. (Abstract). *Gastroenterology.* **124:** 434.
20. Kerem, B., J. M. Rommens, J. A. Buchanan, D. Markiewicz, T. K. Cox, A. Chakravarti, M. Buchwald, and L. C. Tsui. 1989. Identification of the cystic fibrosis gene: genetic analysis. *Science.* **245:** 1073–1080.
21. Muskiet, F. A., J. J. van Doormaal, I. A. Martini, B. G. Wolthers, and W. van der Slik. 1983. Capillary gas chromatographic profiling of total long-chain fatty acids and cholesterol in biological materials. *J. Chromatogr.* **278:** 231–244.
22. Bruzzone, R., P. A. Halban, A. Gjinovci, and E. R. Trimble. 1985. A new, rapid, method for preparation of dispersed pancreatic acini. *Biochem. J.* **226:** 621–624.
23. Bligh, E. G., and W. J. Dyer. 1959. A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
24. Werner, A., D. M. Minich, R. Havinga, V. Bloks, H. Van Goor, F. Kuipers, and H. J. Verkade. 2002. Fat malabsorption in EFA-deficient mice is not due to impaired bile formation. *Am. J. Physiol. Gastrointest. Liver Physiol.* **283:** G900–G908.
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randal. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
26. Kang, J. X., S. F. Man, N. E. Brown, P. A. Labrecque, and M. T. Clandinin. 1992. The chloride channel blocker anthracene 9-carboxylate inhibits fatty acid incorporation into phospholipid in cultured human airway epithelial cells. *Biochem. J.* **285:** 725–729.
27. Freedman, S. D., Blanco, P. G., Shea, J. C., and Alvarez, J. G. 2002. Analysis of lipid abnormalities in CF mice. *Methods Mol. Med.* **70:** 517–524.
28. Eckman, E. A., C. U. Cotton, D. M. Kube, and P. B. Davis. 1995. Dietary changes improve survival of CFTR S489X homozygous mutant mice. *Am. J. Physiol.* **269:** L625–L630.
29. Davidson, D. J., and M. Rolfe. 2001. Mouse models of CF. *Trends Genet.* **17:** S29–S37.
30. Yu, H., S. Z. Nasr, and V. Deretic. 2000. Innate lung defenses and compromised *Pseudomonas aeruginosa* clearance in the malnourished mouse model of respiratory infections in cystic fibrosis. *Infect. Immun.* **68:** 2142–2147.
31. Dorin, J. R., P. Dickinson, E. W. Alton, S. N. Smith, D. M. Geddes, B. J. Stevenson, W. L. Kimber, S. Fleming, A. R. Clarke, and M. L. Hooper. 1992. Cystic fibrosis in the mouse by targeted insertional mutagenesis. *Nature.* **359:** 211–215.
32. Rozmahel, R., M. Wilschanski, A. Matin, S. Plyte, M. Oliver, W. Auerbach, A. Moore, J. Forstner, P. Durie, J. Nadeau, C. Bear, and L. C. Tsui. 1996. Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. *Nat. Genet.* **12:** 280–287.
33. Zeiher, B. G., E. Eichwald, J. Zabner, J. J. Smith, A. P. Puga, P. B. McCray, Jr., M. R. Capecchi, M. J. Welsh, and K. R. Thomas. 1995. A mouse model for the ΔF508 allele of cystic fibrosis. *J. Clin. Invest.* **96:** 2051–2064.
34. Drumm, M. L. 2001. Modifier genes and variation in CF. *Respir. Res.* **2:** 125–128.
35. Zielenski, J. 2000. Genotype and phenotype in CF. *Respiration.* **67:** 117–133.
36. Merlo, C. A., and M. P. Boyle. 2003. Modifier genes in cystic fibrosis lung disease. *J. Lab. Clin. Med.* **141:** 237–241.
37. Zielinski, J., M. Corey, R. Rozmahel, D. Markiewicz, I. Aznarez, T. Casals, S. Larriba, B. Mercier, G. R. Cutting, A. Krebsova, M. Macek, E. Langfelder-Schwind, B. C. Marshall, J. De Celie-Germana, M. Claustres, A. Palecio, J. Bal, A. Nowakowska, C. Ferec, X. Estivill, P. Durie, and L. C. Tsui. 1999. Detection of a cystic fibrosis modifier locus for meconium ileus on human chromosome 19q13. *Nat. Genet.* **22:** 128–129.
38. Dombrowsky, H., G. T. Clark, G. A. Rau, W. Bernhard, and A. D. Postle. 2003. Molecular species compositions of lung and pancreas phospholipids in the $cftr^{tm1HGU/tm1HGU}$ cystic fibrosis mouse. *Pediatr. Res.* **53:** 447–454.