Impact of Nutrition on Phenotype in CFTR-deficient Mice

CHARLES-HENRY COTTART, ELISE BONVIN, COLETTE REY, DOMINIQUE WENDUM, JEAN-FRANÇOIS BERNAUDIN, SYLVIE DUMONT, ELISABETH LASNIER, DOMINIQUE DEBRAY, ANNICK CLÉMENT, CHANTAL HOUSSET, AND MONIQUE BONORA

Inserm UMR-S U680 [C.-H.C., C.R., D.D., C.H.], Inserm UMR-S U719 [E.B., A.C., M.B.], Université Pierre et Marie Curie–Paris 6 Paris, 75012, France; EA3499 [J.-F.B.], Université Pierre et Marie Curie–Paris 6 [D.W., S.D.], Paris, 75012, France; EA 3499 [J.-F.B.], Université Pierre et Marie Curie–Paris 6, Paris, 75020, France; Service d'Anatomie Pathologique [D.W.], Service de Biochimie [E.L.], AP-HP Hôpital Saint-Antoine, Paris, 75012, France

ABSTRACT: To elucidate the impact of nutrition in cystic fibrosis (CF), we compared the phenotypic traits of $Cftr^{-/-}$ mice fed either a lipid-enriched liquid diet (Peptamen) or a standard chow combined with polyethyleneglycol osmotic laxative (PEG), two strategies commonly used to prevent intestinal obstruction in CF mice. Survival, growth, liver, and ventilatory status were determined in $Cftr^{-/-}$ and $Cftr^{+/+}$ mice, followed-up until 120 d. Ventilation was recorded in conscious animals using whole-body plethysmography. We found that the survival rate was similar in Peptamen and PEG $Cftr^{-/-}$ mice. $Cftr^{-/-}$ mice had lower minute ventilation than $Cftr^{+/+}$ mice, whatever the diet. Both $Cftr^{-/-}$ and $Cftr^{+/+}$ mice fed Peptamen displayed preadult growth delay compared with PEG-treated animals. Despite subsequent growth catch-up, $Cftr^{-/-}$ mice remained smaller than $Cftr^{+/+}$ mice, whatever the diet. All Peptamen fed $Cftr^{-/-}$ mice showed hepatomegaly and liver steatosis, which also occurred but to a lesser extent in Peptamen fed $Cftr^{+/+}$ animals. Therefore, while both treatment strategies are similarly efficient to avoid high mortality at weaning, Peptamen induces preadult growth delay and liver steatosis. These effects of diet are important to consider in future animal studies and also prompt to evaluate high-energy diets in CF patients. (*Pediatr Res* 62: 528–532, 2007)

Cystic fibrosis (CF) is among the most common genetic diseases. The organs affected in CF include the lungs, the gastrointestinal tract, the pancreas, and the liver (1). Approximately 15% of CF patients present with meconium ileus (*i.e.* neonatal intestinal obstruction), and more than 80% develop pancreatic insufficiency (2). While both intestinal and pancreatic dysfunctions contribute to malnutrition, there is evidence to indicate that the nutritional status may influence liver and lung injury in these patients (3–5). CF mice with a complete exon-10 knockout of the CFTR gene ($Cftr^{tm1Unc}$) (6) develop severe CF-like intestinal obstruction. This obstruction is lethal at birth or at weaning, unless prevented by special feeding. The most common strategy has been to feed the animals at weaning with Peptamen (Nestlé France, Marne la Vallée, France), a lipid-enriched liquid diet, initially designed to feed infants or young children who suffer malnutrition

(including those born with CF). An alternative strategy to avoid intestinal obstruction in CF mice has been to supply them with an osmotic laxative, such as polyethyleneglycol (PEG) (7). In this latter option, mice are maintained on a standard solid chow. Because the composition of Peptamen differs from that of a standard chow, for example, the proportion of lipids is higher while those of proteins and carbohydrates are lower, we hypothesized that the two regimens result in different nutritional status, and may thus result in different phenotypic traits of CF disease (8). To test this hypothesis and gain insight into the impact of nutrition on CF disease, we herein compared the phenotype of $Cftr^{tm1Unc}$ mice fed either Peptamen or a standard diet with PEG, with particular attention to respiratory and liver alterations, which currently represent the two main causes of death in CF patients.

MATERIALS AND METHODS

Animals. Animals used in this study were male CF mice homozygous for the S489X mutation ($Cftr^{tm1Unc}$) (6) back-crossed into C57BL/6 (three generations) ($Cftr^{-/-}$) and their normal littermates ($Cftr^{+/+}$). Mice were housed and bred at the CDTA-CNRS (Orléans, France) and obtained after weaning at 21–25 d of age. All experiments were performed in accordance with our Institutional Animal Care and Use Department (Direction Départementale des Services Vétérinaires de Paris, agreement no. B75-1201).

Experimental design. Mice were bred on corn cob pellet bedding and maintained in a barrier facility unit under specific pathogen-free conditions with a 12-h light-dark cycle, in accordance with European recommendations. $Cftr^{-/-}$ and $Cftr^{+/+}$ mice were randomly assigned at weaning (21–25 d of age) to one of the following regimens:

1. Standard: solid chow *ad libitum* with free access to water.
2. Standard + PEG: solid chow *ad libitum* with free access to a commercially available osmotic laxative containing PEG-3350 and electrolytes (Transipeg, Roche-Nicholas, Gaillard, France), which was continuously supplied at 6% in the drinking water. Because newly weaned $Cftr^{-/-}$ mice are weak, solid chow was soaked with laxative during the first week after weaning.
3. Peptamen: liquid diet (Nestlé) *ad libitum* with free access to water. Peptamen was changed every 2 d, as previously reported (9). The composition of Peptamen in comparison with that of standard chow is shown in Table 1.

Monitoring of survival and growth. To determine whether survival of $Cftr^{-/-}$ mice was altered by the dietary regimen, survival of $Cftr^{-/-}$ mice fed *ad libitum* either a standard chow ($n = 12$), a standard chow together with PEG ($n = 26$), or Peptamen liquid diet ($n = 18$) was compared with that of $Cftr^{+/+}$ littermates. Survival was calculated every 5 d until 60 d as the percentage of living animals in each group.

Abbreviations: CF, cystic fibrosis; PEG, polyethyleneglycol; V_E , minute ventilation

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Correspondence: Monique Bonora, Ph.D., Inserm, UMR-S U719, Hôpital Saint-Antoine, Bâtiment Kourilsky, 184, rue du Faubourg Saint-Antoine, Paris, 75012, France; e-mail: bonora@st-antoine.inserm.fr

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Table 1. Nutritional information on the main energetic components of Peptamen semi-elemental formula and standard mouse solid chow (AO3)

	Peptamen (Nestlé)	AO3 Chow (Safe)
Proteins (g/100 kcal)	4.0	6.7
Carbohydrates (g/100 kcal)	12.7	16.2
Lipids (g/100 kcal)	3.7	1.6
MC/MC + LC ratio	70%	<5%

MC, medium-chain lipids; LC, long-chain lipids.

Subsequently, part of these animals were followed-up for a longer period of time (120 d), to further examine their growth, liver and ventilatory status.

Respiratory physiology. All data were obtained in 120-d-old mice. Ventilatory parameters were recorded in a whole-body plethysmograph by the barometric method described by Bartlett and Tenney (10). The pressure signal as a result of breathing was detected by a differential pressure transducer (Validyne DP103/12; Validyne, Northridge, CA) connected to the animal chamber (400 mL) and to a reference chamber of the same volume. The spirogram was recorded and stored on a computer using respiratory acquisition software (CIO-DAS 1602/16 interface and ELPHY software) for analysis off-line. Calibration was performed at the beginning of experiments by several injections of 50 μ L of air into the chamber.

Each animal was weighed and placed in the chamber preheated at 28–30°C; a thermistat probe (BIO-BIT14) was inserted rectally and secured in place at the base of the tail and on the wall chamber. A protecting muffle was placed around the mouse allowing it to feel secure and to be rapidly calmed. The ventilatory parameters and colonic and chamber temperature were recorded during 20 min at 5-min interval while the mouse was breathing room air. The CO₂ concentration in the chamber was always <0.5%. The following variables were measured and calculated by a computer-assisted method: minute ventilation (V_E) and its two components: tidal volume (V_T) and respiratory frequency (f_R). For each 5-min recording, values were averaged on 50–100 contiguous breaths.

Blood and tissue collection. Mice aged 120 d were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) (Virbac, Carros Cedex, France) and xylazine (20 mg/kg) (Rompun, Bayer, Leverkusen, Germany) and the abdominal cavity was opened. Blood was collected from the vena cava for biochemical tests. The liver was harvested, immediately weighed, and immersed in 10% buffered formalin for 24 h before paraffin embedment. The chest was then opened, the trachea exposed, and the lungs perfused *in situ* with 10% (vol/vol) neutral-buffered formalin under 5 cm H₂O pressure as recommended by Kent (personal communication). Thereafter, the trachea was ligated; the lungs were excised and immersed in 10% formalin during 5 d before paraffin embedment.

Biochemical tests. Serum transaminase activities, *i.e.* aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were measured at 37°C on an Olympus AU 640 analyzer, using bioMerieux (Marcy l'Etoile, France) reagent.

Histology. All tissue samples were paraffin-embedded and cut into 4- μ m-thick sections. Tissue sections were stained with hematoxylin and eosin, and examined under light microscopy, by an experienced pathologist blinded for the genotype and dietary regimen. Liver steatosis was graded according to the percentage of hepatocytes containing cytoplasmic vacuoles, as follows: mild ($\leq 10\%$); moderate ($>10\%$ and $\leq 30\%$); and severe ($>30\%$).

Statistical analysis. All values are given as means $\pm$ SD. Statistical comparisons were performed using a nonparametric Wilcoxon-Mann-Whitney test, which does not assume a normal distribution. A *p* value <0.05 was considered as statistically significant.

RESULTS

Survival. In the group of Cfr^{-/-} mice, 10 out of 12 animals died within 10 d after weaning, when maintained on standard chow. By contrast, 77% and 73% of Cfr^{-/-} mice fed Peptamen or a standard chow + PEG, respectively, were alive after 60 d (Fig. 1). All age-matched Cfr^{+/+} mice, whatever was the regimen, were alive after 60 d. Therefore, the lifespan of Cfr^{-/-} mice was greatly prolonged by either one of Peptamen or PEG regimens. In the animals followed-up for a longer period of time, survival was stable up to 120 d.

Figure 1. Survival rates from weaning until 60 d. Survival rates from weaning until 60 d of Cfr^{+/+} mice with or without treatment ($\blacklozenge$, *n* = 35), standard chow fed Cfr^{-/-} mice ($\diamond$, *n* = 12), standard + PEG Cfr^{-/-} mice ($\square$, *n* = 26), and Peptamen Cfr^{-/-} mice (∇ , *n* = 18). **Figure 2.** Weight gain between 30 and 120 d of age. Weight gain from 30 d until 120 d of age in (A): standard ($\bullet$, *n* = 13), Peptamen (∇ , *n* = 5), and standard + PEG ($\square$, *n* = 8) Cfr^{+/+} mice; and (B): standard Cfr^{+/+} mice ($\bullet$, *n* = 13), Peptamen (∇ , *n* = 7) and standard + PEG ($\square$, *n* = 7) Cfr^{-/-} mice. **p* < 0.05 compared with standard Cfr^{+/+} mice; \$*p* < 0.05 compared with standard + PEG Cfr^{-/-} mice. Body weights are expressed as means $\pm$ SD.

Growth. The growth of Cfr^{-/-} mice that were fed either Peptamen or a standard chow + PEG, was compared with that of age-matched Cfr^{+/+} mice that were under the same regi-

men or under standard chow (without PEG), this latter group being taken as a reference (Fig. 2). While all $Cftr^{+/+}$ mice had similar body weights at the age of 30 d, during subsequent preadult period (between 30 and 60 d), weight gain was significantly lower in the subgroup of Peptamen-fed animals, than in those under standard or PEG regimen ($+5.4 \pm 1.5$ g versus 9.2 ± 2.6 g and $+11.6 \pm 1.5$ g, respectively). Later on (from 60 to 120 d), a growth catch-up occurred in Peptamen-fed $Cftr^{+/+}$ animals that gained 7.8 ± 1.9 g versus 4.8 ± 1.8 g and 4.5 ± 1.6 g, in animals under standard and PEG regimens, respectively (Fig. 2A).

Figure 2B shows that, at 30 d of age, $Cftr^{-/-}$ mice were significantly smaller than standard $Cftr^{+/+}$ mice. Subsequent weight gain during the entire preadult and adult period was not significantly different between Peptamen-fed $Cftr^{-/-}$ ($+12.7 \pm 2.1$ g), PEG-treated $Cftr^{-/-}$ ($+13.5 \pm 1.9$ g), and standard $Cftr^{+/+}$ mice ($+14.0 \pm 2.0$ g), but during preadult period (30–60 d), weight gain was significantly lower in Peptamen than in PEG $Cftr^{-/-}$ mice ($+7.8 \pm 1.5$ g versus $+10.1 \pm 2.1$ g, respectively). However, after 120 d, neither Peptamen nor PEG $Cftr^{-/-}$ mice had caught-up growth on standard $Cftr^{+/+}$ mice, and $Cftr^{-/-}$ mice in both groups were smaller than standard $Cftr^{+/+}$ mice.

These data indicate that mice with $Cftr^{-/-}$ genotype failed to catch-up growth retardation present at weaning. Unexpectedly, lipid-enriched diet, although designed for malnutrition, induced a growth delay during the preadult period in both $Cftr^{+/+}$ and $Cftr^{-/-}$ mice.

Lung phenotype. Ventilation was recorded in conscious mice breathing room air, at the age of 120 d. As shown in Figure 3A, baseline minute ventilation (V_E) was not different between the three groups of $Cftr^{+/+}$ mice, fed either standard chow, standard chow with PEG, or Peptamen. In $Cftr^{-/-}$ mice, V_E was significantly lower than in $Cftr^{+/+}$ mice, without any difference between PEG- and Peptamen-fed animals.

Because minute ventilation is generally dependent upon body weight, we analyzed the relationship between both parameters in $Cftr^{+/+}$ and $Cftr^{-/-}$ mice (Fig. 3B). This analysis showed a significant correlation between both parameters in $Cftr^{+/+}$ animals ($r = 0.54$, $p < 0.05$) while in $Cftr^{-/-}$ mice, V_E did not increase with body weight. As a consequence, we did not find it appropriate to use V_E to body weight ratios for comparison between the two groups. To minimize the impact of body weight, we considered a narrow range of overlapping body weights (24–30 g) (Fig. 3B, dotted lines). Within this range, the difference between $Cftr^{-/-}$ and $Cftr^{+/+}$ mice remained highly significant ($V_E \pm SD = 44.3 \pm 6.1$ versus 57.5 ± 6.2 , $p < 0.001$).

Histopathological examination of the lungs in the different groups did not show any inflammation, luminal obstruction, or fibrosis. Only a few distended areas of alveolar air spaces, mainly centrally located, were occasionally observed in both Peptamen- and PEG-treated $Cftr^{-/-}$ mice (data not shown).

We conclude that $Cftr^{-/-}$ genotype by itself causes a ventilation defect, with little or no influence of inflammation, nutritional, or growth status.

Liver phenotype. The occurrence of liver damage was first evaluated by measuring the liver/body weight ratio. In com-

Figure 3. Minute ventilation in 120-d-old mice. (A) Mean values ($\pm$ SD) of minute ventilation (mL/min) measured in standard $Cftr^{+/+}$ mice ($\blacksquare$, $n = 10$), Peptamen and standard + PEG $Cftr^{+/+}$ mice ($\blacksquare$, $n = 5$ and $n = 6$, respectively) and Peptamen and standard + PEG $Cftr^{-/-}$ mice ($\square$, $n = 7$ and $n = 8$, respectively). (B) Relationship between minute ventilation (mL/min) and body weight (g) in standard, Peptamen and standard + PEG $Cftr^{+/+}$ mice ($\bullet$, $n = 21$) and Peptamen and standard + PEG $Cftr^{-/-}$ mice ($\circ$, $n = 15$); dotted lines limit a restricted body weight range (24–30 g) for which mean values ($\pm$ SD) of minute ventilation and body weight for $Cftr^{+/+}$ $\blacksquare$ and $Cftr^{-/-}$ $\square$ mice are given. The regression lines fitted for values of each group of mice with correlation coefficients (r) and p value are shown (B). All measurements were performed in 120-d-old animals. * $p < 0.05$ compared with standard $Cftr^{+/+}$; § $p < 0.05$ compared with Peptamen or standard + PEG $Cftr^{+/+}$ mice.

parison with $Cftr^{+/+}$ mice on standard diet, neither $Cftr^{+/+}$ nor $Cftr^{-/-}$ mice treated with PEG showed significant differences in liver/body weight ratios (Fig. 4). Peptamen feeding by itself did not induce any significant change in liver/body weight ratios in $Cftr^{+/+}$ mice, but caused hepatomegaly in $Cftr^{-/-}$ mice (Fig. 4). While the liver was histologically normal in all $Cftr^{+/+}$ ($n = 6$) and $Cftr^{-/-}$ ($n = 6$) animals treated with PEG (Fig. 5, A and C), all $Cftr^{-/-}$ and some $Cftr^{+/+}$ mice fed Peptamen displayed macrovacuolar periportal steatosis. Out of five Peptamen-fed $Cftr^{+/+}$ mice, three showed steatosis,

Figure 4. Liver/body weight ratio in 120-d-old mice. Liver/body weight ratios (mean values $\pm$ SD) in 120-d-old $Cftr^{+/+}$ animals under standard (■, $n = 4$), Peptamen or standard + PEG $Cftr^{+/+}$ mice (□, $n = 5$ and $n = 7$, respectively); Peptamen and standard + PEG $Cftr^{-/-}$ mice (□, $n = 6$ in both groups). * $p < 0.05$ compared with standard $Cftr^{+/+}$ mice; § $p < 0.05$ compared with Peptamen $Cftr^{+/+}$ mice.

Figure 5. Histologic examination of the liver of 120-d-old mice. Hematoxylin/eosin staining of the liver from 120-d-old mice, showing (A) normal liver histology in standard + PEG $Cftr^{+/+}$ mice; (B) mild periportal macrovacuolar steatosis in Peptamen $Cftr^{+/+}$ mice; (C) normal liver histology in standard + PEG $Cftr^{-/-}$ mice; and (D) moderate periportal macrovacuolar steatosis in a Peptamen $Cftr^{-/-}$ mice. Scale bar = 200 μ m. CV, central vein; P, portal tract.

which was graded as mild in two (Fig. 5B) and moderate in one. Steatosis was graded as moderate ($>10\%$ and $\leq 30\%$) in three out of six Peptamen-fed $Cftr^{-/-}$ animals, as illustrated in Figure 5D, and as mild ($\leq 10\%$) in the remaining three. Peptamen also induced steatosis in $Cftr^{+/+}$ animals, although to a lesser extent than in $Cftr^{-/-}$. No other histologic abnormality, including biliary obstruction or portal tract inflammation, was detected. Moreover, there was no difference in the serum levels of transaminases (AST and ALT) between the different groups.

These results indicate that the lipid-enriched liquid diet Peptamen induces liver steatosis, which is associated with hepatomegaly and tends to be more severe, in $Cftr^{-/-}$ genotypic background.

DISCUSSION

On the basis of analyses in $Cftr^{-/-}$ mice, the present study provides evidence that CFTR genetic defect may cause 1) growth failure, 2) hypoventilation, and 3) predisposition to liver steatosis. Premature death at weaning to standard diet in $Cftr^{-/-}$ mice, which was previously shown to occur as a result of intestinal obstruction, was herein prevented with similar efficiency by an osmotic laxative (PEG) and a lipid-enriched liquid diet (Peptamen). While the degree of hypoventilation was similar in animals fed either Peptamen or a standard chow with PEG, the dietary regimen had an impact on growth development and on the occurrence of liver steatosis.

It has been reported that the high mortality rate of CFTR knockout mice soon after weaning was related to intestinal obstruction with solid food. The administration of Peptamen, an easily digestible low-residue liquid diet, was first proposed by Eckman *et al.* (11) to prevent intestinal obstruction. These authors demonstrated that this liquid diet markedly increased the lifespan in $Cftr^{-/-}$ mice, and that 88% of treated mice reached maturity. Subsequently, it was shown that the addition of an osmotic laxative (PEG) in the drinking water also greatly prolonged survival of $Cftr^{-/-}$ mice (7). The present study shows that Peptamen and PEG used in the same experimental conditions have similar beneficial effects on survival, in CFTR knockout mice.

Under both dietary regimens, however, surviving $Cftr^{-/-}$ mice remain consistently smaller than $Cftr^{+/+}$ controls. This finding is reminiscent of the failure to thrive observed in CF children (3,12–14). In addition, weight growth was significantly delayed in the preadult period in Peptamen-fed CF mice compared with PEG-treated CF mice, indicating that Peptamen diet is not the best option at least in mouse species.

In most studies, lung pathology in $Cftr^{-/-}$ mice has been assessed histologically. Only minor lesions of the respiratory tract have been reported, almost exclusively in congenic $Cftr^{-/-}$ mice (15,16). However, the consequences of CFTR defect on pulmonary function have been rarely investigated in mouse models (9,17,18). In the present study, pulmonary function in $Cftr^{-/-}$ mice was tested by measuring the basal minute ventilation (V_E) in breathing air. The results show that V_E is lower in $Cftr^{-/-}$ compared with $Cftr^{+/+}$ mice, irrespective of the dietary regimen. Although V_E and body weight are normally positively correlated (19) as observed here in $Cftr^{+/+}$ animals, the decrease in ventilation noted in $Cftr^{-/-}$ mice cannot be entirely attributed to their underweight. First, V_E was not correlated with body weight in these mice. Second, within a restricted range of similar body weights, V_E values from $Cftr^{-/-}$ were still significantly lower than those from $Cftr^{+/+}$ animals. Low minute ventilation associated with CFTR defect may thus alter the control of breathing and contribute to lung disease. We previously showed that, while the ventilatory response to hypoxia was clearly diminished in juvenile (60-d-old) $Cftr^{-/-}$ mice, baseline minute ventilation was similar to that of their $Cftr^{+/+}$ littermates (17). The decrease in baseline minute ventilation found in 120-d-old $Cftr^{-/-}$ mice may be explained by a progressive impairment in

respiratory mechanics that occurs with aging, as previously suggested (18).

While life expectancy continuously improves in CF patients, hepatic manifestations become increasingly problematic. After cardiorespiratory and transplantation complications, liver disease represents a significant cause of death, in these patients (20). The type and severity of CF hepatic manifestations are both highly variable and their mechanisms are poorly understood. It is hypothesized that biliary-type lesions, resulting from the defective expression of CFTR in cholangiocytes (21–24), are the major pathogenic event leading to end-stage liver disease in CF. However, the most common hepatic lesion recognized in CF patients (23–67%) at any age, is steatosis (20,25). Even though its pathogenesis is poorly understood, liver steatosis is generally ascribed to epigenetic mechanisms related in particular to nutritional deficiencies (e.g. in essential fatty acids) (25). Consistent with this view, we show here that nutritional factors are required for steatosis to develop. No steatosis was noted in PEG treated mice irrespective of the genotype. In contrast, hepatic steatosis developed in Peptamen-fed *Cftr*^{-/-} and *Cftr*^{+/-} mice suggesting that steatosis results from the high fat content of this liquid diet. Furthermore, *Cftr*^{-/-} mice tend to be more severely affected than *Cftr*^{+/-} mice, in agreement with the results of Beharry *et al.* (26). This finding, together with the fact that hepatomegaly was restricted to *Cftr*^{-/-} animals, suggests that the loss of CFTR contributes to the development of liver steatosis. No inflammatory changes were seen in the liver of CFTR knockout animals, but in the same knockout mouse model bred into a congenic C57BL/6J background, Durie *et al.* (27) demonstrated that the severity of liver disease progressed with aging and that pathologic changes in the liver, including steatosis, cholangitis, and fibrosis, became evident after 3 months of age. Although steatosis has long been considered as a benign condition, we hypothesize that on a particular genetic background or that with aging, steatosis may progress to more serious fibrotic liver disease as reported in non-CF adult patients (28,29).

The importance of diet in CF patients has been previously outlined (3,30). Optimizing growth with a high-energy diet is recommended. The present findings indicate that both PEG and Peptamen strategies are similarly efficient to avoid the high mortality at weaning, but a high-fat diet may induce growth retardation during the preadult period and also triggers liver steatosis. These effects of diet are important to consider in future animal studies and also prompt to evaluate different types of high-energy diets in CF patients.

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