Original Research

Low linoleic and high docosahexaenoic acids in a severe phenotype of transgenic cystic fibrosis mice

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Impact statement

In translational research, animal models are important to investigate the effect of genetic mutations in specific diseases and their metabolism. Special attention has to be given to differences in physiology and metabolism between species and humans, which otherwise can hazard the conclusions. Our work illustrates that the different synthesis capacity in mice and humans for DHA would explain different results in different models for cystic fibrosis and different influences of diets. To avoid disappointing clinical results, these facts have to be considered before extensive clinical studies are started based on results from single animal studies.

Abstract

Low linoleic acid concentration is a common finding in patients with cystic fibrosis and associated with severe clinical phenotype. Low docosahexaenoic and arachidonic acids are more inconsistently found in patients, but arachidonic/docosahexaenoic ratio is usually high. In animal models with *cftr* mutations or KO animals for the *cftr* gene, linoleic acid deficiency has not been consistently reported and some report docosahexaenoic deficiency as the major fatty acid abnormality. We hereby describe fatty acid profile in a severe clinical cystic fibrosis phenotype in mice with a duplication of exon 3 generated in the cystic fibrosis gene of C57B1/6J mice ($cftr^{m1Bay}$ allele). In 43/50 animals, plasma phospholipid fatty acids were repeatedly analyzed (mean three times/animal) covering ages between 7 and 235 days. Linoleic acid concentrations were significantly lower in *cftr*−/− mice compared to heterozygotes ($P = 0.03$) and wild type mice ($P < 0.001$). Females had significantly lower linoleic acid than males, not related to age. Arachidonic acid did not differ but docosahexaenoic acid was higher in *cftr*−/− than in wild type mice ($P < 0.001$). The arachidonic/docosahexaenoic acid ratio did not differ but arachidonic/linoleic acid ratio was higher in *cftr*−/− mice compared to wild type mice ($P = 0.007$). Similar to clinical studies, type of mutation is important for lipid abnormality with low linoleic acid most consistently found in the animals. Rodents differ in metabolism by synthesizing docosahexaenoic acid more efficiently comparing to humans, suggesting greater influence by diet. Precaution seems important when comparing animal and humans.

Keywords: CFTR, transgenic mice, genetics, arachidonic acid, oleic acid, heterozygotes, homozygotes

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Introduction

Cystic fibrosis (CF) is generally characterized by pancreatic insufficiency and pulmonary disease although careful examinations reveal abnormalities in many more tissues, also when not presenting with clinical symptoms.[1–5] Low linoleic acid (LA, 18:2n-6) concentration has been described in CF since more than 50 years[6] and slight abnormalities have also been described in heterozygotes and in patients with CF without pancreatic insufficiency.[7,8] Mutations associated with more severe phenotype seem to be associated with more expressed LA deficiency.[9,10]

Before the gene was discovered, linking the disease to mutations related to a defective chloride channel, the cystic fibrosis transmembranous conductance regulator (CFTR), research in animals and patients suggested that much of the clinical symptomatology would be related to LA deficiency.[11–15] This was further suggested to be associated with an increased arachidonic (AA, 20:4n-6) release, which by increasing the turnover was reflected in the low serum levels of LA[16–20] and high eicosanoid metabolites.[21] Low LA concentrations are associated with increase of Δ5- and Δ6 desaturase expressions[22] supported by findings

of inverse associations between LA and AA.[23–25] Docosahexaenoic acid (DHA, 22:6n-3) was first reported low in tissues in CF at autopsy.[26] In patients with CF plasma DHA has been reported low,[8,10,27,28] or not different from controls,[7,9,26,29] and the most common abnormality related to DHA was an increased ratio between AA and DHA.[9,27,30] In patients with CF related liver disease, the concentrations of both LA and DHA have been reported low.[31,32]

In the 90s, new interest was directed to the lipid abnormality when a study of *cftr−/−* mice showed that lung, pancreas, and ileum had low DHA concentrations related to morphology and that these changes could be improved by high dose DHA supplementation.[33] This turned all interest to this fatty acid resulting in many clinical studies supplementing DHA to patients with CF, but the results were mainly disappointing regarding any influence on the symptomatology.[34] Later a long-term study of similar *cftr−/−* mouse strain could not repeat the beneficial effect of DHA supplementation, but showed a protection of liver abnormality.[35]

The development of murine models opened a possibility to study organ pathophysiology in CF, but the investigations have shown different results depending on models with null alleles or those with more or less CFTR function related to mutation strategy.[36–39] Much of the differences might also be related to the different mouse strains and to species and illustrate that the basic metabolism may have an impact on the model. Some models are more suitable for studying special symptomatology[36,39] and very seldom fatty acid concentrations are documented. In some mouse models, no fatty acid abnormality was reported[40] or only a low AA/DHA ratio.[35] In some studies, LA was low without a decrease or even with an increase of DHA.[41] Analyses of different phospholipids in different tissues showed decrease in plasma and pancreas of molecular species containing AA and increase in pancreas for those containing DHA.[42] However, some studies of *cftr−/−* mice and cell culture studies based on CFTR sense/antisense cells reported low DHA concentration as the major finding[43](Table 1).

Most transgenic mouse studies are performed by manipulations of exon 10, corresponding to the dF508 mutations in humans. However, several mutations related to severe clinical diagnosis of CF are associated with exon 3, like 394delTT, E60X, and R75Q.[44] In patients with CF, a similar fatty acid profile is obtained in patients with mutations dF508 and 394delTT.[9] The aim of this paper was to investigate if a similar fatty acid profile was obtained in a transgenic mice strain with duplication of exon 3 as previously described in those with mutations dF508.

Material and methods

Animals

Mice with clinically severe phenotype of CF, characterized by severe intestinal obstruction causing 40% death within one week of life, were analyzed for plasma phospholipid fatty acids. The mice had a duplication of exon 3 generated

Table 1. Results of fatty acid analysis in reported CF mouse strains or cells compared to the present study.

Laboratory origin	Basic mouse strain	Mutation	Tissue	Fatty acid analysis	Reference
Erasmus Medical Center, Rotterdam	C57B1/6J/129	ΔF508/ΔF508, *cftr−/−*^{tm1CAM} *Exon 10*	Different tissues	No different conc.; no abn. transformation(^{13}C-labels)	40
Animal Facility, Univ Louvain	129/FVB	Homolog recomb F508del *cftr*^{tm1EUR}	Different tissues	LA_{low}, AA_{high}, DHA_{high}a	41
Beth Israel Deaconess Med Center (Jackson Lab)	UNC(C57)	Exon 10 *cftr−/−*^{UNC} Inframe stop	Different tissues	AA_{high} DHA_{low} LA not reported	33
Dr Pamela Davis, Cleveland – Am type culture coll (Manassas, VA)	Bronchial epithelial cells (16HBE) – IB3-1 and C38 cells	First 131 nucleotides of CFTR in sense antisense F508/W1282X and C38 corrected with WT CFTR	Cell analyses	LA_{low} DHA_{low} LA_{low} DHA_{low}	43
Jackson Laboratory., Bar Harbor, ME	C57B1/6J	*Exon 10 cftr*^{tm1UNC} Inframe stop	Plasma and red blood cell membranes	Ratio AA/DHA_{low} LA not reported	35
Edinburg/Hannover	ZTM:MF-1	*cftr*$^{tm1HGU/tm1HGU}$ *Exon 10 insertion*	Different tissues	Plasma: PC 16:0/20:4, PC 18:0/20:4, 16:0/$22:6_{low}$ Pancreas: PE, PS, PI 18:0/$22:6_{high}$ and 18:0/$20:4_{low}$	42
Baylor College of Medicine	C57B1/6J	*Dupl exon 3 cftr−/−*^{tm1Bay}	Plasma phospholipids	LA_{low}, DHA_{high}	Present

Note: Only significant differences are reported.
[a]Only in duodenum-jejunum.
LA: linoleic acid; AA: arachidonic acid; DHA: docosahexaenoic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine; PI: phosphatidylinositol.

in the CF gene of C57B1/6J mice (*cftr*^{m1Bay} allele) and are previously described.[45] The CF mice showed a high mortality, and expressed no *cftr* mRNA expression (<1–2%). They had to be fed a liquid diet to survive due to severe intestinal obstructions. They were kept under constant conditions of humidity, temperature, and light. They were divided by sex and pair-fed in cages ad libitum (homozygote, heterozygote together with wild type (WT)) to equal as much as possible the feeding between the different genotypes and ages.

Fifty mice were included in the study; 15 wild type, 13 heterozygotes and 22 homozygotes for the mutation. Females constituted 7, 6, and 10 animals and males 8, 7, and 12, respectively. Plasma samples were obtained in the morning by tail bleeding, in three animals only once and in all others at a mean of three times per animal (range 2–6). The samples were obtained between 7 and 235 days of age, median serial time was 48, 39, and 38 days for WT, heterozygotes, and homozygotes, respectively.

The animals were treated according to ethical rules and the North American legislation and regulation of the use of live animals for scientific research.

Fatty acid analysis

Blood samples were collected from the tip of the tails in the animals in each cage at the same time. Plasma were kept frozen at −70°C and transported to the laboratory in carbon ice until analyses as previously described.[46] Lipids were extracted from the plasma with chloroform–methanol 2:1 (v/v) containing 0.01% butylated hydroxytoluene. The lipids were fractionated on a single SEP-PAK aminopropyl cartridge (Waters Corp., Milford, MA, USA) and the fraction of phospholipids was transmethylated in methanolic-HCL-3N at 90°C over 4 h. The fatty acid methyl esters (FAME) were extracted with *n*-hexane and, thereafter, washed with water until neutral, dried with MgSO4, and then dried with nitrogen. The FAME were separated by capillary gas–liquid chromatography in a Hewlett-Packard, 6890 gas chromatograph equipped with a 30 m × 0.25-mm SP-2380 column, film thickness 20 μm. Helium at 2.0 ml/min was used as carrier gas, and a split less injection was used. The injector and detector temperatures were 300°C and 250°C, respectively. The column oven temperature was programmed from 50°C to 230°C at a heating rate of 20°C/min up to 180°C and, thereafter, 2°C/min. The separation was recorded with HP GC Chem Station software (HP GC, Wilmington, DE). C21:1 was used as internal standard and the FAME identified by comparison with retention times of pure reference substances (Sigma Aldrich Sweden AB, Stockholm, Sweden).

Statistical analysis

We used a three-level mixed model to account for the fact that mice were pair-fed within cages and also the fact that several measurements were taken on the same mouse. Restricted maximum likelihood was used to calculate parameter estimates and standard errors.[47] Our outcome variables were LA, AA, DHA, and oleic acid (18:1n-9, OA) and the ratios AA/DHA, AA/LA, and OA/LA.

We created bar charts illustrating the predicted values for the different fatty acids in the mice at the median age at measurement, 118 days (Supplementary Tables 1 and 2). To model the *cftr* gene, we used three categories, one for WT, one for the recessive homozygotes, and one for the heterozygotes. WT was used as the reference category. Adjustments were made for sex and age (in $\log_e$ days), and we also allowed for an interaction between sex and age. The significance of sex and age in the models was evaluated by testing the coefficient of both main term and interaction term simultaneously (Figure 1). The level of significance was chosen as alpha = 0.05.

We also indicated all measurements in a population based model (Figure 2) by generating locally weighted scatterplot smoothing (LOWESS)[48] with a bandwidth of 0.8. These figures thus show the trend in the population over time. Smoothing was performed separately for each combination of allele and gender. Thus, Figure 1 is based on the individual depicted values with adapted curves illustrating differences by age but showing similar results as the other model (Figure 2), which illustrates the trend of the population over time.

Results

The concentration of LA was significantly higher for the WT compared to the heterozygotes ($P = 0.03$) and the *cftr−/−* mice ($P < 0.001$). LA was also higher for males than females ($P = 0.006$) (Figure 3). LA did not change significantly with age ($P = 0.32$) (Figures 1 and 2).

The concentration of AA increased significantly with age ($P = 0.004$) without difference between sex ($P = 0.54$) (Figures 1 and 2). There were no significant differences between alleles ($P = 0.34$) (Figure 4). The AA/LA-ratio was significantly higher for *cftr−/−* mice compared to the WT ($P = 0.006$) (Figures 1, 2, and 4). The difference between heterozygotes and WT was not significant ($P = 0.65$), and neither between sex ($P = 0.65$), nor age ($P = 0.06$).

The concentration of DHA was significantly higher for *cftr−/−*mice compared to the WT ($P < 0.001$) (Figure 4). The difference between heterozygotes and WT was not significant ($P = 0.66$). No differences were found regarding sex ($P = 0.17$) or age ($P = 0.15$) (Figures 1 and 2). The AA/DHA-ratio increased with age for males, but decreased with age for females (Figures 1 and 2). This interaction between age and sex was significant ($P = 0.008$). The ratio did not differ significantly between alleles ($P = 0.88$) (Figure 4).

The concentration of OA decreased significantly with age ($P = 0.004$), but did not differ between sex ($P = 0.30$). The heterozygote had significantly higher values than the WT ($P = 0.015$) (Supplementary Table 3), but the difference between homozygotes and WT was not significant ($P = 0.19$). The ratio OA/LA was significantly higher in both heterozygotes and homozygotes compared to WT ($P = 0.03$ and $P = 0.001$, respectively).

Supplementary Figure 1 illustrates the results of animals in different cages. LA was constantly low and DHA normal or high in homozygotes in comparison with the WT and heterozygotes.

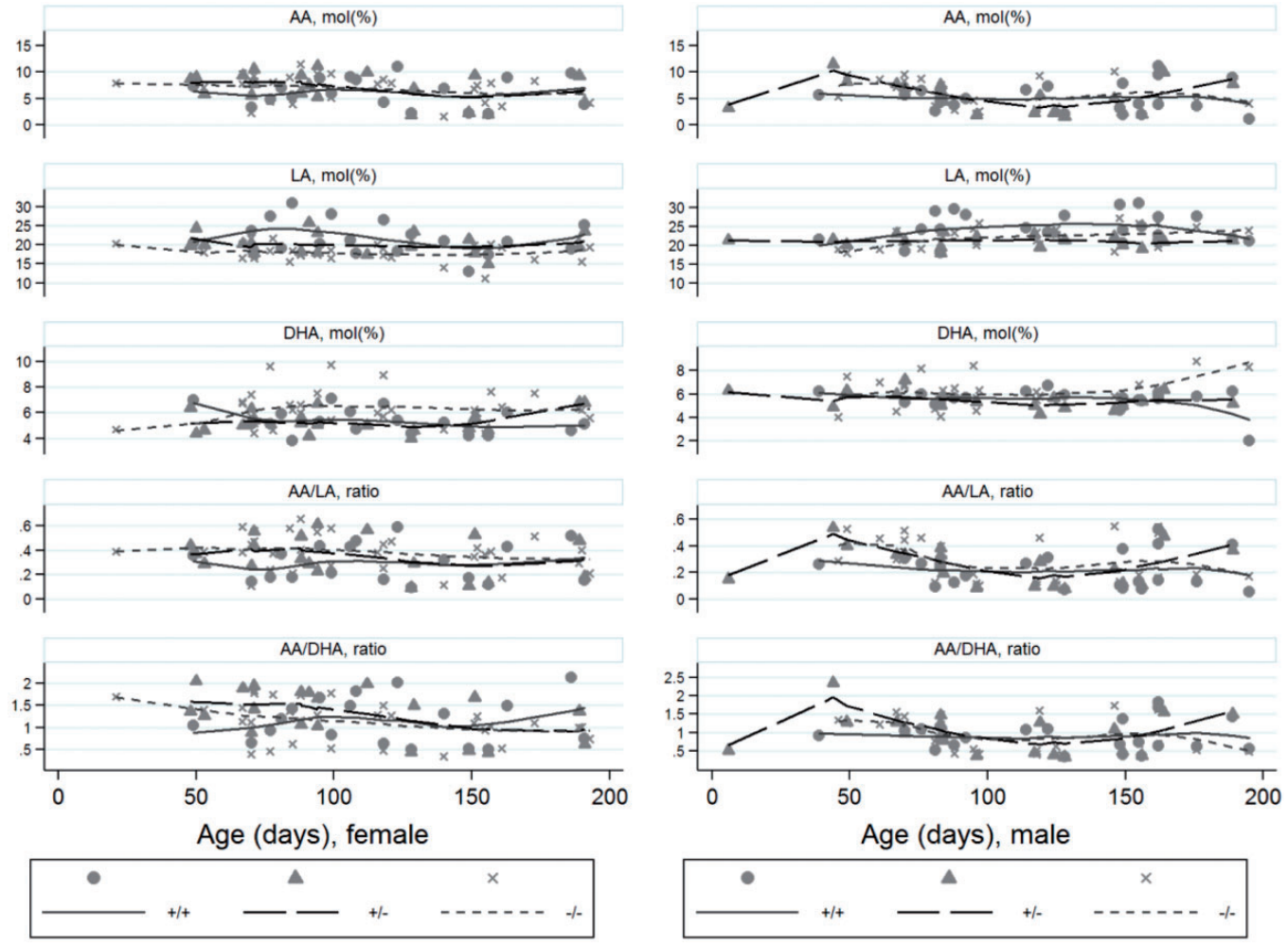

Figure 1. The longitudinal trend of serum phospholipid concentrations based on individual mice and separated by alleles and sex for wild type (+/+), heterozygotes (+/−) and homozygotes (−/−) for the *cftr*^{m1Bay} allele. Arachidonic acid (AA); docosahexaenoic acid (DHA); linoleic acid (LA) and ratios.

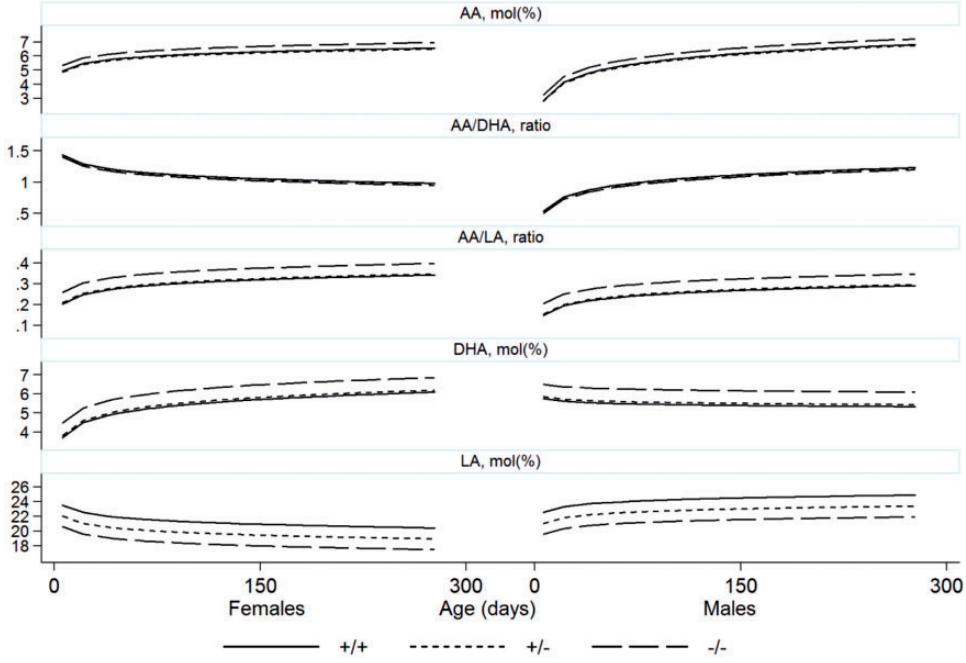

Figure 2. The longitudinal trend of serum phospholipid concentrations based on population of mice and separated by alleles and sex for wild type (+/+), heterozygotes (+/−) and homozygotes (−/−) for the *cftr*^{m1Bay} allele. Arachidonic acid (AA); docosahexaenoic acid (DHA); linoleic acid (LA) and ratios.

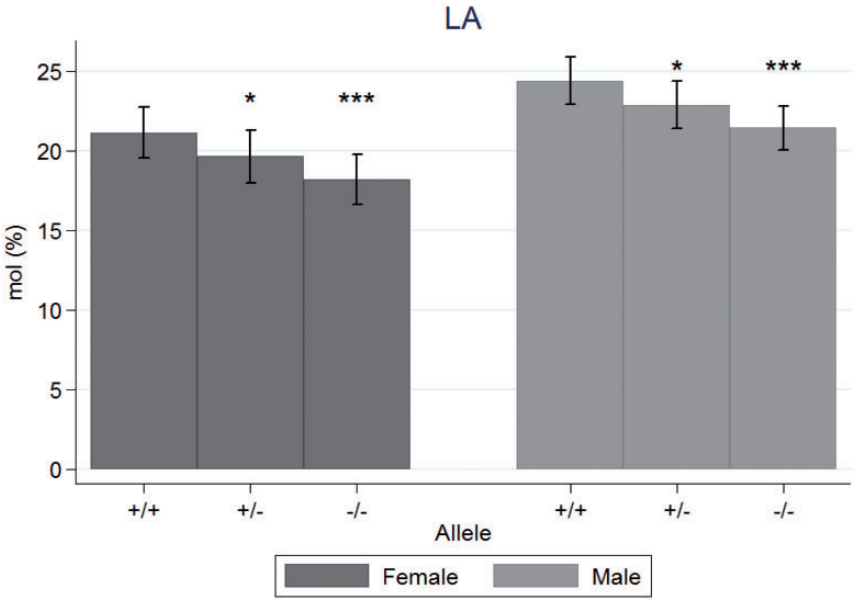

Figure 3. Mean concentrations of linoleic acid in plasma phospholipids at day 118 in wild type (+/+), heterozygotes (+/−) and homozygotes (−/−) for the *cftr*^{m1Bay} allele, divided by sex. * Indicates $P < 0.05$ and *** $P < 0.001$ compared to wild type. The differences were significant also between gender.

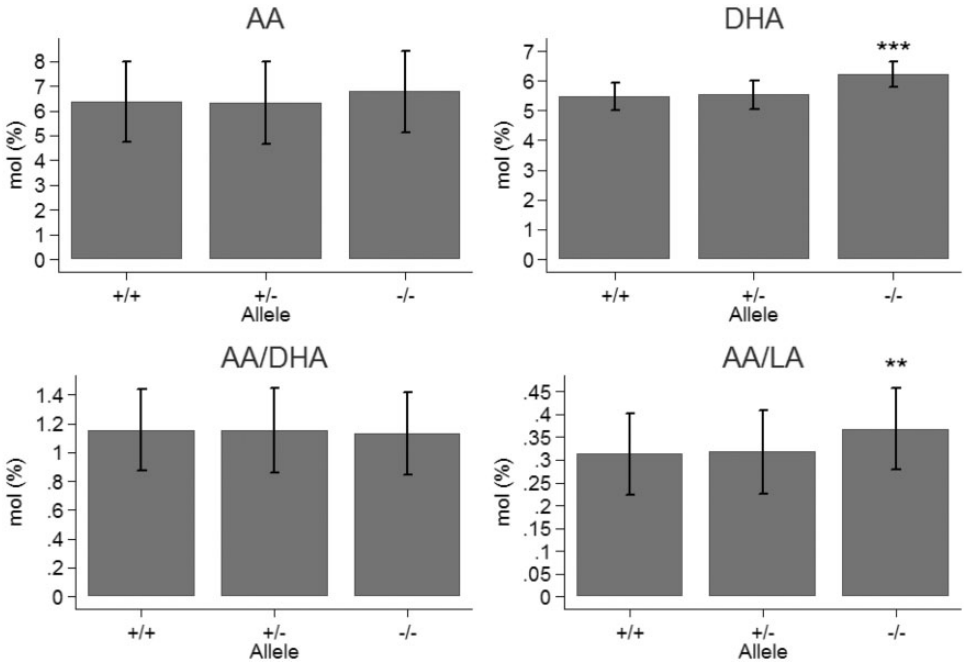

Figure 4. Mean concentrations arachidonic acid (AA), docosahexaenoic acid (DHA) and the ratios AA/DHA and AA/linoleic acid (LA) in plasma phospholipids at day 118 in wild type mice (+/+), heterozygotes (+/−) and homozygotes (−/−) for the *cftr*^{m1Bay} allele. Significant differences to wild type and heterozygotes were only found for DHA ($P < 0.001$) and the ratio AA/LA ($P < 0.01$).

Discussion

This study shows that mice with a severe genotype/phenotype showed low LA concentrations, similar to patients with CF. Contrary to patients with CF, these *cftr*−/− mice had a normal or high DHA concentration and did not have increased AA/DHA ratio in serum phospholipids, which is commonly seen in patients with CF. One possible explanation to this discrepancy might be if AA is excessively liberated from membranes[16] and transformed to eicosanoids.[21,49,50] A compensatory upregulation of the enzyme systems would also increase transformation to DHA, which is considered more efficient in rodents than in humans.[51] The difference in DHA with time between male and female is in agreement with the well-known increased capacity of female to synthesize DHA.[52] The high mortality of the mice made consistent individual longitudinal studies impossible, but the pair feeding strengthened the results since the

differences between the WT, heterozygotes, and CF mice remained the same (Supplementary Figure 1).

The fatty acid abnormality differs in different CF mouse strains with different mutations, but our study corroborates an earlier study showing low LA and high DHA concentration in another mouse model.[41] This illustrates that differences in fatty acid analyses might not only be related to the basic strain for the CFTR knockout models or in which strain the *cftr* mutation is inserted, the latter might theoretically have some CFTR function although usually clinically equal to KO animals. Furthermore, there is not a full homology between the mouse *cftr* and the human CFTR and also tissue distribution differs.[53] The study of Mimoun *et al.*[41] illustrates the impact of dietary fat composition given to the mice on the body tissue composition as well as on survival. Mice in contrast to humans can better synthezise DHA[51] and therefore diets containing alpha-linoleic acid or other omega-3 fatty acids can influence the difference between results. In the study showing the beneficial effect of DHA in the *cftr* −/− mice,[33] Peptamen® was used, the fat content of which according to manufacturer's information mainly consists of medium chain fatty acids and for essential fatty acid supply only soybean oil, which is mainly rich in omega-6 fatty acids. It cannot therefore be excluded that these mice had an initial deficiency of DHA which explained the beneficial effect of the very high dose supplementation. It illustrates why comparison between different models is difficult and the difference in DHA concentrations in different mice models may be related to both background strain, kind of and locus of mutation and diets (Table 1). A limitation of our study is that we did not have the fatty acid composition of the diet. Since we kept animals with different genotypes in cages with supply of the same diet, the consistent difference in fatty acid profile between the genotypes in the cages would indicate no impairment of the *cftr*−/− mice to synthesize DHA.

The finding that OA was not increased in the homozygotes was unexpected since it is a common feature in patients with CF with low LA concentrations, usually interpreted as compensatory, since LA is important for membranes.[7,54,55] One explanation to the found difference might be the high DHA concentration, since it is well known that high concentration of polyunsaturated fatty acids, especially the long-chain polyunsaturated fatty acids, inhibits Δ9-desaturase, stearoyl-CoA desaturase.[56] Such explanation is supported by the lack of difference in DHA concentration between WT and heterozygotes, which thus would explain the significantly higher OA concentration in the latter despite a decrease of LA.

The differences in different models of transgenic CF mice might illustrate the divergence sometimes seen in phenotype/symptomatology between siblings with CF carrying the same mutations and might reflect presence of modifying genes. It might also reflect different dietary intake. Epigenetic factors may as well be of importance, but that has not been studied in the context of CF.[57] The study suggests that low DHA might not be a characteristic finding in the CF model, but as in humans be mainly dependent on diet; in rodent not only DHA but also its substrates. It is a limitation that we could not illustrate that due to impossibility to analyze the food. In conclusion, our study confirms a low LA concentration in CF but also the difference in metabolism between mice and humans, which has to be considered when comparing mouse models with human patients.

Authors' contributions: Participating in research design: BS and WKO. Conducted experiments: WKO and BS.

Performed data analysis: BS, WKO, MAA, UH. Contributing to the writing of the manuscript: BS, WKO, UH.

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DECLARATION OF CONFLICTING INTERESTS

The authors have no conflict of interest to declare with respect to research, authorship and publication of this article.

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