CLINICAL RESEARCH ARTICLE

In a pilot study, reduced fatty acid desaturase 1 function was associated with nonalcoholic fatty liver disease and response to treatment in children

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BACKGROUND: FADS1 gene encodes delta 5 desaturase, a rate-limiting enzyme in the metabolism of n-3 and n-6 polyunsaturated fatty acids (PUFAs). Minor alleles of FADS1 locus polymorphisms are associated with reduced FADS1 expression and intra-hepatic fat accumulation. However, the relationship between FADS1 expression and pediatric nonalcoholic fatty liver disease (NAFLD) risk remains to be explored.
METHODS: We analyzed FADS1 transcription levels and their association with intra-hepatic fat and histology in children, and we performed pathway enrichment analysis on transcriptomic profiles associated with FADS1 polymorphisms. We also evaluated the weight of FADS1 alleles on the response to combined docosahexaenoic acid, choline, and vitamin E (DHA-CHO-VE) treatment.
RESULTS: FADS1 mRNA level was significantly and inversely associated with intra-hepatic fat ($p = 0.004$), degree of steatosis ($p = 0.03$), fibrosis ($p = 0.05$), and NASH ($p = 0.008$) among pediatric livers. Transcriptomics demonstrated a significant enrichment of a number of pathways strongly related to NAFLD (e.g., liver damage, fibrosis, and hepatic stellate cell activation). Compared to children who are common allele homozygotes, children with FADS1 minor alleles had a greater reduction in steatosis, fibrosis, and NAFLD activity score after DHA-CHO-VE.
CONCLUSION: This study suggests that decreased FADS1 expression may be associated with NAFLD in children but an increased response to DHA-CHO-VE.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in children but its pathogenesis remains incompletely understood. The prevalence of pediatric NAFLD in the U.S. was estimated to be 8–17% but up to 38% among obese children.[1] NAFLD is defined as a spectrum of chronic liver disorders, beginning as an accumulation of fat in the liver without significant alcohol consumption that can gradually progress to steatohepatitis (nonalcoholic steatohepatitis (NASH)) and even cirrhosis in children, leading to substantial liver injury.[2,3] Also, NAFLD in children is highly associated with a series of early metabolic conditions, including obesity, insulin resistance, glucose intolerance, and cardiovascular abnormalities,[4] exerting an enormous medical challenge for pediatric care.[5] Understanding the etiology of pediatric NAFLD and NASH is poor especially when compared to extensive studies in adults.[1,5] Given the current rapid growing population of NAFLD and its co-morbidities in children, it is an urgent need to elucidate the principle disease etiology and develop safe and effective treatment for pediatric NAFLD.

It is well recognized that n-3 polyunsaturated fatty acid (PUFA) deficiency is associated with many metabolic perturbations, including NAFLD.[6–9] In humans, particularly due to the high n-6/n-3 PUFA ratio in Western diet, n-3 PUFA deficiency has been associated with NAFLD and NASH in both adults and children.[10–19] Small clinical studies in children suggest that n-3 PUFA supplementation may improve NAFLD.[20] However, recent large clinical trials of n-3 PUFA in adults with NAFLD have yielded mixed results.[21–24] While inter-individual variability has been recognized as a key factor influencing the response to n-3 PUFA treatment,[25–27] the clinical trials testing the effectiveness of n-3 PUFA in NAFLD have not considered genetic variability in n-3 PUFA metabolism among study participants.

Both n-6 and n-3 PUFA metabolism are controlled by Δ5 and Δ6 desaturases (D5D, D6D) encoded by FADS1 and FADS2 genes, respectively. D5D is the rate-limiting enzyme in converting α-linolenic acid, the major dietary n-3 PUFA precursor, into the bioactive forms eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Our recent studies indicated that FADS1 is significantly involved in hepatic fat accumulation and NAFLD.[28,29] Notably, among the genes involved in this pathway, FADS1 harbors genetic alleles in strong linkage disequilibrium (LD; allele frequency ranging from 32% to 43%[30] consistently identified as the most

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important determinants for FADS1 (i.e., D5D) activity and endogenous n-3 PUFA levels in humans.[30–32] In addition, increasing evidence suggests that *FADS1* low-function alleles can influence both the pharmacokinetics and pharmacodynamics of supplemented n-3 PUFA, potentially leading to inter-patient differences in response to its intake.[26,27] Unfortunately, no clinical study thus far has considered these alleles in testing the effectiveness of n-3 PUFA in treating NAFLD. Further delineating this pharmacogenetic mechanism may lead to a more effective n-3 PUFA treatment for NAFLD.

In this proof-of-concept study, we evaluated the relationship between *FADS1* expression and liver histology in pediatric liver samples and explored the molecular pathway associated with low-function *FADS1* alleles. Further, we examined the relationship between *FADS1* polymorphism and clinical response to DHA supplementation combined with choline and vitamin E (DHA-CHO-VE) in a small cohort of children with NAFLD who have completed a randomized clinical trial registered[33] at ClinicalTrials.gov (ID: NCT01934777).

METHODS AND MATERIALS

Samples
Samples used in this study as well as the data analysis plan using the samples are reported in Fig. 1.

The liver tissue samples were described previously.[28] Briefly, these are leftover tissue samples collected from liver transplantation donors who are deceased individuals but were willing to donate their organs for transplantation purposes. Demographic information of the donors was demonstrated in Table 1. Transcriptomic data from 55 pediatric liver tissue samples (aged 0–18 years) were collected previously, among which only 22 were characterized for histologic evaluation. Genotypic data of 6 *FADS1* single-nucleotide polymorphisms (SNPs; rs174576, rs1535, rs174546, rs102275, rs174537, and rs174556) for all 55 pediatric liver samples were previously collected.[28]

Total hepatic fat content was measured using a protocol established in our previous study.[34] The degree of steatosis was visually assessed based on the hematoxylin–eosin–stained tissue sections and the total collagen content was quantified by Sirius red staining. The presence of NAFLD and NASH was defined based on the previously established method by NASH-CRN.[35]

Study population from clinical trial
Nineteen children with NAFLD belonging to the treatment arm of DHA-CHO-VE trial were included in the study. In particular, these children presented a liver biopsy-proven diagnosis of NASH. Other causes of liver diseases were excluded before the enrollment in
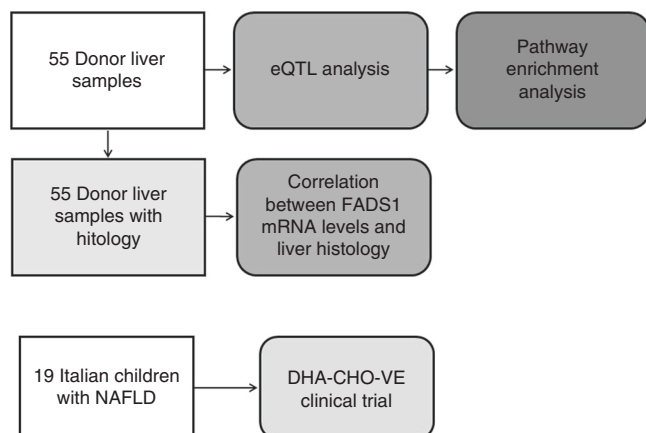
Table 1. Biochemical and histological characteristics of the donor liver tissue samples (n = 22)

Male sex, n (%)	12 (54.5)
Age (years)	
Mean ± SD	6.1 ± 4.4
Median (IQR)	4.5 (2.25–9)
BMI (kg/m²)	
Mean ± SDᵃ	17.7 ± 5.9
Median (IQR)	16.5 (15.5–17.6)
Race	
White	17 (77.3)
Black	5 (22.7)
Biochemical features	
Sirius red, mean ± SD (%)	8.2 ± 4.4
Histological features	
Total hepatic fat, mean ± SD (mg fat/mg tissue)	0.020 ± 0.0088
Steatosis (%, >5%)	4 (18.2)
NASH	4 (18.2)

The quantification of Sirius red staining is based on the percentage of the stained area of the liver tissue section
SD standard deviation, *BMI* body mass index, *IQR* interquartile range
ᵃMissing information for six participants.

the trial. These included: the presence of liver disease due to any of the following: Wilson disease, hepatitis B and C, acute systemic disease, autoimmune hepatitis, hypothyroidism, cystic fibrosis, celiac disease, and suspicion of muscular dystrophy, alpha-1-antitrypsin deficiency, and metabolic inherited diseases. Patients were also excluded if body weight and carbohydrate metabolism were altered by the use of parenteral nutrition, protein malnutrition, previous gastrointestinal surgery, structural abnormalities of the gastrointestinal tract, or neurological impairment. Finally, the use of nonsteroidal anti-inflammatory drugs, antibiotics, probiotics, or antisecretory drugs capable of causing achlorhydria within the 2 months preceding enrolment were considered additional exclusion criteria. Details of the trial have been already reported.[33] However, briefly, all children in the treatment arm received, every day for 6 months, pearls combining 250 mg of DHA, 39 UI of vitamin E, and 201 mg of choline (DHA-CHO-VE). Pearls were provided by DMF Dietetic Metabolic food (Italy). Concomitantly, all patients received recommendation for hypocaloric diet (25–30 kcal/kg/day) and twice weekly 1-h physical activity during the treatment and for further 6 months of follow-up. Compliance to treatments and diet was monitored through monthly visit by counting the amount of investigational product left in the bottle.

Symptoms and side effects were assessed in each visit by the investigator, who was blind to medication assignment. Complete medical histories were recorded for all participants. Collection of anthropometrical data, biochemical data, and second biopsy were performed at baseline and after 12 months from trial start. Patients and investigators were blinded before and after the assignment of the intervention. The study was registered at ClinicalTrials.gov, with the number ID: NCT01934777 on 30 August 2013. However, we underline that the current primary end point differs from the original protocol submitted to ClinicalTrials.gov as well as described in the previous study.[33] The Bambino Gesù Ethics Research Committee approved the study, in accordance with the Declaration of Helsinki, and parents of the included patients gave their written informed consent to therapies and the tests performed for research purpose.

Fig. 1 Diagram of sample sets used in this study as well as the data analysis plan

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Anthropometrics

Anthropometric data were collected at baseline and after 12 months. Weight was measured by a conventional scale with a precision of 100 g and height was measured by a Harpenden stadiometer with a precision of 1 mm. Body mass index was expressed in kilograms per square meters (kg/m^2).

Laboratory parameters

Blood were collected at baseline and at month 12 after an overnight fasting and immediately processed to perform the analysis of alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl-transferase, total triglycerides and cholesterol, high-density lipoprotein, low-density lipoprotein, glucose, and uric acid by standard laboratory methods. Demographic information of the donor individuals are reported in Table 3.

Genotyping

Genotyping of 17 SNPs (rs174545, rs174546, rs174547, rs174548, rs174549, rs174550, rs174555, rs174556, rs174560, rs174561, rs174562, rs174568, rs1535, rs174574, rs174576, rs174577, and rs174581) across the *FADS* locus in the NAFLD children treated with DHA-CHO-VE were performed using with Sequenom MassARRAY iPLEX Gold assays (Sequenom, San Diego, CA). However, given the high LD level among these 17 FADS SNPs (pairwise LD $r^2 > 0.8$), we chose the FADS variant rs174576 as a representative SNP for all subsequent analyses. We chose this SNP also because it was associated with fatty acid denaturation pathway activity, whole-body fat oxidation, and DHA/EPA index in young individuals.[36,37]

Statistical analyses

The association between six *FADS1* SNPs and transcriptome data of donor liver tissues ($n = 55$) was performed using R package Matrix eQTL.[38] Each SNP in *FADS1* region was assessed for the association with liver transcriptome using multiple linear regression model, where age, gender, and ancestry were adjusted for the association as covariates. The SNP was coded using dominant genetic model, i.e., AA: 0, AB and BB: 1. We selected genes significantly ($p < 0.05$) associated with all the six *FADS1* SNPs for pathway analysis. Note that, given the small sample size, we used a liberal nominal p value (0.05) as a statistical significance cutoff for selecting genes for the pathway analysis so that critical pathways may not be missed. Significantly ($p < 0.05$) enriched pathways were identified using Ingenuity Pathway Analysis (IPA®, QIAGEN, Redwood City, www.qiagen.com/ingenuity).

Demographic, biochemical, and histological features of the NAFLD children ($n = 19$) were compared between groups using Wilcoxon rank-sum test for continuous variables. Multiple logistic regression model was used to test the association between *FADS1* genotype and the response to the treatment in terms of steatosis reduction. Genotypes were coded in dominant genetic model. Age, gender, and the genotypes of *PNPLA3* (rs738409)[39] and *TM6SF2* (rs58542926)[40] were adjusted for the association. Logistic regression was performed using the package PLINK 1.07.[41] All the other analyses were performed using R statistical packages (www.r-project.org).

RESULTS

Association between FADS1 mRNA and liver histology in children

We have characterized biochemical, and histologic features of donor liver tissue samples of 22 children. As summarized in Table 1, we found that 4 out of 22 individuals possess a ≥5% steatosis in their livers, and all these 4 patients were characterized as NASH or borderline NASH. The total fat content of all 22 livers is 0.02 ± 0.0088 mg fat per mg tissue, and total collagen content is $8.2 \pm 4.4\%$.

We aimed to understand the relationship between *FADS1* transcription and liver histology in children. We found that *FADS1* mRNA level was significantly and inversely associated with total
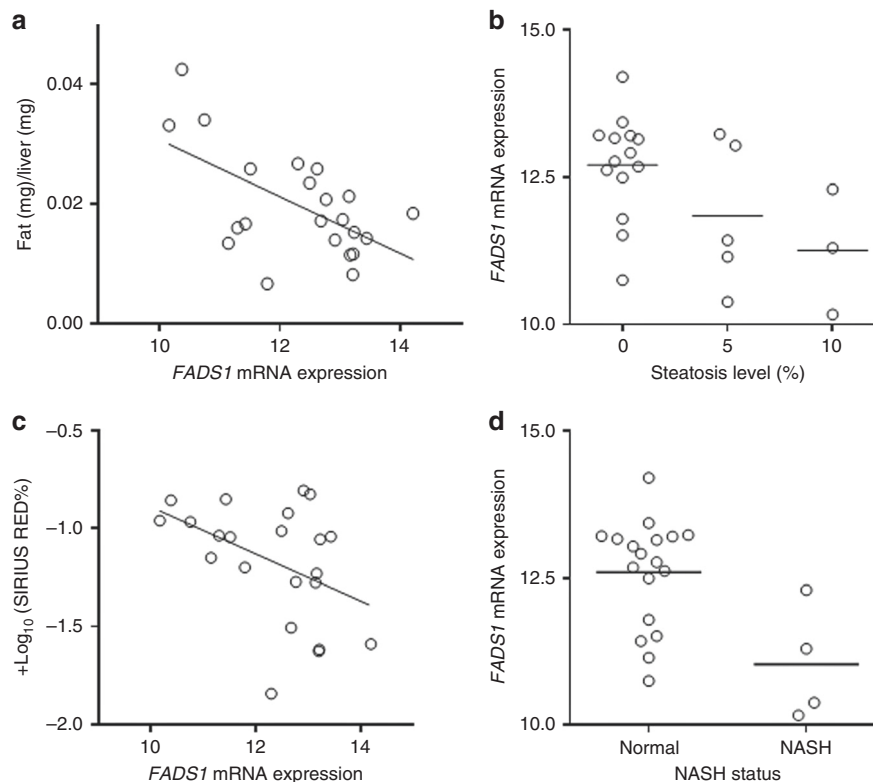

Fig. 2 Correlation between FADS1 mRNA level and total fat content (**a**), steatosis level (**b**), fibrosis level (**c**), and NASH status (**d**)

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hepatic fat content ($p = 0.004$), degree of steatosis ($p = 0.03$), fibrosis ($p = 0.05$), and NASH ($p = 0.008$) among pediatric livers (Fig. 2).

Pathway enrichment of genes associated with FADS1 polymorphism

Our previous study has demonstrated that the minor alleles of *FADS1* variants are associated with decreased hepatic *FADS1* transcription.[28] We hypothesized that these *FADS1* low-function alleles may lead to an altered transcriptomic profile where certain pathways involved in nonalcoholic fatty liver disease may be affected. In order to test this hypothesis, we performed a genome-wide analyses on the association between *FADS1* variant genotypes and mRNA expression. At a nominal significance level ($p < 0.05$), we identified 619 genes whose mRNA expression is significantly associated with *FADS1* genotype (Supplemental Table S1 (online)). We further conducted a pathway enrichment analysis on these genes using the IPA package. Our analysis revealed a significant enrichment for a number of pathways strongly related to NAFLD: hepatic fibrosis/hepatic stellate cell Activation) ($p = 0.01$), protein kinase A signaling ($p = 0.01$), and VDR/RXR activation ($p = 0.002$) as well as multiple interleukin signaling pathways ($p < 0.05$) (Supplemental Table S2 (online)). Given the association between the minor alleles of *FADS1* variants and the low *FADS1* expression and function, we sought to explore whether the liver transcriptome profile associated with these minor alleles is also more likely associated with NAFLD. We then performed IPA toxicity function pathway analysis specifically for the genes whose expression is positively associated with the *FADS1* minor alleles. As demonstrated in Table 2, critical toxicity function pathways, e.g., activation of liver fibrosis ($p = 0.01$), hepatic fibrosis ($p = 0.014$), and liver damage ($p = 0.02$), are particularly enriched. Also, hepatic fibrosis ($p = 0.006$) and increased liver damage ($p = 0.044$) are also top pathways significantly enriched among the toxicity Lists. Again, triacylglycerol biosynthesis ($p = 0.046$) and hepatic fibrosis/hepatic stellate cell activation ($p = 0.047$) are significantly enriched top canonical pathways (Table 2).

Association between FADS1 alleles and clinical response to n-3 lipid supplementation in children

We postulated that, while the low-function *FADS1* alleles may increase the susceptibility to NAFLD, supplementation n-3 lipids to NAFLD patients would lead to a better response in improving the liver histology. We therefore genotyped the *FADS1* variants among NAFLD children who previously received DHA-CHO-VE treatment. Given the high LD level among these variants in the study cohort ($r^2 > 0.8$), we selected rs174576 as a representative variant for the analyses. We then assessed the association between rs174576 minor allele and the improvement in liver histology after the DHA-CHO-VE treatment. We found that, as compared to the carriers of the major allele homozygotes, the minor allele carriers (heterozygotes and minor allele homozygotes) possessed a significant reduction in steatosis, fibrosis, ballooning, and NAFLD activity score ($p < 0.02$ for all tests; Table 3). As other NAFLD-associated genetic variants, i.e., *PNPLA3* and *TM6SF2*, may confound this association,[39,40] we further performed a multivariate analysis using a linear regression model by incorporating the genotype of these two genes. We found that the rs174576 minor allele remained to be significantly ($p < 0.05$) associated with more steatosis reduction after the DHA-CHO-VE treatment (Supplemental Table S3 (online)).

DISCUSSION

Our study demonstrated that reduced hepatic *FADS1* transcription is associated with increased risk to NAFLD in children. The low-function alleles of *FADS1* are particularly associated with alteration of molecular pathways involved in NAFLD. In addition, children with NAFLD who are carriers of *FADS1* low-function alleles are the best responders to n-3 lipid supplementation. Our findings together indicate an important role of FADS1 function in the pathogenesis of NAFLD in children. Moreover, *FADS1* genetic variants may be a useful pharmacogenetic marker capable of distinguishing the patients who are more likely to respond to n-3 lipid supplementation as a therapeutic strategy for NAFLD/NASH in children.

Our results provide new evidence supporting the association between the insufficiency of n-3 PUFA and NAFLD/NASH. While this association may be largely attributed to the imbalanced n-3/n-6 PUFA in the current Western diet, certain genetic perturbations of fatty acid desaturation process may exacerbate this issue. As compared to extensive dietary research, the relationship between genetic variability in fatty acid desaturation and NAFLD/NASH was less clear. This issue could be crucial in pediatric NAFLD since genetic factors may play a more significant role in disease susceptibility at the earlier stage of human development, as compared to the dietary and other environmental factors. Our previous studies have revealed that minor alleles of multiple variants across the *FADS1* locus lead to reduced hepatic transcription level of FADS1, which is further associated with reduced desaturation flux of long chain fatty acids but increased total intra-hepatic fat accumulation and susceptibility to liver steatosis.[28,29] Accordingly, our current study demonstrated that reduced FADS1 function in the liver is highly correlated with not only increased steatosis but also with fibrosis and NASH. This correlation may be mediated by the altered molecular pathways involved in lipid homeostasis, as well as in hepatic fibrogenesis, suggesting that insufficient FADS1 function can lead to both molecular and pathological changes in the liver even at the early developmental stages of the disease. Besides the central function of FADS1 in the liver, the gut–liver axis derangement may also be involved in the lipid homeostasis in the liver.[42] Gut microbiota was found to produce PUFA[43]; our recent study demonstrated that FADS1 genotype may also influence the oxidized derivatives of linoleic acid that are associated with metabolic syndrome and adverse lipid protein profiles in children.[44] It is thus possible that FADS1 alleles play a role in multiple systems in increasing the susceptibility to NAFLD.

Our findings also have implications in clinical management of NAFLD in children. Given the increased understanding of the key role of n-3 PUFA in NAFLD, supplementation of n-3 PUFA to NAFLD patients has long been a potential treatment and preventive option for NAFLD/NASH. Our study revealed a potential mechanism that leads to inter-patient variability in response to n-3 PUFA intake. Our findings suggest that *FADS1* variants can be a pharmacogenetic marker that is useful for selecting NAFLD patients who are most likely to respond to n-3 PUFA supplementation. Our study is consistent with previous studies where *FADS1* minor alleles are associated with significantly better tissue accumulation of n-3 PUFA after supplementation.[26,27] This hypothesis is also supported by the recent large cohort study on using n-3 PUFA supplementation in pregnant women to prevent asthma in their children where carrying *FADS1* minor alleles in the pregnant women was found to be predictive of better response in their children.[45] As a particular biomarker for n-3 PUFA treatment for NAFLD has not been established, our study warranted further validation of the potential predictive value of *FADS1* variants in the treatment or prevention of NAFLD in children and beyond, in particular via prospective clinical studies.

Our study was limited in several aspects. First, the small sample size leads to a low power in testing the relationship between *FADS1* alleles, gene expression, and NAFLD histology or drug responses in our sample sets. A post hoc power calculation indicated that the majority of our tests is potentially underpowered (data not shown). Therefore, our findings should be limited to the observations in our cohorts and the result should be

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Table 2. Top IPA enriched pathways for upregulated genes ($p < 0.05$)

Enriched pathways	p Value	Molecules	Functions	Disease or function annotation
Tox functions				
Liver enlargement, liver hepatomegaly	0.0038	STAT6, ESR2, MIF, GBA	Hepatomegaly	Hepatomegaly
Heart failure	0.0073	IL16	Heart failure with preserved left ventricular ejection fraction	Heart failure with preserved left ventricular ejection fraction
Nephrosis	0.0073	TTC21B	Nephronophthisis type 12	Nephronophthisis type 12
Liver fibrosis	0.0100	IL1A, LEPR, MIF	Activation	Activation of hepatic stellate cells
Cardiac necrosis/cell death	0.0145	TBX5	Apoptosis	Apoptosis of endocardial cells
Increased levels of CRP	0.0145	IL2	Localization	Increased localization of CRP
Liver fibrosis	0.0145	IL2	Hepatic fibrosis	Mild hepatic fibrosis
Liver fibrosis	0.0145	IL2	Hepatic fibrosis	Moderate hepatic fibrosis
Congenital heart anomaly	0.0201	PSEN2, TBX5	Failure of heart looping	Failure of heart looping
Liver damage	0.0213	STAT6, MIF	Damage	Damage of liver cells
Liver proliferation	0.0217	SKP2	Growth	Arrest in growth of hepatocytes
Renal inflammation, Renal nephritis	0.0217	IL16	IgA nephropathy	Primary IgA nephropathy
Kidney failure	0.0217	GBA	Progressive myoclonic epilepsy type 4 with renal failure	Progressive myoclonic epilepsy type 4 with renal failure
Renal damage	0.0328	IL2, ITGB6, LEPR, MIF	Damage	Damage of kidney
Cardiac necrosis/cell death	0.0359	PIN1	Cell death	Cell death of cardiac stem cells
Cardiac inflammation	0.0392	IL2, STAT6	Inflammation	Inflammation of heart
Renal atrophy	0.0460	AGA, OVOL1	Atrophy of kidney	Atrophy of kidney
Renal proliferation	0.0499	SKP2	Proliferation	Proliferation of renal tubular epithelial cells
Tox lists				
Hepatic fibrosis	0.0060	IL2, IL1A, CD40, SDC1		
Increases renal damage	0.0214	IL2, ITGB6, MIF		
Aryl hydrocarbon receptor signaling	0.0302	GSTO2, DHFR, IL1A, ESR2		
Increases liver damage	0.0437	IL2, CD40, MIF		
Canonical pathways				
Crosstalk between dendritic cells and natural killer cells	0.0043	IL2, ICAM3, CD40, TREM2		
dTMP de novo biosynthesis	0.0046	DHFR, DHFRL1		
Amyloid processing	0.0065	PSEN2, APH1A, CAPN2		
Granzyme A signaling	0.0091	HIST1H1E, H1FX		
Dendritic cell maturation	0.0095	IL1A, COL5A3, LEPR, CD40, TREM2		
Chondroitin Sulfate degradation (metazoa)	0.0110	GM2A, MGEA5		
Dermatan sulfate degradation (metazoa)	0.0110	GM2A, MGEA5		
Regulation of the epithelial–mesenchymal transition pathway	0.0112	WNT8A, PSEN2, CLDN3, APH1A, TWIST1		
Estrogen-mediated S-phase entry	0.0132	SKP2, ESR2		
Antiproliferative role of TOB in T cell signaling	0.0151	IL2, SKP2		
T helper cell differentiation	0.0158	IL2, STAT6, CD40		
CDP-diacylglycerol biosynthesis I	0.0166	AGPAT3, AGPAT1		
Aryl hydrocarbon receptor signaling	0.0224	GSTO2, DHFR, IL1A, ESR2		
Phosphatidylglycerol biosynthesis II (non-plastidic)	0.0240	AGPAT3, AGPAT1		
Altered T cell and B cell signaling in rheumatoid arthritis	0.0263	IL2, IL1A, CD40		
Communication between innate and adaptive immune cells	0.0282	IL2, IL1A, CD40		
Notch signaling	0.0309	PSEN2, APH1A		

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Table 2 continued

Enriched pathways	p Value	Molecules	Functions	Disease or function annotation
Inhibition of matrix metalloproteases	0.0347	MMP26, SDC1		
Granulocyte adhesion and diapedesis	0.0417	CLDN3, IL1A, MMP26, SDC1		
Hematopoiesis from pluripotent stem cells	0.0457	IL2, IL1A		
Triacylglycerol biosynthesis	0.0457	AGPAT3, AGPAT1		
Hepatic fibrosis/hepatic stellate cell activation	0.0479	IL1A, COL5A3, LEPR, CD40		

Table 3. Characteristics of Italian children with NAFLD who received DHA-CHO-VE treatment

Demographics and clinical features	FADS genotype (rs174576)					
	CC (n = 9)			CA+AA (n = 10)		
	Before	After	p Value	Before	After	p Value
Age						
Mean ± SD	13.00 ± 2.45	14.00 ± 2.45	0.32	13.81 ± 2.13	14.81 ± 2.13	0.21
Median (IQR)	13.00 (11.00–14.00)	14.00 (12.00–15.00)		13.00 (12.00–15.50)	14.00 (13.00–16.50)	
Male sex, n (%)	5 (55.56)	5 (55.56)	1	8 (80.00)	8 (80.00)	1
BMI (kg/m^2)						
Mean ± SD	26.47 ± 4.09	26.45 ± 4.28	0.89	29.29 ± 4.07	29.06 ± 4.64	0.96
Median (IQR)	28.89 (22.84–29.88)	27.79 (22.84–30.00)		30.12 (26.37–30.71)	28.44 (26.29–33.04)	
BMI (percentile)	95.7 ± 4.1	97.2 ± 2.4	0.35	94.1 ± 4.6	94.5 ± 5.9	0.21
WC (cm)						
Mean ± SD	84.54 (11.62)	84.69 (10.55)	0.97	87.56 (8.89)	92.09 (6.85)	0.19
Median (IQR)	89.00 (78.00–90.00)	84.00 (76.00–89.00)		89.50 (83.00–92.30)	94.00 (84.50–95.25)	
WC (percentile)	83.5 ± 18.9	82.3 ± 20.3	0.95	83 ± 12.8	86.8 ± 9.5	0.43
	Before	After	p Value	Before	After	p Value
Biochemical features						
Col Tot (mg/dL)	149.78 ± 17.38	149 ± 16.43	0.75	132.63 ± 29.16	142.51 ± 27.40	1
HDL (mg/dL)	48.33 ± 8.75	47.22 ± 8.59	0.82	47.8 ± 7.48	43.76 ± 5.00	1
LDL (mg/dL)	83.78 ± 26.54	80.11 ± 20.99	0.89	76.7 ± 25.84	94.33 ± 18.59	0.51
TG (mg/dL)	97.78 ± 55.80	112.22 ± 68.01	0.69	96.36 ± 52.66	94.33 ± 59.83	0.76
Uric acid (mg/dL)	5.33 ± 0.89	5.13 ± 0.87	0.47	5.22 ± 1.07	13.57 ± 24.92	0.87
AST (UI/mL)	33.44 ± 8.90	32.89 ± 7.45	0.69	34.72 ± 14.58	37.33 ± 24.17	0.67
ALT (UI/mL)	48.33 ± 22.94	45.67 ± 21.23	0.79	47.9 ± 32.70	32.41 ± 15.54	0.25
GGT (UI/mL)	16.67 ± 7.00	16.67 ± 6.94	1	17.54 ± 9.27	17.33 ± 8.98	0.56
Glycemia (mg/dL)	86.89 ± 7.49	84.22 ± 8.43	0.93	85.63 ± 7.21	79.33 ± 22.09	0.9
Histological features						
Fibrosis Index	1.78 ± 0.44	1.44 ± 0.52	0.17	1.81 ± 0.40	1.11 ± 0.33	**0.00057**
Steatosis Index	1.56 ± 0.73	1.33 ± 1.22	0.64	1.45 ± 0.93	1 ± 1.00	**0.011**
Ballooning	1.33 ± 0.50	0.78 ± 0.83	0.12	1.18 ± 0.87	0.56 ± 0.52	**0.0054**
Lobular inflammation	1.11 ± 0.60	0.89 ± 0.33	0.36	1.18 ± 0.40	1.11 ± 0.33	0.58
Portal inflammation	1.33 ± 0.50	1.22 ± 0.44	0.64	1.45 ± 0.52	1.22 ± 0.66	0.82
NAS	4 ± 1.32	3.38 ± 1.92	0.52	3.9 ± 1.64	2.66 ± 1.50	**0.0071**

Measurement is expressed in mean ± SD, Wilcoxon's rank-sum test is used for the comparison of before and after treatment
In bold the significant values for p <0.05
SD standard deviation, *BMI* body mass index, *IQR* interquartile range, *WC* waist circumference, *Col Tot* total cholesterol, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *TG* triglycerides, *AST* aspartate aminotransferase, *ALT* alanine transaminase, *GGT* gamma-glutamyl transferase, *NAS* NAFLD activity score

further validated in larger-scale sample sets. However, we have previously reported a strong association between FADS1 minor alleles and decreased hepatic FADS1 gene expression, whereas increased total liver fat content.[28] Therefore, the findings in this study indeed validated at least in part our previous observations. Moreover, noteworthy, in our study the consistent association between FADS1 and clinical features as well as drug responses, albeit weak, collectively generated a new interesting hypothesis

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worth further testing in the future. Second, the age group of the donor cohort (6.1 ± 4.4 years) is different from that of the clinical study (13.00 ± 2.45 years). Therefore, the biological effect observed in the former population may not reflect that of the latter. Third, the NAFLD patients in our study were treated with a combined choline, DHA, and VE rather than n-3 PUFA as a single agent. The result thus could be confounded by the pharmacological effects of other compounds. It is thus necessary to design prospective clinical studies in a large population to specifically investigate the relationship between *FADS1* alleles and clinical outcomes of n-3 PUFA supplementation. Nevertheless, our study generated interesting directions and hypotheses for understanding the pathogenesis and developing therapeutic/preventive strategies for pediatric NAFLD.

ADDITIONAL INFORMATION
The online version of this article (https://doi.org/10.1038/s41390-018-0132-7) contains supplementary material, which is available to authorized users.

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REFERENCES
1. Mencin, A. A., Loomba, R. & Lavine, J. E. Caring for children with NAFLD and navigating their care into adulthood. *Nat. Rev. Gastroenterol. Hepatol.* **12**, 617–28 (2015).
2. Feldstein, A. E., Charatcharoenwitthaya, P., Treeprasertsuk, S., Benson, J. T., Enders, F. B. & Angulo, P. The natural history of non-alcoholic fatty liver disease in children: a follow-up study for up to 20 years. *Gut* **58**, 1538–44 (2009).
3. Alisi, A., Manco, M., Vania, A. & Nobili, V. Pediatric nonalcoholic fatty liver disease in 2009. *J. Pediatr.* **155**, 469–74 (2009).
4. Bonci, E., Chiesa, C., Versacci, P., Anania, C., Silvestri, L. & Pacifico, L. Association of nonalcoholic fatty liver disease with subclinical cardiovascular changes: a systematic review and meta-analysis. *Biomed. Res. Int.* **2015**, 213737 (2015).
5. Nobili, V., Alkhouri, N. & Alisi, A. et al. Nonalcoholic fatty liver disease: a challenge for pediatricians. *JAMA Pediatr.* **169**, 170–6 (2015).
6. Masterton, G. S., Plevris, J. N. & Hayes, P. C. Review article: omega-3 fatty acids - a promising novel therapy for non-alcoholic fatty liver disease. *Aliment. Pharmacol. Ther.* **31**, 679–92 (2010).
7. Di Minno, M. N., Russolillo, A., Lupoli, R., Ambrosino, P., Di Minno, A. & Tarantino, G. Omega-3 fatty acids for the treatment of non-alcoholic fatty liver disease. *World J. Gastroenterol.* **18**, 5839–47 (2012).
8. Parker, H. M., Johnson, N. A., Burdon, C. A., Cohn, J. S., O'Connor, H. T. & George, J. Omega-3 supplementation and non-alcoholic fatty liver disease: a systematic review and meta-analysis. *J. Hepatol.* **56**, 944–51 (2012).
9. Pacifico, L., Giansanti, S., Gallozzi, A. & Chiesa, C. Long chain omega-3 polyunsaturated fatty acids in pediatric metabolic syndrome. *Mini Rev. Med. Chem.* **14**, 791–804 (2014).
10. Zelber-Sagi, S., Nitzan-Kaluski, D. & Goldsmith, R. et al. Long term nutritional intake and the risk for non-alcoholic fatty liver disease (NAFLD): a population based study. *J. Hepatol.* **47**, 711–7 (2007).
11. Shapiro, H., Tehilla, M., Attal-Singer, J., Bruck, R., Luzzatti, R. & Singer, P. The therapeutic potential of long-chain omega-3 fatty acids in nonalcoholic fatty liver disease. *Clin. Nutr.* **30**, 6–19 (2011).
12. St-Jules, D. E., Watters, C. A. & Brunt, E. M. et al. Estimation of fish and omega-3 fatty acid intake in pediatric nonalcoholic fatty liver disease. *J. Pediatr. Gastroenterol. Nutr.* **57**, 627–33 (2013).
13. Papandreou, D., Karabouta, Z., Pantoleon, A. & Rousso, I. Investigation of anthropometric, biochemical and dietary parameters of obese children with and without non-alcoholic fatty liver disease. *Appetite* **59**, 939–44 (2012).
14. Puri, P., Baillie, R. A. & Wiest, M. M. et al. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* **46**, 1081–90 (2007).
15. Elizondo, A., Araya, J. & Rodrigo, R. et al. Polyunsaturated fatty acid pattern in liver and erythrocyte phospholipids from obese patients. *Obesity (Silver Spring)* **15**, 24–31 (2007).
16. Zhu, Q. Q., Lou, D. J. & Si, X. W. et al. [Serum omega-3 polyunsaturated fatty acid and insulin resistance in type 2 diabetes mellitus and non-alcoholic fatty liver disease]. *Zhonghua Nei Ke Za Zhi* **49**, 305–8 (2010).
17. Kishino, T., Ohnishi, H. & Ohtsuka, K. et al. Low concentrations of serum n-3 polyunsaturated fatty acids in non-alcoholic fatty liver disease patients with liver injury. *Clin. Chem. Lab. Med.* **49**, 159–62 (2011).
18. Zheng, J. S., Xu, A., Huang, T., Yu, X. & Li, D. Low docosahexaenoic acid content in plasma phospholipids is associated with increased non-alcoholic fatty liver disease in China. *Lipids* **47**, 549–56 (2012).
19. Petit, J. M., Guiu, B. & Duvillard, L. et al. Increased erythrocytes n-3 and n-6 polyunsaturated fatty acids is significantly associated with a lower prevalence of steatosis in patients with type 2 diabetes. *Clin. Nutr.* **31**, 520–5 (2012).
20. Nobili, V., Carpino, G. & Alisi, A. et al. Role of docosahexaenoic acid treatment in improving liver histology in pediatric nonalcoholic fatty liver disease. *PLoS ONE* **9**, e88005 (2014).
21. Scorletti, E., Bhatia, L. & McCormick, K. G. et al. Effects of purified eicosapentaenoic and docosahexaenoic acids in non-alcoholic fatty liver disease: results from the *WELCOME study. *Hepatology* **60**, 1211–21 (2014).
22. Sanyal, A. J., Abdelmalek, M. F., Suzuki, A., Cummings, O. W. & Chojkier, M., EPE-A Study Group. No significant effects of ethyl-eicosapentanoic acid on histologic features of nonalcoholic steatohepatitis in a phase 2 trial. *Gastroenterology* **147**, 377–84 (2014). e1.
23. Argo, C. K., Patrie, J. T. & Lackner, C. et al. Effects of n-3 fish oil on metabolic and histological parameters in NASH: a double-blind, randomized, placebo-controlled trial. *J. Hepatol.* **62**, 190–7 (2015).
24. Dasarathy, S., Dasarathy, J. & Khiyami, A. et al. Double-blind randomized placebo-controlled clinical trial of omega 3 fatty acids for the treatment of diabetic patients with nonalcoholic steatohepatitis. *J. Clin. Gastroenterol.* **49**, 137–44 (2015).
25. Nording, M. L., Yang, J. & Georgi, K. et al. Individual variation in lipidomic profiles of healthy subjects in response to omega-3 Fatty acids. *PLoS ONE* **8**, e76575 (2013).
26. Roke, K. & Mutch, D. M. The role of FADS1/2 polymorphisms on cardiometabolic markers and fatty acid profiles in young adults consuming fish oil supplements. *Nutrients* **6**, 2290–304 (2014).
27. Al-Hilal, M., Alsaleh, A. & Maniou, Z. et al. Genetic variation at the FADS1-FADS2 gene locus influences delta-5 desaturase activity and LC-PUFA proportions after fish oil supplement. *J. Lipid Res.* **54**, 542–51 (2013).
28. Wang, L., Athinarayanan, S., Jiang, G., Chalasani, N., Zhang, M. & Liu, W. Fatty acid desaturase 1 gene polymorphisms control human hepatic lipid composition. *Hepatology* **61**, 119–28 (2015).
29. Mirkov, S., Myers, J. L., Ramirez, J. & Liu, W. SNPs affecting serum metabolomic traits may regulate gene transcription and lipid accumulation in the liver. *Metabolism* **61**, 1523–7 (2012).
30. Ameur, A., Enroth, S. & Johansson, A. et al. Genetic adaptation of fatty-acid metabolism: a human-specific haplotype increasing the biosynthesis of long-chain omega-3 and omega-6 fatty acids. *Am. J. Hum. Genet.* **90**, 809–20 (2012).
31. Lemaitre, R. N., Tanaka, T. & Tang, W. et al. Genetic loci associated with plasma phospholipid n-3 fatty acids: a meta-analysis of genome-wide association studies from the CHARGE Consortium. *PLoS Genet.* **7**, e1002193 (2011).
32. Tanaka, T., Shen, J. & Abecasis, G. R. et al. Genome-wide association study of plasma polyunsaturated fatty acids in the InCHIANTI Study. *PLoS Genet* **5**, e1000338 (2009).
33. Zohrer., E. et al. Efficacy of docosahexaenoic acid-choline-vitamin E in paediatric NASH: a randomized controlled clinical trial. *Appl. Physiol. Nutr. Metab.* **42**, 948–954 (2017).
34. Li, M., Song, J., Mirkov, S., Xiao, S. Y., Hart, J. & Liu, W. Comparing morphometric, biochemical, and visual measurements of macrovesicular steatosis of liver. *Hum. Pathol.* **42**, 356–60 (2011).
35. Kleiner, D. E., Brunt, E. M., Van Natta, M. & Behling, C. et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **41**, 1313–21 (2005).
36. Abdelmagid, S. A., Clarke, S. E. & Roke, K. et al. Ethnicity, sex, FADS genetic variation, and hormonal contraceptive use influence delta-5- and delta-6-desaturase indices and plasma docosahexaenoic acid concentration in young Canadian adults: a cross-sectional study. *Nutr. Metab.* **12**, 14 (2015).
37. Roke, K., Jannas-Vela, S., Spriet, L. L. & Mutch, D. M. FADS2 genotype influences whole-body resting fat oxidation in young adult men. *Appl. Physiol. Nutr. Metab.* **41**, 791–4 (2016).
38. Shabalin, A. A. Matrix eQTL: ultra fast eQTL analysis via large matrix operations. *Bioinformatics* **28**, 1353–8 (2012).

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39. Romeo, S., Kozlitina, J. & Xing, C. et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat. Genet.* **40**, 1461–5 (2008).

40. Kozlitina, J., Smagris, E. & Stender, S. et al. Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease. *Nat. Genet.* **46**, 352–6 (2014).

41. Purcell, S., Neale, B. & Todd-Brown, K. et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81**, 559–75 (2007).

42. PoetaM., PierriL. & VajroP. Gut-liver axis derangement in non-alcoholic fatty liver disease. *Children* **4**, E66 (2017).

43. Druart, C., Bindels, L. B. & Schmaltz, R. et al. Ability of the gut microbiota to produce PUFA-derived bacterial metabolites: proof of concept in germ-free versus conventionalized mice. *Mol. Nutr. Food Res* **59**, 1603–13 (2015).

44. Trico, D., et al. Oxidized derivatives of linoleic acid in pediatric metabolic syndrome: is their pathogenic role modulated by the genetic background and the gut microbiota? Antioxid. Redox Signal. 2017, Apr 7. https://doi.org/10.1089/ars.2017.7049.

45. Bisgaard, H., Stokholm, J. & Chawes, B. L. et al. Fish oil-derived fatty acids in pregnancy and wheeze and asthma in offspring. *N. Engl. J. Med.* **375**, 2530–9 (2016).