Scandinavian Journal of Clinical and Laboratory Investigation

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/iclb20

Reduced gut microbial diversity in familial hypercholesterolemia with no effect of omega-3 polyunsaturated fatty acids intervention – a pilot trial

Christopher Storm-Larsen, Liv Nesse Hande, Martin Kummen, Hilde Thunhaug, Beate Vestad, Simen Hyll Hansen, Anders Hovland, Marius Trøseid, Knut Tore Lappegård & Johannes R. Hov

To cite this article: Christopher Storm-Larsen, Liv Nesse Hande, Martin Kummen, Hilde Thunhaug, Beate Vestad, Simen Hyll Hansen, Anders Hovland, Marius Trøseid, Knut Tore Lappegård & Johannes R. Hov (2022) Reduced gut microbial diversity in familial hypercholesterolemia with no effect of omega-3 polyunsaturated fatty acids intervention – a pilot trial, Scandinavian Journal of Clinical and Laboratory Investigation, 82:5, 363-370, DOI: 10.1080/00365513.2022.2102540

To link to this article: https://doi.org/10.1080/00365513.2022.2102540

© 2022 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

View supplementary material

Published online: 01 Aug 2022.

Submit your article to this journal

Article views: 638

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at https://www.tandfonline.com/action/journalInformation?journalCode=iclb20

SCANDINAVIAN JOURNAL OF CLINICAL AND LABORATORY INVESTIGATION
2022, VOL. 82, NO. 5, 363–370
https://doi.org/10.1080/00365513.2022.2102540

ORIGINAL ARTICLE

OPEN ACCESS

Reduced gut microbial diversity in familial hypercholesterolemia with no effect of omega-3 polyunsaturated fatty acids intervention – a pilot trial

Christopher Storm-Larsen[a,b,c], Liv Nesse Hande[d,e], Martin Kummen[a,b,c,f], Hilde Thunhaug[d], Beate Vestad[a,c], Simen Hyll Hansen[a,b,c], Anders Hovland[d,g], Marius Trøseid[a,c,h], Knut Tore Lappegård[d,g] and Johannes R. Hov[a,b,c,i]

[a]Institute of Clinical Medicine, University of Oslo, Oslo, Norway; [b]Department of Transplantation Medicine, Division of Surgery, Inflammatory diseases and Transplantation, Norwegian PSC Research Center, Oslo University Hospital, Oslo, Norway; [c]Division of Surgery, Inflammatory diseases and Transplantation, Research Institute of Internal Medicine, Oslo University Hospital, Oslo, Norway; [d]Division of Internal Medicine, Nordland Hospital, Bodø, Norway; [e]Faculty of Health Sciences, University of Tromsø, Tromsø, Norway; [f]Department of Oncology, Oslo University Hospital, Oslo, Norway; [g]Institute of Clinical Medicine, University of Tromsø, Tromsø, Norway; [h]Section of Clinical Immunology and Infectious diseases, Division of Cancer Medicine, Surgery and Transplantation, Oslo University Hospital, Oslo, Norway; [i]Department of Transplantation Medicine, Section of Gastroenterology, Oslo University Hospital, Oslo, Norway

ABSTRACT
Individuals with familial hypercholesterolemia (FH) undergo an aggressive treatment with cholesterol-lowering drugs to prevent coronary heart disease. Recent evidence suggests an interplay between the gut microbiota, blood lipid levels and lipid-lowering drugs, but this has yet to be studied in individuals with FH. The objective of the study was to characterize the gut microbiota of individuals with familial hypercholesterolemia and examine if effects of omega-3 polyunsaturated fatty acids (PUFAs) on blood lipids act through modification of the gut microbiome. The gut microbiota composition of individuals with FH ($N = 21$) and healthy controls ($N = 144$) was analyzed by extracting DNA from stool samples and sequencing of the V3–V4 region of the 16S rRNA gene. A subgroup ($n = 15$) of the participants received omega-3 polyunsaturated fatty acids (PUFAs) supplementation or placebo in a crossover manner, and the effect of PUFAs on the gut microbiota was also investigated. Individuals with FH had a different gut microbiota composition compared to healthy controls, characterized by reduced richness ($p = .001$) and reduction of several genera belonging to Clostridia and Coriobacteriia. Patients using ezetimibe in addition to statins appeared to have lower richness compared to those only using statins ($p = .01$). Intervention with omega-3 PUFAs had negligible impact on the microbiota composition. Positive effects on blood lipids after intervention with omega-3 PUFA were not associated with baseline gut microbiota composition or gut microbial changes during treatment. Further, patients with FH have an altered gut microbiota compared to healthy controls, possibly driven by the use of ezetimibe.

ARTICLE HISTORY
Received 9 January 2022
Revised 6 June 2022
Accepted 19 June 2022

KEYWORDS
Gut microbiota; dysbiosis; familial hypercholesterolemia; fatty acids; omega-3; triglycerides; statin; ezetimibe; dyslipidemia

Introduction

Individuals with familial hypercholesterolemia (FH) have a high risk of early cardiovascular disease (CVD), premature mortality and increased morbidity compared to the general population [1]. The most common cause of FH is a mutation in the gene encoding for the low-density lipoprotein (LDL)-receptor protein, causing reduced clearance of LDL-c from the circulation [2]. Lowering of LDL-c is crucial to prevent premature morbidity and mortality, but FH individuals have a high residual risk of CVD despite effective treatment with statins [3]. Thus, finding efficient means to reduce this residual risk might improve patient outcome.

Recent studies have reported statistical associations between the gut microbiota composition and lipid levels in the blood, as well as clinical outcomes related to CVD [4,5]. Fu et al. correlated gut microbiota composition and levels of blood lipids in ∼900 individuals, demonstrating that the gut microbiome would explain 6% of the variation in triglycerides, but little effect on low-density lipoproteins or total cholesterol [6]. Furthermore, in a placebo-controlled randomized study involving 114 subjects with elevated cholesterol levels, Jones et al. reported a decrease in total cholesterol levels by 9% after daily consumption of yogurt containing the probiotic bacteria *Lactobacillus reuteri* [7]. However, little is known about the gut microbiota of individuals with FH and how it may influence the regulation of lipid levels.

There is also an evolving interest in pharmacomicrobiomics, that is, how drugs act on the microbiota and vice

CONTACT Johannes R. Hov j.e.r.hov@medisin.uio.no Department of Transplantation Medicine, Oslo University Hospital Rikshospitalet, Postboks 4950, Nydalen, Oslo, 0424, Norway

Supplemental data for this article is available online at https://doi.org/10.1080/00365513.2022.2102540.

© 2022 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (http://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.

versa. Some common drugs may even act, at least in part, by targeting the microbiota [8]. Statin therapy ranks second among drug classes associated with microbiota composition in population based studies, and has been associated with reduced alpha diversity and increased anti-inflammatory bacteria in cross-sectional studies [4,9]. In a recent randomized controlled trial with rosuvastatin, we found a reduction in inferred microbial genes related to metabolism and transport of pro-atherogenic metabolites in the Trimethylamin-N-Oxide (TMAO)-pathway, but limited effect on the gut microbiota composition [10]. Further, interventional trials have demonstrated enhanced lipid-lowering effect of statins in individuals with increased gut microbial diversity and higher abundance of Firmicutes, *Lactobacillus* and *Bifidobacterium* [11,12]. Similarly, regarding omega-3 PUFAs, data from mouse studies suggest that supplementation leads to gut microbiota alterations [13,14]. In humans, a recent randomized controlled trial demonstrated a reversible increase in several short-chain fatty acid-producing bacteria after eight weeks supplementation with omega-3 PUFAs in 22 healthy volunteers with normal cholesterol levels [15]. These findings warrant further studies on individuals with hyperlipidemia.

Therefore, in this study, we aimed to (1) characterize the gut microbiota of individuals with familial hypercholesterolemia, and (2) investigate if supplementation of omega-3 polyunsaturated fatty acids would alter the gut microbial composition in these individuals.

Patients and methods

Trial design and participants

We investigated a subgroup from a previously published interventional study. For a complete methodology, we refer to the original study [16]. In brief, individuals with genetically verified heterozygote FH aged 18–75 and using statins >12 months were recruited from Nordland Hospital's lipid clinic (Bodø, Norway). All individuals had mutations in the gene for the LDL-receptor. The study was designed as a double-blind, placebo-controlled crossover study. In random order, all individuals were treated for 3 months with omega-3 PUFA supplement (2 g b.i.d) and 3 months placebo (olive oil, 2 g b.i.d), separated by a 3-month washout period (Figure 1). The marine omega-3 PUFA supplement was a 1000 mg capsule consisting of 460 mg of eicosapentaenoic acid (EPA) and 380 mg docosahexanoic acid (BASF, Lysaker, Norway). Blood and stool samples were collected at baseline and after each study period (3, 6 and 9 months). According to the returned medication in the original study, compliance was around 90% in both treatment groups.

In addition, we designed a case-control analysis, comparing the gut microbiota of individuals with FH and healthy controls. The baseline sample was included for all individuals with FH except for one sample with very low microbial genetic read count, for which the baseline after the washout was included. To serve as control material, data from $n = 144$ healthy controls from a previously published study were included [17]. The controls were recruited from the Norwegian Bone Marrow Donor Registry and all sample processing and sequencing steps were identical to the process with the FH samples.

Endpoints

The primary outcome was change in the gut microbiota composition after treatment with omega-3 PUFAs, described as change in the relative abundance of any taxa from phylum to genus level or change in alpha or beta diversity. Secondary outcomes were relations between blood lipids and changes in gut microbiota composition, and exploratory outcomes were effects of omega-3 PUFAs on the inferred metagenome and differences of gut microbiota composition between individuals with familial hypercholesterolemia and healthy controls.

Ethics

The study was conducted according to the guidelines of the declaration of Helsinki, and was registered at ClinicalTrials.gov (identifier NCT01813006, 18/03/2013), approved by the Regional Ethics Committee of Northern Norway (REK2011/899) and the Norwegian Medicines Agency (EUDRACTNR 2012- 000505-68). Informed written consent was obtained from all individuals included in the study.

Gut microbiota

Stool samples were collected by the patients at home in tubes with a preservative liquid (Stool Collection Tubes with Stool DNA Stabilizer (Stratec Molecular GmbH, Berlin, Germany), shipped to the central study laboratory and

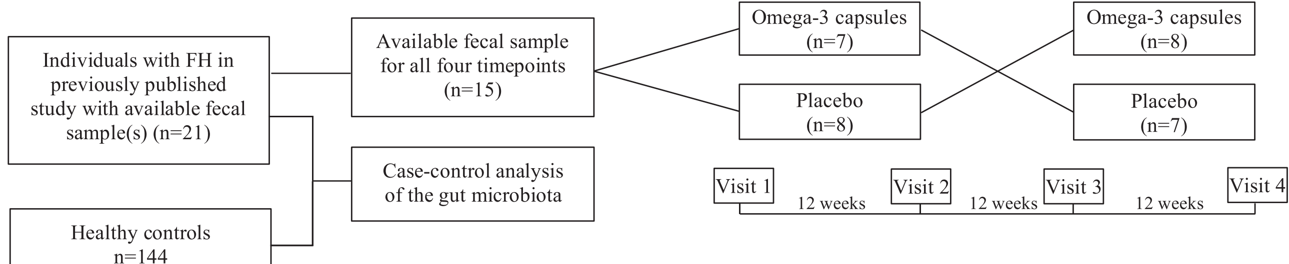

Figure 1. Flowchart of the study design.

stored at −80 °C. Microbial DNA was extracted by using the PSP Spin Stool Plus DNA Kit (Stratec Molecular GmbH) and libraries were generated from PCR amplicons of the V3-V4 region of the 16S rRNA gene [18]. Sequencing was performed at the Norwegian Sequencing Centre (Oslo, Norway) on Illumina MiSeq using the v3 kit (San Diego, CA, USA), set at 300 bp paired-end reads. The sequenced reads were filtered, demultiplexed, trimmed and merged as previously described [19]. Further processing was performed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) version 2018.8 [20], where sequences were taxonomically classified using a naïve bayes classifier trained on a 99% sequence similarity version of the SILVA database version 132 [21]. Reads were rarefied to an OTU count of 6145 per sample for the case-control analysis and 3678 reads per sample for the analysis of the intervention, and all analyses were performed on these rarefied datasets. The PICRUSt2 plugin for QIIME2 2018.8 was used to predict the gut microbial functional profiles based on the 16S rRNA sequencing data [22].

Lipid analysis

Fasting blood samples were collected at each visit and analyzed consequently. Total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol were analyzed on an ADVIA1800 system (Siemens Medical Solutions Diagnostics, Japan) using reagents from Siemens Healthcare Diagnostics Ltd.

Power estimates

The study was designed as a follow-up exploratory pilot study of the original intervention trial, hence *a priori* power analysis was not performed when designing the microbiota profiling experiment. However, a sensitivity power analysis was applied to determine the minimal detectable effect (MDE). We had 80% power to detect a Cohen's D effect size of 0.83, corresponding to $a \geq 15\%$ change in microbial richness [23]. This would correspond to an approximate power of detecting ≥20% change in taxa with arc-sin square root transformed relative abundance ≥0.2 or ≥50% change in taxa with relative abundance ≥0.01, correction for multiple comparisons not taken into account.

Statistical analysis

All continuous variables are presented as median and interquartile range (IQR) if otherwise not stated and non-parametric statistics were applied. The statistical approach included: (1) Case-control analysis of baseline FH samples compared with healthy individuals, using Mann–Whitney *U* test on all phylogenetic levels from phylum to genus, corrected with Benjamini–Hochberg false discovery rate (FDR) within each phylogenetic level. (2) We analyzed the treatment effect in the crossover study by grouping the data into the four timepoints (before/after intervention with placebo/omega-3), irrespective of treatment order. Then, Wilcoxon paired test was applied between the change from corresponding baseline to after intervention with placebo versus the change after treatment with omega-3. All values, except taxa being significantly altered in the initial cross-sectional analysis, were corrected for multiple testing using FDR within each phylogenetic level.

Statistical analyses were performed in SPSS Statistics v27.0 (IBM Corporation, Armonk, New York, USA). Graphical presentations were made using Prism V9.0d software (GraphPad, San Diego, California, USA).

Results

Baseline characteristics

Fifteen patients with familial hypercholesterolemia (FH) were included for analysis after intervention with omega-3 PUFA and placebo. In addition, six patients with FH and 144 healthy controls (HC) were included for case-control analysis at baseline. One sample after the washout period were excluded due to sequencing failure (baseline sample for placebo). Hence, fourteen patients with FH were available for analysis after intervention, as well as 21 patients with FH and 144 healthy controls for case-control analysis at baseline (Table 1, Figure 1).

Lower intraindividual diversity in patients with familial hypercholesterolemia

Individuals with FH had lower alpha diversity compared to healthy controls, as measured by a lower richness of the gut microbiota ($p = .001$, Figure 2(A)), while the combined richness/evenness measure Shannon index was numerically reduced ($p = .10$, data not shown). Individuals with FH taking ezetimibe in addition to statins ($n = 7$) had significantly lower richness compared to individuals with FH only taking

Table 1. Baseline characteristics.

	FH ($n = 21$)	HC ($n = 144$)	*p*-Value
Age	46 (38–54)	46 (44–47)	.86
Gender (female), *n* (%)	12 (57%)	93 (65%)	.68
BMI (kg/m^2)	26.9 (24.5–29.3)	26.4 (25.7–27.0)	.47
Smoking, yes	3 (14%)	13 (9%)	.50
Established CVD, yes	5 (24%)	–	–
Triglycerides, mmol/L	0.9 ± 0.5	–	–
LDL-c, mmol/L	3.0 ± 0.5	–	–
HDL-c, mmol/L	1.39 ± 0.56	–	–
Total cholesterol, mmol/L	4.79 ± 0.81	–	–
Drugs			
PPI, *n* (%)	0	10 (7%)	–
Beta blocker, *n* (%)	3 (14%)	–	–
Antidiabetic, *n* (%)	(0)	–	–
Antihypertensives[a], *n* (%)	3 (14%)	8 (5%)	–
Statin, *n* (%)	21 (100%)	–	–
Atorvastatin, *n* (%)	14 (67%)	–	–
Simvastatin, *n* (%)	1 (5%)	–	–
Rosuvastatin, *n* (%)	5 (24%)	–	
Statin + ezetimibe, *n* (%)	7 (33%)	–	–

BMI: body mass index; CVD: cardiovascular disease; PPI: proton-pump inhibitors; LDL-c: low-density lipoprotein cholesterol; HDL-c: high-density lipoprotein cholesterol. Data are presented as mean ± standard deviation. The *p*-value was calculated using standard student *T*-test. [a]Angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers, calcium channel blockers and diuretics.

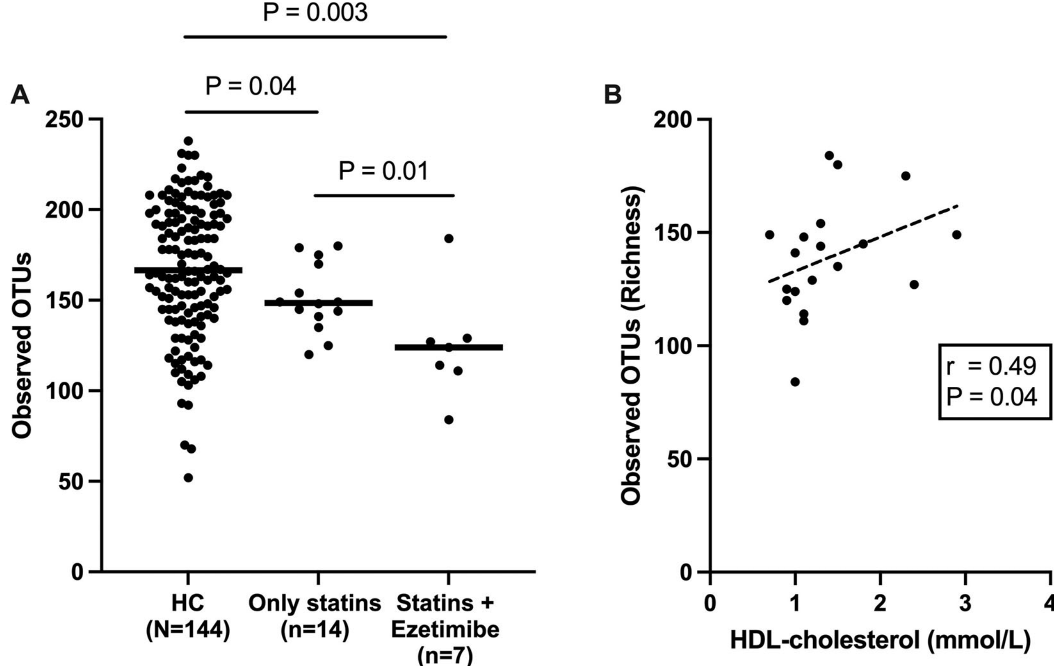

Figure 2. Baseline differences in microbial richness. (A) Individuals with familial hypercholesterolemia (FH) had lower microbial richness (observed OTUs) compared to healthy controls and this effect was more prominent in individuals with FH taking ezetimibe in addition to statins. (B) Microbial richness in individuals with FH was positively correlated to serum levels of HDL-cholesterol. OTUs: observed operational units; HC: healthy controls; FH: familial hypercholesterolemia; HDL: high-density lipoprotein. Mann–Whitney non-parametric *U*-test and Spearman's rank-order correlation.

statins ($n = 14$, $p = .01$, Figure 2(A)). When only analyzing individuals with FH not taking ezetimibe, we still observed a significant lower richness compared to healthy controls ($p = .04$, Figure 2(A)). In patients with FH, the gut microbial richness was positively correlated to serum levels of HDL-cholesterol ($r = 0.49$, $p = .04$, Figure 2(B)), but not with LDL-c (r=-0.21, $p = .38$, Supplementary Table 1). The global microbial composition (beta diversity) of patients with FH differed from HC (Supplementary Figure 1(A), Pseudo-F 2.77, $p = .002$). Moreover, individuals with FH taking statins and ezetimibe clustered differently compared to patients with FH only taking statins (Supplementary Figure 1(B), Pseudo-F 3.32, $p = .003$).

Lower actinobacteria in patients with FH compared to HC

Considering all taxonomic levels, fourteen taxa were different between FH and healthy controls at $Q_{FDR}<0.2$, the majority being less abundant in patients with FH compared to healthy controls (Figure 3). This includes lower relative abundance of the phylum Actinobacteria (0.8 vs. 2.1%, Q_{FDR}=0.03) as well as lower abundance of the family *Veillonellaceae* (<0.1 vs. 2.0%, Q_{FDR}=0.09). No differences were observed in the Firmicutes: Bacteroidetes-ratio (data not shown). Except for a negative correlation between the phylum Tenericutes, class Mollicutes, and serum triglycerides (r=-0.53, $p = .02$), no significant correlations between altered microbial taxa in individuals with FH and serum levels of lipids were identified (Supplementary Table 1). No significant differences were observed in the inferred microbial gene content after FDR correction (Supplementary Table 2).

Subanalysis with matched controls

For validation of the main findings from the comparison between FH-patients and healthy controls, we created a subgroup of gender- and age-matched controls from the same dataset, using a 1:3 matching. Using this subgroup of control, individuals with FH still had lower richness compared to the matched controls ($p = .01$), lower relative abundance of Actinobacteria ($p = .01$) as well as lower relative abundance of the family *Veillonellaceae* ($p = .002$).

No change in microbial taxa after intervention with omega-3 PUFA

As previously reported in the original study, we found a significant decrease in LDL-c, total cholesterol and triglycerides after intervention with omega-3 PUFAs, but no changes in BMI (Table 2) [16]. When analyzing the treatment effect of omega-3 PUFAs on the gut microbiota, no effect was observed on alpha diversity ($p = .26$ for Shannon index, $p = .9$ for observed OTUs/richness, Figure 4(A)) compared to placebo. Further, the intervention with omega-3 PUFAs was not associated with significant changes of any taxa at any taxonomic level from phylum to genus level, when correcting for multiple testing within each taxonomic level and using a significance level of

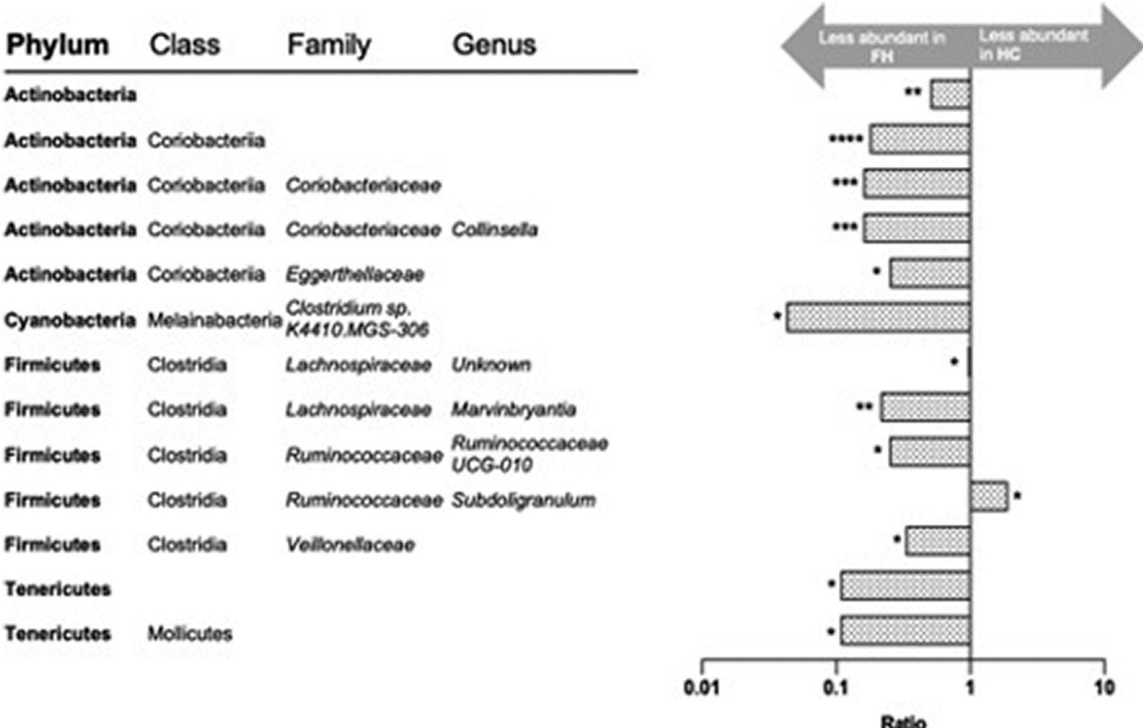

Figure 3. Differences in microbial taxa between patients with familial hypercholesterolemia (FH) and healthy controls (HC) at different taxonomic levels, illustrated by the ratio of the mean relative abundance between FH and HC on a logarithmic scale. Mann–Whitney *U*-test with correction for multiple testing using Benjamini–Hochberg false discovery rate (Q_{FDR}). *$Q_{FDR}<0.20$ **$Q_{FDR}<0.05$ ***$Q_{FDR}<0.01$ ****$Q_{FDR}<0.001$.

Table 2. Effects of omega-3 fatty acids intervention on clinical parameters in the sub-cohort included in the current study, data are previous published in the larger cohort [16].

	Baseline	After omega-3 fatty acids	*p*-Value
BMI (kg/m^2)	27.6 ± 5.0	27.6 ± 5.2	.16
Total cholesterol, mmol/L	4.9 ± 0.8	4.6 ± 0.7	.03
LDL-C, mmol/L	3.1 ± 0.7	2.7 ± 0.7	.01
HDL-C, mmol/L	1.4 ± 0.5	1.4 ± 0.4	.72
Triglycerides, mmol/L	1.1 ± 0.7	0.8 ± 0.4	.003

BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides. Data are presented as mean ± standard deviation. The *p* value was calculated as the difference between baseline values and after omega-3 fatty acids intervention period using Wilcoxon Matched Pairs Signed Rank test.

$p<.05$ and $Q_{FDR}<0.20$ (Supplementary Table 3). Watson et al. reported increased butyrate-producing bacteria after intervention with omega-3 PUFA in healthy individuals [15]. When specifically investigating common butyrate-producing families *Lachnospiraceae* and *Ruminococcaceae,* we found no trend of a change of butyrate-producing bacteria ($p=.85$ and .45, respectively). Also, no change in the Firmicutes: Bacteroidetes ratio was observed (Figure 4(B)). Considering taxa being altered at baseline compared to healthy controls, the low baseline relative abundances of *Eggerthellaceae* and Coriobacteriia was increased after treatment with omega-3 PUFAs ($p=.01$ and $p=.04$, respectively, Figure 4(C–D)). No correlations were observed between the treatment effect of taxa with $p<.05$ ($N=14$, $Q_{FDR}>0.20$ of all) and the treatment effect on blood lipids levels (Supplementary Table 4). No changes were seen in the inferred microbial gene content after intervention with omega-3 PUFAs (Supplementary Table 5).

Discussion

The main findings of this study are (1) Individuals with FH have an altered gut microbial composition compared to healthy individuals, characterized by a lower alpha diversity, different global microbial composition and shifts in individual taxa. These changes were apparent in all patients but were more pronounced in individuals taking the cholesterol-lowering drug ezetimibe in addition to statins. (2) Intervention with daily marine omega-3 fatty acids for 3 months did not cause major shifts in the gut microbiota compared to treatment with placebo.

We observed an association between microbial diversity and HDL-c, but not with LDL-c, which is the key factor in FH. The literature on hypercholesterolemic patients *per se* and gut microbiota is scarce, but our observations are confirmed in studies of lipids and microbiota in population-cohorts [24]. Using 16S data from ∼900 individuals from the Dutch population, excluding those using statins, Fu et al. observed a positive association between microbial richness and HDL-c, a negative association with triglycerides and no association with LDL or total cholesterol [6]. A study on obese and nonobese individuals demonstrated dyslipidemia, as measured with low HDL-c and high triglycerides, in individuals with low microbial richness [5]. Further, a Korean study on ∼1100 healthy individuals demonstrated that increased levels of triglycerides was associated with lower microbial diversity, but no association with LDL- or HDL-c [25]. Focusing on the association between serum lipids and the fourteen taxa being altered in patients with FH, only Tenericutes was negatively correlated with triglycerides in our study. Tenericutes is a low-abundant phylum,

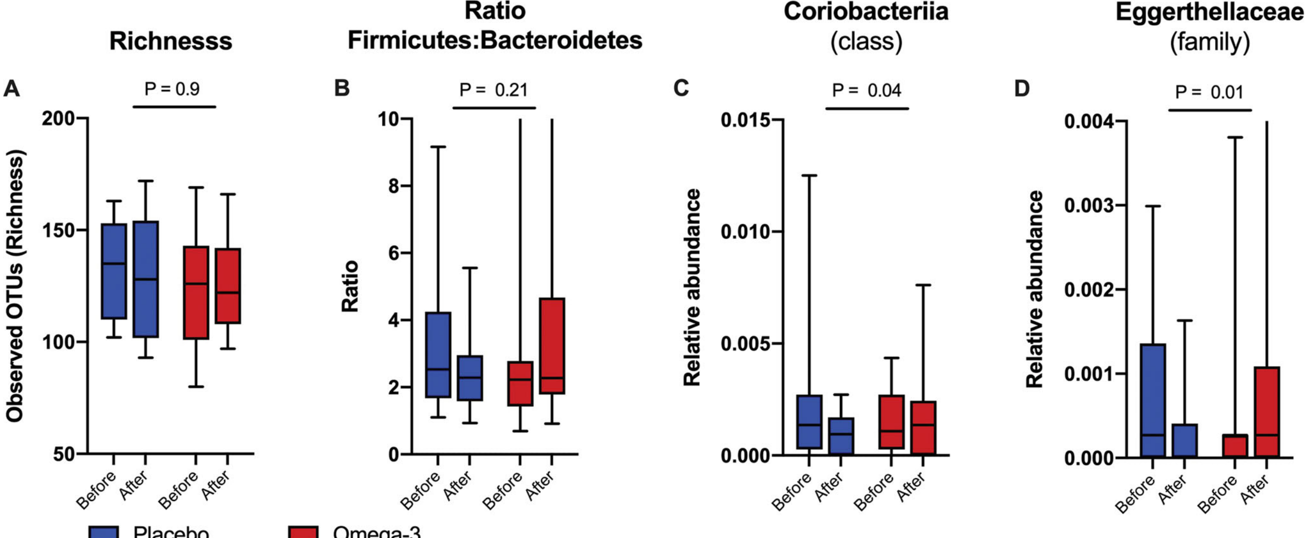

Figure 4. Intervention with omega-3 PUFAs in patients with FH were not associated with changes in microbial richness (A) or the ratio of Firmicutes: Bacteroidetes (B), but the lower baseline abundance of Coriobacteriia (C) and *Eggerthellaceae* (D) was significant increased after the intervention. The *p*-values are calculated using Wilcoxon paired test on the treatment effect, i.e. the change from corresponding baseline to after intervention with placebo versus the change after treatment with omega-3. Boxes in the boxplots represent 25–75% percentiles and whisker represent min to max.

previously associated with metabolic health, including in the study from Fu et al. where low abundance of Tenericutes was associated with increased levels of triglycerides, lower levels of HDL-c and higher BMI [26,6]. Taken together, this suggest that alterations of the microbiome are related to dyslipidemia of HDL-c and triglycerides, but variance in LDL-c is more likely determined by genetic factors.

A study of treated patients with FH will inevitably be affected by strong confounding factors influencing both lipids and the microbiota. Both genetic and environmental factors are well-established contributors to plasma cholesterol levels [27,28]. Diet has a strong impact on the gut microbiota, but is also a major risk factor for hyperlipidemia, thereby increasing the challenge of demonstrating a direct link between hyperlipidemia and the gut microbiota in cross-sectional studies [29]. By focusing on generally healthy individuals with genetically verified hyperlipidemia, we exclude some of the confounder related to a sedentary lifestyle. The included patients had the same BMI as the controls, normal levels of triglycerides and no diabetes.

Although we possibly reduce some of the diet-induced confounders by focusing on patients with FH, still, our observations do not establish a causal link. All of the patients with FH were using daily statins, in contrast to none in the healthy control group. A growing number of studies have demonstrated direct effects of statins on microbial pathogens in infectious diseases, probably contributing the observed gut microbiota alterations associated with statin use [30]. In obese individuals, treatment with statins was associated with lower prevalence of the pro-inflammatory gut microbial enterotype *Bact2*, but the mechanism and direction of this association remains unknown [31]. Jackson et al. demonstrated reduced microbial diversity in individuals using statins, Zhernakova et al. reported an association between statin-use and overall bacterial composition in a large population-based cohort, but not with gene richness or alpha diversity, while Vila et al. did not observed any association between the use of statins and microbial diversity or overall bacterial composition in a similar study [4,32,33]. Analyzing microbiota collected from participants in a recent RCT, we found no major effect on the gut microbial composition after a 6-month intervention with rosuvastatin in females undergoing coronary angiography, although alterations in the inferred metagenome related to the TMAO-pathway and a trend towards increasing diversity were observed [10]. While we lack conclusive data to explain how or to what degree statins modify the gut microbiota, for the present study it is reasonable to conclude that we cannot separate potential effects on the gut microbiota in FH from the effects of statins [34].

Interestingly, when we did a separate analysis on the one third of patients taking ezetimibe in addition to statins, we found that this group had a more pronounced dysbiosis than individuals with FH only taking statins. The numbers are low ($n = 7$) and need to be interpreted cautiously but based on these findings, one could hypothesize there is a direct association between ezetimibe and the gut microbiome. Niemann-Pick C1-Like 1 (NPC1L1) protein is essential for intestinal cholesterol absorption in the small intestine and is the molecular target of ezetimibe. Inhibiting the intestinal uptake of cholesterol increases the luminal cholesterol available for metabolization by colon bacteria, which could be speculated to change the microbial composition [35]. Mice lacking NPC1L1 have been demonstrated to have an altered gut microbial composition compared to their wild-type littermates [36]. To our knowledge, only one study has researched the effect of ezetimibe on the microbiome, showing increased levels of *Lactobacillus* spp. in mice treated with ezetimibe [37]. Ezetimibe is recommended as a second-line treatment for patients with FH or overt atherosclerotic disease, who do not reach treatment goals on treatment with statins alone [3]. In studies of the gut

microbiome and the therapeutic effect of statins, a decreased gut microbial diversity was associated with reduced cholesterol-lowering effect of statin, consistent with murine studies [11,12,38]. One could therefore hypothesize that the lower microbial diversity observed in patients using ezetimibe is not an effect of the drug, but a cause for the use of the drug because the low microbial diversity leads to therapy failure on statins alone. This finding provides a rationale for further studies on how the gut microbiota influence the effect of statins in individuals with FH, but possible direct effects of ezetimibe on gut microbes should also be addressed in future studies.

The literature on the effect of omega-3 PUFAs on the microbiota in adults is sparse [39]. In the present study, we were not able to detect any major effects on the gut microbiota after intervention with omega-3 PUFAs. This is in line with the results of a crossover-study including 25 patients with risk of metabolic syndrome, who were given a diet enriched with omega-3 PUFAs for 30 days [40]. However, in healthy individuals, Watson et al. administered supplementation of 2 g b.i.d. omega-3 PUFAs for 8 weeks to 20 individuals, demonstrating small, reversible effects on the gut microbiota, including a significant decrease in *Coprococcus* [15]. While the duration and the statistical power of both studies are comparable, the conflicting results could possibly be explained by differences in the study populations, or the possibility that observed effects occurred by chance.

The main limitation of the study is the small number of individuals with FH both in the case-control study and in the intervention trial, with subsequent low statistical power. The cross-over design does however double the numbers in both treatment arms, and sensitivity power analysis suggest we have adequate power to detect major effects of the intervention. The use of 16S rRNA sequencing does only permit detection of larger microbial shifts down to the genus level, whereas the use of inferred microbial gene content has its limitations in evaluating the microbial functions. Also, fecal microbiota mostly reflects luminal colonic species and neglects the potential role of mucosal bacteria or bacteria residing in the small intestines, which could be relevant for lipid homeostasis. Furthermore, a higher omega-3 dose, or longer duration of treatment might be necessary to observe an effect.

In this study of the gut microbiota in patients with FH, we found reduced microbial diversity and changes in the abundance of multiple taxa in individuals with FH compared to healthy controls, but it is not possible to delineate whether these are secondary to FH or due to drug treatment in our study. Furthermore, we detected no effect of supplementation with omega-3 PUFAs for 12 weeks. We speculate that the observations in FH are caused by the complex interactions between gut bacteria and cholesterol-lowering drugs, but due to lack of power, further studies on the effect of cholesterol-lowering drug on the microbiota, and vice versa, are needed.

Acknowledgements

The authors would like to thank Hanne Guldsten for expert technical assistance and Liv Wenche Thorbjørnsen for biobank assistance. Pronova/BASF supplied study medication and placebo free of charge. The company received the manuscript 14 days prior to submission but had no influence on the writing process. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

MT, MK, AH, KTL and JRH conceptualized the study. LNH, HT, MK, BV and CSL contributed to collection of samples and clinical data. BV, CSL and HT performed laboratory analysis. SHH performed the post-sequencing data processing. CSL, SHH and JRH analyzed and interpreted the data. CSL and JRH wrote the original draft and LNH, MK, SHH, AH, MT and KTL contributed to the writing of the manuscript. All authors read, critically revised for important intellectual content, and approved the final manuscript.

Disclosure statement

JRH has served on advisory boards and/or given lectures for Orkla Health, Novartis, and Roche, and received research support from Biogen, all unrelated to the present study. CSL, LNH, MK, HT, BV, SHH, AH, MT, KTL: Nothing to disclose. No potential conflict of interest was reported by the author(s).

ORCID

Christopher Storm-Larsen http://orcid.org/0000-0003-4499-1371
Johannes R. Hov http://orcid.org/0000-0002-5900-8096

References

[1] Svendsen K, Krogh HW, Igland J, et al. 2.5-fold increased risk of recurrent acute myocardial infarction with familial hypercholesterolemia. Atherosclerosis. 2021;319:28–34.
[2] Defesche JC, Gidding SS, Harada-Shiba M, et al. Familial hypercholesterolaemia. Nat Rev Dis Primers. 2017;3:17093.
[3] Mach F, Baigent C, Catapano AL, ESC Scientific Document Group, et al. 2019 ESC/EAS guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk: the task force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS). Eur Heart J. 2020;41(1):111–188.
[4] Zhernakova A, Kurilshikov A, Bonder MJ, LifeLines cohort study, et al. Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. Science. 2016;352(6285):565–569.
[5] Le Chatelier E, Nielsen T, Qin J, MetaHIT Consortium, et al. Richness of human gut microbiome correlates with metabolic markers. Nature. 2013;500(7464):541–546.
[6] Fu J, Bonder MJ, Cenit MC, et al. The gut microbiome contributes to a substantial proportion of the variation in blood lipids. Circ Res. 2015;117(9):817–824.
[7] Jones ML, Martoni CJ, Parent M, et al. Cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active *Lactobacillus reuteri* NCIMB 30242 yoghurt formulation in hypercholesterolaemic adults. Br J Nutr. 2012;107(10):1505–1513.
[8] Wu H, Esteve E, Tremaroli V, et al. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. Nat Med. 2017;23(7):850–858.
[9] Khan TJ, Ahmed YM, Zamzami MA, et al. Atorvastatin treatment modulates the gut microbiota of the hypercholesterolemic patients. OMICS. 2018;22(2):154–163.
[10] Kummen M, Solberg OG, Storm-Larsen C, et al. Rosuvastatin alters the genetic composition of the human gut microbiome. Sci Rep. 2020;10(1):5397.

[11] Sun B, Li L, Zhou X. Comparative analysis of the gut microbiota in distinct statin response patients in east China. J Microbiol. 2018;56(12):886–892.

[12] Liu Y, Song X, Zhou H, et al. Gut microbiome associates With Lipid-Lowering effect of rosuvastatin *in vivo*. Front Microbiol. 2018;9(530):530.

[13] Robertson RC, Seira Oriach C, Murphy K, et al. Omega-3 polyunsaturated fatty acids critically regulate behaviour and gut microbiota development in adolescence and adulthood. Brain Behav Immun. 2017;59:21–37.

[14] Caesar R, Tremaroli V, Kovatcheva-Datchary P, et al. Crosstalk between gut microbiota and dietary lipids aggravates WAT inflammation through TLR signaling. Cell Metab. 2015;22(4):658–668.

[15] Watson H, Mitra S, Croden FC, et al. A randomised trial of the effect of omega-3 polyunsaturated fatty acid supplements on the human intestinal microbiota. Gut. 2018;67(11):1974–1983.

[16] Hande LN, Thunhaug H, Enebakk T, et al. Addition of marine omega-3 fatty acids to statins in familial hypercholesterolemia does not affect *in vivo* or *in vitro* endothelial function. J Clin Lipidol. 2019;13(5):762–770.

[17] Kummen M, Holm K, Anmarkrud JA, et al. The gut microbial profile in patients with primary sclerosing cholangitis is distinct from patients with ulcerative colitis without biliary disease and healthy controls. Gut. 2017;66(4):611–619.

[18] Fadrosh DW, Ma B, Gajer P, et al. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the illumina MiSeq platform. Microbiome. 2014;2(1):6.

[19] Storm-Larsen C, Myhr KM, Farbu E, et al. Gut microbiota composition during a 12-week intervention with delayed-release dimethyl fumarate in multiple sclerosis - a pilot trial. Mult Scler J Exp Transl Clin. 2019;5(4):2055217319888767.

[20] Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37(8):852–857.

[21] Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41(Database issue):D590–6.

[22] Douglas GM, Maffei VJ, Zaneveld JR, et al. PICRUSt2 for prediction of metagenome functions. Nat Biotechnol. 2020;38(6):685–688.

[23] Faul F, Erdfelder E, Lang A-G, et al. G*power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007;39(2):175–191.

[24] Villette R, Kc P, Beliard S, et al. Unraveling host-gut microbiota dialogue and its impact on cholesterol levels. Front Pharmacol. 2020;11(278):278.

[25] Yun KE, Kim J, Kim MH, et al. Major lipids, apolipoproteins, and alterations of gut microbiota. J Clin Med. 2020;9(5):1589.

[26] Yuan X, Chen R, McCormick KL, et al. The role of the gut microbiota on the metabolic status of obese children. Microb Cell Fact. 2021;20(1):53.

[27] Hoffmann TJ, Theusch E, Haldar T, et al. A large electronic-health-record-based genome-wide study of serum lipids. Nat Genet. 2018;50(3):401–413.

[28] Mente A, Dehghan M, Rangarajan S, et al. Association of dietary nutrients with blood lipids and blood pressure in 18 countries: a cross-sectional analysis from the PURE study. Lancet Diabetes Endocrinol. 2017;5(10):774–787.

[29] Rothschild D, Weissbrod O, Barkan E, et al. Environment dominates over host genetics in shaping human gut microbiota. Nature. 2018;555(7695):210–215.

[30] Hennessy E, Adams C, Reen FJ, et al. Is there potential for repurposing statins as novel antimicrobials? Antimicrob Agents Chemother. 2016;60(9):5111–5121.

[31] Vieira-Silva S, Falony G, Belda E, MetaCardis Consortium, et al. Statin therapy is associated with lower prevalence of gut microbiota dysbiosis. Nature. 2020;581(7808):310–315.

[32] Jackson MA, Verdi S, Maxan M-E, et al. Gut microbiota associations with common diseases and prescription medications in a population-based cohort. Nat Commun. 2018;9(1):2655.

[33] Vich Vila A, Collij V, Sanna S, et al. Impact of commonly used drugs on the composition and metabolic function of the gut microbiota. Nat Commun. 2020;11(1):362.

[34] Dias AM, Cordeiro G, Estevinho MM, The Clinical Pharmacology Unit, São João Hospital University Centre, et al. Gut bacterial microbiome composition and statin intake-A systematic review. Pharmacol Res Perspect. 2020;8(3):e00601.

[35] Kriaa A, Bourgin M, Potiron A, et al. Microbial impact on cholesterol and bile acid metabolism: current status and future prospects. J Lipid Res. 2019;60(2):323–332.

[36] Zhong C-Y, Sun W-W, Ma Y, et al. Microbiota prevents cholesterol loss from the body by regulating host gene expression in mice. Sci Rep. 2015;5(1):10512.

[37] Catry E, Pachikian BD, Salazar N, et al. Ezetimibe and simvastatin modulate gut microbiota and expression of genes related to cholesterol metabolism. Life Sci. 2015;132:77–84.

[38] Wang L, Wang Y, Wang H, et al. The influence of the intestinal microflora to the efficacy of rosuvastatin. Lipids Health Dis. 2018;17(1):151.

[39] Costantini L, Molinari R, Farinon B, et al. Impact of omega-3 fatty acids on the gut microbiota. Int J Mol Sci. 2017;18(12):2645.

[40] Pu S, Khazanehei H, Jones PJ, et al. Interactions between obesity status and dietary intake of monounsaturated and polyunsaturated oils on human gut microbiome profiles in the canola oil multicenter intervention trial (COMIT). Front Microbiol. 2016;7(1612):1612.