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Abdominalzentrum der Universität zu Köln
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The relationship between the dietary omega-6/omega-3 ratio, gut microbiota composition and disease severity in patients with non-alcoholic fatty liver disease

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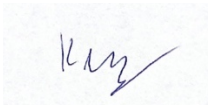
Unterschrift: 26.11.2022 

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Danksagung

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Widmung

Table of content

Abbreviations

ALA, alpha-linolenic acid;
ARA, arachidonic acid;
BMI, body mass index;
DHA, docosahexaenoic acid;
EPA, eicosapentaenoic acid;
FMT, fecal microbiota transfer
HCC, hepatocellular carcinoma;
IL-6, interleukin-6;
LA, linoleic acid;
LPS, lipopolysaccharides;
MS, metabolic syndrome;
NAFLD, non-alcoholic fatty liver disease;
NAFL, non-alcoholic fatty liver;
NASH, non-alcoholic steatohepatitis;
n-3, omega-3 fatty acid;
n-6, omega-6 fatty acid;
PAMP, pathogen-associated molecular pattern molecule;
PNPLA3, Patatin-like phospholipase domain-containing 3;
PPARα, peroxisome-proliferated receptor alpha;
PUFA, polyunsaturated fatty acids;
ROS, reactive oxygen species;
SCFA, short chain fatty acids;
SREBP1, sterol regulatory element-binding protein-1;
TLR, toll-like-receptor
TNF-α, tumor necrosis factor alpha

1 Summary

Due to the modern western style diet, the consumed Omega-6 (n-6) to Omega 3 (n-3) fatty acids ratio has notably increased. Mechanisms like increased lipogenic activity, impaired oxidation of fatty acids and increased production of proinflammatory eicosanoids may link an increased dietary n-6/n-3 ratio to NAFLD. The objective of this cross-sectional study with a cohort of biopsy proven NAFLD patients, was to point out associations between the n-6 and n-3 fatty acids consumption, the gut bacterial composition and the disease severity [1].

NAFLD study participants (n=101) were enrolled to fill out a food and activity record for 14 days. All study participants provided stool samples evaluated with the use of 16S rRNA gene sequencing. The personal dietary composition of each patient was determined using Ebispro 2016 professional software. This allowed us to divide the patients into quantiles with a low (Q1), moderate (Q2) or high (Q3) dietary n-6/n-3 ratio of <6.1 (n=34), 6.1–7.8 (n=33), >7.8 (n=34), respectively. The gut microbiota composition was analyzed using Spearman correlation coefficients as well as principal coordinate analysis. Within our total study collective, the data of biopsy proven NAFLD patients (n=63) was collected to draw conclusions regarding the histological disease severity [1].

Median age of all study participants was 53 years (range: 19-80 years) and 47% were female. The median n-6/n-3 fatty acid ratio in the groups Q1, Q2 and Q3 was 4.9 (IQR 1.4); 6.7 (IQR 0.8) und 9.4 (IQR 1.9), respectively. The presence of certain bacteria displayed a positive (*Catenibacterium* (genus); *Lactobacillus ruminis* (species)) or negative (*Clostridium* (genus)) correlation with the dietary intake of n-6 fatty acids. However, we could not show any significant associations between the n-6/n-3 ratio in the diet, the gut microbiota composition, and the histological disease severity by comparing the three quantiles [1].

As a result, the exact relation between specific human gut bacteria and n-6 fatty acids in NAFLD development remains an open field of research. In this study there is currently no evidence that an increased consumption of n-6/n-3 fatty acids represents a major contributor in NAFLD development [1].

Zusammenfassung

In der modernen westlichen Ernährung ist die Relation von Omega-6- (n-6) zu Omega-3-Fettsäuren (n-3) deutlich angestiegen. Ein hohes Verhältnis wird mit der nichtalkoholischen Fettlebererkrankung (NAFLD) in Verbindung gebracht, da es die Lipogenese und Fettsäureoxidation beeinträchtigen kann, zur Produktion von entzündungsfördernden Eicosanoiden führt und die Zusammensetzung der Darmmikrobiota beeinflusst. In dieser Querschnittsstudie wurde die Beziehung zwischen dem n-6/n-3 Verhältnis in der Nahrung und der Zusammensetzung der bakteriellen Darmikrobiota sowie dem Schweregrad der Erkrankung bei Patienten mit nichtalkoholischer Fettlebererkrankung (NAFLD) untersucht [1].

An der Studie nahmen 101 NAFLD-Patienten teil, wobei 63 davon bioptisch gesicherte NAFLD Fälle waren. Alle 101 Patienten führten ein 14-tägiges Ernährungs- und Aktivitätsprotokoll. Zusätzlich wurden anthropometrische, demografische und klinische Daten für alle Patienten erhoben. Die Software Ebispro 2016 Professional wurde verwendet, um die Aufnahme der einzelnen Makro- und Mikronährstoffe zu berechnen. Die Patienten wurden in drei Quantile (Q1-3) entsprechend einem niedrigen <6.1 (n=34), moderaten 6.1–7.8 (n=33) und hohen >7.8 (n=34) n-6/n-3-Verhältnis in der Nahrung eingeteilt. Die Stuhlproben wurden mittels Sequenzierung des 16S rRNA-Gens analysiert. Die Unterschiede in der bakteriellen Zusammensetzung der Darmmikrobiota wurden mittels Spearman-Korrelationskoeffizienten und Hauptkoordinatenanalyse analysiert [1].

Das mediane Alter aller Patienten betrug 53 Jahre, 47 % waren weiblich. Medianes n-6/n-3 Verhältnis aller Patienten war 6,7 (2,9 IQR). Das n-6/n-3 Verhältnis in den Gruppen niedrig, moderat und hoch betrug im median jeweils 4,9 (IQR 1,4); 6,7 (IQR 0,8) und 9,4 (IQR 1,9). Im Vergleich der drei Gruppen wurden keine signifikanten Unterschiede in der Zusammensetzung der Darmmikrobiota oder eine Assoziation mit einer fortgeschritteneren Verlaufsform der Erkrankung beobachtet. Es wurde jedoch festgestellt, dass der Konsum von n-6 Fettsäuren mit der Häufigkeit spezifischer Bakterien wie Catenibacterium (Gattung) oder Lactobacillus ruminis (Art) positiv und der Häufigkeit von Clostridium (Gattung) negativ korreliert [1].

Zusammenfassend scheint ein hohes Verhältnis von n-6 zu n-3 in der Nahrung wahrscheinlich kein ausschlaggebender Faktor für die Entstehung der NAFLD in dieser Kohorte zu sein. Weitere Studien sind erforderlich, um die Bedeutung der

Wechselwirkungen zwischen Teilen der Darmmikrobiota und n-6-Fettsäuren für die Pathophysiologie der NAFLD zu klären [1].

2 Introduction

2.1 Non-alcoholic fatty liver disease (NAFLD)

2.1.1 Definition

Non-alcoholic fatty liver Disease (NAFLD) is a chronic liver disease with a growing health impact worldwide. NAFLD is histologically divided into the two phenotypes; non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). NAFL describes a simple steatosis with storage of extra liver fat with a hepatocyte fat concentration at the minimum of 5%. An additional inflammation and injured ballooned hepatocytes define the state of a NASH [2]. NAFLD patients with progressive disease severity are at higher risk to develop further liver damages like fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [3]. In addition, NAFLD seems to be closely related to the metabolic syndrome (MS)., as the MS is reported to be prevalent in around 30% of NAFLD patients. Therefore, NAFLD has recently been quoted “the hepatic manifestation of the MS” [3,4]. Although NAFLD occurs more frequently in obese subjects, NAFLD can also affect lean individuals [5]. This reflects the complex pathogenesis of NAFLD and that the MS is one of multiple factors involved in the disease development.

2.1.2 Epidemiology

The overall prevalence of NAFLD around the world is approximitly 25%. The prevalence in Europe ranges around 20-30% with a prevalence of NASH of 3% [4]. The prevalence of NAFLD may vary significantly depending on other comorbidities. In North America, the NAFLD prevalence in morbidly obese patients and diabetic (type 2) patients can rise up to 90% and 70%, respectively [6]. With the rising obesity rate children are also at risk to develop NAFLD. Thus, the worldwide prevalence for pediatric NAFLD is approximately 7.6% [7]. Unfortunately, with an increasing prevalence in the United States of America, NASH has emerged as a major reason for liver transplantation [8].

2.1.3 Pathogenesis of NAFLD

The “multiple parallel hits hypothesis” describes the complex development of NAFLD as an interaction of several factors [9]. The human adipose tissue is the first important factor. Fueled by excess body weight, insulin resistance and lipolysis, increased amounts free fatty acids are being released from human fat cells [10]. Free fatty acids function as a substrate for triglycerides synthesis stored in the liver [11]. Increased de-novo lipogenesis as well as an impaired hepatic fatty acid oxidation contribute to the accumulation of triglycerides in the liver [10]. Additionally, inflammatory cytokines like interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) are set free from the adipose tissue and transported to the liver [12]. Eventually, this interaction may result in hepatic steatosis, hepatocyte injury via recruiting of further immune cells and liver fibrosis due to activated hepatic stellate cells [13]. Secondly, the diet is a fundamental aspect in the development of NAFLD. Consuming the western-style diet loaded with saturated fatty acids and sugar likely increases the risk for NAFLD [14]. Furthermore, dietary components have a direct influence on the host’s gut microbiota [15]. In fact, another important pathway in the pathogenesis of NAFLD is an imbalanced gut microbiota composition, also known as dysbiosis [16]. Finally, genetic pathways are also risk factors for NAFLD disease manifestation. For example, studies linked a sequence variation of patatin-like phospholipase domain-containing 3 (PNPLA3), to increased hepatic steatosis [17].

2.1.4 Treatment and Prognosis

Lifestyle modification is the first step in NAFLD treatment. Dietary interventions resulting in weight loss are most effective with a relative reduction of hepatic triglycerides up to 80% [18]. To reduce liver steatosis a weight loss of at least 5% needs to be achieved [19]. To improve liver fibrosis a higher weight loss of at least 10% should be aspired [20]. Although any hypocaloric diet can lead to weight loss, the Mediterranean diet is widely recommended as the best option to reduce liver fat and can also improve type 2 diabetes or cardiovascular disease [21,22]. Alongside the dietary intervention, physical exercise for at least 3 hours weekly has been proven to show positive effects on hepatic triglyceride content and visceral adiposity [21,23]. So far, no pharmacologic agents have been approved for the treatment of NAFLD. Medication in NAFLD is only recommended within the treatment of common comorbidities such as diabetes (e.g.,

metformin and glucagon-like peptide-1 receptor agonists) or hyperlipidemia (e.g., statins) [24]. Surgical interventions like bariatric surgery can be considered if patients present with Class III obesity (BMI $\geq$ 40 kg/m^2), no comorbidities and failure of conservative therapy [24]. At last, patients with decompensated liver cirrhosis should be listed for liver transplantation [8].

2.2 Gut Microbiota

2.2.1 Definition and impact on human health

The microbiota is the collective of all microorganisms existent in a particular ecosystem. The microbiome, on the other hand, refers to the total genome of all microorganisms in a given ecosystem. Furthermore, an unbalanced composition of gut microbiota is referred to as a gut microbiota dysbiosis [25]. In a healthy gut microbiota, the most abundant intestinal bacterial phyla are Firmicutes and Bacteroidetes [26]. From the beginning in utero the human fetus is in contact with microbiota [27]. As the human grows so does the individual microbiota composition influenced by numerous factors such as delivery mode, diet or infections [25,28-30]. The human gut microbiota has been studied intensively during the past years. The close relation between the gut microbiota the human health has now become clear. Gut microbiota influence or even enable various functions in the human body and therefore several diseases have been linked to an intestinal dysbiosis. For instance, the gut microbiota take part in the maturation of a healthy immune system [31]. In this context, the early use of antibiotics has been linked to an impaired immune function and can be a risk factor for childhood atopy [32]. Further, the gut microbiota decisively assists the host with food absorption and helps to digest complex carbohydrates and increases energy harvest. Turnbaugh et al. demonstrated a notable increase in body fat content when transferring gut microbiota from obese mice into receiving germ-free mice [33]. This indicates the great influence of intestinal microbiota on the metabolism of food and thus the emerge of obesity.

2.2.2 Gut microbiota and NAFLD

The gut microbiota is described to be one crucial “hit” in the development of NAFLD (Figure 1). Current research identified several pathways linking gut microbiota and liver damage. A gut microbiota dysbiosis has been shown to impair intestinal tight junctions leading to a disrupted intestinal barrier. This enables pathogen-associated molecular

pattern molecules (PAMPs) like lipopolysaccharides (LPS) to pass into the blood circulation. LPS for example are known to be part of the outer cell wall of gram-negative bacteria [34]. PAMPs transported by the portal vein are then recognized by hepatic toll-like-receptors (TLR). As part of the hepatic immune response kupffer and stellate from the liver are being activated leading to intrahepatic inflammation via secretion of proinflammatory cytokines like IL-6 and TNF-α [35]. The term 'gut liver axis' best describes this important connection between the intestine and the liver [36]. Recently the gut microbiota has been linked to enhancing de-novo lipogenesis and therefore increasing the availability of lipids for liver fat deposit. Acetate is a metabolite from the carbohydrate digestion produced by the gut microbiota and functions as a precursor in the production of triglycerides [37]. The choline metabolism in humans is an additional factor in liver disease. NAFLD is associated with the abundance of trimethylamine-N-oxide, a substance occurring in the metabolic pathway of choline when broken down by intestinal microbiota [38]. Furthermore, a dysbiosis is associated with an increased abundance of alcohol producing bacteria. This endogenous alcohol production leads to further hepatic injury as part of the NAFLD pathogenesis [39]. Controversial findings have been published discussing short chain fatty acids (SCFA) as part of the pathogenesis of NAFLD. SCFA are bacterial metabolites from carbohydrate digestion (e.g., Acetate). As opposed to healthy individuals, studies with NAFLD patients noticed elevated levels of fecal SCFA, which has been linked to hepatic steatosis via de-novo lipogenesis, inflammation and disease severity [37,40]. On the other hand, supplementing SCFA to mice has led to increased fatty acid oxidation [41]. This reflects the complexity of SCFA and the exact role of SCFA in NAFLD development is still discussed. Lastly, there has been a growing interest in the role of bile acids and their metabolism in NAFLD development. Overall, bile acids are important for the absorption of fats. The gall bladder functions as a storage for the bile acids derived by the liver, which are then further secreted by the bile duct. Gut microbiota can break down liver derived primary bile acid to secondary bile acids [42]. Especially secondary bile acids have been linked to a disturbed signaling of the farnesoid X receptor that can lead to liver damage and intestinal inflammation. Patients with NAFLD exhibit not only generally higher levels of bile acids but also an imbalance of primary to secondary bile acids [43,44].

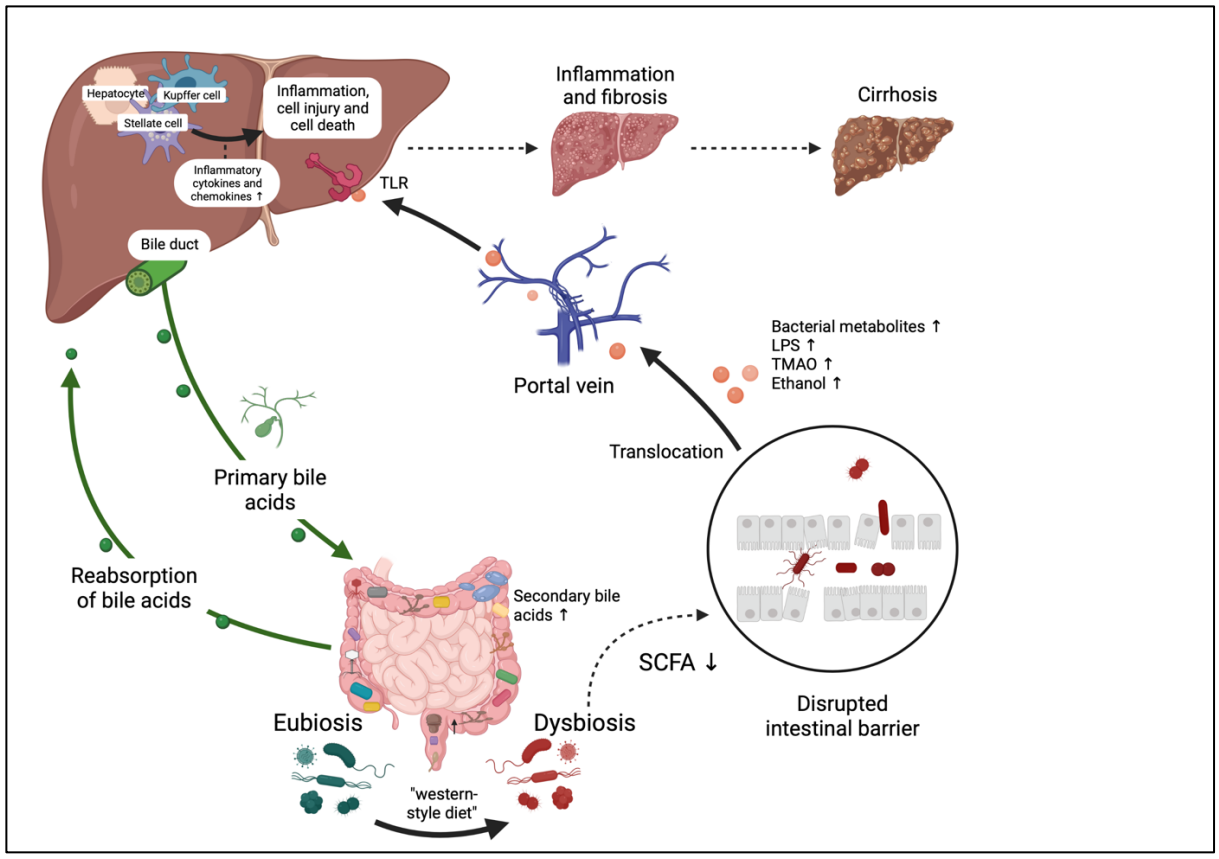

Figure 1. Gut-Liver-Axis. SCFA: short chain fatty acids; LPS: lipopolysaccharides; TMAO: trimethylamine-N-oxide; TLR: toll-like-receptors. * Created with biorender.com

2.2.3 Gut microbiota as a therapeutic target

As the liver health is influenced by the hosts gut microbiota, new therapeutic approaches focusing on the intestinal dysbiosis are being discussed (Figure 2). The main target is to promote healthy and beneficial gut microbiota and to improve overall metabolic homeostasis. Possible treatment options are (a) fecal microbiota transfer (FMT). Thereby, the entire gut microbiota of a healthy donor is transplanted to restore the gut microbiota dysbiosis in patients. This approach has already been successfully applied in patients suffering from Clostridioides difficile (C. difficile) infections [45]. (b) the use of pre-, pro- and synbiotics. Prebiotics are non-digestible dietary compounds that enhance the growth of beneficial bacteria. Probiotics are living bacteria that can be supplemented to promote gut health. A combination of pro- and prebiotics is called synbiotics [46]. (c) engineered bacteria. This describes a new approach to artificially design bacterial strains able to target harmful compounds and provide beneficial metabolites [47]. (d) Bacteriophage-mediated modulation. Here, the aim is to precisely target and eradicate pathogenic bacteria with the aid of engineered bacteriophages [48].

(e) antimicrobials. This refers to the known use of antibiotics or antifungals to eradicate harmful bacteria and fungi [46].

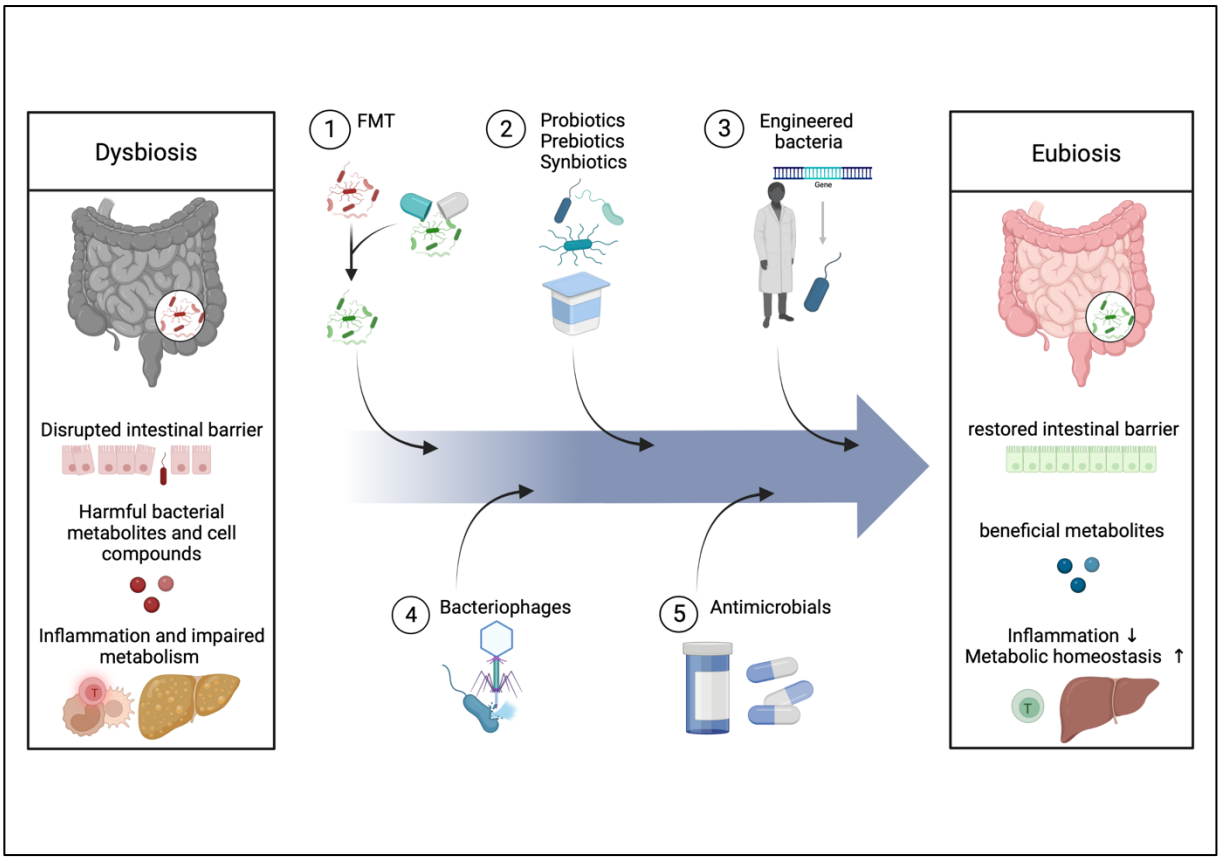

Figure 2. Visualization potential therapeutic options to target the gut microbiota. FMT: fecal microbiota transfer. * Created with biorender.com

2.3 Omega-6 and Omega-3 fatty acids

2.3.1 Definition

Omega-6 (n-6) and Omega-3 (n-3) fatty acids are considered essential polyunsaturated fatty acids (PUFA), which means they need to be obtained from the diet as the human body is not able to produce them by itself. N-6 fatty acids are mainly represented by linoleic acid (LA) (18:2ω6), mostly present in plant oils (e.g., sunflower oil), and its elongated form arachidonic acid (ARA) (20:4ω6), present in eggs, dairy products and meat. Alpha-linolenic acid (ALA) (18:3ω3) together with docosahexaenoic acid (DHA) (22:6ω3) and eicosapentaenoic acid (EPA) (20:5ω3) represent important n-3 fatty acids, although the last two exhibit higher bioactivites in the human body. ALA can be found in plant oils or seeds while DHA and EPA are present in fatty fish or fish oils [49]. Although the intake of both Omega fatty acids is

necessary for humans, different properties of the two fatty acids have been discussed. Overall, positive health properties have been attributed to n-3 fatty acids in recent years. For example, studies reported beneficial effects on inflammation, cardiovascular disease, type 2 diabetes and dementia [50-53]. However, it has to be mentioned that due to controversial findings the exact role of n-3 fatty acids is still subject of current research [54]. On the contrary, for n-6 fatty acids effects with negative health outcome have been discussed. This is mainly reasoned by proinflammatory eicosanoids generated in the metabolic pathway of the n-6 fatty acids ARA [49]. As these eicosanoids induce a proinflammatory state, n-6 fatty acids have been linked to diseases such as cardiovascular disease or NAFLD [55,56]. In this context there was a rising interest not only in the individual omega fatty acids intake but a healthy n-6/n-3 ratio. A healthy ratio enables an essential omega fatty acid intake while promoting a balanced omega fatty acid metabolism without excessive pro-inflammatory compounds. However, the estimated n-6/n-3 ratio in the early days of mankind is thought to be around 1:1, while the modern day Western-style diet, with excess fats and sugars, is changing this ratio up to 20:1 [49].

2.3.2 Omega-6/Omega-3 ratio and NAFLD

Several pathways have been explored by which the n-6/n-3 fatty acid ratio may impact liver health. Interestingly, NAFLD patients have shown a decreased level of total n-3 fatty acids together with an increased hepatic and adipose tissue n-6/n-3 fatty acid ratio [57]. Firstly, enhanced hepatic de-novo lipogenesis by a depletion of n-3 fatty acids may interfere with the sterol regulatory element-binding protein-1 (SREBP1) signaling. Physiologically, n-3 fatty acids suppress the expression of SREBP1, a protein that activates lipogenic genes. Hence decreased n-3 fatty acid levels promote fatty acid synthesis [58]. Secondly, a depletion of n-3 fatty decreases fatty acid oxidation. Peroxisome proliferator-activated receptor alpha (PPARα) is a transcription factor and can by activated by n-3 leading to increased fatty acids oxidation. The depletion of n-3 fatty acids will impair this pathway of lipid metabolism [58]. Thirdly an elevated n-6/n-3 ratio can increase hepatic oxidative stress. Ghazali et al. have revealed that an elevated n-6/n-3 ratio can the abundance of reactive oxygen species (ROS) in human hepatoma cells [59]. Additionally, Kaliannan et al. discovered a disrupted intestinal barrier function, a higher liver fat content, fibrosis of the liver and an unfavorably shift in the gut microbiota composition to a pro-inflammatory state and elevated

inflammatory parameters in association with an elevated tissue n-6/n-3 fatty acid ratio in a transgenic mouse model [1,60].

2.3.3 Omega fatty acids and the gut microbiota

A balanced intestinal bacterial composition is a necessary prerequisite for a healthy gut. Moreover, diet is a factor that significantly influences the composition of intestinal bacteria [61]. In general, a high fat diet has shown to alter the gut microbiota composition by increasing Firmicutes (phyla) and decreasing Bacteroidetes (phyla) as well as the overall microbiota richness [62,63]. A high Firmicutes/ Bacteroidetes ratio has been related to excess body weight [64]. Studies have also analyzed the impact of n-6 and n-3 fatty acids on the gut microbiota and have identified several associations. Ghosh et al. fed mice a high n-6 fatty diet over 5 weeks. He observed a n-6 induced gut microbiota dysbiosis, enhanced colonic inflammatory cell invasion as well as a disrupted barrier function with increased bacterial translocation [65]. In agreement, another study observed that an elevated tissue n-6/n-3 ratio is linked to circulating LPS levels due to the increase of LPS producing bacteria. They also described a higher abundance of bacteria known to be proinflammatory like *Enterobacteriaceae* (genus) or *Escherichia coli* (genus) [66]. On the other hand, n-3 fatty acid intake has shown a positive influence of the host's gut microbiota and even the ability to restore n-6 fatty acid induced dysbiosis [65,66]. Watson et al. recognized an increase of SCFA producing bacteria by supplementing n-3 fatty acids. In this context, SCFA can be helpful to control mucosal inflammation [67]. Furthermore, n-3 supplementation has shown to decrease the abundance of Firmicutes, therefore counteracting against an obesity promoting gut microbiota composition [68]. Nonetheless, studies in this field do present contradictory results with the need of additional research focusing on the relationship between gut microbiota and n-3 or n-6 fatty acids.

2.4 Aims of the Study

This cross-sectional study includes a NAFLD cohort with detailed clinical data. To our knowledge an association between the n-6/n-3 ratio in the diet of NAFLD patients and the disease severity as well as the gut bacterial composition has not yet been defined. By analyzing the diet of NAFLD patients and especially their n-6 and n-3 fatty acids intake, we aimed to reveal associations between the dietary n-6/n-3 ratio, the

histological disease severity, and the gut microbiota composition. We hypothesized that the gut microbiota composition and the histological disease severity can be affected by a high dietary n-6/n-3 ratio in NAFLD patients [1].

3 Publication

The original publication can be seen in the following pages. The original article can be found online in the August 6th , 2022 publication in Nutrition Research.

Heinzer K, Lang S, Farowski F, Wisplinghoff H, Vehreschild MJGT, Martin A, Nowag A, Kretzschmar A, Scholz CJ, Roderburg C, Mohr R, Tacke F, Kasper P, Goeser T, Steffen HM, Demir M. Dietary omega-6/omega-3 ratio is not associated with gut microbiota composition and disease severity in patients with nonalcoholic fatty liver disease. *Nutr Res* 2022; **107**: 12-25.

And is available at the following URL:

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In addition to the introduction and discussion, the article presents the materials and methods underlying the study and its results.

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Dietary omega-6/omega-3 ratio is not associated with gut microbiota composition and disease severity in patients with nonalcoholic fatty liver disease

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ABSTRACT

In this cross-sectional study, we hypothesized that a high dietary ratio of omega-6 (n-6) to omega-3 (n-3) fatty acids could be associated with an altered gut bacterial composition and with the disease severity in patients with nonalcoholic fatty liver disease (NAFLD). A total of 101 NAFLD patients were included in the study, of which 63 underwent a liver biopsy. All 101 patients completed a 14-day food and activity record. Ebispro 2016 professional software

Abbreviations: ALA, alpha-linolenic acid; ARA, arachidonic acid; BMI, body mass index; BMR, basal metabolic rate; CAP, controlled attenuation parameter; DHA, docosahexaenoic acid; EI, energy intake; EPA, eicosapentaenoic acid; HYA, 10-Hydroxy-cis-12-octadecenoic acid; IQR, interquartile range; LA, linoleic acid; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; NGS, next-generation sequencing; n-3, omega-3 fatty acid; n-6, omega-6 fatty acid; PCoA, principal coordinate analysis; PUFAs, polyunsaturated fatty acids; TNF, tumor necrosis factor.

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was used to calculate individual macronutrients and micronutrients consumed. Patients were grouped into 3 quantiles (Q) according to a low (Q1: <6.1, n = 34), moderate (Q2: 6.1-7.8, n = 33), or high (Q3: >7.8, n = 34) dietary n-6/n-3 ratio. Stool samples were analyzed using 16S rRNA gene sequencing. Spearman correlation coefficients and principal coordinate analysis were used to detect differences in the bacterial composition of the gut microbiota. The median dietary n-6/n-3 ratio of all patients was 6.7 (range, 3.1-14.9). No significant associations between the dietary n-6/n-3 ratio and the gut microbiota composition or disease severity were observed. However, the abundance of specific bacteria such as *Catenibacterium* or *Lactobacillus ruminis* were found to be positively correlated and the abundance of *Clostridium* were negatively correlated with dietary n-6 fatty acid intake. The results indicate that a high dietary n-6/n-3 ratio is probably not a highly relevant factor in the pathogenesis of human NAFLD. Further studies are needed to clarify the importance of interactions between gut bacterial taxa and n-6 fatty acids in the pathophysiology of NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) has evolved as the leading cause of chronic liver disease worldwide [1] with an estimated prevalence of 23% in Europe [2]. Histologically NAFLD can be separated into 2 phenotypes: simple steatosis (nonalcoholic fatty liver), defined by lipid deposits in at least 5% of hepatocytes, and nonalcoholic steatohepatitis (NASH), characterized by inflammation and injury of hepatocytes predominantly via hepatocellular ballooning. Patients may develop fibrosis or hepatocellular carcinoma with progressive disease severity [3]. Because NAFLD is often accompanied by metabolic and cardiovascular diseases, it is considered the hepatic manifestation of the metabolic syndrome [4]. NAFLD patients often consume a Western-style diet, characterized by high concentrations of simple carbohydrates and saturated fatty acids, which contributes to obesity and liver disease [5].

Human gut microbiota has a major influence on the host's health. A dysregulated microbiota composition, dysbiosis, can affect diseases such as obesity and chronic liver disease [6,7]. This dysbiosis impairs the function of intestinal tight junctions with disturbed intestinal barrier permeability. Consequently, endotoxins like lipopolysaccharides from the cell wall of gram-negative bacteria can enter the bloodstream and induce intrahepatic inflammation via Toll-like-receptors and secretion of inflammatory cytokines (e.g., interleukin-6, tumor necrosis factor α) [8,9]. This close relationship between the gut and the liver is known as the gut–liver axis. An impaired gut permeability and the translocation of microbe-associated molecular patterns to the liver are likely important factors in the pathogenesis of chronic liver diseases such as NAFLD [10]. The human gut microbiota is noticeably influenced by the host's diet. In fact, a Western-style diet can lead to gut bacterial dysbiosis [11] that is considered to be 1 of the multiple "hits" contributing to NAFLD [12] and eventually advanced liver disease [13].

Omega-3 (n-3) fatty acids and in particular omega-6 (n-6) fatty acids are essential polyunsaturated fatty acids (PUFAs) typically present in the Western-style diet [14]. Dietary n-6 PUFAs, found in various plant oils, are mainly represented by linoleic acid (LA) (18:2ω6) and its metabolite arachidonic acid (ARA) (20:4ω6). N-3 PUFAs are found in oils like flaxseed or walnut oil and are represented by alpha-linolenic acid (ALA) (18:3ω3), which can be metabolized to the more significant bioactive forms eicosapentaenoic acid (EPA) (20:5ω3) and docosahexaenoic acid (DHA) (22:6ω3). ARA is predominantly present in meat products, dairy, and eggs, whereas fish oils are a well-known source for EPA and DHA [15].

Whereas mankind has evolved with a n-6/n-3 ratio of almost 1:1, the modern Western-style diet is changing the ratio to values close to 20:1 [15]. This imbalanced ratio is associated with increased lipogenic activity [16], impaired hepatic fatty acid oxidation [16], and a promoting role of ARA as a precursor of proinflammatory eicosanoids [17]. A high ratio has been linked to liver diseases [17], cardiovascular diseases [18], and a state of systemic inflammation [19,20].

Overall, a high-fat diet has shown to have an adverse effect on the human gut microbiota by increasing the *Firmicutes/Bacteroidetes* ratio. Furthermore, it can create an environment favoring gram-negative bacteria, which leads to higher levels of circulation lipopolysaccharides. In addition, a high-fat diet can elevate the abundance of potentially unfavorable microbes such as Enterobacteriaceae known to produce endotoxins [21].

Although ARA (n-6) is often associated with adverse metabolic changes, EPA and DHA (n-3) can interfere with these pathways and show anti-inflammatory effects [22,23]. In addition, EPA and DHA can also change the composition of the gut microbiota [24]. Hence, n-3 PUFA supplementation has been discussed as a possible treatment option for NAFLD [25].

Recently, possible effects of high dietary n-6 fatty acids on the gut microbiota and chronic liver disease were examined. High y-linolenic acid (n-6) in human erythrocytes was associated with a lower alpha diversity of gut microbiota, pointing out the potential of n-6 fatty acids to promote gut dysbiosis [26]. In a transgenic mouse model able to overproduce n-6 fatty acids, mice showed signs of hepatic lipid accumulation, liver fibrosis, downregulation of intestinal tight junction proteins, overall increased inflammatory markers, and depletion of beneficial gut microbiota [27].

However, most studies only focused on n-3 PUFAs, whereas associations between the dietary n-6/n-3 ratio, the gut bacterial composition, and the disease severity of NAFLD remained unclear. We hypothesize that a high dietary n-6/n-3 ratio may influence the gut microbiota composition and/or the histo-

logical disease severity in patients with NAFLD. Therefore, we aimed to analyze dietary PUFA intake, the individual gut microbiota composition and parameters indicating disease severity of each patient.

2. Methods and materials

2.1. Study design

This is a cross-sectional study that has been approved by the Ethics Commission of the Faculty of Medicine of the University of Cologne, Germany (reference #15-056). The primary objective of this study was to examine the n-6/n-3 fatty acids consumption of NAFLD patients and to analyze its association with gut microbiota composition and/or histological disease severity in a clinically well characterized cohort of patients with NAFLD.

2.2. Patients

A total of 180 NAFLD patients were recruited at the Clinic of Gastroenterology and Hepatology, University Hospital of Cologne, Germany, during regular check-ups between July 2015 and December 2018. The study cohort has been described previously [28]. As part of the standard clinical check-ups, NAFLD patients were counseled following to the current European Guidelines [29].

A total of 101 NAFLD patients agreed to provide a food and activity record over 14 days to evaluate macro- and micronutrients in the individual diet. Furthermore, fecal samples of these patients were collected to test for differences in the gut microbiota composition. The cohort included 63 biopsy-proven NAFLD patients in whom the association between the histological disease severity and the dietary intake of n-6 and n-3 fatty acids could be assessed. A flow diagram of study participants can be seen in Fig. 1.

NAFLD was defined as hepatic steatosis detected by liver imaging and/or the presence of hepatic steatosis in >5% of hepatocytes on histological examination [30]. Exclusion criteria were age <18 years, alcohol consumption >10 g/d for women and >20 g/d for men, use of steatogenic drugs (e.g., glucocorticoids, methotrexate, amiodarone, tamoxifen), diseases causing secondary steatosis (e.g., HIV infection, celiac disease, inflammatory bowel disease), other chronic liver diseases (e.g., viral hepatitis, autoimmune hepatitis, toxic liver injury, alcoholic liver disease, cholestatic liver disease, Wilson disease, hereditary hemochromatosis), malignant neoplasms, pregnancy, antibiotic treatment or changed dietary habits 6 months before the beginning of this study, loss of >5% body weight through lifestyle modifications 3 months before entering the study and current or prior participation in an interventional NASH study [31]. Written informed consent was obtained from all study participants. This study has been approved by the Ethics Commission of the Faculty of Medicine of the University of Cologne, Germany (#15-056), and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

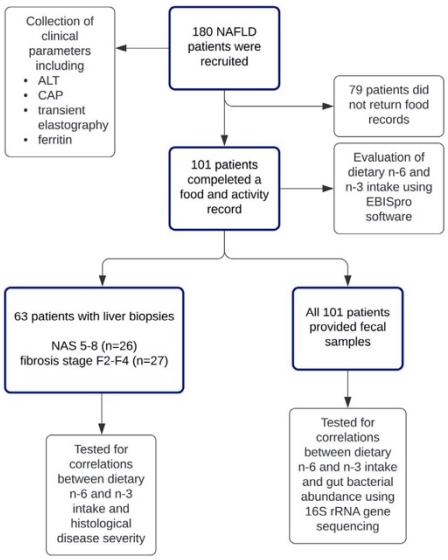

Fig. 1 – Study flow diagram. A total of 180 NAFLD patients were recruited for this study and clinical parameters were collected including ALT, CAP, transient elastography, and ferritin. A total of 101 patients completed a 14-day food and activity record and provided fecal samples to test for differences in the gut microbiota composition. Sixty-three of the 101 NAFLD patients were biopsy-proven NAFLD cases and were used to test for correlations between n-6 and n-3 fatty acid intake and histological disease severity. Dietary data was assessed using EbisPro professional software 2016. Gut microbiota was analyzed using 16S rRNA next generation sequencing. ALT, alanine aminotransferase; CAP, controlled attenuation parameter; n-3, omega-3 fatty acids; n-6, omega-6 fatty acids; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score.

2.3. Clinical data and liver histology

The anthropometric parameters height (cm), weight (kg), waist circumference (cm), and body mass index (BMI; kg/m^2) as well as the demographic data age, sex, current medication, alcohol consumption, and any concomitant diseases were collected once for all study participants during regular follow-up visits. Anthropometrics were assessed by using a tape measure and a scale. Office blood pressure measurements were obtained twice by trained nurses using the DINAMAP GE Carescape V100, GE Healthcare, USA, as a validated and automated oscillometric device. If measurements did not differ more than 10 mmHg, 1 was picked as the definite variable. If measurements differed more than 10 mmHg, the mean value was calculated. All measurements were done in the sitting position after 5 minutes' rest, with adjusted cuff sizes on the nondominant arm. Blood was drawn by trained medical staff during outpatient visit and the most recent values

were used for our analysis. Type 2 diabetes mellitus was defined as glycated hemoglobin ≥6.5% and/or fasting glucose ≥126 mg/dL and/or use of antidiabetic medication. Patients were considered overweight with a BMI ≥25 kg/m^2. Criteria for arterial hypertension were blood pressure ≥140/90 mmHg on ≥2 measurements during ≥2 occasions and/or the use of antihypertensive medication. The diagnosis of metabolic syndrome followed the criteria of the International Diabetes Federation.

If liver biopsy was performed, samples were evaluated by an experienced liver pathologist who was blinded to all clinical and laboratory patient data [31]. The NASH Clinical Research Network histological scoring system was used to assess disease severity for each liver biopsy [32]. The NAFLD activity score (NAS) (0-8) and histological grade of fibrosis (0-4) were evaluated. In the NAS scoring system, points are given for steatosis (0-3), ballooning (0-2), and lobular inflammation (0-3) with a maximum score of 8. Scores ≥5 are defined as definite NASH. Grade of fibrosis was defined as 0, no fibrosis; 1, perisinusoidal/periportal fibrosis; 2, perisinusoidal and portal/periportal fibrosis; 3, bridging fibrosis; and 4, cirrhosis.

Liver imaging with abdominal ultrasound and/or magnetic resonance imaging and/or transient elastography (FibroScan, Echosens, Paris, France) were performed on all patients.

2.4. *Nutrition and activity*

Patients were asked to complete a 14-day, self-administered food and activity record to determine each patient's individual macro- and micronutrient profile as well as the individual activity level. Instructions and examples were attached on how to complete the dietary record. Patients were asked to list all foods and beverages consumed each day. Furthermore, patients were encouraged to give as much detailed information as possible about the kind and amount of food they consumed during the survey period, either by weighing foods (g) or indicating portion sizes. Additionally, patients listed activities performed each day to calculate basal metabolic rate (BMR) and active metabolic rate. Patients were asked not to alter their regular diet or daily activities. EbisPro professional software 2016 (http://www.ebispro.de) was used to calculate and evaluate all parameters. Individual macro- and micronutrients consumed were charted and the median absolute values over all days were determined. Values were presented as the total amount in grams and the relative intake regarding overall energy intake (EI), displayed as energy percent (E%). The nutrient density method was used to calculate the relative intake in relation to the overall EI, as previously described [31]. The physical activity level was defined as the quotient of estimated energy expenditure over BMR [33]. The n-6/n-3 ratio was calculated as the quotient of LA, eicosadienoic acid, and ARA over ALA, EPA, docosapentaenoic acid, and DHA.

EI misreporting is a common problem among nutritional studies with a tendency of dietary underreporting in obese subjects [34]. As an alternative approach, we calculated the ratio between EI and the BMR (EI:BMR ratio) [31]. A total of 42% of the studied patients showed an EI:BMR ratio <1. Thus, the analyses were adjusted for the EI:BMR ratio as described previously [35,36].

2.5. *Stool samples, DNA extraction, and sequencing*

Next-generation sequencing (NGS) was used for the analysis of gut microbiota composition from each patient and performed as described previously [31,37]. Total DNA was isolated from the samples working with the RNeasy Power Microbiome Kit (Qiagen, Hilden, Germany). The amplification of 7 of 9 variable bacterial 16S rRNA regions (pool 1: V2, V4, and V8; pool 2: V3, V6/7, and V9) was conducted using the Ion 16S Metagenomics Kit (Thermo Fisher Scientific, Waltham, MA, USA) with 2 primer pools (an integrated research solution for bacterial identification using 16S rRNA sequencing on the Ion PGM System with Ion Reporter Software (https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/Ion-16S-Metagenomics-Kit-Software-Application-ote.pdf). Pooled amplicons were cleaned using the NucleoMag NGS Clean-up (Macherey-Nagel, Düren, Germany). Amplicon concentration was determined by the Qubit system. The Ion Plus Fragment Library Kit (Thermo Fisher Scientific) was used for library preparation. The amplicon concentration was diluted to 30 ng/mL to prepare templates. To enrich and assemble template-positive Ion Sphere Particles the Ion Chef Kit and the Ion Chef system (both, Thermo Fisher Scientific) were used. The Ion Torrent S5 system (pH-dependent, Thermo Fisher Scientific) was used to sequence the metagenome. All kits were used according to the manufacturers' instructions, unless stated otherwise. Operational taxonomic units were used to cluster amplicon sequences. The MicroSEQ 16S-rDNA Reference Library v2013.1 (Thermo Fisher Scientific) and Greengenes v13.5 databases have been applied for taxonomical alignment. Genus and species level assignment were based on 97% and 99% similarity, respectively. The Ion Reporter metagenomics 16S w1.1 workflow (Thermo Fisher Scientific) was used for data file assignment. The raw data were processed using the programming language R version 3.5.1.

2.6. *Accession numbers sequencing data*

The sequence data were registered at the National Center for Biotechnology Information under BioProject PRJNA540738 and sequence reads are found at the National Center for Biotechnology Information under the following BioSample IDs: SAMN11554417-SAMN11554484, SAMN13895359, SAMN13895361, SAMN13895362, SAMN13895369, SAMN13895370, SAMN13895372, SAMN13895374, SAMN13895377, SAMN13895378, SAMN13895381, SAMN13895382, SAMN13895384, SAMN13895388, SAMN13895391, SAMN13895392, SAMN13895394, SAMN13895400.

2.7. *Statistical analyses*

Data are presented as medians and interquartile ranges (IQRs) for continuous variables and numbers and percentages for categorical variables. Groups were compared using Mann-Whitney-Wilcoxon test for continuous variables and the χ^2 test for categorical variables. NAS and histological fibrosis stage groups were compared regarding their omega fatty acid consumption using the Kruskal-Wallis test for skewed distributions. Spearman correlation coefficient was used to test for

Table 1 – Characteristics and nutritional assessment of patients with NAFLD.

	N/A, n	NAFLD
Total n		101
Demographics		
Age, y		53.0 (23.6)
Female, n (%)		47 (46.5)
Body mass index, kg/m^2		30.2 (6.9)
Type 2 diabetes, n (%)		25 (24.8)
Arterial hypertension, n (%)		63 (62.4)
Metabolic syndrome (International Diabetes		41 (40.6)
Federation criteria), n (%)		
Waist circumference, cm	19	107.0 (19.5)
Noninvasive fibrosis assessment		
Transient elastography (TE), kPa	4	6.1 (6.4)
Laboratory parameters		
Albumin, g/L	2	45.0 (4.0)
Creatinine, mg/dL	2	0.9 (0.3)
Urea mg/dL	2	28.0 (12.0)
Uric acid, mg/dL	2	6.1 (2.0)
AST, U/L	2	36.0 (25.5)
ALT, U/L	2	45.0 (50.0)
GGT, U/L	2	72.0 (98.0)
Alkaline phosphatase, U/L	2	73.0 (30.5)
Bilirubin, mg/dL	3	0.5 (0.4)
Ferritin, µg/L	3	197.0 (190.2)
Triglycerides, mg/dL	2	149.0 (106.5)
Total cholesterol, mg/dL	2	195.0 (51.0)
HDL cholesterol, mg/dL	5	48.0 (15.5)
LDL cholesterol, mg/dL	8	121.0 (48.0)
Platelet count, x1E9/L	2	215.0 (85.5)
INR	2	1.0 (0.1)
HbA1c, %	7	5.5 (0.8)
Fasting glucose, mg/dL	2	96.0 (22.5)
Dietary composition/physical activity		
Physical activity level		1.5 (0.2)
EI:BMR		1.1 (0.4)
EI:BMR <1		42 (41.6)
Daily energy intake, kcal		1622.5 (670.8)
Protein E%		16.9 (4.4)
Protein, g		70.8 (27.0)
Carbohydrates E%		43.7 (10.3)
Carbohydrates, g		182.1 (67.9)
Monosaccharides E%		5.1 (4.3)
Monosaccharides, g		22.1 (16.7)
Disaccharides E%		8.5 (6.6)
Disaccharides, g		35.2 (30.2)
Polysaccharides E%		23.2 (8.5)
Polysaccharides, g		99.2 (42.2)
Fiber E%		1.9 (0.8)
Fiber, g		16.0 (6.8)
Fructose E%		2.9 (2.1)
Fructose, g		11.3 (9.9)
Fat E%		34.6 (9.4)
Fat, g		62.2 (28.2)
PUFA E%		5.0 (2.0)
PUFA, g		8.6 (4.6)
MUFA E%		11.8 (3.6)
MUFA, g		21.4 (9.1)
SFA E%		14.7 (4.9)
SFA, g		27.4 (13.4)
Cholesterol E%		0.1 (0.1)
Cholesterol, mg		255.8 (149.6)
N-3 E%		0.5 (0.2)
N-3, g		1.0 (0.5)
N-6 E%		3.9 (2.1)

(continued on next page)

Table 1 (*continued*)

		N/A, n	NAFLD
N-6, g			6.9 (4.4)
N-6/n-3 ratio			6.7 (2.9)
Linoleic acid (LA) E%			3.8 (2.0)
Linoleic acid, g			6.8 (4.6)
Alpha-linolenic acid (ALA) E%			0.4 (0.1)
Alpha-linolenic acid, g			0.9 (0.4)
Arachidonic acid E%			0.0 (0.1; mean 0.041 ± 0.05)
Arachidonic acid, g			0.1 (0.1)
Eicosapentaenoic acid E%			0.0 (0.0; mean 0.004 ± 0.02)
Eicosapentaenoic acid, g			0.0 (0.0; mean 0.008 ± 0.03)
Docosahexaenoic acid E%			0.0 (0.0; mean 0.024 ± 0.04)
Docosahexaenoic acid, g			0.0 (0.0; mean 0.048 ± 0.09)
LA/ALA ratio			8.4 (3.6)
Alcohol E%			0.0 (0.1)
Alcohol, g			0.0 (0.2)
Liver histology			
Total n			**63**
NAS, n (%)	0-4		37 (58.7)
	5-8		26 (41.3)
Stage of fibrosis, n (%)	F0-F1		36 (57.1)
	F2-F4		27 (42.9)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMR, basal metabolic rate; EI, energy intake; GGT, gamma-glutamyl-transferase; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; INR, international normalized ratio; kPa, kilopascal; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acids, N/A, not applicable; NAFLD, nonalcoholic fatty liver disease; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.
Data are presented as median and interquartile range (IQR) for continuous variables and number and percentage for categorical variables.

correlation between omega fatty acid consumption and clinical parameters. Multivariate regression analyses were performed adjusted for age, sex, type 2 diabetes, arterial hypertension, BMI, protein (E%), fat (E%), PUFA (E%), and the EI:BMR ratio, to evaluate independent associations between the dietary n-6/n-3 ratio and disease severity. Nutrient intake was evaluated as continuous variable (in E%), similar to a previous study [38]. For gut microbiota analysis, patients were grouped into quantiles according to their dietary n-6/n-3 ratio. Quantiles were defined as Q1 low dietary n-6/n-3 ratio (<6.1), Q2 moderate dietary n-6/n-3 ratio (6.1-7.8), and Q3 high dietary n-6/n-3 ratio (>7.8). The abundance of each patient's individual bacterial taxa was detected by normalized operational taxonomic units. Bacterial taxa with at least 2% abundance were included in further statistical analysis. A principal coordinate analysis (PCoA) was performed to distinguish the gut microbiota profiles of the 3 groups. A heatmap analysis was conducted reporting partial spearman's correlations between the gut bacterial taxa and the intake of omega fatty acids. PCoA and heat map analysis with spearman's correlations were adjusted for potentially confounding factors. Statistical analyses were performed using R statistical software, R version 3.5.1, 2018 the R Foundation for Statistical Computing. A P value < .05 was considered as statistically significant.

3. Results

3.1. *Patient characteristics*

Patient characteristics and clinical data of included NAFLD patients (n = 101; 47% female) are presented in Table 1. The median age was 53 years (IQR, 23.6). There were no significant differences in demographic and anthropometric measurements between the n-6/n-3 quantiles (Q) (Table 2). Regarding laboratory parameters, the moderate n-6/n-3 ratio group showed a slightly higher median glycated hemoglobin value (Q1, 5.5 [IQR, 0.6] vs. Q2, 5.8 [IQR, 1.1] vs. Q3, 5.2 [IQR, 0.6], $P = .029$).

3.2. *Dietary Data*

Macro- and micronutrients of all NAFLD patients reported in the food diary are displayed in Table 1. NAFLD patients consumed more n-6 than n-3 fatty acids resulting in a median n-6/n-3 ratio of 6.7 (2.9). The ranges for the intake of n-6 fatty acids and n-3 fatty acids in E% and the n-6/n-3 ratio in all NAFLD patients were 1.05 to 9.88 (minimum-maximum), 0.18 to 2.20 (minimum-maximum), and 3.11 to 14.86 (minimum-maximum), respectively. Table 1 provides further information about the intake of LA, ARA (n-6) and ALA, EPA, and DHA (n-

Table 2 – . Characteristics and nutritional assessment based on low, moderate, and high omega-6/omega-3 ratio in the diet of patients with NAFLD.

	N/A, n	Q1	Q2	Q3	P
Total n		**34**	**33**	**34**	
Age, y		53.7 (22.4)	53.1 (16.1)	48.4 (24.8)	.222
Female, n (%)		14 (41.2)	16 (48.5)	17 (50.0)	.738
Body mass index, kg/m^2		29.0 (4.9)	31.5 (10.6)	30.2 (7.4)	.205
Type 2 diabetes, n (%)		9 (26.5)	9 (27.3)	7 (20.6)	.785
Arterial hypertension, n (%)		21 (61.8)	22 (66.7)	20 (58.8)	.800
Metabolic syndrome (International Diabetes Federation criteria), n (%)		12 (35.3)	15 (45.5)	14 (41.2)	.696
Waist circumference, cm	19	103.5 (22.8)	109.0 (22.0)	107.0 (17.5)	.813
Noninvasive fibrosis assessment					
Transient elastography (TE), kPa	4	5.3 (4.6)	6.2 (8.9)	6.1 (2.7)	.196
Laboratory parameters					
Albumin, g/L	2	45.0 (4.8)	44.0 (4.0)	45.0 (5.0)	.382
Creatinine, mg/dL	2	0.9 (0.3)	0.8 (0.3)	0.9 (0.3)	.574
Urea mg/dL	2	29.0 (7.8)	27.5 (12.0)	27.0 (13.0)	.289
Uric acid, mg/dL	2	6.1 (1.8)	5.8 (2.2)	6.1 (2.0)	.769
AST, U/L	2	35.0 (22.0)	36.5 (24.8)	36.0 (29.0)	.970
ALT, U/L	2	44.0 (57.5)	42.5 (53.8)	51.0 (46.0)	.766
GGT, U/L	2	69.5 (62.2)	67.5 (84.2)	97.0 (117.0)	.575
Alkaline phosphatase, U/L	2	76.5 (25.8)	74.5 (35.5)	70.0 (33.0)	.418
Bilirubin, mg/dL	3	0.5 (0.3)	0.5 (0.5)	0.5 (0.5)	.904
Ferritin, µg/L	3	191.0 (188.0)	225.5 (183.5)	175.0 (150.0)	.399
Triglycerides, mg/dL	2	133.5 (70.8)	167.5 (110.0)	171.0 (124.0)	.338
Total cholesterol, mg/dL	2	193.0 (35.0)	200.0 (63.5)	189.0 (49.0)	.859
HDL cholesterol, mg/dL	5	48.0 (15.0)	49.0 (14.8)	46.0 (15.0)	.807
LDL cholesterol, mg/dL	8	120.0 (37.5)	127.0 (66.0)	115.5 (51.2)	.768
Platelet count, x1E9/L	2	207.0 (73.2)	225.0 (85.0)	212.0 (85.0)	.239
INR	2	1.0 (0.1)	1.0 (0.1)	1.0 (0.0)	.551
HbA1c, %	7	5.5 (0.6)	5.8 (1.1)	5.2 (0.6)	**.029**
Fasting glucose, mg/dL	2	92.5 (22.5)	99.0 (24.2)	93.0 (24.0)	.271
Dietary composition/physical activity					
Physical activity level		1.5 (0.2)	1.5 (0.2)	1.5 (0.2)	.905
EI:BMR		1.0 (0.3)	1.0 (0.4)	1.2 (0.5)	.157
EI:BMR <1		17 (50.0)	14 (42.4)	11 (32.4)	.334
Daily energy intake, kcal		1518.6 (585.3)	1580.4 (465.3)	1812.6 (717.8)	.077
Protein E%		18.4 (3.9)	16.8 (3.7)	15.5 (4.3)	**.019**
Protein, g		71.9 (29.6)	67.7 (22.9)	76.4 (20.5)	.367
Carbohydrates E%		43.9 (11.2)	43.7 (7.7)	41.8 (11.2)	.655
Carbohydrates, g		180.7 (57.4)	179.0 (62.5)	184.7 (99.9)	.409
Monosaccharides E%		5.1 (4.7)	4.8 (4.1)	5.2 (2.9)	.716
Monosaccharides, g		16.5 (19.3)	20.7 (14.0)	22.8 (18.7)	.399
Disaccharides E%		7.6 (6.4)	9.4 (5.9)	8.5 (6.6)	.532
Disaccharides, g		30.7 (33.6)	36.5 (24.6)	36.8 (32.2)	.422
Polysaccharides E%		23.4 (8.6)	24.4 (9.5)	22.8 (5.3)	.452
Polysaccharides, g		93.8 (38.8)	97.2 (46.8)	103.8 (41.1)	.646
Fiber E%		2.0 (1.0)	1.9 (0.7)	1.8 (0.9)	.208
Fiber, g		15.6 (8.2)	16.0 (4.7)	16.7 (8.0)	.519
Fructose E%		2.8 (2.6)	2.9 (1.7)	2.9 (1.8)	.968
Fructose, g		10.5 (11.5)	11.1 (7.1)	11.5 (10.5)	.502
Fat E%		33.0 (7.6)	33.7 (9.1)	37.7 (8.3)	.333
Fat, g		58.9 (22.9)	59.5 (37.1)	71.9 (30.7)	**.023**
PUFA E%		4.0 (1.7)	4.9 (1.6)	5.7 (1.7)	**<.001**
PUFA, g		7.2 (2.8)	8.6 (3.5)	11.5 (4.8)	**<.001**
MUFA E%		11.0 (2.7)	12.1 (2.8)	12.2 (3.4)	.460
MUFA, g		18.8 (7.4)	20.6 (12.3)	22.8 (10.0)	.053
SFA E%		14.6 (4.6)	14.0 (5.3)	14.8 (4.2)	.920
SFA, g		26.5 (10.4)	24.7 (15.3)	30.6 (12.9)	.196
Cholesterol E%		0.1 (0.1)	0.1 (0.0)	0.1 (0.1)	.918
Cholesterol, mg		244.5 (109.7)	239.5 (153.2)	268.5 (131.6)	.439
N-3 E%		0.6 (0.3)	0.6 (0.3)	0.5 (0.1)	**.014**
N-6 E%		2.8 (1.8)	3.5 (1.8)	4.7 (1.5)	**<.001**

(continued on next page)

Table 2 (*continued*)

		N/A, n	Q1	Q2	Q3	P
N-6/n-3 ratio E%			4.9 (1.4)	6.7 (0.8)	9.4 (1.9)	**<.001**
Alcohol E%			0.0 (0.0)	0.0 (0.1)	0.0 (0.1)	.689
Alcohol, g			0.0 (0.0)	0.0 (0.2)	0.0 (0.4)	.649
Liver histology (n = 63)						
Total n			**19**	**23**	**21**	
NAS, n (%)	0-4		11 (57.9)	13 (56.5)	13 (61.9)	.933
	5-8		8 (42.1)	10 (43.5)	8 (38.1)	
Stage of fibrosis, n (%)	F0-F1		10 (52.6)	12 (52.2)	14 (66.7)	.558
	F2-F4		9 (47.4)	11 (47.8)	7 (33.3)	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMR, basal metabolic rate; EI: energy intake; GGT, gamma-glutamyl-transferase; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; INR, international normalized ratio; kPa, kilopascal; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acids, N/A, not applicable; NAFLD, nonalcoholic fatty liver disease; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.
Data are presented as median and interquartile range (IQR) for continuous variables (Wilcoxon-Mann-Whitney test) and number and percentage for categorical variables (χ^2- test). Bold font represents statistical significance ($P < .05$). Comparison between different ratios of omega-6/omega-3 in the diet with Q1: low ratio of <6.1; Q2 moderate ratio of 6.1-7.8; Q3 high ratio of >7.8.

3), and the LA/ALA ratio, to distinguish between the individual omega fatty acids. No omega-3 supplements were taken by any of the patients.

Table 2 displays the dietary assessment of NAFLD patients based on a low, moderate, or high dietary n-6/n-3 ratio. The groups differed significantly only in protein intake (E%). Therefore, further analyses were adjusted for protein intake (E%) as a possible confounding factor. Median n-6/n-3 ratios in the low, moderate, and high group were 4.9 (1.4), 6.7 (0.8), and 9.4 (1.9), respectively. We observed a trend in an increased consumption of saturated fatty acids in grams, concurrent with increasing n-6/n-3 ratios (P = .053).

3.3. *Disease severity*

To detect associations between the dietary n-6/n-3 ratio and disease severity correlations between the n-6/n-3 ratio and severity indicating parameters such as the NAS score, histological fibrosis stage, transient elastography (kPa), controlled attenuation parameter (CAP), alanine transaminase, and ferritin were analyzed. No significant associations between the dietary n-6/n-3 ratio and the NAFLD activity score (NAS 1-8) or the fibrosis stage (0-4) could be detected (Fig. 2A and B). Furthermore, there was no association between the dietary n-6/n-3 ratio and values for transient elastography (kPa), CAP, alanine transaminase, or ferritin (Fig. 3A-D).

To rule out possible confounding factors, multivariate regression analyses were performed adjusted for age, sex, type 2 diabetes, arterial hypertension, BMI, protein (E%), fat (E%), PUFA (E%), and EI:BMR. However, the n-6/n-3 ratio was not significantly associated with higher NAS scores (5-8) or advanced stages of fibrosis (2-4) (Table 3), even after taking other cofactors into account. We also analyzed associations between n-6 and n-3 individually, retesting for associations concerning the NAS score, the fibrosis stage and severity indicating parameters (Supplementary material). However, we still did not observe any statistically significant difference. Thus, correlations between a higher dietary n-6/n-3 ratio and progressive NAFLD disease severity could not be observed in this study cohort.

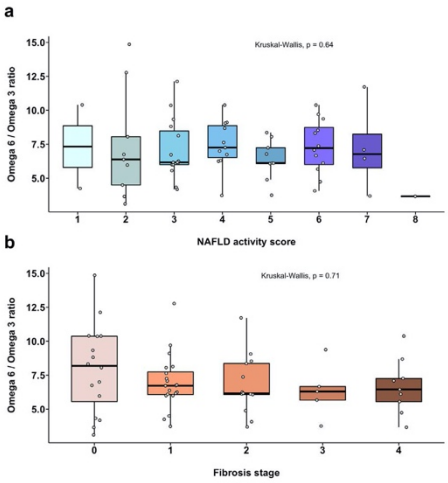

Fig. 2 – Box plots showing associations between the dietary omega-6/omega-3 ratio and histological disease severity. The box plots show the omega-6/omega-3 ratio in the diet of NAFLD patients with liver biopsies (n = 63) for each stage of (A) the NAFLD activity score of 0 to 8 and (B) the fibrosis stage F0-F4. *P* values were obtained from Kruskal-Wallis tests. No significant associations between the dietary n-6/n-3 ratio and the NAFLD activity score (NAS 1-8) or the fibrosis stage (0-4) could be detected. n-3, omega-3 fatty acids; n-6: omega-6 fatty acids; NAFLD, nonalcoholic fatty liver disease.

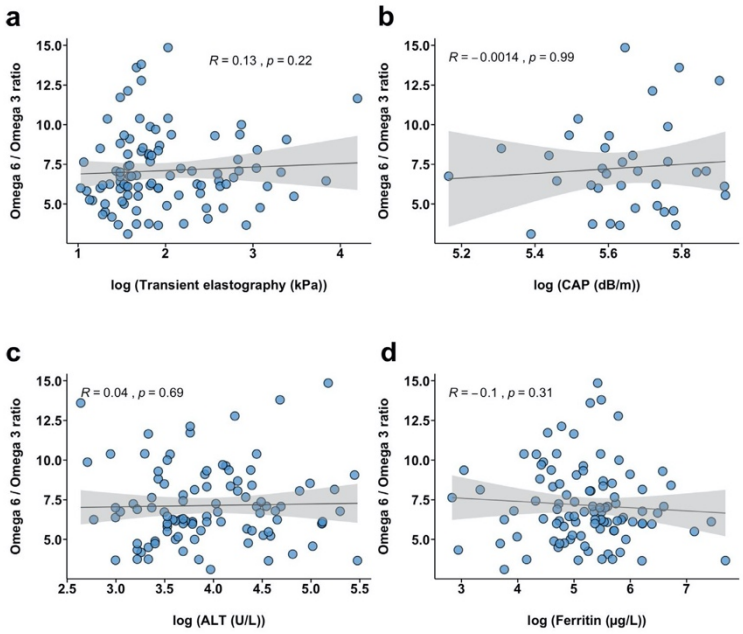

Fig. 3 – Scatter plot showing spearman correlations for dietary omega-6/omega-3 ratio and clinical parameters. Associations between the omega-6/omega-3 ratio in the diet of all NAFLD patients (n = 101) and clinical parameters including (A) transient elastography, (B) CAP, (C) ALT, and (D) ferritin levels. Continuous line represents line of best fit and gray shading displays 95% confidence interval. Spearman's correlations were performed to test for correlations. No significant associations between the dietary n-6/n-3 ratio and clinical parameters could be detected. *P* values were generated using Spearman correlations. ALT, alanine aminotransferase; CAP, controlled attenuation parameter; n-3, omega-3 fatty acids; n-6, omega-6 fatty acids; NAFLD, nonalcoholic fatty liver disease.

Table 3 – Multivariate analysis to evaluate associations between omega-6 fatty acid intake, omega-3 fatty acid intake, and omega-6/omega-3 ratio and definite NASH or F2-F4 fibrosis in patients with NAFLD.

	Outcome: NAFLD activity score 5-8 OR (95% CI, P-value)	Outcome: liver fibrosis F2-F4 OR (95% CI, P-value)
n-6 (%)	0.84 (0.29-2.31, P = .727)	0.58 (0.16-1.80, P = .361)
n-3 (%)	1.05 (0.06-25.14, P = .974)	1.17 (0.06-27.46, P = .916)
n-6/n-3 ratio	0.92 (0.66-1.23, P = .573)	0.86 (0.59-1.19, P = .402)

Abbreviations: BMI, body mass index; BMR, basal metabolic rate; CI, confidence interval; EI, energy intake; E%, energy percent; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.
The multivariate analysis was adjusted for age, sex, type 2 diabetes, arterial hypertension, BMI, protein E%, fat E%, PUFA E%, and the EI:BMR ratio. Sixty-three patients with biopsy proven NAFLD were included into the analysis. A NAFLD activity score ranging from 5-8 (n = 26) indicates definite NASH and a fibrosis stage F2-F4 (n = 27) significant fibrosis.

3.4. *Gut microbiota*

Next, the association of dietary n-6/n-3 ratio with the gut microbiota composition of NAFLD patients was analyzed. Data from all 101 patients was included in the analysis. The mean relative bacterial abundance on genus level was compared between the low, moderate, and high dietary n-6/n-3 ratio groups (Fig. 4A). Within the 3 groups, the most abundant bacteria on genus level were *Bacteroides*, *Blautia*, and *Faecalibacterium*. No significant difference in the mean abundance of any bacterial taxa was observed between the groups. The PCoA revealed no differential clustering of gut microbiota profiles when comparing the groups (P = .375) (Fig. 4B). Additionally, a heatmap analysis reporting partial spearman's correlations was conducted to determine correlations between the consumption of omega fatty acids and the abundance of specific bacterial taxa (Fig. 5). For the analysis, bacteria with at least a 2% relative abundance in the fecal sample were included. The analy-

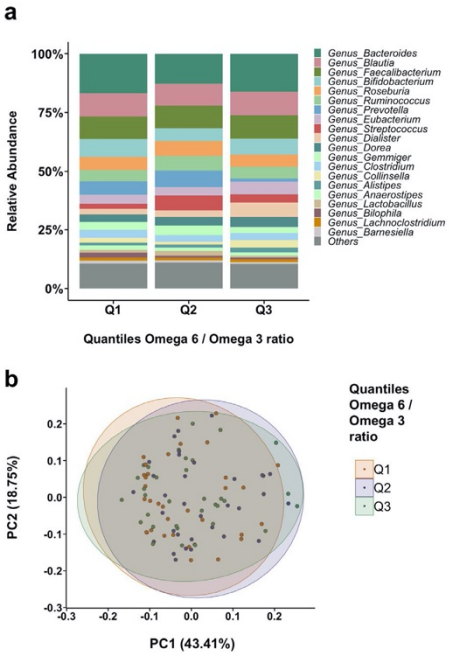

Fig. 4 – Relative abundance and clustering of gut microbiota profiles comparing the groups with a low, moderate, or high dietary omega-6/omega-3 ratio. A total of 101 NAFLD patients were grouped into 3 quantiles (Q) according to the daily intake of omega-6/omega-3 fatty acids. Q1 low ratio of <6.1 (n = 34); Q2 moderate ratio of 6.1-7.8 (n = 33); Q3 high ratio of >7.8 (n = 34). (A) Bar plot showing the mean relative abundance of gut microbial taxa on genus level detected by 16S rRNA gene sequencing. (B) Cluster visualization using principal coordinate analysis (PCoA) to compare gut microbiota profiles in NAFLD patients with a low (Q1: red), moderate (Q2: blue), and high (Q3: green) dietary omega-6/omega-3 ratio. Each dot represents one fecal sample. The PCoA revealed that there is no significant difference in the overall gut bacterial microbiota composition. NAFLD, nonalcoholic fatty liver disease.

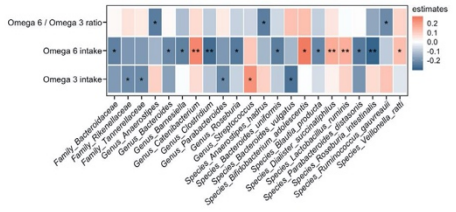

Fig. 5 – Heatmap analysis reporting partial Spearman correlations between dietary omega fatty acids and relative abundance of gut microbial taxa. Heatmap analysis showing partial Spearman correlations between the abundance of gut microbial taxa and the omega-6/omega-3 ratio in the diet of NAFLD patients (n = 101), as well as the relative omega-6 and omega-3 intake. Colors indicate type of correlation (red: positive; blue: negative). *P < .05, P > .01, **P < .01, P > .001. Only bacteria with a mean abundance of at least 2% and significant correlations are shown. P values were adjusted for age, sex, body mass index, type 2 diabetes, arterial hypertension, relative intake of protein, fat and poly-unsaturated fatty acids, and the ratio for energy intake/ basal metabolic rate. Specific bacteria such as *Catenibacterium* or *Lactobacillus ruminis* were positively and other bacteria such as *Clostridium* were negatively correlated with the dietary intake of omega-6 fatty acids. NAFLD, nonalcoholic fatty liver disease.

sis was also adjusted for the confounding variables age, gender, type 2 diabetes, arterial hypertension, BMI, protein (E%), fat (E%), PUFA (E%), and the EI:BMR ratio. Specific bacteria such as *Catenibacterium* or *Lactobacillus ruminis* were positively and bacteria such as *Clostridium* were negatively correlated with the dietary intake of n-6 fatty acids (Fig. 5).

4. Discussion

According to the presented data, we reject our hypothesis as an increased n-6/n-3 ratio in the diet of NAFLD patients is not associated with the gut microbiota composition and disease severity. In our study, NAFLD patients consumed more n-6 than n-3 fatty acids resulting in a median n-6/n-3 ratio of 6.7. Higher ratios were mainly caused by a higher intake of n-6 fatty acids rather than a decreased intake of n-3 fatty acids. With an increased dietary n-6/n-3 ratio, no significant associations concerning the NAS, fibrosis grade and severity indicating parameters were observed. Additionally, the comparison of the low, moderate, or high dietary n-6/n-3 ratio did not reveal differences in the mean relative abundance of gut bacterial taxa or differential clustering of gut microbiota profiles. However, partial Spearman correlation revealed specific associations between the relative abundance of certain gut bacteria and the n-6 or n-3 fatty acid intake, respectively.

Simopoulos et al. [15] reported an increase in the dietary n-6/n-3 ratio over time, beginning with a ratio of 1 in paleolithic times and progressing to values as high as 20:1 with the modern Western-style diet. In contrast, our study could not confirm ratios higher than 15. The general dietary n-6/n-3 ratio consumed in Germany is similar to the median dietary n-6/n-3 ratio of 6.7 observed in our NAFLD cohort, indicating an overall comparable omega fatty acid consumption [39]. Nonetheless, NAFLD patients that were consuming higher dietary n-6/n-3 ratios did not show any associations with proceeding disease severity or the gut microbiota composition. Recommendations for the consumption of n-3 and n-6 fatty acids have been published by several professional societies [40–42]. The median n-3 fatty acid intake of 0.5 E% observed in our study is in accordance with most European and international recommendations for ALA intake (n-3) [41]. European dietary intake of EPA and DHA is often reported to be relatively low in

comparison with current recommendations [43]. In this study cohort, no dietary EPA and DHA intake could be observed. Consequently, no conclusion about the specific effects of EPA and DHA can be drawn. Recommendations for the n-6 fatty acid intake are more varied, depending on the source. The German nutrition association recommends an LA (n-6) intake of 2.5 E% and a n-6/n-3 ratio of 5 [40]. Thus, around 80% of our patients showed a n-6 fatty acid intake higher than recommended, which resulted in n-6/n-3 ratios being greater than 5. The Food and Agriculture Organization of the United Nations [42] have proposed an adequate intake for LA of 2 to 3 E% and an acceptable range for LA intake of 2.5 to 9 E%. Using this metric, the n-6 fatty acid intake of our patients would be within the acceptable range. Taken together, many studies seem to agree on keeping the average daily intake of n-6 fatty acids close to 2 E%, whereas the acceptable maximum intake of n-6 fatty acids still needs to be evaluated.

Several mechanisms have been proposed as to how a high dietary n-6/n-3 ratio may induce liver damage and promote NAFLD development. First, a high ratio enhances hepatic lipogenesis by increasing the expression of lipogenic enzymes or lipogenic genes including stearoyl-CoA desaturase 1 or sterol regulatory element-binding protein-1. Second, hepatic fat oxidation is impaired via the reduced expression of the peroxisome proliferator-activated receptor alpha. Third, a high ratio leads to more oxidative stress with increased levels of reactive oxygen species, which enhance lobular inflammation and progression to NASH [16,44]. Finally, dietary LA is desaturated and elongated to ARA, which acts as a substrate for proinflammatory eicosanoids mediating inflammatory cell activation [17]. However, these data were captured using cell cultures and animal models, but little research is published evaluating these mechanisms in humans. Our study did not confirm any progression in disease severity in conjunction with an increased n-6/n-3 ratio. This may be because even the high dietary n-6/n-3 ratio group consumed a median n-6/n-3 ratio of 9.4. In comparison, preclinical reports defined "high" n-6/n-3 ratios as 15:1 to 25:1 [16,17]. Additionally, the role of n-6 fatty acids is still not completely understood. Research has shown that not all eicosanoids derived from ARA are proinflammatory. In fact, other n-6 fatty acids are generated in the metabolic pathway of LA, with resultant products exhibiting anti-inflammatory properties. Dihomo-γ-linolenic acid (n-6) for example, a PUFA derived from LA, is further metabolized to prostaglandin E1, which is known to suppress inflammation [45]. Inflammation is a key factor contributing to an advanced disease stage in NAFLD. In support of our results, a systematic review failed to prove increased inflammatory markers with a high dietary LA intake [46]. In addition, Enos et al. [47] reported that lowering the n-6/n-3 ratio did not prevent the development of NAFLD when consuming a high-fat diet. On the 1 hand, this may suggest that high n-6 PUFA consumption is of minor relevance for NAFLD pathogenesis. On the other hand, this could also indicate that the potential beneficial and protective effect of n-3 PUFA is less significant than the potential detrimental effects of n-6 PUFA. It needs to be acknowledged that changes in the ratio can be due to increased n-6 or decreased n-3 fatty acids or both. Therefore, it might be difficult to identify the main underlying driver of change. In addition, the abundance of specific PUFAs, such as EPA and DHA, seem to be of particular significance for the observed effects and their abundance in appreciable levels may be just as important as the n-6/n-3 ratio. Certainly, because of the cross-sectional study design in this cohort, no conclusions can be drawn whether a dietary intervention with specific PUFAs at a preclinical state would lead to a different outcome in disease severity.

Of note, there are several other known factors that have been proven to interfere with the gut microbiota and/or may be part of the pathogenesis of NAFLD. An increased level of bile acids as well as an imbalanced ratio of primary to secondary bile acids has been observed in NAFLD patients [48]. Interestingly, mice fed a diet high in n-6 fatty acids showed increased levels of hepatic bile acids [49]. Excessive primary bile acids are then more likely to escape the enterohepatic circulation and are metabolized to secondary bile acids by gut microbiota. Subsequently, secondary bile acid may lead to inflammation and liver injury via impaired farnesoid X receptor signaling [48].

In addition, a diet high in cholesterol can cause a gut dysbiosis as well as an increased disease severity eventually leading to hepatocellular carcinoma in mice [50]. On the other hand, higher levels of dietary fiber seem to have beneficial effects by favoring bacteria able to produce SCFA, consequently interfering with proinflammatory pathways [51]. Nonetheless, by comparing our groups (Q1-Q3) we did not observe a significant difference in the dietary intake of other macro- and micronutrients besides the protein intake, why we adjusted our further analysis for protein intake as a possible confounding factor.

A main objective of this study was to detect if the gut microbiota composition is associated with a high dietary n-6/n-3 ratio. Gut bacterial dysbiosis might be one of multiple hits inducing the development of NAFLD. However, specific gut microbiota signatures associated with an increased dietary n-6/n-3 ratio were not detected in the present NAFLD cohort. Nonetheless, the heat map analysis, showing Spearman correlations, revealed both positive and negative correlations between specific bacterial taxa and dietary n-6 or n-3 fatty acids, respectively (Fig. 5). For example, a significant ($P < .01$) positive correlation between the abundance of *Catenibacterium* and the intake of n-6 fatty acids was observed. Interestingly, Shin et al. [52] found the genus *Catenibacterium* to be elevated in subjects consuming a high-fat diet, especially a diet high in animal fat. Consistent with these results, dietary products derived from grain-fed animals are particularly rich in ARA (n-6) [15]. In addition, the intake of n-6 fatty acids revealed a significant ($P < .01$) positive correlation with the abundance of *Dialister succinatiphilus*. Significantly higher levels of *D succinatiphilus* have been reported in healthy individuals compared with patients with progressive hepatitis B virus infection. The exact function of *D succinatiphilus* still needs to be investigated but its metabolites might be potentially beneficial by initiating an appropriate immune response [53]. Another bacterium that showed a significantly positive correlation ($P < .01$) with the intake of n-6 fatty acids was *Lactobacillus ruminis* (species). As autochthonous members of the gastrointestinal tract, Lactobacilli have beneficial functions like preserving the epithelial gut barrier. *L ruminis* in particular has been shown to protect against rotavirus infections in mice [54]. On the other hand,

L ruminis shows immunostimulating properties via Toll-like-receptors or inducing secretion of tumor necrosis factor in vitro. Whether a small increase in inflammatory activity represents an advantage or disadvantage for the host is still under discussion. However, *L ruminis* has been proposed as an immunoprobiotic bacteria [55]. A recent study from Miyamoto et al. [56] analyzed the gut microbiota of mice fed a high-fat diet supplemented with LA and described an increased abundance of 10-hydroxy-cis-12-octadecenoic acid (HYA) producing *Lactobacilli* compared with controls. HYA has been found to increase the resistance to obesity induced by a high-fat diet. Although LA supplementation still resulted in a proinflammatory ARA cascade, beneficial bacteria like *Lactobacilli* have the ability to convert excess LA into the protective metabolite HYA. Not all species of *Lactobacilli* are capable of producing HYA and to our knowledge, the specific role of *L ruminis* remains unclear. The observations of Miyamoto et al. [56] indicate a detoxifying mechanism carried out by specific gut microbiota and describes a new way of host–microbe interaction. Furthermore, in the present study population, the intake of n-6 fatty acids was negatively correlated ($P < .01$) with the abundance of *Clostridium* (genus) and *Roseburia intestinalis* (species). Seo et al. [57] discovered that the administration of *R intestinalis* lead to improved gut integrity, restored gut microbiota diversity, and reduced hepatic inflammation and steatosis in an alcohol related liver disease model in mice. These positive effects were most likely induced by flagellin, a structural protein of the bacterial flagellum. Thus, a depletion of *R intestinalis* might indicate a disadvantage for the host. On the other hand, previous studies identified *clostridia* as alcohol-producing bacteria. Therefore, a depletion of *clostridia* could be beneficial for the host's liver because it reduces the burden of endogenous alcohol production. As certain correlations can be observed, it needs to be noticed that the specific n-6 fatty acids like ARA or γ-linolenic acid may have diverse effects not only on inflammatory makers but also on the gut microbiota abundance.

Several limitations for our study need to be acknowledged. Conducting a clinical study with food recording and reporting physical activity may attract more patients with an existing interest in food or diet in general. Although there are possibly diverse motives to participate in a medical study this selection bias cannot be ruled out. In addition, as has been reported in previous studies, dietary underreporting, especially with obese subjects, is a limiting factor for dietary assessment and was in fact observed in our study. The lack of a healthy control group also contributes to this limitation. Social desirability bias, as well as limited time and motivation for daily dietary reporting, may also distort the results. Also, concentrating on everyday food recording can create more awareness and therefore may change dietary habits. In addition, portion miscalculation cannot be ruled out, even with detailed instructions given. Furthermore, because of the cross-sectional study design, we cannot prove causality. No conclusions can be drawn as to whether the omega fatty acids influenced the microbiota or the other way around. As the n-6/n-3 ratio cannot distinguish between the different n-6 and n-3 fatty acids other metrics (for example, the omega-3 index in red blood cells) have been proposed as more precise options. Unfortunately, the omega fatty acid content in blood or erythrocytes was not analyzed in this study. Further studies could compare the n-6/n-3 ratio with other metrics and differentiate between the different n-6 (LA, ARA) and n-3 (ALA, DHA, EPA) fatty acids and their specific interactions with gut bacterial taxa as well as their role in pro- or anti-inflammatory processes.

The main strength of our study is the large and clinically well-described cohort of NAFLD patients with detailed anthropometric and demographic data, including biopsy-proven NAFLD cases.

In conclusion, associations between the dietary n-6/n-3 ratio and the gut microbiota composition or histological disease severity in NAFLD could not be ascertained. On the other hand, several specific gut bacteria were positively or negatively correlated with the consumption of either n-6 or n-3 fatty acids, which in turn could modulate disease activity. Further clinical studies are needed to clarify the pro-inflammatory effects of n-6 fatty acids. Although specific amounts of nutrients consumed using dietary assessment can be detected, it is still not clear how exactly these nutrients are metabolized. Factors like the individual gut microbiota composition may induce metabolic pathways that are still not fully understood. More research needs to be done, concentrating on the specific functions of each bacterium and possible interactions with the host's metabolism.

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Author contributions

K.H. provided assistance with the analysis of food records and was responsible for the interpretation of data and writing of the manuscript; S.L. was responsible for collection of samples, statistical analysis, interpretation of data, and editing the manuscript; F.F. was responsible for data preparation and assistance of statistical analysis; H.W. was responsible for sequencing of fecal samples; M.J.G.T.V provided assistance with interpretation of data and editing the manuscript; A.M. was responsible for collection of samples and editing the manuscript; A.N., A.K., and C.J.S were responsible for fecal DNA extraction and sequencing; P.K. provided assistance with collection of samples and editing the manuscript; C.R., R.M., F.T., T.G., and H.-M.S. provided assistance with interpretation of data and editing the manuscript; M.D. was responsible for the study concept and design, collection of samples, analysis of the food records, data collection, interpretation of data, study supervision, and editing the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgment

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nutres.2022.07.006.

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4 Discussion

4.1 Key results

The n-6/n-3 ratio in the diet of this cohort was 6.7 (median). Higher n-6/n-3 ratios were mainly achieved by consuming a higher amount of n-6 fatty acids. In the gut microbiota analysis, the Partial Spearman's correlation detected certain associations between the n-6 or n-3 fatty acid intake and the relative abundance of specific bacteria. The analysis of gut microbiota profiles and mean relative abundance of gut bacterial taxa in the groups of NAFLD patients consuming low, moderate, or high dietary n-6/n-3 ratios could not detect significant distinctions between the groups. Regarding the severity indicating parameters, fibrosis grade and the NAFLD activity score no significant associations could be ascertained in relation to a higher dietary n-6/n-3 ratio. Therefore, in this study an high dietary n-6/n-3 ratio intake in NAFLD patients is not associated with the gut microbiota composition and disease severity [1].

4.2 Dietary intake of Omega fatty acids

This study investigated the dietary intake of NAFLD patients and the median dietary n-6/n-3 fatty acid ratio was found to be 6.7 [1]. Several other studies have also analyzed the dietary PUFA intake. Linseisen et al. presented the data obtained from two German cohorts of the EPIC (European Prospective Investigation into Cancer and Nutrition) study. Here the median ratio was closely 7, although women tend to have slightly lower values than men [69]. Therefore, the n-6/n-3 fatty acid ratio in our cohort appears consistent with the general German population ratio. However, a study with Canadian patients from Da Silva et al. observed a higher n-6/n-3 fatty acid ratio of 9.7 in NAFL patients and 12.6 in NASH patients [70]. In comparison, the median ratio in the group displaying the high dietary n-6/n-3 ratio (Q3) in our study was 9.4 [1]. López-Bautista et al., evaluated the fatty acid intake in NAFLD patients group according to mild, moderate or severe steatosis and reported a median intake of LA in g/day of 6.5, 8.1 and 8, respectively [71]. The median daily intake in gram of 6.8 g/day in our cohort can be found within the described range. However, the intake of ALA and ARA was higher in the described NAFLD cohort compared to our results. EPA and DHA are important bioactive n-3 fatty acids. Notably the research shows that the general European dietary EPA and DHA intake is relatively low [72]. Unfortunately, in this study no dietary EPA

and DHA levels were observed. In consequence we were not able to analyze any particular outcome based on the dietary EPA and DHA levels [1].

The composition of bile acids, as well as other factors like dietary fiber, saturated fatty acids or cholesterol are also known to influence NAFLD and the gut microbiota. For instance, dietary fiber may have the ability to promote the abundance of intestinal bacteria able to produce SCFA which can then exert anti-inflammatory effects [73]. On the contrary, Zhang et al. fed mice a high cholesterol diet for 14 months. He observed advanced liver fat deposit, NASH, fibrosis and an altered gut microbiota composition [74]. Eventually the high cholesterol feeding promoted the development of NAFLD-HCC. In addition, oxidative stress has been identified as a factor contributing to liver injury in NAFLD. In liver cells, a higher dietary intake of saturated fatty acids can lead to additional endoplasmic reticulum (ER) stress [75]. Nonetheless, except the dietary protein intake, no significant distinctions in the group comparison (Q1-Q3) concerning the other dietary macro-and micronutrients could be detected [1].

Multiple recommendations about the proper intake of n-6 and n-3 PUFA have been published. This study cohort displayed an intake for LA and ALA of 3.9 E% and 0.4 E%, respectively [1]. The recommendation as proposed by the German nutrition association (DGE) of the LA (n-6) and ALA (n-3) intake is 2.5 energy percent (E%) and 0.5 E%, respectively [76]. This would indicate an increased dietary LA intake and a slightly decreased dietary ALA intake in our study cohort compared to the recommended intake which explains a higher n-6/n-3 fatty acid ratio. The French Food Safety Agency adds to keep a minimum dietary intake of LA around 2 E% and to limit the maximum intake in order to keep an n-6/n-3 fatty acid ratio under 5 [77]. Furthermore, an appropriate LA and ALA intake of 4 E% and 0.5 E%, respectively is suggested by European guidelines [78]. In cardiovascular disease the adequate intake of EPA plus DHA is set at 250 mg/day [78]. Interestingly, the Food and Agriculture Organization of the United Nations set the span for a reasonable intake of LA between 2.5–9 E% and suggest an appropriate LA intake of 2-3 E% and [1,79]. It seems to remain unclear how to define a maximum intake of n-6 fatty acids. However, guidelines seem to agree on an adequate intake for ALA around 0.5 E% and an appropriate intake of LA around 2 E% [1].

4.3 Omega fatty acids and NAFLD

According to our data no significant associations could be detected when comparing the n-6/n-3 ratio in the diet with parameters indicating disease severity like ferritin, ALT, CAP, transient elastography (kPa) as well as the fibrosis stage (0-4) and NAFLD activity score (NAS 1-8). Therefore, we did not observe an association between a higher dietary n-6/n-3 ratio and disease severity when comparing our groups (Q1-Q3) of NAFLD patients [1].

Interestingly, several other studies could not prove an association linking the n-6/n-3 fatty acid ratio with NAFLD disease severity. For example, Johnson et al. reviewed randomized controlled intervention studies including healthy adults to clarify the influence of dietary LA on inflammatory markers. The review could not confirm a link between increased dietary LA and higher levels of inflammatory markers [80]. Furthermore, a study of Enos et al. could not prove an attenuation of NAFLD progression due to a reduced n-6/n-3 fatty acid ratio. This research fed mice a diet high in fat with different n-6/n-3 fatty acid ratios. Not only did all mice develop the same degree of hepatic steatosis but they were also not able to detect any changes in tight junction protein gene expression as a sign of a disrupted intestinal barrier [81]. The authors discussed how eventually using ALA and not EPA and DHA to decrease the ratio could have influenced the study outcome. Furthermore, research shows not all n-6 fatty acids show negative health implications. In fact, dihomo-γ-linolenic acid (n-6) has even shown anti-inflammatory properties [82]. Considering the effects of individual n-6 PUFA, it becomes more difficult to assign negative health implications to the whole group of n-6 fatty acids.

Another study evaluated the erythrocyte n-3 fatty acid concentration in older adults. In fact, they found a risk reduction of NAFLD with higher abundance of erythrocyte n-3 fatty acids. However, this association was only significant in women and not in men indicating a gender-dependent effect of PUFA in this study [83]. Interestingly, one study with patients exhibiting type 2 diabetes and high C-peptide levels even demonstrated that low n-6/n-3 fatty acid ratios may increase the NAFLD risk [84]. In agreement, Mäkelä et al. reported a lower risk for the development of NAFLD with higher serum total n-6 fatty acid and LA levels [85]. Even if more studies are needed to clarify the findings, all this suggests a complex function of PUFA, which may be dependent on many other factors such as gender, age and comorbidities. In particular, the properties of the

individual n-3 and n-6 PUFA like EPA, DHA and ARA in contrast to the essential PUFA like ALA and LA need to be clarified.

4.4 Omega fatty acids and gut microbiota

The results in our research did not display any associations concerning an increased dietary n-6/n-3 ratio and the composition of gut microbiota [1]. Selmin et al. also investigated if the intake of n-6 fatty acids in mice has an impact on the gut microbiota and observed an association with a gut microbiota dysbiosis. For example, they noticed a depletion in *Lachnospiraceae* (family) and an increased abundance of *Deferribacteraceae* (family) and *Porphyromonadaceae* (family) which are also found in patients with inflammatory bowel diseases [73]. In agreement, proinflammatory and LPS-producing bacteria, like Proteobacteria, were increased due to an elevated n-6/n-3 ratio in mice as part of a transgenic mouse model [60]. Moreover, another study examined the ileum of older mice and found a correlation between increased amounts of dietary n-6 and bacterial overgrowth. In addition to the bacterial overgrowth, they observed that the group with extra n-6 fatty acids in the diet showed an increased Firmicutes to Bacteroidetes ratio and increased bacterial infiltration across the intestinal barrier [86]. Nonetheless, these findings were obtained using animal models and the exact processes in the human body have not yet been researched in sufficient detail. Miao et al. investigated the relationship between the gut microbiota, the n-6 fatty acid level in erythrocytes and type 2 diabetes in Chinese adults. Surprisingly they noticed an association between lower levels of erythrocyte y-linolenic acid (n-6) and a higher alpha-diversity of gut microbiota [1,87]. However, it needs to be acknowledged that due to the different metabolic and environmental factors, a comparison between Asian and European cohorts can be difficult.

Current studies theorize that n-3 fatty acids can help to promote a healthy gut microbiota composition [88]. In this context Ghosh et al. observed that a dysbiosis in mice caused by an increased consumption of n-6 fatty acids could indeed be restored by adding more n-3 fatty acid to the diet. At the same time they noticed an increased mortality in the situation of an acute gut infection. This was most likely caused by the impairment of LPS dephosphorylation by n-3 fatty acids during acute gut infection [65]. This indicates that the definite effects of the individual fatty acids require further clarification and again shows that other factors in the hosts metabolism and environment can have an influence on these pathways.

The dietary n-6 or n-3 fatty acids correlated negatively and positively with the abundance of certain bacterial taxa in our heat map analysis. Notable significant correlations (**P<0.01) could especially be displayed with the intake of n-6 fatty acids [1]. For instance, positive correlations were detected between *Catenibacterium* (genus), *Dialister succinatiphilus* (species) and *Lactobacillus ruminis* (species) and dietary n-6 fatty acids intake. Moreover, *Clostridium* (genus) as well as *Roseburia intestinalis* (species) displayed negative correlations with dietary n-6 fatty acids intake [1].

The abundance of Catenibacterium (genus) has recently shown beneficial correlations to obesity indicating parameters as well as inflammatory markers. In fact, a positive correlation with high-density lipoproteins (HDL) and a negative correlation with body weight, low-density lipoproteins (LDL), and TNF-α has been observed [89]. Thus, an increase in the abundance of *Catenibacterium* (genus) could be advantageous. In the comparison of healthy patients and patients with hepatitis B virus infection, *Dialister succinatiphilus* was detected in the healthy patients, indicating that they may initiate an appropriate immune response [1,90]. However, Yan et al have reported an association between increased *D. succinatiphilus* and high levels of visceral adipose tissue [91]. This indicates that the exact function of *D. succinatiphilus* in the human gut still needs to be investigated. Yang et al. recently reported many favorable effects of *Lactobacillus ruminis* in mice with initially induced colitis. The positive effects of *L. ruminis* were related to the secretion of proinflammatory cytokines, production of SCFA and a restored gut microbiota dysbiosis[92]. This indicates a probable positive outcome with increased levels of *L. ruminis.* The genus *Catenibacterium* harbors beneficial strains that may even have anti-inflammatory associations as well as pathogenic strains able to produce toxins. The function of *Catenibacterium* remains complex and further research is needed to investigate its role in gut microbiota dysbiosis [93]. Another study examined the role of *Roseburia intestinalis* in mice with colitis. *Roseburia intestinalis* and its flagellin have been found to decrease inflammation via the secretion of anti-inflammatory factors. In addition, the production of SCFA helped to maintain gastrointestinal health [94]. Accordingly, a depletion of *Roseburia intestinalis* may be disadvantageous. Although the exact functions of the gut bacteria remain a current topic of research, these findings suggest a significant role of gut microbiota in modulating disease activity.

4.5 Limitations of the study

Several limitations have to be acknowledged for this study. First, the use of food recording and reporting physical activity in clinical studies can be challenging. A selection bias to conduct patients already interested in diet and activity cannot be ruled out. Social desirability bias and the lack of time and motivation may influence the recorded dietary intake as well as activity. If patients are asked to concentrate on the detailed dietary intake every day, this may lead to more awareness and change in the patients' dietary habits. Although patients were given detailed instructions on how to fill out the food and activity diary, miscalculation of portions represents a possible limitation. Dietary underreporting has been observed in dietary assessment. This is particularly noticeable with obese subjects and was also noticed in our research [1].

The absence of heathy control patients is a major limitation in this study. Another limitation is the collection of data on fatty acids through dietary recording. The serum omega fatty acid concentration or the concentration of omega fatty acid in the cell membrane of red blood cells represent more accurate methods to detect the omega fatty acid levels in humans but were not determined in our research. Furthermore, no conclusions can be drawn about the effects of the individual PUFAs alone. A differentiation between the various the n-6 and n-3 fatty acids is not reflected in the -6/n-3 fatty acid ratio. At last, this study design does not allow any conclusions about causality. We cannot determine if the gut microbiota represent the major influence on the omega-fatty acids or whether the fatty acids influence the gut microbiota [1].

The advantage of this research is the cohort with a large number of NAFLD patients, including 63 biopsy-proven NAFLD cases. The Cohort is clinically well described with detailed anthropometric, demographic and clinical data [1].

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6 Anhang

10/2018 **Annahme als Doktorandin**

Beginn der Studienplanung mit Priv.-Doz. Dr. med. Münevver Demir

Einarbeitung in das Thema, Literaturrecherche

10/2018- 04/2019 **Datenerhebung und schriftliche Dokumentation** Daten von 180 NAFLD Patienten, davon 63 mit Leberbiopsie, welche sich im Zeitraum von 07/2015 und 12/2018 in der gastroenterologischen Ambulanz der Uniklinik Köln vorstellten, wurden retrospektiv im krankenhausinternen Informationssystem ORBIS erhoben. Alle Patientenakten wurden gesichtet und folgende Merkmale schriftlich in einer Microsoft®-Excel®-Tabelle dokumentiert:

- Alter, Geschlecht, Gewicht (kg) und Größe (cm)
- PNPLA-3 Genotyp
- Metabolische Risikofaktoren (Übergewicht, Hypertonus, Dyslipidämie, Metabolisches Syndrom, BMI (kg/m²), Taillen- und Hüftumfang (cm))
- Blutdruck (mmHg), Puls (/min)
- Alkoholkonsum (g/Tag)
- Begleiterkrankungen und Medikation
- Labordiagnostik:
    - Elektrolyte
    - Albumin (g/l), AST (U/l), ALT (U/l), GGT (U/l), AP (U/l), CHE (U/l), Bilirubin gesamt/direkt/indirekt (mg/dl), LDH (U/l)
    - Kreatinin (mg/dl), Harnstoff (mg/dl), Harnsäure (mg/dl), GFR (ml/min)
    - Eisen (µmol/l), Transferrin (g/l), Ferritin (µg/l), Transferrinsättigung (%)
    - Amylase (U/l), Lipase (U/l)

- Triglyceride (mg/dl), Cholesterol, HDL (mg/dl), LDL (mg/dl)
- BSG (mm/h), Serumelektrophorese
- WBC (x10^9/l), Erythrozyten (x10^{12}/l), Hämoglobin (g/dl), Hämatokrit (%), MCV (fl), Thrombozyten (x10^9/l)
- Quick (%), INR, APTT (sek)
- Insulin (mU/l), HBA1c (%)
- TSH (mU/l), T3, T4 (ng/l)
- AFP (kU/l), Alpha-1-Antitrypsin (g/l), Ceruloplasmin (g/l), Kupfer (µmol/l)
- CRP (mg/l)
- IgG, IgA, IgM, IgE (g/l)
- Autoimmunmarker (ANA, ASMA, SLA, LKM LC1, AMA, ANCA, pANCA, Transglutaminase Ak, Antigliadin, Endomysium Ak)
- Virusdiagnostik (HAV, HEV, HBsAg, HBV, HCV Ak, HCV RNA, HIV)

▪ Lebersonografie (NAS Score) und Fibroscan (kPa, fibrosis stage)

▪ Leberbiopsie

Auswertung und schriftliche Dokumentation Ernährungs- und Aktivtätstagebücher Daten aus den Ernährungstagebüchern von 101 NAFLD Patienten wurden mit Hilfe von EbisPro Professional Software 2016 ausgewertet und schriftlich in einer Microsoft®-Excel®-Tabelle dokumentiert

05/2019 **Posterbeitrag:** Digestive Disease Week® - DDW 2019, 18. -21. Mai, San Diego, CA, USA

Heinzer K, Lang S, Farowski F, Wisplinghoff H, Vehreschild MJGT, Krawczyk M, Nowag A, Lammert F, Martin A, Goeser T, Steffen H-M, Demir M. No significant impact of fructose consumption on gut microbiota composition in a German cohort of patients with biopsy-proven nonalcoholic fatty liver disease (NAFLD), 2019

10/2019 **Vorstellung eines Abstracts:** Vizeralmedizin 2019 Gastroenterologie Viszeralchirurgie, 2. – 5. Oktober 2019 RMCC – RheinMain CongressCenter, Wiesbaden, Deutschland Heinzer K, Lang S, Farowski F, Wisplinghoff H, Vehreschild MJGT, Krawczyk M, Nowag A, Lammert F, Martin A, Goeser T, Steffen H-M, Demir M. Kein signifikanter Einfluss des Fruktosekonsums auf die Zusammensetzung der Darmmikrobiota bei Patienten mit bioptisch gesicherter nichtalkoholischer Fettlebererkrankung. 2019

10/2019-11/2020 **Literaturrecherche**

Erster Entwurf eines Manuskripts zur Publikation: „Dietary omega-6/omega-3 ratio is not associated with gut microbiota composition and disease severity in patients with non-alcoholic fatty liver disease”

07/2022 **Publikation eines Artikels** in der Fachzeitschrift „Nutrition Research“.

Heinzer K, Lang S, Farowski F, Wisplinghoff H, Vehreschild MJGT, Martin A, Nowag A, Kretzschmar A, Scholz CJ, Roderburg C, Mohr R, Tacke F, Kasper P, Goeser T, Steffen H-M, Demir M. Dietary omega-6/omega-3 ratio is not associated with gut microbiota composition and disease severity in patients with non-alcoholic fatty liver disease, Nutrition Research 2022; https://doi.org/10.1016/j.nutres.2022.07.006

08/2022 **Verfassung der Dissertationschrift**