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Randomized Control Trials

Increase in PUFA and protein, and decrease in carbohydrate intake improves liver fat in 12 months and the role of weight loss as a mediator: A randomized controlled trial

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SUMMARY

Background & aims: Recently, a beneficial effect of high intake of unsaturated fatty acids (UFA) and protein on intrahepatic lipids (IHL) was demonstrated over 12 months within a randomized controlled trial (the NutriAct trial). We now aimed to explore the specific macronutrient components driving this IHL improvement within this trial in middle-aged and elderly subjects (50–80 y) at risk for age-related diseases.

Methods: The NutriAct trial (n = 502) analyzed the effect of a high-protein and high-UFA diet on age related diseases including fatty liver disease. Individuals who completed 3-day food records with available IHL data both at baseline and at month 12 were included in this analysis. The impact of each macronutrient (E%) on IHL (measured by magnetic resonance spectroscopy) was analyzed by linear regression analyses and mediation analysis. Adherence in the intervention group was defined as intake at month 12 of ≥1 g protein/kg bodyweight or ≥25%E UFA intake; in the control group it was defined as intake of ≥15%E protein or ≥17%E UFA.

Results: 248 participants were included in the analyses (34 % male, median age 66 y). Although BMI changed similarly in both groups within 12 months (mean change −0.41 kg/m^2 in the control and −0.70 kg/m^2 in the intervention group, p within groups <0.001, p between groups = 0.09), IHL improved more strongly in the compliant intervention participants than in compliant controls (estimate of relative change 0.21 % (95 % CI 0.01, 0.40), p = 0.03). Participants with stronger increase in protein and PUFA intake and a greater decrease in carbohydrate intake showed a stronger improvement in IHL (estimate for linear relative change −0.04 % (95%CI -0.06, −0.02), estimate 4th quartile vs. 1st quartile −0.40 % (95%CI -0.65, −0.16), and 0.32 % (95%CI 0.05, 0.59), respectively). These associations were partially mediated by BMI changes. Increase in PUFA intake was also directly associated with IHL

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improvement independently of BMI changes (estimate for linear relative change −0.03 % (95%CI -0.05, −0.01)).
Conclusions: Beneficial effects of increased protein and decreased carbohydrate intake on IHL are mediated by BMI changes in middle-aged and elderly subjects. The effect of high PUFA intake on IHL improvement was partly independent of weight loss. These results give insight into the understanding of a macronutrient specific effect on IHL changes in a long-term dietary intervention.
Clinical trial registration: The trial was registered at German Clinical Trials Register (drks.de) as DRKS00010049.

Abbreviations

AFLD	alcoholic fatty liver disease
CVD	cardiovascular diseases
GNDB	German Nutrient Data Base
BMI	body mass index
FGF21	fibroblast growth factor 21
GNS	German Nutrition Society
IHL	intrahepatic lipids
IQR	interquartile range
MUFA	monounsaturated fatty acids
MRS	magnetic resonance spectroscopy
MASLD	metabolic dysfunction-associated steatotic liver disease
PUFA	polyunsaturated fatty acids
SFA	saturated fatty acids
UFA	unsaturated fatty acids
VIF	variation inflation factor

1. Introduction

It is estimated that 20 % of the world's population suffers from metabolic dysfunction-associated steatotic liver disease (MASLD), previously called non-alcoholic fatty-liver disease [1]. In northern Germany its prevalence was reported to be as high as 42 % in the general population [2]. The subsequent stage of MASLD, steatohepatitis, defined as the evidence of liver injury, inflammation and fibrosis, is the most rapidly increasing reason for liver transplantation [1,3]. In addition, persons with MASLD present 26 % higher health care costs [4]. The rapid increase in incidence and prevalence of MASLD is mainly driven by the obesity pandemic and its associated factors, including hypertriglyceridemia, dyslipidemia, sedentary behavior, type 2 diabetes, and metabolic syndrome [1,5]. Interestingly, current evidence indicates that the association between metabolic syndrome and MASLD may be bidirectional, meaning that increased liver fat could also augment features of metabolic syndrome [5]. Importantly, both MASLD and steatohepatitis can be reverted by changes in lifestyle, including dietary changes [5].

The effect of diet on liver fat (intrahepatic lipids, IHL) seems to be dependent on weight loss induced by reduced total energy intake [6]. However, there are apparently also some macronutrient-specific effects. The effect of carbohydrate intake on IHL content seems to be different in a hypo-from in an iso- and hyper-energetic diet [7–11]. Hypoenergetic diets show a low-carbohydrate diet to reduce IHL more intensely than a traditional low energy diet in short periods (up to 2 weeks), but after that time there seems to be no difference between the effect of those two diets in changes in IHL [7–9,12]. In contrast, isoenergetic trials (up to 4 weeks) showed IHL to decrease in low-fat-high-carbohydrate diets and increase in high-fat-low-carbohydrate-diets [7,10,11,13]. The balance of saturated fatty acids (SFA), unsaturated fatty acids (UFA) and proteins seems of relevance, too [6]. Overfeeding with SFA but not with PUFA seems to increase IHL content more markedly than overfeeding with carbohydrates [7,14]. Furthermore, a 3-week hypoenergetic diet high in proteins showed to decrease liver fat compared to a hypoenergetic low-protein diet in subjects with obesity [15]. Additionally, a beneficial effect on liver fat was demonstrated by short-term isoenergetic interventions focusing on increased protein intake [16–19].

However, the actual body of literature studying isocaloric dietary interventions and liver fat changes is extremely limited, includes mainly short-term dietary interventions (up to 10–12 weeks) and limited sample sizes (less than 100 participants) [10,11,13]. Thus, the effect of dietary composition on liver fat, including fat quality, especially in the long term, is not sufficiently clarified yet. Recently we analyzed the long-term effects of a dietary intervention based on high-protein and high-UFA intake in a large sample size of elderly subjects (NutriAct trial) [20]. Thereby we demonstrated a beneficial effect of this diet on liver fat over 12 months in those subjects who were more adherent to the proposed intervention [21]. This was independent of weight changes [21]. Given the modulation of fat composition as well as changes in protein and carbohydrate intake, the specific impact of the different macronutrient components on IHL is not clear.

We now aimed to investigate the specific impact of the different macronutrients on the observed effect on IHL content. Secondly, we aimed to explore the role of small weight changes as a potential mediator of these associations. For population prevention strategies as well as for clinical practice, finding a dietary component that can reduce IHL and is implementable for long periods would be essential for improving current strategies for primary prevention as well as for treatment of MASLD.

2. Materials and methods

2.1. Study design

The NutriAct is a multi-centered randomized controlled trial that took place from 2016 to 2021 in Berlin and Potsdam. Participants (502 at baseline) were followed during the entire intervention period of 36 months by means of phenotyping visits and phone interviews. The NutriAct Trial was approved by the Institutional Review Board of the Charité Medical School. The study was conducted in accordance with the Declaration of Helsinki. All participants provided informed consent. Recruitment and study visits were performed in two different study sites: at the Metabolic Research Unit of the Clinic of Endocrinology, Diabetes and Metabolism, Charité – Universitätsmedizin Berlin and the Human Study Center of the German Institute of Human Nutrition Potsdam-Rehbruecke (DIfE). Phenotyping included a medical interview,

physical examination, blood sampling, assessment of food intake by food diaries, and assessment of liver fat by magnetic resonance spectroscopy (MRS). After initial phenotyping, an adaptive randomization was performed to assign participants either to intervention group focusing on NutriAct food pattern or to control group focusing on usual care. Sample size was calculated for a log-rank test in accordance with the primary NutriAct endpoint. Details of allocation and sample size calculation are described in the Supplementary Appendix. For the present study, data from baseline and from month 12 were included.

2.2. Participants and setting

Participants were included if they were aged between 50 and 80 years, had an intermediate risk profile for age-related diseases, which included cardiometabolic diseases, reduced cognition and/or impaired muscle strength. Detailed data collection information as well as inclusion and exclusion criteria are described elsewhere [20]. In brief, eligible men and women were included if they had at least one risk factor for unhealthy aging as follows: arterial hypertension (systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or medical history of hypertension or use of antihypertensive medication), known cardiovascular disease (including stroke, myocardial infarction, coronary heart disease and peripheral artery disease), heart failure (defined by New York Heart Association ≥ II or N-terminal pro-B-type natriuretic peptide >300 ng/l in absence of atrial fibrillation), cognitive impairment (Montreal Cognitive Assessment Score <26) or decreased physical function (Short Physical Performance Battery Score <10). Candidates were excluded if they had one of the following conditions: acute severe CVD, including unstable CVD, a recent cardiovascular event or surgery ≤3 months, uncontrolled arterial hypertension (systolic blood pressure >180 mmHg and/or diastolic blood pressure >110 mmHg), type 1 diabetes, insulin-dependent type 2 diabetes, life expectancy <1 year, prevalent cancer, severe hepatic or renal diseases (GFR <50 ml/min/1.73 m^2), severe gait disturbance diseases (for example Parkinson's disease, stroke with paresis), severe systemic infection, severe immune diseases, severe food allergy, severe malabsorption disease, oral glucocorticoid treatment, untreated active endocrine diseases, severe psychiatric disorders, severe drug and/or alcohol abuse or known eating disorder.

From the 502 NutriAct participants included at baseline, the ones who did not fill in their food records at baseline and/or at month 12 (n = 93), who did not undergo MRS at baseline and/or at month 12 (n = 212), and the ones with significant alcohol consumption according to the sex-specific threshold of the German Nutrition Society (GNS) recommendation at baseline (30 g/d for males and 20 g/d for females, n = 57) were excluded from the present analyses (exclusion reasons overlap). A total of 248 participants (124 control and 124 intervention) were included in the present analyses (Supplemental Fig. 1). Reasons for not undergoing an MRS both at baseline and at after 12 months were either medical contraindications, refusal from the participant or technical problems.

2.3. Intervention and control

2.3.1. Intervention: NutriAct dietary pattern

Detailed information on the NutriAct dietary pattern was already described elsewhere [20]. In summary, the NutriAct intervention consists of a daily intake of a) 35–40 % energy (%E) total fat with ≤10 % of total daily energy intake (%E) saturated fatty acids (SFA), 15–20%E MUFA and 10–15%E PUFA. A reduced supply of saturated SFA, achieved by replacing them with UFA, consuming rapeseed oil instead of butter and cream, and supplying them with specific designed NutriAct foods (e.g. pasta enriched with fiber and protein and flaxseed meals). b) 15–25%E protein, with preference to vegetable protein sources, c) 35–45%E carbohydrates with low glycemic index and d) more than 30 g fibers/day. Nutritional counselling took place 11 times in the first 12 months of intervention and was performed in groups of 4–8 participants. Intervention participants were supplied with 1 L of rapeseed oil per month, as well as fiber-enriched high-protein foods. Participants were instructed to keep their bodyweight stable over the trial [20].

2.3.2. Control group: usual care

The control group based on usual care including nutritional counselling three times within the first 12 months. During those sessions, dietary recommendations were given in accordance to the GNS [22]. This includes 30%E total fat, 55%E carbohydrates and 15%E proteins, and a consumption of 30 g fibers/d. As for the intervention group, participants were instructed to keep their bodyweight stable over the trial [20].

3. Dietary assessment

Dietary intakes were assessed by means of open food records on three consecutive days including one weekend day within 10–14 days before each visit. These data were computed according to the German Nutrient Data Base (GNDB) in PRODI® (6.5 Expert; Nutri-Science GmbH, Freiburg, Germany) and converted into intake of macronutrients [20].

4. Assessment of IHL by MRS

Magnetic resonance spectroscopy was performed on a 1.5-T whole-body scanner (Magnetom Avanto, Siemens Healthcare, Erlangen, Germany) for quantification of IHL at baseline and after 12 months. Participants were placed on the six-channel spine array coil of the manufacturer in supine position with the spine along the symmetry axis of the coil system. An additional body array coil with two segments was placed symmetrically on the chest in order to obtain nearly homogeneous signal sensitivity inside the entire liver, as well as comparable receiver characteristics on the left and right abdominal sides. To access the topography of the liver, T1-weighted gradient-echo images were recorded. Analysis of MRS data was carried out in cooperation with the Institute for Diabetes Research and Metabolic Diseases (IDM) of the Helmholtz Center Munich at the University of Tübingen. IHL are quantified by a single voxel STEAM technique (stimulated echo acquisition mode) with a voxel (volume of interest, VOI) size of 30 × 30 × 20mm^3 in the posterior part of segment 7 [23] and IHLs are given as ratio of fat (methylene + methyl resonances at 1.3 and 0.9 ppm, respectively) divided by the sum of water (at 4.7 ppm) and fat resonances, respectively [20]. MASLD was defined as IHL >5.56 % in the absence of significant alcohol intake (see thresholds above) [24].

5. Statistical analyses

Baseline participants characteristics are described as medians [interquartile range (IQR)] if continuous, and as number (percentages) if categorical. Changes in macronutrients intake (in % of total energy intake) were calculated as the absolute difference between intake at baseline and at month 12 (%E month 12 - %E baseline). These were then categorized into quartiles; the first quartile was used as the reference group. IHL data were $\log_e$-transformed and the difference between the two time points was calculated (month 12 - baseline). Absolute changes of BMI were calculated as BMI at month 12 − BMI at baseline. Spearman correlation coefficients were computed among change in macronutrients intake at baseline

and at month 12 (%E). A linear regression analysis was performed to compare changes (month 12 - baseline) in BMI and in IHL between intervention- and control-groups as well as in adherent intervention- and control-subjects (age-, sex-, baseline BMI- and baseline IHL-adjusted). Adherence in the intervention group was defined as intake at month 12 of ≥1 g protein/kg bodyweight or ≥25%E UFA intake; in the control group adherence was defined as intake of ≥15%E protein or ≥17%E UFA. From the control group, 118 participants fulfilled the criteria for adherence, while 87 participants intervention participants were considered adherent. Reasons for the chosen cutoffs are described elsewhere [21]. Boxplots were built to illustrate these changes in both groups. To explore the association between changes in macronutrients intake (linear as well as quartiles) and change in IHL, we used multivariate linear regression models adjusted for age, sex, macronutrient intake at baseline and IHL at baseline (Model 1), and additionally adjusted for change in BMI (Model 2). For these analyses participants were regarded independently of randomized intervention arm. We analyzed the variation inflation factors (VIF) of models that contained more than one macronutrient in order to control for multicollinearity. Models with VIF greater than 2 for any variable were considered not appropriate and are not shown.

To test the hypothesis that changes in BMI could mediate the association between changes in macronutrients intake and changes in liver fat (Supplemental Fig. 2), we conducted causal mediation analyses using the approach proposed by Valeri and VanderWeele [25,26,]. The R package "*mediate*" was used. Therefore, separate models including one of the macronutrients (carbohydrates, protein or PUFA) as well age, sex, baseline IHL, baseline respective macronutrient intake as well as BMI changes were built. Due to multicollinearity, a multivariate model containing all three macronutrients was not built. A 2-sided p value < 0.05 denoted statistical significance; all statistical analyses were conducted in R (version 4.0.0) for Windows.

6. Results

From the total of 248 NutriAct participants included in this study, 34 % were males, median age at baseline was 66 [IQR 62, 71] years and 42.8 % of participants had obesity (BMI≥30 kg/m^2). MASLD was prevalent in 34.7 % of participants at baseline. One hundred and twenty-four participants were randomized to the control and 124 to the intervention group. Baseline characteristics were similar between participants from both groups (all p > 0.05) (Table 1). Baseline characteristics of the 248 participants included in this study did not differ substantially from the participants excluded from the current analyses (n = 254) (Supplemental Table 1).

At baseline, increased fiber intake was associated to lower IHL (estimate −0.02 % (95%CI -0.04, −0.01), p = 0.024 after adjustment for age, sex and baseline BMI). No other cross-sectional associations were found between baseline macronutrient intake and IHL (data not shown). A substantial increase of PUFA (intervention +85 % vs. control +24 %; p < 0.001 for between group difference) and of protein (intervention +17 % vs. control +7 %; p < 0.01 for between group difference) was observed in the intervention group, while intake of carbohydrate (intervention −7% vs. control −0.2 %; p < 0.01 for between group difference) declined. Changes in total energy intake (kcal/kg bodyweight) did not differ between the intervention and control group over time (intervention −92 kcal vs. control −182 kcal, p for between groups difference = 0.16). Several correlations were found between the changes in macronutrients from baseline to month 12 (ρ range −0.75, 0.83) (Supplemental Fig. 3). In accordance with previously reported results [22],

Table 1
Baseline participants' characteristics.

Characteristics	Total (n = 248)	Control Group (n = 124)	Intervention Group (n = 124)	P-Value
Males	83 (33.5)	40 (32.3)	43 (34.7)	0.788
Age (years)	65.50 [62.00, 71.00]	66.00 [61.00, 71.00]	65.00 [63.00, 70.00]	0.693
BMI (kg*m^{-2})	29.11 [25.93, 32.52]	29.09 [26.04, 32.25]	29.39 [25.88, 32.54]	0.767
Normal (18–24.9 kg*m^{-2})	46 (18.5)	24 (19.4)	22 (17.7)	0.246
Overweight (25–29.9 kg*m^{-2})	96 (38.7)	50 (40.3)	46 (37.1)	
Obesity I° (30–34.9 kg*m^{-2})	80 (32.3)	37 (29.8)	43 (34.7)	
Obesity II° (35–39.9 kg*m^{-2})	22 (8.9)	13 (10.5)	9 (7.3)	
Obesity III° (≥40 kg*m^{-2})	4 (1.6)	0 (0.0)	4 (3.2)	
ASCVD	43 (17.3)	21 (16.9)	22 (17.7)	1.000
Diabetes mellitus	44 (17.7)	20 (16.1)	24 (19.4)	0.618
Arterial hypertension	211 (85.1)	106 (85.5)	105 (84.7)	1.000
Smoking status (current, %)	19 (7.7)	7 (5.6)	12 (9.7)	0.340
Alcohol intake (g*d^{-1})	1.88 [0.06, 8.73]	1.20 [0.05, 8.66]	2.67 [0.07, 9.10]	0.315
Dietary pattern				
Protein intake (%E*d^{-1})	16.01 [14.36, 18.39]	16.31 [14.87, 18.49]	15.72 [13.93, 18.14]	0.056
Protein (g*kg body weight^{-1})	0.95 [0.75, 1.13]	0.94 [0.74, 1.16]	0.95 [0.76, 1.11]	0.876
Carbohydrates intake (% E*d^{-1})	41.42 [36.19, 45.39]	41.02 [36.40, 45.11]	41.62 [36.11, 45.53]	0.691
Total fat intake (% E*d^{-1})	37.88 [33.12, 42.84]	37.58 [33.16, 42.52]	38.73 [33.12, 42.89]	0.570
Saturated fatty acid intake (% E*d^{-1})	15.66 [13.32, 18.20]	15.68 [13.75, 18.22]	15.53 [12.94, 18.20]	0.607
MUFAs intake (% E*d^{-1})	12.91 [11.37, 15.13]	13.02 [11.32, 15.24]	12.72 [11.42, 14.88]	0.628
PUFAs intake (% E*d^{-1})	5.70 [4.54, 7.47]	5.44 [4.43, 7.71]	5.79 [4.60, 7.30]	0.753
Fiber intake (g*d^{-1})	21.79 [17.56, 27.22]	21.63 [16.52, 26.08]	21.79 [18.39, 27.90]	0.322
Energy (kcal*d^{-1})	1882.26 [1574.03, 2243.20]	1865.91 [1564.05, 2232.72]	1941.43 [1602.68, 2250.76]	0.354
Energy/bodyweight (kcal*kg^{-1}*d^{-1})	23.19 [19.49, 27.86]	22.93 [19.18, 27.88]	23.64 [19.56, 27.77]	0.772
MRS parameters				
IHL (%)	3.79 [1.41, 7.70]	3.62 [1.61, 7.32]	3.95 [1.35, 8.23]	0.638
NAFLD	86 (34.7)	40 (32.3)	46 (37.1)	0.505

Data are described as median [interquartile range] for continuous variables and as n (%) for categorical variables. P-values for the differences between intervention and control groups. Dietary components are described as % from the total daily energy intake, except for fiber, which is described as g/d. Protein is also shown as g/kg bodyweight. Abbreviations: BMI body mass index, ASCVD atherosclerotic cardiovascular diseases, MRS magnetic resonance spectroscopy, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids, IHL intrahepatic lipids, NAFLD non-alcoholic fatty liver disease.

findings in the subgroup with IHL and data on food intake revealed a reduction of IHL as well as of BMI in both groups which was not statistically significantly different between groups (Supplemental Figure 4A and B). However, when considering adherent-control and -intervention participants only, IHL improvement was more pronounced in the adherent intervention group (Supplemental Fig. 4C), and the decrease in BMI was more pronounced in the intervention group (Supplemental Fig. 4D).

Figure 1 shows the associations between changes in intake of macronutrients and relative changes in IHL. A greater increase in protein and in PUFA intake, as well as a stronger reduction in carbohydrate intake was associated with improvement of IHL. This could be also confirmed by linear regression analyses (Supplemental Table 2). Supplemental analyses additionally adjusted for a) baseline energy intake and b) baseline and month 12 energy intake were carried out, where no material changes in the results were observed (Supplemental Table 3).

Stratified analyses by sex showed that the effect of protein intake changes on IHL were only statistically significant in male but not in female, while of carbohydrates and PUFA were statistically significant in female and not in male. Effect of carbohydrate, protein and PUFA intake changes on IHL were similar among in participants with obesity and without (Supplemental Table 4).

As there was a markedly attenuation of macronutrients` effects on IHL upon adjustment for changes in BMI (Model 2), we hypothesized that changes in BMI could be at least in part a potential mediator of these associations. Mediation analyses are described in Table 2 and show a consistent mediation effect of changes in BMI in the association between the increase in protein and PUFA, as well as the decrease in carbohydrate intake and IHL improvement. Most interestingly, an additional direct effect between increase in PUFA intake and improvement in IHL could be found ($p = 0.01$), which was apparently independent of BMI changes.

7. Discussion

We have previously shown that a successful implementation of a diet focusing on high intake of UFA, protein, fiber as well reduced consumption of carbohydrate [27] results in an improvement in liver fat [21]. The specific macronutrient changes that drove IHL improvement were yet still unclear. The main findings of the present analyses are that the increase in dietary content of protein and PUFA, as well as the dietary decrease of carbohydrate was associated with IHL improvement over a 12-month period in middle-aged and elderly participants. In this context, a direct determinant of IHL improvement was only observed for the increased

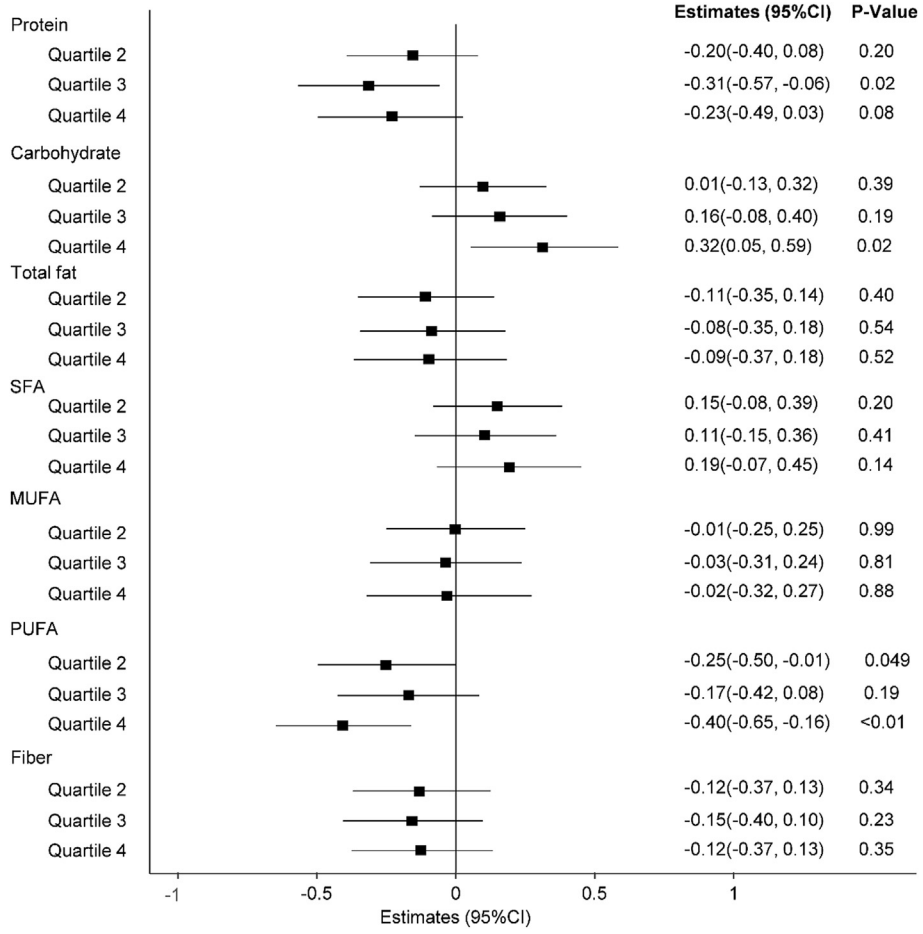

Fig. 1. Associations between changes in intake of macronutrients and changes in liver fat over 1 year (n = 248).
Legend Fig. 1. Estimates and 95 % confidence intervals of the associations between changes in macronutrients intake and relative changes in IHL over 12 months in 248 NutriAct participants. Reference is quartile 1. Models adjusted for age, sex, baseline intake of the respective macronutrient, baseline IHL and BMI change (BMI at month 12 − BMI at baseline). Abbreviations: CI confidence interval, SFA saturated fatty acid, MUFA monounsaturated fatty acid, PUFA polyunsaturated fatty acid. Changes in macronutrient intake calculated as % of total daily energy intake (%E) at month 12 − at baseline, except for fiber, where g/d was used.

Table 2
Direct and indirect effects of changes in macronutrient intake on intrahepatic lipid content. Mediator: absolute change in BMI.

	Direct effect		Indirect effect	
	Beta coefficient (95 % confidence interval)	P-Value	Beta coefficient (95 % confidence interval)	P-Value
Protein	−0.02 (-0.04, 0.01)	0.27	−0.02 (-0.04, −0.01)	<0.001
Carbohydrate	0.01 (-0.01, 0.02)	0.12	0.01 (0.01, 0.01)	<0.01
PUFA	−0.03 (-0.05, −0.01)	0.01	−0.01 (-0.02, −0.01)	<0.001

Mediator: change in BMI (BMI month 12 - BMI baseline). Abbreviations: PUFA polyunsaturated fatty acids. Direct effect corresponds to the association between the variable described in the first column and intrahepatic lipid (IHL) change; the mediated effect represents the same association, but considering change in BMI as a mediator (Supplemental Fig. 2). Model adjusted for age, sex, baseline IHL and baseline respective macronutrient intake.

intake of PUFA in the mediation analyses, although parts of the PUFA effects were also weight-loss-mediated. In contrast, the beneficial effect of increased protein and decreased carbohydrate intake on liver fat predominantly depends on concomitant weight loss. These data shed light into the different role of an increase in protein and PUFA intake and decrease in carbohydrate intake on liver fat in the performed 12 months dietary intervention within the NutriAct trial.

The current recommendations of the German nutrition society include daily 0.8 g/kg bodyweight of protein for adults between 19 and 65 years, and 1 g/kg bodyweight for people older than 65 years [28]. Total fat should represent 30%E and carbohydrates more than 50 % of the daily total energy intake [28]. Although we know that the compliance in the intervention group was good [27], some participants did not reach the recommended targets. Therefore, we created the intervention compliant and control compliant groups, hoping to be able to address participants who reached the aimed targets for the most important macronutrients in both groups [21]. In parallel, analyses on changes in macronutrients intake may be helpful to explore the potential effects of improvement of diet also when not achieving certain human-defined targets.

To date, changes in liver fat over time have mostly been studied in hyper- or hypocaloric or relatively short-term studies [7,29,30]. The current evidence on hypo- and hypercaloric studies shows energetic intake to be related to liver fat. In contrast, isocaloric studies generated conflicting results, are heterogeneous in terms of type of intervention and study participants, were mostly no longer than 10 weeks in duration and included small sample sizes [7,29,30]. Some of them compared low-fat vs. high-fat diets, without specifying fat subtypes i.e. MUFA, PUFA or SFA, a fact potentially affecting the results [11,13,31]. Actually, one rather isocaloric intervention reported a decline in liver fat by a high-PUFA (n-6 PUFA) diet in a 10-week intervention (n = 67) [32]. However, adjustment for weight changes had no effect in the results in this trial [32]. This is also supported by our analysis, as the effect of increase in PUFA intake on liver fat was partly mediated by very small BMI changes observed in our cohort. However, at least for PUFA, a direct effect was revealed as well. This indicates a beneficial effect of increase in PUFA intake on liver fat also without any alterations of body weight.

Also, dietary supplementation with omega-3 PUFA was already shown to demonstrate beneficial effects on IHL in persons with prevalent MASLD [33–35]. Although the majority of the population analyzed in our study did not have MASLD at baseline, this fact might also affect our findings, as several of the products applied to subjects of the intervention group reflect a balance of omega-3 and omega-6 PUFA. Currently, we can only speculate about the underlying mechanisms of the reported associations. The reported effect of a high-PUFA diet on weight loss and on improvement of liver fat may be partially driven by brown fat compartment and its thermogenic effects, which results in increased energy expenditure [36,37]. Animal models have shown n-3 PUFA to stimulate brown adipose tissue thermogenesis [38,39]. In adult humans, 20 % of the energy expenditure is estimated to come from non-shivering adaptive thermogenesis from metabolic active brown adipose tissue [38]. Human studies reported a higher post-prandial thermogenesis after a PUFA-rich meal compared to SFA- or MUFA-rich meals [40–42]. This link may represent a potential pathophysiological explanation and should be clearly evaluated in future studies. Alternatively, the decrease in hepatic fat accumulation under high-PUFA intake could be due to a decrease in *de novo* lipogenesis, as 6-n-PUFA is a suppressor of lipogenic gene expression [43]. In line with such an assumption, the inhibitory impact of increased PUFA intake on hepatic gene expression of lipogenic enzymes have been repeatedly reported in animal as well as in human studies [15,44–48]. Further human intervention studies investigating the effect of increased PUFA-intake in the expression of lipogenic enzymes in the liver are still needed.

Our results, showing no associations between change in MUFA intake and IHL, contrast with two previous studies: Bozzetto et al. found an association between increased MUFA intake and a decrease in IHL, which was reported during an isoenergetic study independent from changes in body weight among patients with type 2 diabetes [49]. In a recent study in 27 male subjects with obesity, Kruse et al. showed a reduction in IHL during an isocaloric diet including rapeseed oil (rich in PUFA) in comparison to olive oil (rich in MUFA) [50]. The population differences - in the first study participants with type 2 diabetes, and in the second participants with obesity - could explain parts of the reported differences compared to our study [49,50].

In addition to the beneficial effects of increase in PUFA intake, increase in protein consumption was also related to the decline in liver fat content. Such an effect is supported by numerous other small short-term studies [15–17,19,30,51]. An isocaloric trial comparing animal with plant-protein in 37 subjects with type 2 diabetes showed a decrease in IHL in both groups after 6 weeks [17]. This effect was reported to be independent from BMI changes. In contrast, our data indicate that the protein effect was mostly driven by protein-induced small changes in body weight than protein intake itself. Interestingly, the small but significant weight loss observed in the mentioned study of Markova and coworkers was comparable to the BMI changes observed in both groups of our trial [17]. The described differences could be partly explained by the smaller sample size or population differences, as only 37 participants were included in the mentioned study and all had type 2 diabetes as well as MASLD while 82 % of the NutriAct participants did not have diabetes and 65 % did not have MASLD [17].

Mechanistically, besides the effect of increased protein intake on body weight [52,53], the effect on IHL might be driven by numerous other processes. First, a high-protein diet increases body amino acid degradation through AMP-activated protein kinase, whose catabolism in the liver is energy demanding [54,55]. This increase in liver energy expenditure may increase hepatic lipid oxidation, leading to a decrease in IHL, potentially mediated by fibroblast growth factor 21 (FGF21) [54,56]. Secondly, increase in protein intake increases bile acid production and, therefore, lipid oxidation [57]. Third,

glucagon secretion, which is stimulated by high-protein ingestion, stimulates intrahepatic lipolysis through an INSP3R1-and CAMKII-dependent process [57–59]. Further research is clearly required to analyze the role of these pathways in modulation of liver fat.

The reported association between decreased carbohydrate intake and IHL reduction is in accordance with a previous iso-energetic study performed in 45 subjects with type 2 diabetes for an 8-week period comparing a high-carbohydrate-high-fiber to a high-MUFA diet [49]. However, a recently published systematic review and meta-analysis containing the previously cited study, among others, showed no effect of a low-carbohydrate high-fat diet in relation to a high-carbohydrate low-fat diet in reducing liver fat content [30]. It is, however, important to notice, that the heterogeneity between the studies in the meta-analysis was substantial (68 %) [30]. Additionally, classifying "high-fat low-carbohydrate" without further specification regarding the types of fat or considering the protein intake may lead to erroneous conclusions, as shown in the same meta-analysis, where high-UFA improved liver fat compared to high-SFA and a high-protein low-carbohydrate diet favors liver fat reduction compared to a low-protein high-carbohydrate [30]. In the present results, though, we show that the effect of reduction of carbohydrate intake in IHL was predominantly mediated by weight changes, even though the observed weight loss was very slight. Thus, we would speculate that there might be no substantial weight loss independent effect of low carbohydrate intake on liver fat.

7.1. Strengths and limitations

To the best of our knowledge, this is the longest and largest randomized controlled trial investigating the effects of a high-UFA and high-protein (predominantly plant-based) diet on liver fat in community-dwelling elderly subjects, which disentangles the impact of the different macronutrient components as well as of weight loss as a mediator in this context. Most importantly, a successful implementation of the intended diet with a significant change in the dietary habits of the intervention group was achieved in the first 12 months of the study [27]. Furthermore, measurement of intrahepatic lipids was performed using state-of-the-art MRS phenotyping in a large cohort, when compared to the present body of literature. Nevertheless, our study has some limitations that must be acknowledged. Although the NutriAct is a randomized controlled trial, the present analyses considered all participants irrespective of randomised arm, but rather in regards of changes in their dietary (macronutrient) intake. Additionally, even though we intended to consider multiple factors affecting our results, we cannot exclude residual confounding such as physical activity and not measured social factors. As expected, changes in macronutrients intake are correlated to one another, as they represent a percentage of the total daily energy intake. This may have affected the results. Even though we calculated the VIF and in the presented results it was always lower than 2, the inclusion of multiple macronutrients in the same regression model represented a significant increase in multi-collinearity. Furthermore, only 248 out of the 502 NutriAct participants had nutritional and IHL data both at baseline and at month 12. As the reasons, why participants did not undergo an MRS were mostly technical problems and/or due to MR-contraindications or participants refusal, we do not expect selection bias due to this fact. In fact, no substantial differences in baseline characteristics between the whole cohort ($n = 502$) and the present subcohort ($n = 248$) were seen. Nevertheless, with 248 subjects included it is one of the largest cohorts of IHL data observed over 12 months to date in the described dietary regime.

Additionally, as this is a community-dwelling study, we cannot rule out that other foods than the reported were consumed. Such misreporting is expected to be non-differential between the two intervention groups and should therefore not affect the validity of the results or the overall conclusions. Hence, we do not expect significant bias due to this fact. Finally, the presented results may not be generalizable to other populations.

8. Conclusions

In the present study we found that, in middle-aged and elderly subjects at risk for age-related diseases, especially a high intake of PUFA was associated with a greater reduction in liver fat during a 12-month nutritional intervention. This effect was partly independent from weight changes. In contrast, the beneficial effects of changes in protein and carbohydrate intake were mainly mediated via alterations of body weight. These data increase our understanding how different macronutrients affect liver fat outside from weight loss interventions. Further mechanistic research is essential to increase our understanding of underlying mechanism. This should include studies exploring the effect of increased PUFA intake on thermogenesis, lipid metabolism, inflammatory markers and body composition.

Authors contributions

KM, SH, AFHP and JP designed research, KM, LPB, CW, JM, NMTM, THN, AP, SH and CG conducted research, LPB and KM analyzed data, wrote the manuscript and have primary responsibility for final content. All authors read and approved the final version of the manuscript.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of interest

LPB was employed by Charité-Universitätsmedizin Berlin during the conduct of the study, but as of the 1st of August 2023 is employed by and holds shares of Novo Nordisk.

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Appendix A. Supplementary data

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