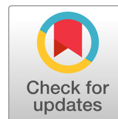

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Dietary rapeseed oil supplementation reduces hepatic steatosis in obese men – a randomized controlled trial

Michael Kruse [1,2], Margrit Kemper [1,2,3], Sofiya Gancheva [3,4,5], Martin Osterhoff [1,2], Dirk Dannenberger [6], Daniel Markgraf [3,5], Jürgen Machann [3,7,8], Johannes Hierholzer [9], Michael Roden [3,4,5], Andreas F. H. Pfeiffer [1,2,3]

[1]German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany, [2]Department of Endocrinology, Diabetes and Nutrition, Charité University Medicine Berlin, Germany, [3]German Center for Diabetes Research (DZD), München-Neuherberg, Germany, [4]Division of Endocrinology and Diabetology, Medical Faculty, Heinrich-Heine University, Düsseldorf, Germany, [5]Institute for Clinical Diabetology, German Diabetes Center Düsseldorf, Leibniz Center for Diabetes Research at Heinrich-Heine University, Düsseldorf, Germany, [6]Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany, [7]Institute for Diabetes Research and Metabolic Diseases (IDM) of the Helmholtz Center Munich at the University of Tübingen, Germany, [8]Section of Experimental Radiology, Department of Diagnostic and Interventional Radiology, University Hospital Tübingen, Germany, [9]Department of Diagnostic and Interventional Radiology, Ernst von Bergmann Hospital, Potsdam, Germany

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Hepatic steatosis, obesity, rapeseed oil, olive oil, serum lipids.

Corresponding author: Michael Kruse, c/o Department of Clinical Nutrition, German Institute of Human Nutrition Potsdam-Rehbruecke, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany, tel.: +49 33200882796, fax: +49 33200882777, Email: Michael.Kruse@charite.de

Abbreviations:

AA: arachidonic acid, ALA: alpha linolenic acid, ALT: alanine aminotransferase, apoB-48: apolipoprotein B-48, AST: aspartate aminotransferase, AU: arbitrary units, ChREBP: carbohydrate response element binding protein, DHA: docosahexaenoic acid, DPA: docosapentaenoic acid, EGP: endogenous glucose production, EPA: eicosapentaenoic acid, FFA: free fatty acids, γGT: γ-glutamyl-transpeptidase, ^{1}H-MRS: proton magnetic resonance spectroscopy, IHL: intrahepatic lipid content, LA: linoleic acid, MUFA: monounsaturated fatty acids, NAFLD: Non-alcoholic fatty liver disease, NASH: non-alcoholic steatohepatitis, OL: olive oil, PPARα: peroxisome proliferator-activated receptor alpha, PUFA: polyunsaturated fatty acids, RA: rapeseed oil, SFA: saturated fatty acid, SREBP-1c: sterol regulatory element binding protein 1c, T2DM: type 2 diabetes mellitus, WHR: waist-to-hip ratio

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Abstract

Scope: Obesity is associated with non-alcoholic fatty liver disease (NAFLD) but effective treatment is limited. Dietary supplementation of n-3 polyunsaturated fatty acids, specifically alpha linolenic acid (ALA), can resolve intrahepatic lipid content (IHL). This study investigated the effect of daily supplementation of either refined rapeseed (RA), which contains high amounts of ALA, or refined olive (OL) oil on IHL and glucose metabolism in NAFLD patients.

Methods and results: 27 obese men consumed an isocaloric diet including either 50 g of RA or OL daily for eight weeks. Hepatic proton magnetic resonance spectroscopy, hyperinsulinemic-euglycemic clamp studies and blood tests were performed before and at the end of the study. BMI did not change for RA or OL. At eight weeks a significant reduction in IHL was observed for RA (13.1±1.6 before vs. 11.1±1.6 % after intervention) versus OL (13.3±2.5 before vs. 15.7±2.7 % after intervention). For RA, a 21% reduction ($P<0.02$) in serum free fatty acids (FFA) and a 1.68-fold increase ($P=0.03$) of serum interleukin-6 (IL-6) was observed after eight weeks.

Conclusion: RA has a beneficial effect on hepatic lipid metabolism as shown by reduced IHL and serum FFA. RA induced IL-6 production seems to be liver protective confirming previous results.

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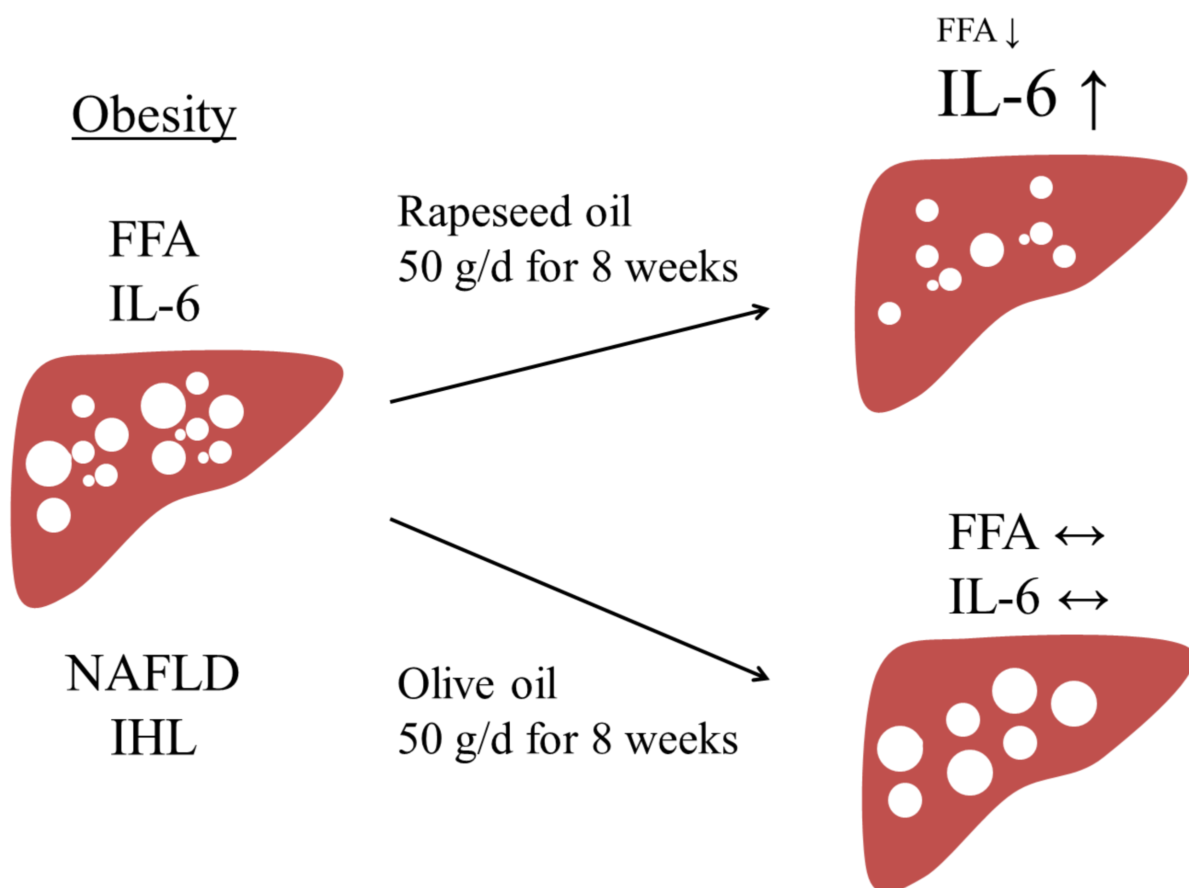
Obesity is associated with non-alcoholic fatty liver disease (NAFLD). This study shows that isocaloric dietary supplementation with 50 g/ day of rapeseed oil, containing n-3 polyunsaturated fatty acids, for eight weeks reduces intrahepatic lipid content (IHL), serum free fatty acids (FFA), but increases serum interleukin-6 (IL-6) in obese men. These effects were not observed when an isolcaloric diet containing 50 g/ day of olive oil was given.



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1. **Introduction**

Non-alcoholic fatty liver disease (NAFLD) is the most frequent liver disease in industrialized nations with a prevalence of 25 % in the adult population worldwide.[1,2] Fatty liver has a high probability to progress into non-alcoholic steatohepatitis (NASH), liver cirrhosis, liver cancer and subsequently liver failure.[1,3] Clinical studies have shown that NAFLD is strongly associated with obesity, metabolic syndrome and type 2 diabetes mellitus (T2DM).[4,5] Over-eating of diets rich in refined carbohydrates and saturated fats leads to obesity, hyperlipidemia and insulin resistance which subsequently can result into T2DM, cardiovascular diseases and liver steatosis. Additionally, NAFLD has been identified as a cardiovascular risk factor on its own.[6] Morphologically hepatic steatosis is characterized by intrahepatic accumulation of fatty acids and triacylglycerols as a result of an imbalance between increased fatty acid uptake or de-novo lipogenesis and reduced removal of lipids by β-oxidation.[7,8,9] Lipids are delivered to the liver by chylomicrons from the diet or as fatty acids which are massively released from adipose tissue in the state of insulin resistance thus overwhelming hepatic fat metabolism.[9] Many studies have shown that hepatic insulin resistance is a result of steatosis, however, over recent years this association has been discussed controversially indicating a more complex pathophysiologic mechanism of NAFLD.[10] Clinical and animal studies have shown that increased hepatic fat does not necessarily lead to hepatic insulin resistance, depending on the type of lipid and cellular compartmentation of lipid storage. There is a line of evidence that hepatic lipid accumulation can selectively impair the branch of the insulin signaling cascades that inhibits glucose production whereas the lipogenic branch is unaffected.[11] Lipid synthesis in the liver requires the activation of the transcription factors sterol regulatory element binding protein 1c (SREBP-1c) [12] and carbohydrate response element binding protein (ChREBP) [13] whereas fatty acid oxidation requires

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the activation of peroxisome proliferator-activated receptor alpha (PPARα).[14] The activity of these transcription factors can be influenced by n-3 polyunsaturated fatty acids (PUFA). Expression of SREBP-1c is decreased and of PPAR-α is increased, respectively, by high intrahepatic n-3 PUFA content. Remarkably, intrahepatic reduction of n-3 PUFA, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been associated with hepatic steatosis.[15]

Regarding the alarming epidemiological data and severe consequences of NAFLD it is essential starting treatment as early as possible and as sufficient as possible. However, until now effective treatment is limited. Therapeutic options usually recommend healthy diets and physical activity.[16] Clinical studies have shown that nutritional supplementation of PUFA can reduce liver fat content: Consuming 1 g/ day of the n-3 fatty acids EPA and DHA could improve hepatic steatosis in NAFLD patients.[17] EPA and DHA are conversion products of alpha-linolenic acid (ALA) which is highly enriched in rapeseed oil (RA).[18] However, the elongation of ALA into long-chain n-3 PUFA appears to be very limited in adult humans even if consumed in large amounts).[19,20] Approximately 5% of ALA are converted into EPA and less than 0.5% are converted into DHA).[19] Since ALA itself has beneficial effects on cardiovascular diseases it might have also direct effects on hepatic steatosis. Studies investigating the effect of dietary n-6 PUFA could also demonstrate reduced hepatic steatosis in humans when compared to dietary saturated fatty acids (SFA).[21,22] Additionally, mainly animal studies have shown a reduction in hepatic steatosis with dietary ALA.[23]

Based on this observation we used a nutritional approach to find an easy, efficient and cost effective therapeutic option for the treatment of hepatic steatosis. Since RA contains high amounts of the n-3 PUFA ALA and the n-6 PUFA linoleic acid (LA) we compared the effect of an isocaloric diet supplemented with either 50 g RA or olive oil (OL) daily over eight weeks on liver fat content,

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endogenous glucose production, total body insulin sensitivity and serum lipids in obese humans suffering from NAFLD.

2. **Experimental Section**

2.1. Study Participants

This randomized controlled trial with an open-label, parallel-arm study design was approved by the local ethical committee of the University of Potsdam (project number: 12/2014) and was in accordance with the Declaration of Helsinki of 1975, as revised in 1983. It was registered at www.clinicaltrials.gov (NCT02458586). All participants gave their written consent after the study design was explained to them in detail.

A total number of 27 overweight/ obese (BMI 28 – 46 kg/m²) men, age 27 – 72 years, were randomly assigned to the OL or the RA arm of the study. Only men were included in this study to exclude confounding influences that might result from variable estrogen levels. Besides this aspect the inclusion criteria were: Adiposity with BMI > 28 kg/m², age 18 – 75 years, intrahepatic lipid content (IHL) > 5 %. Exclusion criteria were: BMI < 28 kg/m², age < 18 or > 75 years, IHL < 5 %, T2DM, any illness that would interfere with the study results, medication which would influence the results, especially medication influencing lipid metabolism, liver metabolism or inflammation. Parameters used for randomisation were age (years), body weight (kg), height (m) and waist circumference (cm). A computerized program was used to assure that both groups were equally balanced in terms of parameters used for randomization and group size. After randomization one individual had to be excluded from the rapeseed arm because of acute illness that was not related to the study. At the

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end of the study, 11 participants completed the OL arm and 15 the RA arm, respectively. A subgroup of five participants of each group completed the hyperinsulinemic-euglycemic clamp study.

2.2. Study Design

On the screening day a physical examination was performed and height (m), body weight (kg), waist and hip circumference (cm) were taken and body mass index (BMI) and waist-to-hip-ratio were calculated. Total body fat was determined using an air-displacement plethysmography system).[24] An ultrasound examination was performed to estimate hepatic fat content. Individuals with hepatic steatosis were further evaluated by proton magnetic resonance spectroscopy (^{1}H-MRS) to determine the exact amount of IHL. Blood was collected after an overnight fast for further analysis. An oral glucose tolerance test was performed to exclude impaired glucose tolerance or T2DM. Base line characteristics for the individuals of the OL and the RA group are shown in Table 1. After randomization all individuals were asked to fill out a three-day food record, with one day being a weekend day, regarding their dietary habits and individuals were invited for an initial detailed counselling. Energy requirements were calculated based on food records and levels of activity, the appropriate oil was provided and individuals were given detailed instructions for daily food consumption. They were instructed to consume 50 g (18 E%) per day of either refined OL (Henry Lamotte Oils, Bremen, Germany) or refined RA/ Canola Oil (containing low amounts of erucic aid) (Kunella Feinkost, Cottbus, Germany) for eight weeks but not to use it for baking or frying. The amount of oil given to the participants was determined from our previous study.[18] Prior to the oil consumption a hyperinsulinemic-euglycemic clamp with isotope labelled glucose was performed. For each individual the study started on the day after the initial nutritional counselling. Every two weeks participants were asked again to fill out a three-day food record and see our unit for body weight

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recording. If body weight was different more than 1 kg from the initial body weight participants were instructed to modify their energy intake accordingly. Participants received a second in-depth nutritional counselling with anthropometric measurements and blood analysis after four weeks of the study. At the end of the study a second hyperinsulinemic-euglycemic clamp, hepatic ^{1}H-MRS, anthropometric measurement and blood collection were performed. IHL was defined as primary outcome measure and total body and hepatic insulin sensitivity, basal endogenous glucose production and serum lipids were defined as secondary outcome measures. Estimated from data of the literature we expected an average IHL of approximately 10% at baseline in our study population of obese male patients. Also estimated from the literature we expected a 30% difference from baseline of IHL after an eight week dietary oil intervention. Based on a power calculation with a value of 80% power a size of 7 per group was estimated. The study design is illustrated in Figure 1. Data were analysed on the basis of a per-protocol analysis, since this study was designed as a pilot study with a limited number of participants.

2.3. Proton Magnetic Resonance Spectroscopy

For quantification of IHL, a single voxel STEAM technique was applied on a 1.5T whole-body imager (Magnetom Avanto, Siemens Healthineers, Erlangen, Germany) with the subjects in supine position. After morphological imaging, a cubic volume of interest of 3x3x2cm³ was placed in the posterior part of segment 7. Spectra were recorded with a short echo time (TE) of 10 ms and a long repetition time (TR) of 4 s in order to avoid relaxation-based inaccuracies. Thirty-two acquisitions were acquired in order to obtain a sufficient signal-to-noise ratio. Post-processing was performed by integration of the water resonance at 4.7 ppm (serving as internal reference) as well as lipids resonances

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(methylene at 1.3 ppm and methyl at 0.9 ppm). The ratio of lipids/(water+lipids) was calculated and IHL are given in % of the total signal).[25]

2.4. Hyperinsulinemic-euglycemic Clamp

Participants underwent two-step hyperinsulinemic-euglycemic clamps after an overnight fast as shown previously).[26] In brief, two intravenous catheters were inserted to contralateral forearm veins. From -120 min, a continuous infusion (0.036 mg • [kg body weight]$^{-1}$ • min^{-1}) of D-[6,6-$^{2}H_2$]glucose (99% enriched in ^{2}H glucose; Cambridge Isotope Laboratories, Andover, MA, USA) was given after a priming bolus (0.36 mg • [kg body weight]$^{-1}$ • min^{-1} • [mg/dL fasting plasma glucose]) for 5 min. At zero time a hyperinsulinemic-euglycemic clamp was started with 20 mU/min/m^2 (low-dose) followed by 40 mU/min/m^2 (high-dose) insulin infusions. Somatostatin was infused in parallel (250 µg/h) for suppression of endogenous pancreatic insulin secretion. Plasma glucose was adjusted to 90 mg/dL using 20% glucose (B. Braun AG, Melsungen, Germany) enriched with 2% D-[6,6-$^{2}H_2$]glucose.

2.5. Blood Analysis

Serum parameters (total cholesterol, HDL, triacylglycerols, FFA, AST, ALT, γGT) were measured using standard techniques in a certified laboratory for clinical chemistry. LDL-cholesterol was calculated from the above data. Serum insulin was measured using commercially available ELISA (Mercodia, Uppsala, Sweden). Glucose concentrations were measured in venous serum samples (ABX Pentra 400; ABX Diagnostics, Montpellier, France).

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Serum IL-6 and IL-8 were measured using a human IL-6 Quantikine high-sensitive ELISA KIT and a human IL-8/CXCL8 Quantikine ELISA Kit, respectively (both R&D Systems, Wiesbaden, Germany). Serum cytokeratin 18 fragments (CK 18) were measured using a human CK 18 ELISA Kit (CUSABIO, Houston, Texas, USA). Serum apolipoprotein B-48 was measured using an Apo B-48 Human ELISA Kit (BioVendor, Brno, Czech Republic).

Serum parameters and serum glucose concentrations were measured at fasted conditions before and at four and eight weeks after the dietary intervention.

2.6. Analysis of Plasma Fatty Acids

Blood plasma samples were dropwise added to 8 mL chloroform/methanol (2:1, v/v) at room temperature. The solution contained C19:0 as an internal standard. The detailed sample preparation procedure has been recently described.[27] Briefly, the extraction mixtures were stirred two times for 15 minutes and stored at 5°C for 18 h in the dark and subsequently washed with 0.02% $CaCl_2$ solution. For transesterification 1 ml of 0.5 M sodium methoxide in methanol was added to the samples, which were shaken in a 60ºC water bath for 10 minutes. Subsequently, 0.5 mL of 14% boron trifluoride (BF_3) in methanol was added to the mixture, which was then shaken for an additional 10 minutes at 60ºC. The fatty acid methyl esters (FAMEs) were extracted three times in 2 mL of *n*-hexane. The FAMEs were resuspended in 100 µL of *n*-hexane and stored at -18 ºC until used for gas chromatography (GC) analysis. The fatty acid analysis of blood plasma lipids was performed using capillary GC with a CP-Sil 88 CB column (100 m x 0.25 mm, Agilent, Santa Clara, CA, United States) that was installed in a PerkinElmer gas chromatograph CLARUS 680 with a flame ionisation detector and split injection (PerkinElmer Instruments, Shelton, United States). The detailed GC conditions were described previously.[28] Hydrogen was used as the carrier gas at a flow rate of 1 mL

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min^{-1}. The split ratio was 1:20, and the injector and detector were set at 260°C and 280°C, respectively. The quantification of fatty acids was done by the use of C19:0 as internal standard. For the calibration procedure the reference standard mixture 'Sigma FAME' (Sigma-Aldrich, Deisenhofen, Germany), the methyl ester of C18:1*cis*-11, C22:5*n*-3 and C18:2*cis*-9,*trans*-11 (Matreya, PA, USA), C22:4*n*-6 (Sigma-Aldrich, Deisenhofen, Germany) and C18:4*n*-3 (Larodan, Limhamn, Sweden) were used. The five-point calibration of single fatty acids ranged between 16 and 415 mg/mL and was checked after GC analysis of five samples.

2.7. Statistics and Calculations

All data are given as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 20.0 (SPSS, Chicago; IL, USA). Normal distribution was tested using the Kolmogorov-Smirnov test. ANOVA with repeated measurements and Greenhouse-Geisser correction was performed. In case of normally distributed data a paired, two-tailed unpaired Student's *t* test and two-tailed paired Student's *t* test was used to test for statistical significance between OL and RA groups or within one experimental group, respectively. Otherwise a Wilcoxon matched-pairs signed rank test was applied. A two-sided P-value < 0.05 was considered significant.. Basal (fasting) endogenous glucose production (EGP) was calculated by dividing the tracer infusion rate times the tracer enrichment by the steady-state tracer enrichment of plasma glucose before the clamp and subtracting the tracer infusion rate. During the clamp, rates of appearance (Ra) and disappearance (whole-body glucose disposal, Rd) of glucose were calculated from Steele's equations).[29] EGP suppression was calculated as the percent suppression of EGP rates during clamp steady-state. Homeostatic Model Assessment (HOMA) was used to provide a proxy of insulin resistance and allow

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comparisons with other studies by calculating the product of fasting serum insulin concentrations (µU/ml) and fasting serum glucose concentration (mg/dl) divided by 405).[30]

3. Results

3.1. Body Composition, Energy Intake, Plasma Fatty Acid Concentrations

Supplementation of either 50g/day of OL or RA did not change body weight, body mass index (BMI), total body fat or waist-to-hip ratio (WHR) of the participants over the time of intervention. As shown in Figure 2 body weight was 106.4±4.66 kg at the beginning, 106.1±4.84 kg at 4 wks and 106.6±4.93 kg at 8 wks, respectively, for OL and 103.2±43.27 kg at the beginning, 102.3±3.13 kg at 4 wks and 102.4±3.44 kg at 8 wks, respectively, for RA. BMI was 33.1±1.44 kg/m² at the beginning, 33.0±1.53 kg/m² at 4 wks and 33.2±1.56 kg/m² at 8 wks, respectively, for OL and 32.0±0.76 kg/m² at the beginning, 31.7±0.67 kg/m² at 4 wks and 31.6±0.74 kg/m² at 8 wks, respectively, for RA. Total body fat content was 35.4±1.84 % at the beginning, 35.4±1.60 % at 4 wks and 35.4±1.97 % at 8 wks, respectively, for OL and 34.0±1.16 % at the beginning, 34.5±1.49 % at 4 wks and 34.8±1.57 % at 8 wks, respectively, for RA. For WHR we obtained 1.03±0.02 arbitrary units (AU) at the beginning, 1.02±0.02 AU at 4 wks and 1.03±0.02 AU at 8 wks, respectively, for OL and 1.01±0.01 AU at the beginning, 1.01±0.01 AU at 4 wks and 1.01±0.02 AU at 8 wks, respectively, for RA.

Oil supplementation over the time of intervention resulted in a slight increase in relative fat intake for OL (40.0±1.7% at beginning vs. 46.1±1.4% at 8 wks) and RA (39.3±2.7% at beginning vs. 43.7±1.5% at 8 wks) (Table 2). However, no significant differences in relative fat intake between OL

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and RA, neither at the beginning nor at 8 wks (Table 2) and no effects on BMI (Figure 2B) were observed.

Consumption of RA led to a 1.69-fold increase (P<0.001) of plasma ALA concentration after 4 wks and a 1.53-fold increase (P<0.01) after 8 wks, respectively, when compared to baseline (Figure 3A). For EPA a 27% reduction (P=0.011) was seen for OL and a 33% reduction (P=0.008) for RA, respectively, at 8 wks compared to baseline (Figure 3B). Consumption of OL led to a 15% reduction of docosapentaenoic acid (DPA) at 4 wks (P<0.001) and to a 14% reduction at 8 wks (P=0.011), respectively, compared to baseline (Figure 3C). For RA a 18% reduction (P=0.007) of plasma DHA was observed at 8 wks compared to baseline (Figure 3D). Daily intake of RA increased plasma levels of the n-6 fatty acid linoleic acid (LA) 1.14-fold (P=0.025) at 4 wks and 1.10-fold (P=0.044) at 8 wks, respectively, compared to daily intake of OL. Also, a 1.07-fold (P=0.038) increase in plasma concentration of LA was seen at 4 wks compared to baseline for RA (Figure 3E). Consuming OL led to a slight increase (1.14-fold, P=0.020) of the n-6/n-3 fatty acid ratio at 8 wks when compared to baseline (Figure 3F).

3.2. Intrahepatic Lipid Content

Prior to oil supplementation IHL determined by ^{1}H-MRS was 13.3±2.5 % in participants assigned to OL and 13.1±1.6 % assigned to RA (P=0.947), respectively (Figure 4). At the end of the study at 8 wks we observed a reduction in IHL for RA (11.1±1.6 %) which was close to significance when compared to baseline of this group (P=0.055). For OL a slight increase in IHL (15.7±2.7 %, P=0.245 compared to baseline) was seen. However, a significant reduction in IHL was observed for RA when compared to OL at the end of intervention at 8 wks (P=0.038, Figure 4).

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3.3. Serum Lipids, Glucose, Insulin, HOMA IR, CRP, Liver Enzymes

For RA, we observed a 21% reduction ($P<0.02$) in serum free fatty acids (FFA) after eight weeks of oil consumption whereas no significant changes were seen for OL (Figure 5). CRP levels were significantly higher in OL compared to RA (4.72±1.6 mg/L vs. 1.56±0.3, $P=0.034$) at baseline, however below the reference range (< 6 mg/L) for both groups. We did not see any significant difference over the time of intervention in serum levels of triacylglycerols, total cholesterol, LDL, HDL, apolipoprotein B-48, serum glucose, insulin and HOMA IR (Figure 5 and Table 3). AST, ALT and γ-GT did not change over the time of intervention (Table 3).

3.4. IL-6, IL-8 and CK 18 Serum Concentrations

Serum concentrations of IL-6 were significantly increased 1.68-fold in RA after 8 wks of oil supplementation ($P=0.03$, data individually normalized to baseline for each participant, Figure 6A). For OL only a slight non-significant increase ($P=0.14$) was seen for IL-6 after 8 wks. No significant changes of IL-8 or CK 18 (Figure 6B and 6C, respectively) serum concentrations were observed over the time of oil supplementation in either OL or RA.

3.5. Insulin Sensitivity

Rates of whole body insulin sensitivity and EGP were assessed from hyperinsulinemic-euglycemic clamps with isotopic dilution of deuterated glucose in a subgroup of the participants. After 8 wks of oil supplementation whole body insulin sensitivity showed a declining tendency in RA (18.3%,

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P=0.141) in the setting of insulin concentrations at basal level during the low-dose insulin clamp (Figure 7A). Also, basal EGP (12.7%, P=0.357) showed a tendency to decline after 8 wks in RA (Figure 7B). No differences in hepatic insulin sensitivity were seen prior to and after the oil supplementation in either OL or RA (Figure 7C).

4. Discussion

The aim of this dietary interventional study was to find a nutritional option that could reduce hepatic fat content (intrahepatic lipids, IHL) and be practically implemented in daily life. In light of the high prevalence of NAFLD, its deleterious consequences and limited therapeutic options there is a strong need for novel curative strategies. The main findings of this study are a reduction of IHL and decreased FFA serum concentrations in obese men who received an isocaloric diet supplemented daily with 50 g of RA over eight weeks compared to the supplementation of 50 g of OL. Notably, in the RA group we observed a significant increase in IL-6 serum concentration that was not seen in the OL group.

RA and OL are two favourable oils recommended for a healthy diet [18] however, they differ in their fatty acid profile as shown by the analysis of the oils used in this study (Supporting Information Table S1). RA and OL contain predominantly large amounts of MUFA, but RA contains less saturated fatty acids and higher PUFA including approximately 8-9% of n-3 ALA and 19-20% of n-6 LA.[31] ALA is almost absent in OL. A study in rats showed that a diet high in ALA with 55 % energy from fat over 12 wks was able to reduce hepatic lipid content of 20 % and to increase the relative amount of intra hepatic n-3 PUFA compared to a lard-fed control group.[23] Intra hepatic n-3 PUFA are critical for liver

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fat accumulation, since its reduction resulted in hepatic steatosis.[15] Another study showed that feeding rats with vegetable oils containing high amounts of ALA caused increased hepatic n-3 PUFA concentration and increased hepatic fatty acid oxidation.[32] Moreover this study demonstrated increased DNA-binding of PPARα and decreased DNA-binding of SREBP-1c, which are transcription factors involved in lipid oxidation and synthesis, respectively,[12,14] indicating a modulation of molecular mechanisms of hepatic lipid metabolism by dietary ALA consumption. Additionally, ALA and its conversion products EPA and DHA are precursors for resolvins and protectins which have anti-inflammatory functions and can induce resolution of obesity induced steatohepatitis.[33] In our clinical study participants of the RA group had a robust increase in plasma ALA after 4 and 8 wks of oil consumption, which was absent in the OL group as expected. At the end of our study we observed a difference of 4.59 % in IHL between OL and RA. At this point it is speculative, however very likely that these mechanisms might be responsible for the reduction in liver fat in the RA group. A clinical study showed that an isocaloric diet high in n-6 PUFA consumed for 10 weeks was able to reduce liver fat content by 16% in obese individuals when compared to a diet high in SFA.[22] We were also able to detect a significant increase in plasma LA concentrations in the RA group.

The n-6/n-3 fatty acid ratio is crucial for the development of NAFLD specifically in obese patient since overnutrition in these individuals results in increase liver oxidative stress associated with reduced hepatic n-3 long chain PUFA.[34] Therefore it seems favourable to reduce plasma n-6/n-3 fatty acid ratio in the long term for the prevention of NAFLD. In our study we did not see a significant difference in plasma n-6/n-3 fatty acid ratio for RA, but interestingly a significant slight increase for OL at the end of the study compared to baseline.

Consuming diets high in MUFA and PUFA as a replacement of saturated fats lowers blood lipids, reduces cardiovascular risk and has beneficial effects on glucose and insulin homeostasis.[35,36] A RA

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enriched diet has beneficial effects on triacylglycerols, total cholesterol and LDL cholesterol, in comparison of a common, saturated fats containing diet.[37] We observed a significant 21 % reduction of serum FFA after 8 wks in the RA group. The increased delivery of serum FFA to the liver and their reduced hepatic elimination are key factors of the development of NAFLD.[9] The amount of serum FFA levels is mainly dependent on the composition of the diet and on lipolysis from adipose tissue. In our study the reduction of serum FFA after RA supplementation appears to be a direct dietary effect, since we did not observe any changes in whole body insulin sensitivity that would affect adipose tissue lipolysis. It might be possible that β-oxidation of fatty acids in the liver is increased since we observed a decrease in IHL that possibly would occur prior to improved hepatic insulin sensitivity. However, it appears that NAFLD is associated with high rates of β-oxidation in general [38] while changes of lipolysis and reduced lipogenesis explained the reduction of liver fat by high protein diets.[39]

Lipid transport is crucial in obesity. Elevated remnant cholesterol in triglyceride-rich lipoproteins is known to be a causal risk factor for ischemic heart disease.[40] Apolipoprotein B-48 (apoB-48) is a marker for transportation of triglycerides in the form of chylomicrons from the gut and an increase indicates higher risk for cardiovascular events.[41] Apolipoprotein B-100 (apoB-100) indicates VLDL transport from the liver.[41] In our pilot study we did not observe any significant differences in fasting apoB-48 serum levels after consuming OL or RA. However, further work is needed to analyse possible alterations of lipid transport.

Interleukin-6 is commonly seen as a pro-inflammatory cytokine that promotes insulin resistance in obesity since serum IL-6 levels correlate well with adiposity in humans.[42] However, its effects on metabolism are now being discussed controversially. It was shown that physical exercise induces a massive short-term release of IL-6 from the myocytes leading to improved insulin sensitivity.[43] In a

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mouse model knocking out IL-6 was associated with obesity, hepatic inflammation and insulin resistance.[44] Another study observed reduced body weight and hepatic steatosis in mice fed a high fat diet for 6 wks when IL-6 was experimentally overexpressed using a gene transfer technique of an IL-6 plasmid construct.[45] Inhibition of IL-6 signalling by injecting an IL-6 receptor antibody increased hepatic lipid content and SREBP-1 gene expression and decreased PPARα gene expression in a mouse NASH model.[46] In our study we observed a significant 1.68-fold increase of serum IL-6 concentration in RA at the end of the oil intervention which is in line with the results obtained from the animal studies. Thus together with the observed reduction in IHL this increased IL-6 serum concentration apparently indicates a beneficial stimulus rather than a negative cytokine effect. Serum IL-8 which is a strong pro-inflammatory cytokine and is positively associated with NASH [47] did not change with either RA or OL over the time of intervention. Although IL-6 has been associated with hepatic insulin resistance [48] it may need to be integrated into the context of the cytokine profile [49] and the hepatic exposure to FFA since according to the study by Perry et al.,[48] IL-6 was a major driver of adipose tissue lipolysis and delivery of FFA while IL-6 was increased in the context of reduced FFAs in the present study.

It has been discussed that consuming large amounts of LA could induce inflammation because of the subsequent formation of arachidonic acid (AA) as a precursor of eicosanoids.[50] Because of limitation in the data available this hypothesis has not been confirmed or rejected.[50] It is speculative if the observed increase in plasma LA in the RA group in our study might contribute to the increased IL-6 levels. We did not observe any changes in plasma AA over the time of intervention in either OL or RA group (data not shown).

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CK 18 fragments are cleaved in the liver during hepatocyte apoptosis and viewed as a marker of hepatic inflammation.[51] However, CK 18 as well as IL-8 serum levels were unchanged during our study in OL an RA groups.

It was shown that NAFLD is associated with peripheral and hepatic insulin resistance [52,53] and that weight loss led to reduced hepatic fat content and an improvement of peripheral and hepatic insulin resistance.[54,55] However, in most studies this benefit was associated with a massive reduction of IHL (e. g. approximately 80 %) mainly due to an experimental dietary regime that is impossible to maintain in daily life in the long term.[56] In our study we did not observe any significant changes in hepatic or peripheral insulin sensitivity or in hepatic glucose production. Also the HOMA IR index was not significantly different between OL and RA over the time of intervention. We attribute this to the fact that in this pilot study, that was explicitly designed for a practicable daily use without any change in body weight, we were not able to achieve this massive decrease in IHL as seen in studies where weight loss was observed. We also did not observe any significant differences in %-energy (Table 2) from protein, carbohydrates and fat in the diet between OL and RA at the beginning and at the end of the study. Therefore it seems unlikely that the content of macronutrients might be responsible for differences in IHL at the end of the study.

In our study aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the serum were within the normal range or only very moderately increased, respectively. In clinical practice increased serum liver enzymes are commonly viewed as an indication for liver damage. However, it was shown recently that serum levels of the aminotransferases AST and ALT are not necessarily indicating the presence of NAFLD or hepatic insulin resistance.[57] Elevated serum gamma-glutamyltransferase is known as an indicator of NAFLD [58] and was elevated in OL and RA throughout

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our study with no significant differences between the beginning and the end of the study in both groups.

Limitations of our pilot study are the limited number of individuals included in the entire study and the subgroup that underwent the clamp studies. Studies in a larger cohort, including male and female participants, and for longer time periods are needed.

In conclusion our results indicate that the supplementation of RA in an isocaloric manner on a daily basis should be considered as a nutritional option for patients suffering from NAFLD since we could show beneficial effects on hepatic steatosis and serum FFA. Further studies should consider unveiling the underlying mechanism.

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Data available on request from the authors.

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Author Contributions

The authors' responsibilities were as follows: MKr, MKe and AFHP were responsible for concept, design and conduciton of the study. MKe, DD, DM and JH performed experiments and data acquisition. MKr, SG, MO, and JM performed statistical data analysis. MKr and AFHP were responsible for interpretation of the data and drafting of the manuscript. MKr, MKe, SG, MO, DD, DM, JM, JH, MR, and AFHP contributed to the critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

Conflict of Interest

Dr. Kruse, Dr. Kemper and Dr. Pfeiffer report grants from UFOP (Union zur Foerderung von Protein- und oelpflanzen e. V.) during the conduct of the study. Dr. Roden reports personal fees from Bristol-Myers Squibb, grants and personal fees from Eli Lilly, personal fees from Gilead Sciences, grants and personal fees from NovoNordisk, personal fees from Servier Laboratories, personal fees from Target Pharmasolutions, personal fees from Terra Firma, grants and personal fees from Sanofi, grants and personal fees from Boehringer Ingelheim, personal fees from AstraZeneca, grants from Nutricia/Danone outside the submitted work. Dr. Gancheva, Dr. Osterhoff, Dr. Dannenberger, Dr. Markgraf and Dr. Machann declare no conflicts of interest.

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Table 1: Baseline charactaristics of the participants.

	OL (*n*=11)	RA (*n*=15)	*P*
Age (years)	54±3.95	58±2.64	0.457
Body height (m)	1.73±0.06	1.79±0.01	0.233
Body weight (kg)	106.4±4.66	103.2±3.27	0.567
BMI (kg/m²)	33.1±1.44	32.0±0.76	0.475
Body fat content (%)	35.38±1.84	34.04±1.16	0.529
Intrahepatic lipid content (%)	13.29±2.52	13.09±1.61	0.947
Waist/Hip-Ratio	1.03±0.02	1.01±0.01	0.403
Serum total cholesterol (mmol/L)	5.08±0.34	5.48±0.31	0.399
Serum LDL cholesterol (mmol/L)	3.39±0.30	3.44±0.28	0.917
Serum HDL cholesterol (mmol/L)	0.98±0.06	1.10±0.04	0.098
Serum triacylglycerol (mmol/L)	1.55±0.21	2.06±0.25	0.149
Serum free fatty acids (mmol/L)	0.49±0.08	0.56±0.04	0.383
Serum glucose (mg/dL)	92.5±3.1	89.7±2.3	0.460
Insulin (mU/L)	14.4±1.1	15.2±1.4	0.671

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HOMA IR index	3.78±0.35	3.94±0.45	0.796
Serum aspartate aminotransferase (U/L)	27.2±2.80	26.88±2.32	0.938
Serum alanine aminotransferase (U/L)	37.40±5.84	37.69±2.95	0.963
Serum γ-glutamyl-transpeptidase (U/L)	59.15±18.77	81.81±22.96	0.476
CRP (mg/L)	4.72±1.60	1.56±0.31	0.034

Values are means ± SEMs. Between-group differences were determined with the use of a 2-tailed unpaired *t* test in each group. * $P < 0.05$ for differences between the compared groups. OL, olive oil; RA, rapeseed oil.

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Table 2: Average of energy consumption related to nutrients at the beginning, at 4 weeks and at 8 weeks of the intervention.

	Nutrients OL			Nutrients RA			*P*-value OL vs. RA		
	Start	4 weeks	8 weeks	Start	4 weeks	8 weeks	Start	4 weeks	8 weeks
Protein (E%)	16.3±0.8	14.8±0.7	13.8±0.9	16.8±0.7	16.4±0.8	17.5±1.2	0.639	0.172	0.036
Protein (g)	86.5±5.6	88.5±8.1	78.5±6.5	99.4±6.0	80.9±4.6	95.5±6.5	0.142	0.387	0.084
CH (E%)	42.5±1.4	35.5±1.7	40.7±1.6	43.3±2.3	44.0±2.6	37.2±1.6	0.790	0.022	0.157
CH (g)	227.1±13.7	221.1±19.9	236.3±22.0	268.6±30.3	216.5±13.4	205.3±12.0	0.279	0.818	0.197
Fat (E%)	40.0±1.7	48.2±1.7	46.1±1.4	39.3±2.7	37.7±3.0	43.7±1.5	0.839	0.015	0.284
Fat (g)	97.8±8.0	127.9±8.1	118.7±7.1	109.1±10.9	87.7±9.1	108.5±5.2	0.443	0.005	0.243
Energy (kcal)	2255±131	2488±172	2417±161	2573±210	2103±110	2322±108	0.252	0.060	0.620

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SFA (%)	33.1±0.9	31.9±0.9	31.6±1.0	32.0±0.7	29.6±0.8	30.2±0.5	0.342	0.065	0.159
MUFA (%)	27.3±1.0	30.2±1.0	30.7±1.2	27.6±0.9	28.5±0.9	29.0±0.9	0.836	0.229	0.263
PUFA (%)	39.4±1.7	37.8±1.5	37.6±1.5	40.2±1.3	41.8±1.4	40.7±1.2	0.707	0.067	0.108

Intake of nutrients is shown relatively in energy-percent (E%) and absolutely in grams (g) for protein, fat and carbohydrates and in kcal for total energy intake. For SFA, MUFA and PUFA data are shown in percent of plasma fatty acids. Values are means ± SEMs. Between-group differences were determined with the use of a 2-tailed unpaired *t* test in each group. * $P < 0.05$ for differences between the compared groups. MUFA, monounsaturated; OL, olive oil; PUFA, polyunsaturated; RA, rapeseed oil; SFA, saturated fatty acids; CH, carbohydrates.

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Table 3: Blood glucose, insulin, HOMAR IR index, CRP and liver enzymes at the beginning, at 4 weeks and at 8 weeks of the intervention.

	OL			RA		
	Start	4 weeks	8 weeks	Start	4 weeks	8 weeks
Glucose (mg/dL)	105.4±3.1	108.6±3.8	110.0±3.3	103.9±3.0	107.3±2.4	108.5±4.7
Insulin (mU/L)	14.35±1.1	16.25±1.4	16.01±2.2	15.15±1.4	15.98±1.3	17.76±3.0
HOMA IR index	3.78±0.4	4.38±0.4	4.44±0.7	3.94±0.4	4.24±0.4	3.94±0.4
CRP (mg/L) (NR < 6 mg/L)	4.72±1.6	2.52±0.7	3.97±1.3	1.56±0.3	1.51±0.3	5.99±3.3
AST (U/L) (NR <37 U/L)	27.2±2.8	31.65±7.0	26.04±3.6	26.88±2.3	28.55±2.2	26.59±2.8
ALT (U/L) (NR <40 U/L)	37.40±5.8	42.80±7.9	36.28±5.5	37.69±3.0	34.65±2.7	35.00±2.2
γGT (U/L) (NR <55 U/L)	59.15±18.8	98.21±45.7	65.46±23.4	81.81±23.0	81.83±25.4	80.95±24.2

Values are means ± SEMs. Between-group differences were determined with the use of a 2-tailed unpaired *t* test in each group. * $P < 0.05$ for differences between the compared groups. OL, olive oil; RA, rapeseed oil; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, γ-glutamyl-transpeptidase; NR, normal range.

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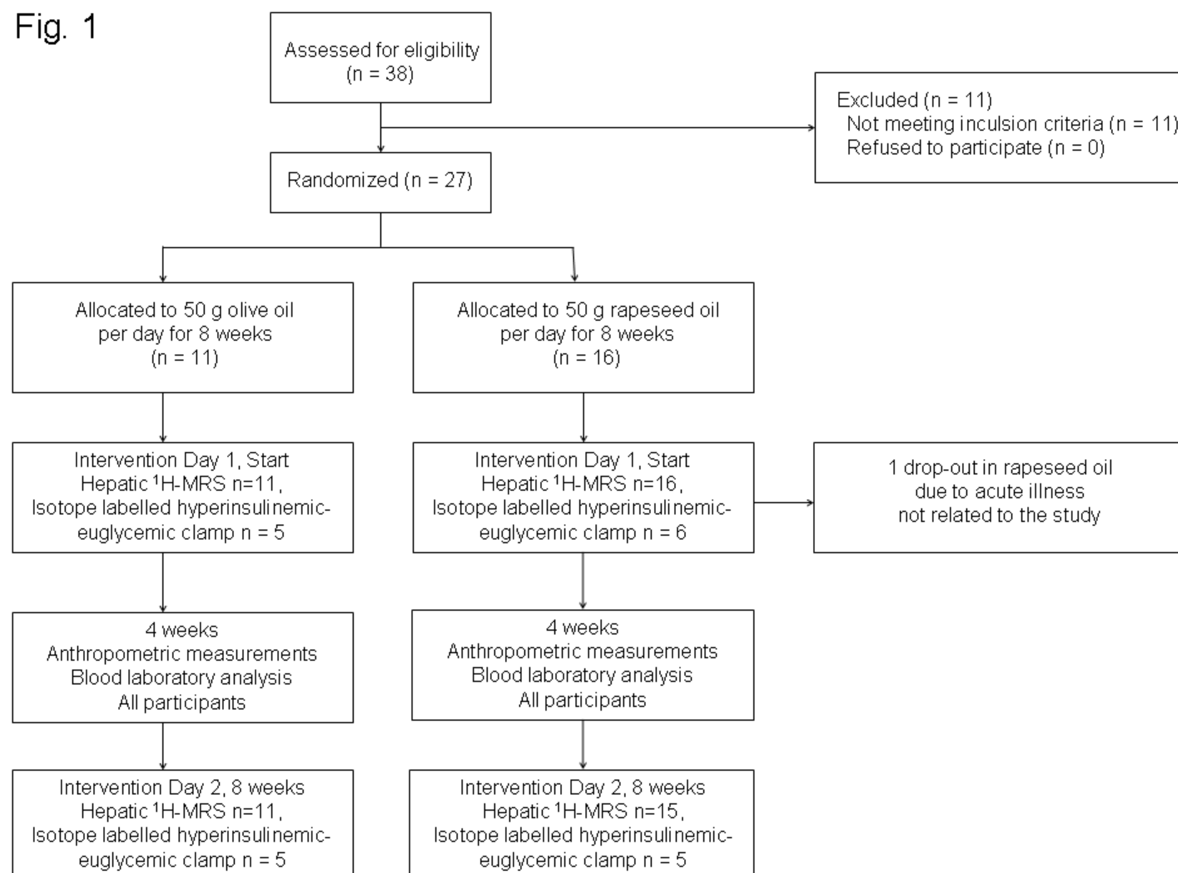

Figure 1: Schematic of the study. Participants were randomized to either olive oil or rapeseed oil. At the beginning and at the end of the study all participants underwent anthropometric measurements, laboratory blood tests and hepatic ^{1}H-MRS to assess intrahepatic lipid content. At four weeks of the study anthropometric measurements and laboratory blood tests were performed on all participants. In a subgroup of five participants of each group additional isotope labelled hyperinsulinemic-euglycemic clamps with isotope labelled glucose were performed at the beginning and at the end of the study. ^{1}H-MRS: Hepatic proton magnetic resonance spectroscopy.

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Fig. 2

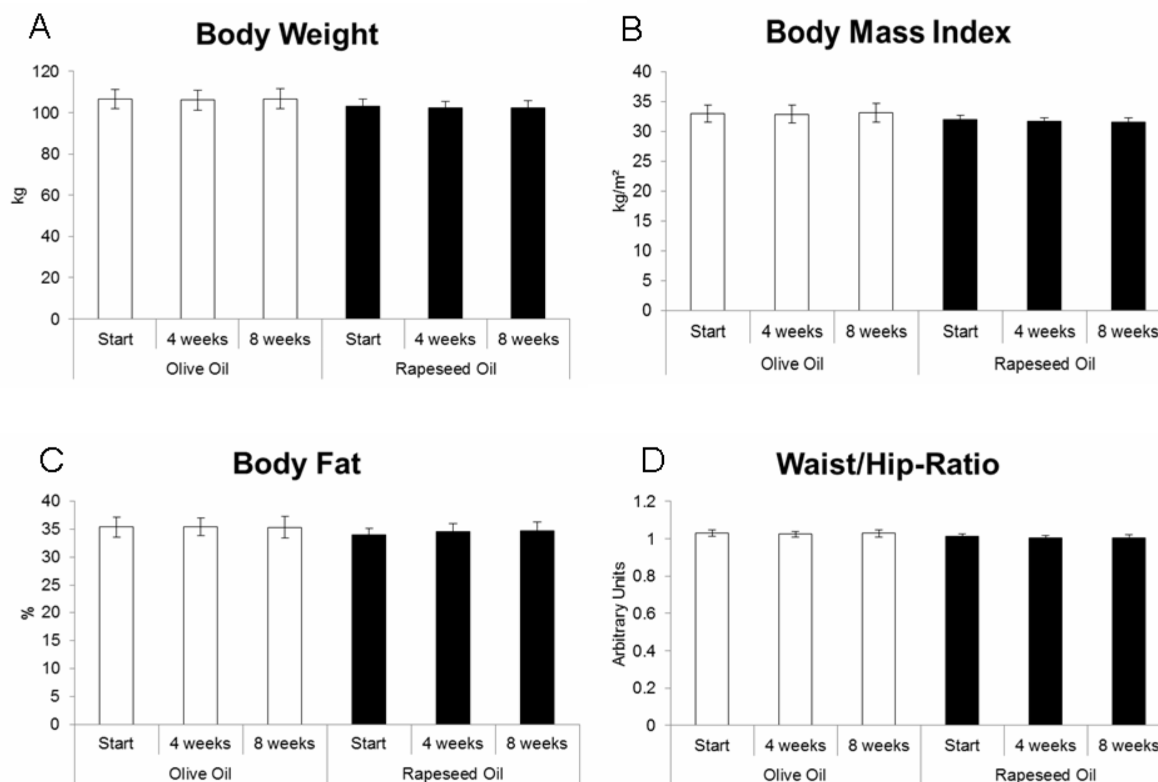

Figure 2: Body weight (A), Body mass index (B), Body fat (C) and Waist-to-Hip ratio (D). Body weight, Body mass index, Body fat and Waist-to-Hip ratio were measured at the beginning, at four weeks and at the end (eight weeks) of the study. Values are means ± SEMs. Differences between the beginning and four and eight weeks of the study, respectively, were determined with the use of a 2-tailed paired *t* test within each group.

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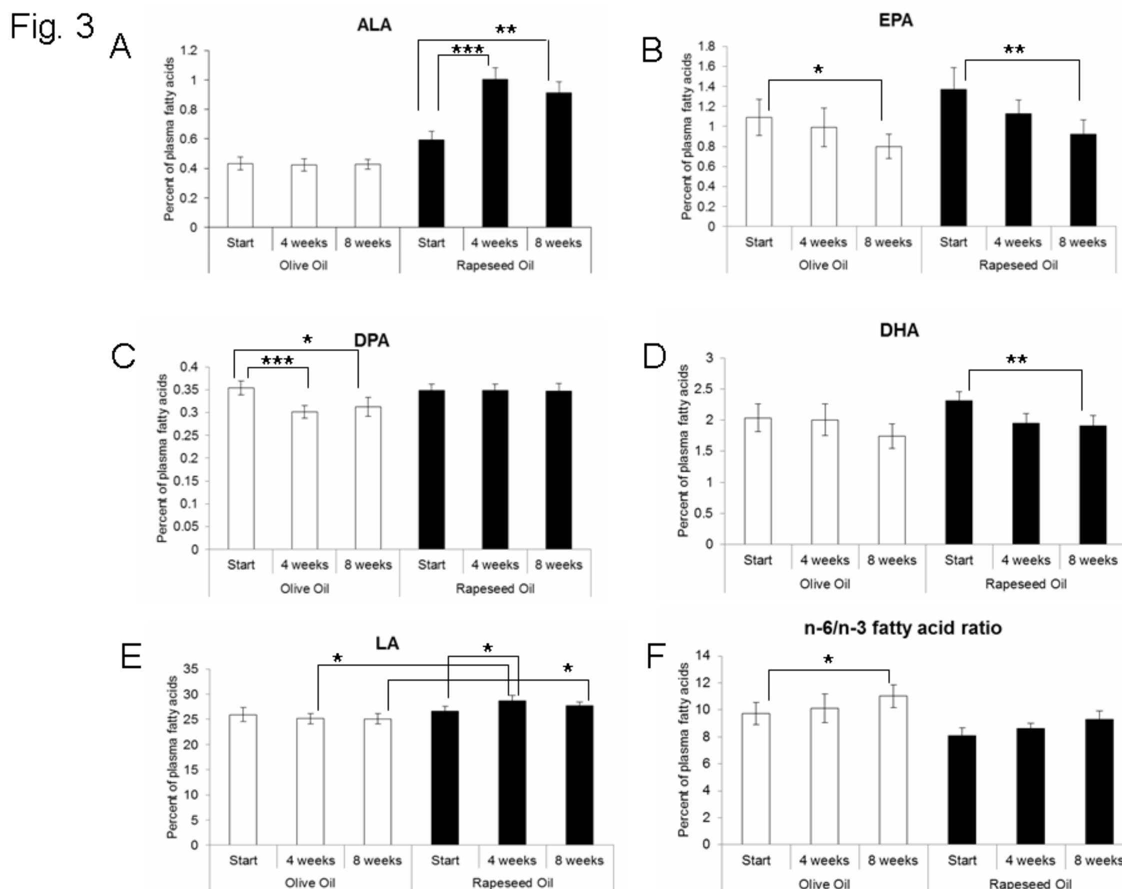

Figure 3: Plasma fatty acid concentrations. The percentage of alpha linolenic acid and its conversion products **(A, B C, D)** and of linoleic acids **(E)** of total plasma fatty acids in was determined at four weeks and at the end (eight weeks) of the study. Additionally, the n-6/n-3 fatty acid ratio was calculates for these time points **(F)**. Values are means ± SEMs. Differences between the beginning and four and eight weeks of the study, respectively, were determined with the use of a 2-tailed paired *t* test within each group or a 2-tailed unpaired *t* test between OL and RA. * $P<0.05$, ** $P < 0.01$, *** $P < 0.001$ for differences between the compared groups. ALA, alpha linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA: linoleic acid.

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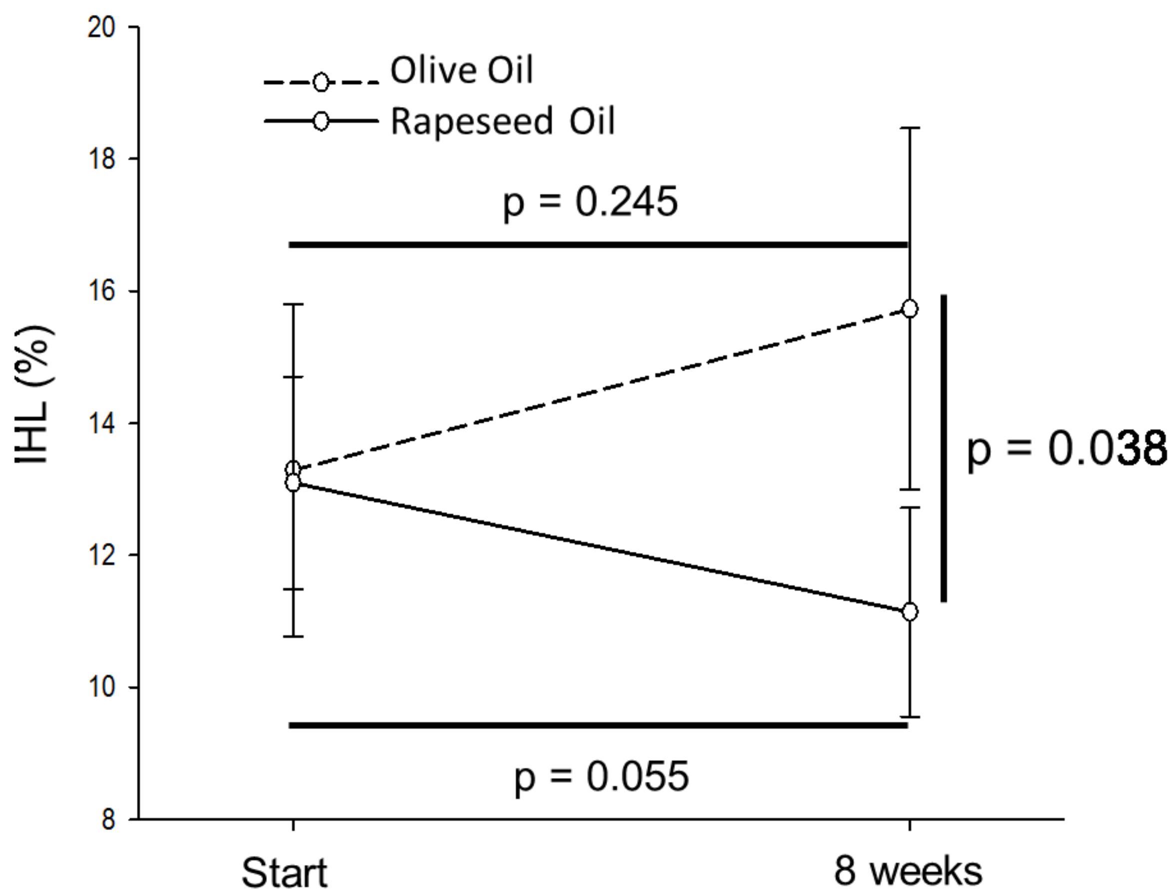

Figure 4: Intrahepatic lipid content. Intrahepatic lipid content was determined prior to (Start) and after (8 weeks) dietary supplementation with olive or rapeseed oil, respectively. Values are means ± SEMs. Differences in IHL between the beginning and eight weeks of the study within each group were determined with the use of a 2-tailed paired *t* test. Differences in IHL between the two groups at the beginning and at eight weeks of the study were determined with the use of a 2-tailed unpaired *t* test. IHL, intrahepatic lipid content.

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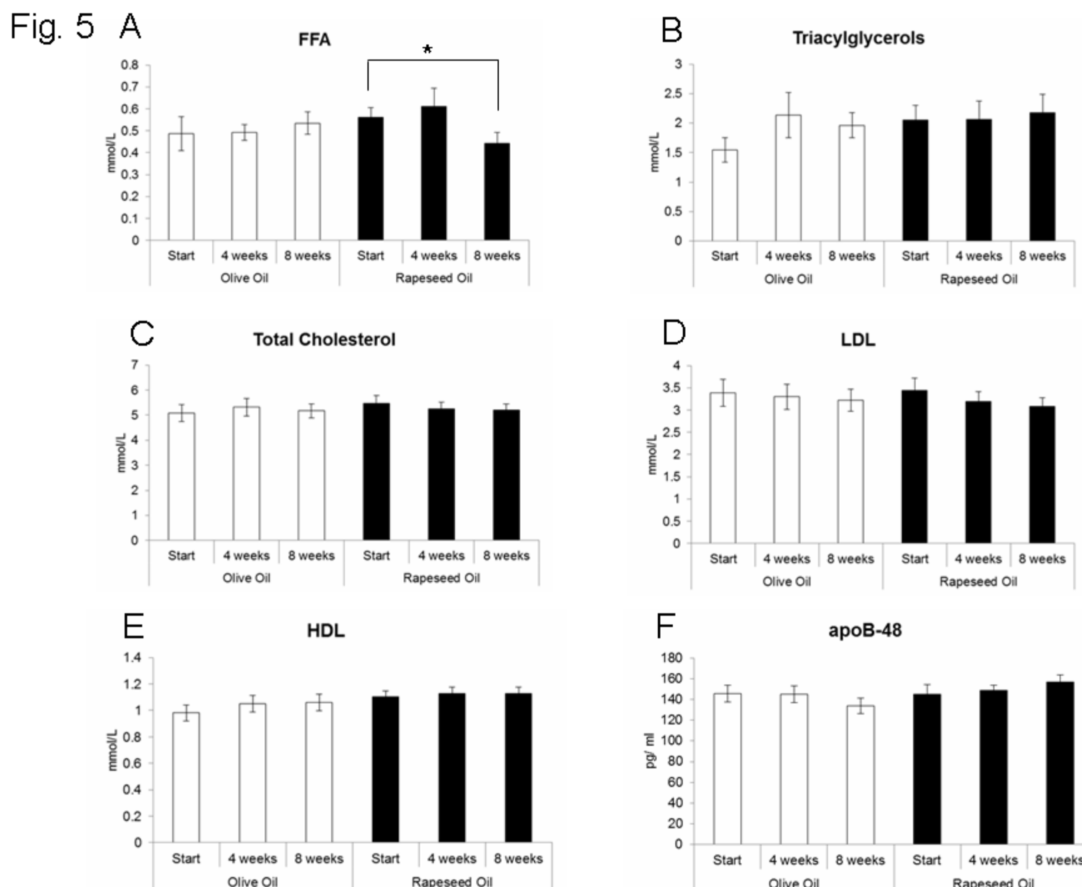

Figure 5: Measurement of serum lipids. Serum free fatty acids (A), triacylglycerols (B), total cholesterol (C), LDL cholesterol (D), HDL cholesterol (E) and apolipoprotein B-48 (F) were measured at the beginning, at four weeks and at the end (eight weeks) of the study. Values are means ± SEMs. Differences between the beginning and four and eight weeks of the study, respectively, were determined with the use of a 2-tailed paired *t* test within each group. * $P < 0.05$ for differences between the compared groups. apoB-48, apolipoprotein B-48; FFA, free fatty acids.

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Fig. 6

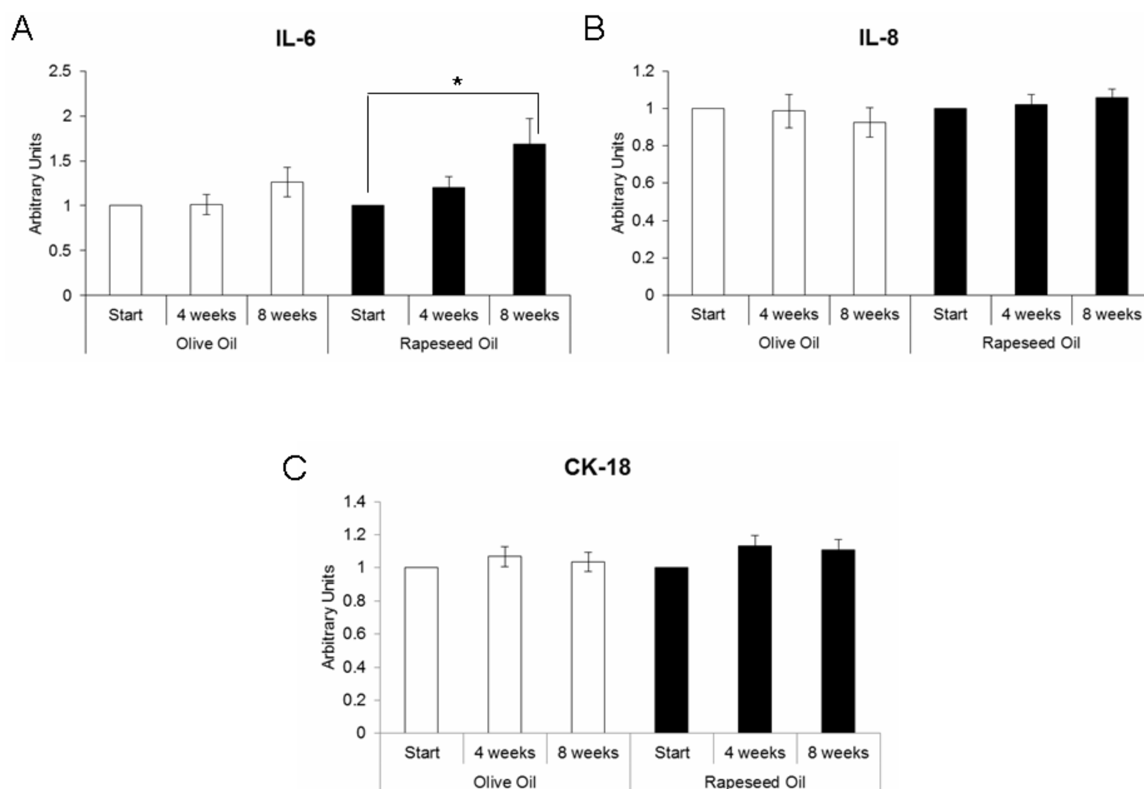

Figure 6: Measurement of serum cytokines and cytokeratin 18. Interleukin-6 (A), interleukin-8 (B) and cytokeratin 18 (C) were measured at the beginning, at four weeks and at the end (eight weeks) of the study. Values are means ± SEMs and expressed relatively compared to the beginning (Start) of the study. Differences between the beginning and four and eight weeks of the study, respectively, were determined with the use of a 2-tailed paired *t* test within each group. * $P < 0.05$ for differences between the compared groups.

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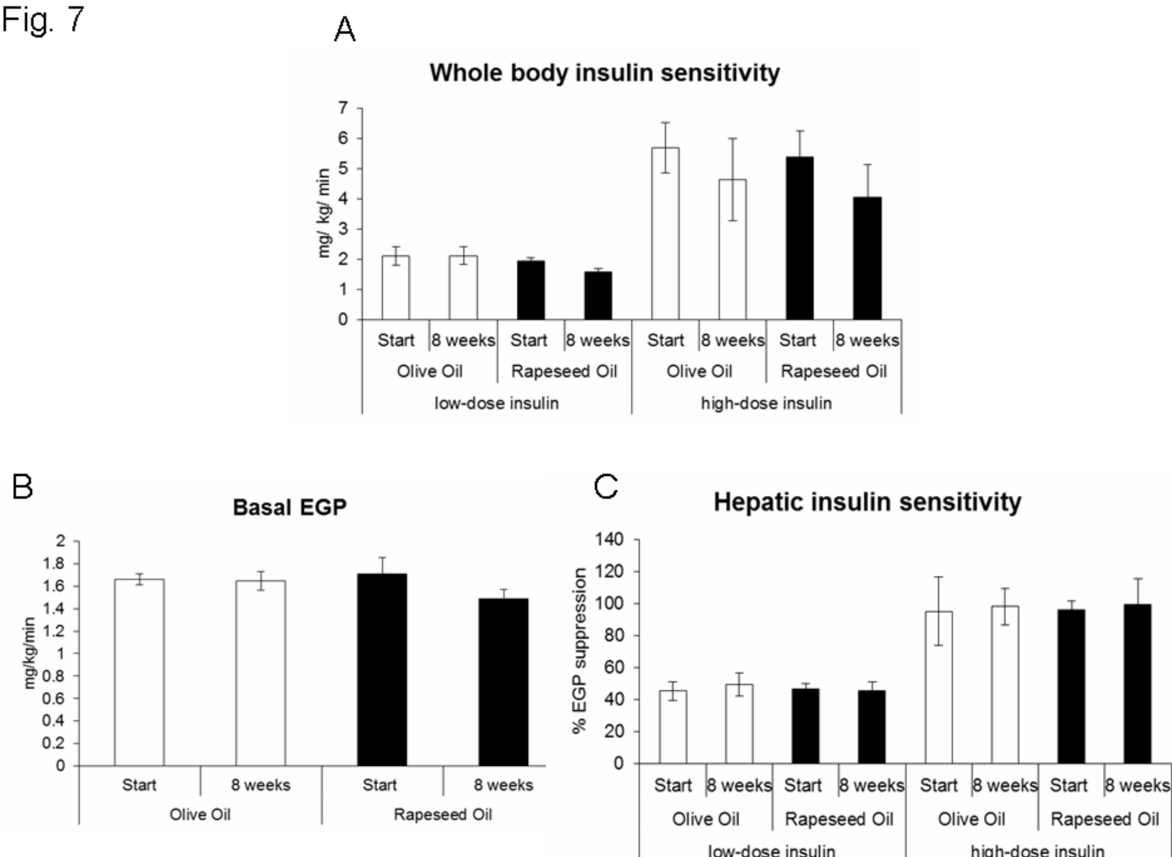

Figure 7: Two-step hyperinsulinemic-euglycemic clamps. Whole body (A) and hepatic (C) insulin sensitivity were assessed using hyperinsulinemic-euglycemic clamps with 20 mU/min/m2 (low-dose) followed by 40 mU/min/m2 (high-dose) insulin infusion at the beginning and at the end of the study. Basal endogenous glucose production (B) was measured with isotope labelled glucose infusion at the beginning and at the end of the study. Values are means ± SEMs. Differences between the beginning and the end of the study were determined with the use of a 2-tailed paired *t* test within each group. EGP: endogenous glucose production.

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