Association of dietary macronutrient composition and non-alcoholic fatty liver disease in an aging population: The Rotterdam Study

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Abstract

Introduction A healthy lifestyle is the first-line treatment in NAFLD, but specific dietary recommendations are lacking. Therefore, we aimed to determine whether dietary macronutrient composition is associated with NAFLD.

Methods Participants from the Rotterdam Study were assessed on 1) average intake of macronutrients (protein, carbohydrate, fat, fibre) using a Food Frequency Questionnaire, and 2) NAFLD presence using ultrasonography, in absence of excessive alcohol, steatogenic drugs, and viral hepatitis. Macronutrients were analysed using the nutrient density method and ranked (Q1-Q4). Logistic regression analyses were adjusted for sociodemographic, lifestyle, and metabolic covariates. Moreover analyses were adjusted for and stratified by BMI (25kg/m²). Also, substitution models were built.

Results In total, 3882 participants were included (age 70±9, 58% female). NAFLD was present in 1337 (34%) participants of whom 132 were lean and 1205 overweight. Total protein was associated with overweight NAFLD after adjustment for sociodemographic and lifestyle covariates ($OR_{Q4vs.Q1}$ 1.40; 95%CI 1.11–1.77). This association was driven by animal protein ($OR_{Q4vs.Q1}$ 1.54; 95%CI 1.20–1.98). After adjustment for metabolic covariates, only animal protein remained associated with overweight NAFLD ($OR_{Q4vs.Q1}$ 1.36; 95%CI 1.05–1.77). Mono and disaccharides were associated with lower overall NAFLD prevalence (OR_{Q4vsQ1} 0.66; 95%CI 0.52–0.83) but this effect diminished after adjustment for metabolic covariates and BMI. No consistent associations were observed for fat subtypes or fibre. There were no substitution effects.

Conclusion This large population-based study shows that high animal protein intake is associated with NAFLD in overweight, predominantly aged Caucasians, independently of well-known risk factors. Contrary to previous literature, our results do not support a harmful association of mono and disaccharides with NAFLD.

Introduction

Since the first case description by Ludwig et al. in 1980, the prevalence of non-alcoholic fatty liver disease (NAFLD) increased expeditiously paralleling the obesity epidemic.[13,237] NAFLD is characterised by fat deposition in the liver in absence of excessive alcohol consumption or established liver disease.[13] It is referred to as the hepatic manifestation of the metabolic syndrome, and has now become the most common liver disease affecting an estimated one-third of adults in the general population of developed countries.[6] In high-risk populations with type 2 diabetes and metabolic comorbidities, prevalence of NAFLD even reaches up to 70%.[238] Progression of NAFLD can lead to fibrosis, cirrhosis, hepatocellular carcinoma and liver failure with corresponding life-threatening complications. The end-stage disease often requires liver transplantation. Indeed NAFLD already constitutes the second most common indication for liver transplantation in the United States and is predicted to become the number one indication soon.[22] In addition to the above-mentioned, NAFLD also contributes to an increased risk for metabolic and cardiovascular morbidity and mortality.[239] Hence, NAFLD has emerged as a great global health threat and subsequently, prevention and treatment thereof are of strong public interest.

NAFLD is more common in people with an unhealthy lifestyle, i.e. with an unhealthy diet and physical inactivity.[13] Although there are several hundreds of promising pharmacological trials ongoing, there is still no registered drug for the treatment of NAFLD. Therefore, in daily practice, lifestyle modification remains the first-line treatment in NAFLD.[55] At present, weight loss of 5–7% or more is recommended, based on two prospective (randomised controlled) trials in overweight patients.[240,241] However, not all overweight individuals will develop NAFLD, and likewise, not all individuals with NAFLD are overweight.[242] This gives food for thought on whether dietary quality, rather than dietary quantity, is important in the pathogenesis and treatment of NAFLD. Current dietary recommendations include caloric restriction and adherence to the macronutrient composition of the Mediterranean diet.[55] However, evidence on the mono-unsaturated fatty acid (MUFA)–rich Mediterranean diet for NAFLD is limited by small study populations (N=12–90 subjects), suboptimal nutritional analyses, or use of surrogate primary endpoints (i.e. liver transaminases) rather than imaging diagnosis of NAFLD.[59] Moreover, health recommendations on fat and carbohydrate consumption have been widely debated.[243,244] Only a minority of studies examined the effect of all macronutrients combined, and those who did, showed conflicting results.[57,245-247] In addition, these studies too are hampered by small sample size (N=56–349 subjects) and/or by suboptimal methodology (e.g. not correcting for energy intake, BMI, overall dietary quality, or other potential confounders).

So far, no study has examined macronutrient composition in relation to NAFLD on a large scale using comprehensive nutritional analyses methods including energy density and substitution models, taking into account potentially important sociodemographic, lifestyle,

and metabolic risk factors. We therefore conducted a large population-based study in elderly Caucasians, who completed a validated 389-item food frequency questionnaire (FFQ) and underwent hepatic ultrasound, to determine whether macronutrient intake is associated with NAFLD independently of total energy intake and a large number of potentially confounding traits.

Methods

Study population

The Rotterdam Study is a large ongoing population-based cohort of predominantly elder participants residing in a suburb of Rotterdam, the Netherlands. The design and rationale of this population-based study have been described in detail previously.[248] In short, the study commenced in 1989 and comprises three different cohorts (RS I, RS II, and RS III). All residents aged 55 (RS I, RS II) or 45 (RS III) and above were invited to participate. Participation rate of these cohorts were 78%, 67% and 65%, respectively. Liver imaging is part of the core protocol since 2009. Hence, all participants who underwent abdominal US between January 2009 and June 2014 were included. The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus MC University Medical Centre Rotterdam and by the review board of The Netherlands Ministry of Health, Welfare, and Sports. Written informed consent was obtained from the participants.

Dietary Data

Participants were asked to complete an externally validated semi-quantitative 389-item FFQ developed for Dutch adults during their visit at the research centre.[98,249] This questionnaire included detailed questions on food item consumption over the last month and addressed frequency, portion size, type of food item, and preparation methods. Servings were estimated in grams per day using standardised household measures,[250] and macronutrient intake was extracted from the questionnaires using the Dutch Food Composition Table (NEVO v2011) that includes information on nutrient content per gram or serving per product. Incomplete or unreliable FFQs, defined as caloric intake of less than 500 or more than 7500 kilocalories (kcal), were excluded. To correct for potential measurement error and to examine the relative contribution of a macronutrient to the diet, we adjusted for energy intake using the nutrient density method.[251] For example, 1 gram of protein equals 4 kcal, hence to calculate the energy percent of protein (E%) = (total protein intake(g)*4/ total kcal intake)*100. Similarly, the E% of carbohydrates (4kcal/g), fats (9kcal/g), fibre

(2kcal/g) and alcohol (6kcal/g) were determined. Subsequently, all E% were ranked into quartiles (Q1=lowest quartile).
Additionally, to account for confounding by overall dietary quality, the Dutch Healthy Diet Index (DHDI) was derived from the FFQ and added to the multivariable analyses.[100] A higher DHDI indicates stricter adherence to the Dutch dietary guidelines. DHDI is an adherence score designed specifically for the Netherlands, but correlates highly with the perhaps more familiar (Alternate)- Healthy Eating Index, the (A)HEI ($r \geq 0.60$).[252] For the purpose of this study the DHDI was modified in multivariable models to avoid multicollinearity (e.g. macronutrient analyses of fat is adjusted for DHDI minus the trans fatty acid and saturated fat components of the DHDI).

Liver imaging

Steatosis was assessed using abdominal ultrasound, which was carried out by a certified and experienced technician on Hitachi HI VISION 900. All participants were unaware of the presence of steatosis before completing the FFQs. Ultrasound images were stored digitally and re-evaluated by a single hepatologist with over 10 years of experience (RK). The ultrasound technician and hepatologist were blinded for the FFQ data. Diagnosis of steatosis was determined dichotomously according to Hamaguchi et al.,[104] as presence or absence of hyperechogenic liver parenchyma. Participants with possible secondary causes for steatosis were excluded from this study, i.e. 1) excessive alcohol consumption (>30g/day for men and >20g/day for women) as assessed by the FFQ; 2) use of steatogenic drugs, i.e. amiodarone, systemic corticosteroids, methotrexate, or tamoxifen, extracted from linked pharmacy data; and 3) viral hepatitis, based on hepatitis B surface antigen and anti-hepatitis C virus serology, as measured by an automatic immunoassay (Roche Diagnostic GmbH). Of note, for participants from RS-III, there was a median time-gap of 5.5 years between completing the FFQ (before introduction of liver ultrasound) and the performance of ultrasound. Because dietary data are known to be stable over time, RS-III was included in the total study population.[109]

Biochemistry and additional covariates

All blood samples were collected after overnight fasting just before abdominal US. Blood lipids, platelet count, glucose, alanine aminotransferase (ALT), aspartate aminotransferase, gamma-glutamyl transferase (GGT), and total bilirubin were measured using automatic enzyme procedures (Roche Diagnostic GmbH, Mannheim, DE). Insulin was determined using an automatic immunoassay (Roche Diagnostic GmbH).
Data concerning demographics, physical activity, smoking, educational level, and comorbid conditions were obtained during an extensive home interview by trained interviewers. An-

thropometric measurements were carried out by well-trained research assistants measuring height (m), weight (kg) and waist circumference (WC in cm). Blood pressure measurements (mmHg) were obtained at a single visit using two subsequent measurements in upright position after a minimum of 5 minutes rest. BMI was calculated as weight/height² (kg/m²) and considered lean if <25 kg/m² and overweight if ≥25 kg/m².

Insulin resistance was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR), as fasting glucose (mmol/dl) multiplied by fasting insulin (mU/L) divided by 22.5.[106] The metabolic syndrome was diagnosed when at least three of the following traits were present: 1) abdominal obesity, defined as WC ≥102 cm in men and ≥88 cm in women; 2) serum triglycerides ≥150 mg/dl (1.7 mmol/L), or drug treatment for elevated triglycerides; 3) serum high-density lipoprotein cholesterol (HDL-C) ≤40 mg/dl (1.0 mmol/L) in men and ≤50 mg/dl (1.3 mmol/L) in women, or drug treatment for low HDL-C; 4) blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure; and 5) fasting plasma glucose (FPG) ≥100 mg/dl (5.6 mmol/L) or drug treatment for elevated blood glucose.[28]

Statistical Analyses

After excluding participants with either missing or unreliable FFQs and participants with more than 30% missing study variables, the remaining missing values (range of 0.02–10.79% within covariates) were imputed using multiple imputation (fully conditioned specification) to reduce bias due to missing data.[253] Ten imputed datasets were created using the R-package mice and the analyses were performed in each dataset before the results were pooled by Rubin's rules, to take into account uncertainty with the prediction of missing data.[254] The imputation process is described in more detail in the *Supplementary Methods*.

Descriptive statistics were used to describe population characteristics. Continuous data were presented as mean ± standard deviation (SD) or median with 25th and 75th percentile (P25-P75) according to the distribution of the variable. Categorical data were presented as percentage. Chi-square test, Student's T test, or Wilcoxon Rank Sum test were used to evaluate differences in categorical, normally distributed, and not-normally distributed data, between subjects with and without NAFLD. In order to give more insight in the composition of the different macronutrients, we created 49 relevant food groups out of the 389 food items and performed a Spearman correlation to identify the 3 topmost correlations between macronutrients and food groups.

All macronutrients were analysed continuously (in E%) using standardised values (increase per 1SD) as well as in quartiles using Q1 as reference. We used three separate multivariable logistic regression models to assess the associations of macronutrients with NAFLD. The first 'socio-demographic' model (model 1) includes adjustment for age, gender, education level (low/moderate/high), and study cohort. The second 'lifestyle confounding' model

(model 2) is additionally adjusted for smoking status (never vs. past/current), alcohol in E%, energy intake (kcal), physical activity (metabolic equivalent of task (MET) hours/week), and DHDI. Finally, model 3, the 'metabolic' model, was additionally adjusted for presence of diabetes, metabolic syndrome, and total cholesterol (mg/dL). Moreover, analyses with carbohydrates were adjusted for fibre intake and vice versa, and all subtypes of one macronutrient were adjusted for each other. Results were presented as odds ratio (OR) with 95% confidence interval (95%CI).

BMI is an important covariate, it could act as potential mediator (in the pathway between the exposure [diet] and the outcome [NAFLD]), as a confounder (affecting both exposure and outcome, causing a false association), as a collider (a covariate that is not in the pathway but is influenced by both the exposure and the outcome, it may create a non-existing association between the exposure and outcome), or as effect modifier (indicating different associations in subgroups of patients). Also, participants with NAFLD and a normal BMI (lean NAFLD) could have a different pathophysiological pathway from overweight NAFLD, e.g. through genetic predisposition or body composition. In addition, measurement error, eating habits, and hence, macronutrient associations with NAFLD could have differed between lean and overweight individuals. Therefore, we used the following approaches to account for BMI: First, we evaluated the linearity of BMI in model 3 in relation to steatosis using cubic splines and found a non-linear effect using log-likelihood ratio testing. The figure of the spline showed that a log-shaped form could improve the fit of the model, we therefore created a model 4, adding log-transformed BMI as covariate to the metabolic model to evaluate changes in effect estimates. We believe the effect of BMI can be studied best in this model 4, because here we already adjusted for all other potential confounding factors. Second, we tested for interaction between BMI and each macronutrient (e.g. BMI x total protein). And third, all analyses were stratified for lean and overweight participants at a cut-point of 25 kg/m², while this is a widely used cut-off as established by the WHO and because there was no clear cut-off point visible in the cubic splines.

Also, to evaluate whether the observed associations were due to higher intake of that specific macronutrient rather than lower intake of another macronutrient, we performed substitution analyses in the metabolic model.[255] For example, the substitution model of replacing total protein for total fat included the following dietary covariates, total protein, total carbohydrates, total fibre, and alcohol, but not total fat, in addition to the above-mentioned metabolic and environmental covariates. The obtained estimate from the regression coefficient for protein from this model reflect the theoretical effect of replacing all fat intake completely with protein intake (in E%). Additional sensitivity analyses were performed comparing the analyses of imputed data to that of the complete case, and assessed differences between the group with and without missing cases in order to assess the robustness of our data. In addition, we excluded the third cohort from the final analyses as they filled in their FFQ 5.5 years prior to liver imaging.

To correct for the inflated type I error that arises due to multiple testing we applied the method proposed by Sidák,[256] adapted as described in Galwey et al.,[257] using the effective number of tests instead of the actual number of tests. This adaptation is necessary to take into account that dietary exposures inter-correlate (i.e. the intake of individual macronutrients are not fully independent from each other), and hence, the corresponding tests are not independent from each other. The resulting corrected significance level for all macronutrient analyses was $P<0.021$. All analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA) and R version 3.4.1.

Patient involvement

Participants were not involved in setting the research question or the outcome measures, nor were they involved in developing plans for recruitment, design, or implementation of the study. Participant were not asked for advise on the interpretation of results. All participants were regularly updated on study outcomes via a home-sent newsletter and the study website.

Results

Study Characteristics

The flowchart of the study population is depicted in *Figure 1*. 5967 participants were eligible for this study. First, we excluded participants due to missing, incomplete, or unreliable FFQs (n=1173; 19.7%). These participants were younger (mean age 68.5 vs. 69.6 years old; $P<0.01$), less often of Caucasian origin (95.0% vs. 98.0%; $P<0.01$), and had a slightly higher BMI (27.5 vs. 26.9 kg/m²; $P<0.01$) than the included study group, whereas gender and frequency of steatosis were similar (55.8% vs. 57.5% female; $P=0.27$ and 37.2% vs. 35.5% steatosis; $P=0.27$). Second, we excluded participants with more than 30% of missing study variables (0.8%), and participants with secondary causes for steatosis (18.3%). Hence, the total study population included 3882 participants. Population characteristics are presented in *Table 1* and original and imputed data in *Supplementary Table 1*. In short, mean age was 69.7 ± 8.8, 58.3% were female, the majority of participants were of Caucasian origin (97.6%), and median BMI was 26.9 (24.5 – 29.7) kg/m². NAFLD prevalence was 34.4% (n=1337). Participants with NAFLD had lower education level, were more often current or former smokers, performed less physical activity, had higher BMI, more comorbidities, and more deviant mean or median laboratory values albeit within the normal range.

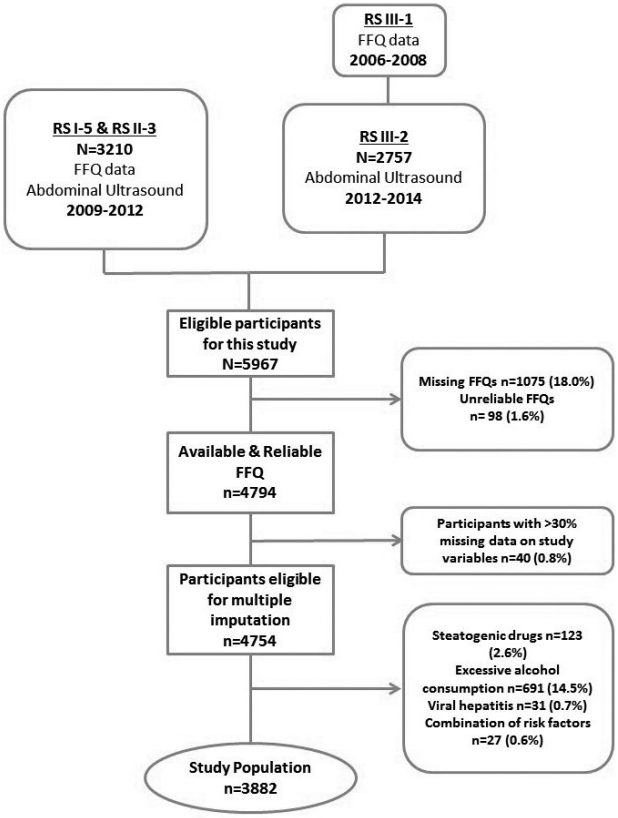

Figure 1: Flowchart of the study
Legend: RS is Rotterdam Study, I-III is number of cohort, 1–5 is number of times cohort visited. FFQ is Food Frequency Questionnaire.

Dietary Data

Dietary characteristics are presented as relative consumption (E%) in *Table 1*. Animal protein correlated most with red meat (r=0.35), refined meat (r=0.25), and fish (r=0.24); and vegetable protein with whole grain, rice, bread and pasta (r=0.50), cruciferous vegetables (r=0.27), and vegetarian food products (r=0.24). Mono and disaccharides correlated most with fruit (r=0.63), sweets (r=0.30), and fruit juice (r=0.24); and polysaccharides with whole grain, rice, bread and pasta (r=0.39), refined grain, rice, bread and pasta (r=0.29), and potatoes (r=0.27). Lastly, saturated fat correlated most with full fat cheese/ cream (r=0.50), full/ non-fluid fat (r=0.42), and desserts (0.27); MUFAs with sauce (r=0.29), fried snacks (r=0.29), and oil fats (r=0.29); poly-unsaturated fatty acids (PUFA) with diet/ fluid fats (r=0.35), full/ non-fluid fats (r=0.32), and peanuts (r=0.25); and trans fatty acid with full fat cheese/cream (r=0.39), full/ non-fluid fat (r=0.38), and desserts (r=0.32). NAFLD

Table 1: Characteristics of the study population

	Total Population N=3882	No NAFLD n=2545 (65.6%)	NAFLD n=1337 (34.4%)	*P*-value*
Demographics				
Age (years)	69.7 ± 8.8	69.6 ± 9.2	70.0 ± 8.2	0.164
Female (%)	58.3	59.1	56.8	0.184
Caucasian (%)	97.6	97.4	98.0	0.458
Education Level (%)				
Low	48.4	45.0	54.9	<0.001
Intermediate	30.2	31.4	28.1	
High	21.3	23.6	17.0	
Smoking status (%)				
Never	36.1	38.3	31.8	<0.001
Past or Current	63.9	61.7	68.2	
Alcohol (units/d)	0.45 (0.05 – 1.20)	0.45 (0.06 – 1.21)	0.43 (0.05 – 1.19)	0.422
Physical Activity†	40.6 (15.7 – 77.6)	43.8 (17.2 – 81.3)	34.6 (13.5 – 70.0)	<0.001
Physical examination				
BMI (kg/m²)	26.9 (24.5 – 29.7)	25.8 (23.7 – 28.0)	29.3 (27.0 – 32.3)	<0.001
Lean	30.2	40.9	9.9	<0.001
Overweight	69.7	59.1	90.1	
Waist Circumference (cm)				
Men	98.2 ± 10.6	95.0 ± 9.1	104.1 ± 10.5	<0.001
Women	89.1 ± 12.2	85.0 ± 10.5	97.4 ± 11.3	<0.001
Biochemistry				
AST (U/L)	24 (21 – 28)	24 (21 – 28)	25 (21 – 29)	<0.001
ALT (U/L)	18 (15 – 24)	17 (14 – 22)	21 (16 – 29)	<0.001
GGT (U/L)	23 (17 – 34)	21 (15 – 30)	28 (20 – 39)	<0.001
Platelets (*10^9/L)	262 (223 – 305)	260 (222 – 303)	266 (225 – 310)	0.031
HOMA-IR	2.6 (1.7 – 4.1)	2.1 (1.5 – 3.1)	4.1 (2.7 – 6.1)	<0.001
Total Cholesterol (mmol/L)	5.4 ± 1.1	5.5 ± 1.1	5.4 ± 1.1	0.002
HDL-C (mmol/L)	1.5 ± 0.4	1.5 ± 0.4	1.3 ± 0.4	<0.001
Triglycerides (mmol/L)	1.3 (1.0 – 1.7)	1.2 (0.9 – 1.5)	1.6 (1.2 – 2.1)	<0.001
Comorbidities				
Metabolic Syndrome	51.9	40.8	73.0	<0.001
-WC>88cm (♀) or >120cm (♂)	43.2	29.1	69.9	<0.001
- Triglycerides >150mg/dL	46.0	39.0	59.4	<0.001
- HDL-C <40mg/dL (♂) or 50mg/dL (♀)	44.7	38.3	56.7	<0.001
- Blood pressure ≥130/85mmHg	84.3	80.5	91.5	<0.001
- FPG>100mg/dL	41.5	31.1	61.2	<0.001
Diabetes Mellitus (%)	13.1	7.5	23.7	<0.001
Hypertension (%)	74.0	68.9	83.7	<0.001

Table 1 (*continued*)

	Total Population N=3882	No NAFLD n=2545 (65.6%)	NAFLD n=1337 (34.4%)	*P*-value*
Dietary Data (E%)				
Total Kilocalories/ day	2031 (1620 – 2515)	2052 (1642 – 2537)	1996 (1579 – 2456)	0.003
Total Protein	15.6 (14.0 – 17.4)	15.4 (13.8 – 17.2)	16.0 (14.3 – 17.8)	<0.001
Animal protein	9.2 (7.5 – 11.2)	9.0 (7.2 – 10.9)	9.5 (7.9 – 11.6)	<0.001
Vegetable protein	6.3 (5.5 – 7.1)	6.3 (5.5 – 7.1)	6.2 (5.4 – 7.1)	0.076
Total Fat	31.9 (27.9 – 36.1)	31.7 (27.8 – 35.9)	32.4 (28.2 – 36.5)	0.039
Saturated fat	11.5 (9.7 – 13.3)	11.4 (9.7 – 13.2)	11.6 (9.9 – 13.5)	0.018
MUFA	10.7 (9.2 – 12.4)	10.7 (9.1 – 12.3)	10.8 (9.3 – 12.6)	0.055
PUFA	6.4 (5.3 – 7.6)	6.4 (5.4 – 7.6)	6.4 (5.3 – 7.6)	0.276
Trans fatty acid	0.51 (0.41 – 0.62)	0.50 (0.41 – 0.61)	0.53 (0.42 – 0.64)	0.002
Total Carbohydrate	46.2 (41.9 – 50.7)	46.6 (42.2 – 51.0)	45.5 (41.5 – 50.3)	<0.001
Mono & disaccharide	22.8 (18.2 – 27.7)	23.2 (18.7 – 28.0)	21.9 (17.5 – 26.9)	<0.001
Polysaccharide	22.7 (19.8 – 26.0)	22.7 (19.7 – 26.0)	22.7 (19.9 – 25.9)	0.683
Fibre	2.6 (2.2 – 3.0)	2.6 (2.2 – 3.1)	2.6 (2.1 – 3.0)	0.013
Total Alcohol	1.4 (0.2 – 3.4)	1.4 (0.2 – 3.4)	1.3 (0.1 – 3.5)	0.939

Pooled data based on 10 imputations represent % for categorical variables and for continuous variables mean ± SD or median (P25-P75).*P-value is based on T-test, Wilcoxon rank sum test, Chi-square test or Fisher's exact test and is a comparison between the no NAFLD and NAFLD columns. †Physical activity in metabolic equivalent task hours/week. Abbreviations ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; FPG: fasting plasma glucose; GGT: gamma-glutamyltransferase; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; MUFA: mono-unsaturated fatty acid; PUFA: poly-unsaturated fatty acid

participants reported lower median caloric consumption than participants without NAFLD (1996 vs. 2052 kcal, *Table 1*). The same was observed for BMI (overweight individuals reported 2006 kcal vs 2089 kcal in lean individuals; *P*<0.01). NAFLD participants reported higher median total protein, animal protein, total fat, saturated fat, and trans fatty acid intake (all in E%, *Table 1*). Moreover, they reported lower total carbohydrate and mono and disaccharide consumption, and marginally lower fibre consumption (*Table 1*). Absolute consumption of macronutrients in grams and energy percentage per quartile are given in *Supplementary Table 2*.

Protein consumption and NAFLD

Both total and animal protein were associated with higher odds for NAFLD in the first 3 models (*Table 2*). Vegetable protein was not associated with NAFLD in any of the models. After adjustment for log-transformed BMI none of the associations remained. However, effect modification by BMI was suggested: interaction with BMI was *P*=0.10 for total protein, *P*=0.04 for animal protein, and *P*=0.19 for vegetable protein. Indeed,

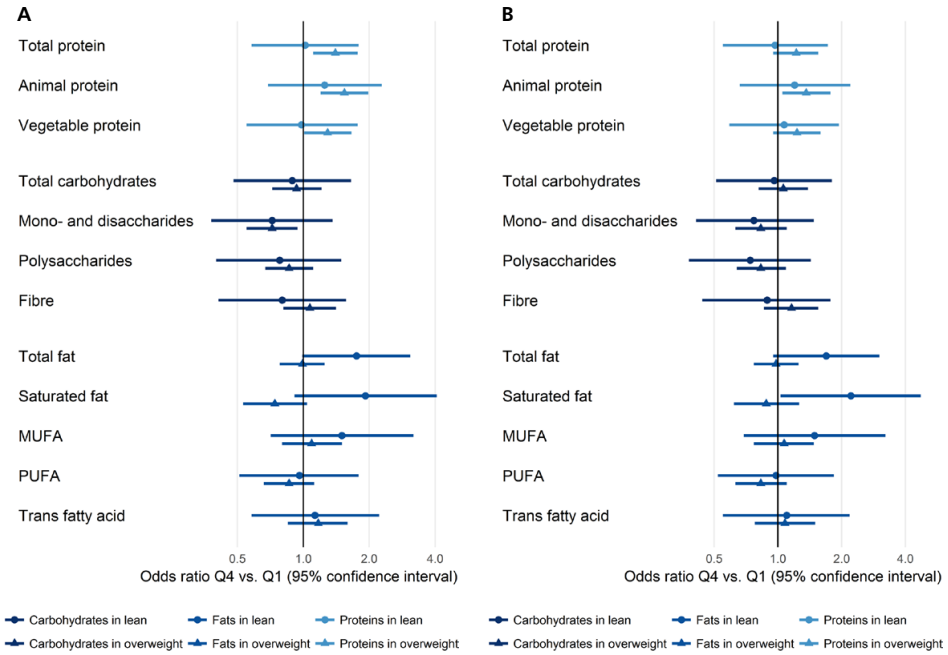

Figure 2: Stratified multivariable analyses for the association of macronutrients and its subtypes with NAFLD (OR with 95%CI)

Results of multivariable logistic regression of the association of the highest quartile (Q4) versus the lowest quartile (Q1) of consumption of a particular (subtype) of macronutrient with NAFLD as dependent outcome. A) Odds ratios are adjusted for age, gender, education level, study cohort, past or current smoking, alcohol in E%, physical activity and DHDI. B) Odds ratios are adjusted for age, gender, education level, past or current smoking, alcohol in E%, physical activity, DHDI, diabetes mellitus, cholesterol and metabolic syndrome. Y-axis: all macronutrients and the P-value for trend over quartiles. X-axis: multivariable OR (95%CI) of Q4 vs. Q1 on a semi-log scale, circle bullets reflect lean participants and triangle bullets reflect overweight participants.

stratified analyses by BMI revealed that both total protein and animal protein intake were associated with overweight NAFLD in model 1 and 2 (*Figure 2A)*. Vegetable protein was associated with overweight NAFLD as well, but only in model 2. In model 3, the association with overweight NAFLD attenuated but remained significant for animal protein ($OR_{Q4vs.Q1}$ 1.36, 95%CI 1.05–1.77; $P_{for\ trend}$=0.09) while not for total protein ($OR_{Q4vs.Q1}$ 1.22, 95%CI 0.95–1.55; $P_{for\ trend}$=0.09), as shown in *Figure 2B*. Detailed results of the stratified models are displayed in *Supplementary Table 3*.

Carbohydrate consumption and NAFLD

Total carbohydrate intake and mono and disaccharide intake were inversely related with NAFLD prevalence in both model 1 and 2, but the associations attenuated in the third and fourth models (*Table 2*). In contrast, polysaccharide consumption was not associated in the first two models, but after adjustment for metabolic traits in model 3, there was an

Table 2: Stepwise logistic regression models between macronutrients as independent variables and NAFLD as dependent variable using quartile 1 as reference in the total study population

	Q2	Q3	Q4	*P* for trend	Continuous (per SD increase)
Model 1 (Sociodemographic)					
Total protein	**1.24 (1.02 – 1.51)**	**1.49 (1.23 – 1.81)‡**	**1.71 (1.41 – 2.07)‡**	**<0.001‡**	**1.22 (1.14 – 1.30)‡**
Animal protein	**1.45 (1.19 – 1.77)‡**	**1.41 (1.16 – 1.72)‡**	**1.90 (1.55 – 2.33)‡**	**<0.001‡**	**1.24 (1.15 – 1.33)‡**
Vegetable protein	1.06 (0.88 – 1.28)	0.98 (0.81 – 1.20)	1.12 (0.92 – 1.38)	0.411	1.05 (0.97 – 1.13)
Total carbohydrate	0.94 (0.78 – 1.14)	**0.76 (0.62 – 0.92)‡**	**0.74 (0.60 – 0.91)‡**	**0.001‡**	**0.87 (0.81 – 0.93)‡**
Mono-disaccharide	**0.73 (0.61 – 0.88)‡**	**0.67 (0.55 – 0.82)‡**	**0.60 (0.49 – 0.75)‡**	**<0.001‡**	**0.85 (0.78 – 0.92)‡**
Polysaccharide	0.99 (0.82 – 1.20)	0.94 (0.77 – 1.15)	0.83 (0.67 – 1.03)	0.088	0.96 (0.89 – 1.04)
Fibre	0.84 (0.69 – 1.02)	0.93 (0.76 – 1.13)	0.93 (0.75 – 1.15)	0.745	1.01 (0.94 – 1.09)
Total fat	0.96 (0.79 – 1.16)	1.13 (0.94 – 1.37)	1.17 (0.97 – 1.41)	**0.037**	1.05 (0.99 – 1.13)
Saturated fat	0.94 (0.77 – 1.15)	0.98 (0.78 – 1.22)	0.94 (0.71 – 1.24)	0.759	0.91 (0.80 – 1.04)
MUFA	1.07 (0.87 – 1.31)	1.07 (0.85 – 1.33)	1.25 (0.95 – 1.64)	0.141	**1.15 (1.03 – 1.29)‡**
PUFA	0.98 (0.81 – 1.19)	0.89 (0.73 – 1.08)	**0.79 (0.63 – 1.00)**	**0.033**	**0.89 (0.81 – 0.98)‡**
Trans fatty acid	0.95 (0.78 – 1.17)	1.16 (0.93 – 1.44)	1.25 (0.96 – 1.63)	**0.044**	1.11 (0.99 – 1.25)
Model 2 (Lifestyle Confounding)					
Total protein	**1.23 (1.01 – 1.50)**	**1.46 (1.20 – 1.78)‡**	**1.61 (1.31 – 1.97)‡**	**<0.001‡**	**1.20 (1.11 – 1.29)‡**
Animal protein	**1.44 (1.18 – 1.76)‡**	**1.38 (1.12 – 1.69)‡**	**1.80 (1.45 – 2.23)‡**	**<0.001‡**	**1.21 (1.12 – 1.31)‡**
Vegetable protein	1.10 (0.91 – 1.33)	1.03 (0.84 – 1.26)	1.18 (0.95 – 1.47)	0.218	1.07 (0.99 – 1.16)
Total carbohydrate	**0.97 (0.83 – 0.97)‡**	0.82 (0.67 – 1.01)	0.82 (0.66 – 1.03)	**0.037**	**0.90 (0.83 – 0.97)‡**
Mono-disaccharide	**0.76 (0.63 – 0.93)‡**	**0.73 (0.59 – 0.89)‡**	**0.66 (0.52 – 0.83)‡**	**0.001‡**	**0.89 (0.81 – 0.97)‡**
Polysaccharide	0.99 (0.82 – 1.20)	0.93 (0.76 – 1.14)	0.81 (0.65 – 1.01)	0.057	0.95 (0.88 – 1.03)
Fibre	0.89 (0.72 – 1.08)	1.00 (0.81 – 1.25)	1.03 (0.81 – 1.32)	0.532	1.06 (0.97 – 1.16)
Total fat	0.94 (0.78 – 1.14)	1.09 (0.90 – 1.32)	1.07 (0.87 – 1.32)	0.293	1.03 (0.95 – 1.11)
Saturated fat	0.91 (0.74 – 1.12)	0.91 (0.73 – 1.15)	0.83 (0.62 – 1.11)	0.266	**0.86 (0.75 – 0.99)**
MUFA	1.07 (0.87 – 1.32)	1.06 (0.84 – 1.33)	1.24 (0.94 – 1.63)	0.176	**1.17 (1.05 – 1.32)‡**
PUFA	1.00 (0.82 – 1.21)	0.89 (0.73 – 1.09)	**0.79 (0.63 – 1.00)**	**0.032**	**0.89 (0.81 – 0.98)‡**
Trans fatty acid	0.95 (0.78 – 1.17)	1.15 (0.93 – 1.44)	1.22 (0.93 – 1.60)	0.069	1.11 (0.99 – 1.24)
Model 3 (Metabolic)					
Total protein	1.19 (0.97 – 1.47)	**1.33 (1.08 – 1.64)‡**	**1.34 (1.08 – 1.67)‡**	**0.004‡**	**1.13 (1.04 – 1.22)‡**
Animal protein	**1.40 (1.14 – 1.73)‡**	1.23 (0.99 – 1.52)	**1.53 (1.22 – 1.92)‡**	**0.002‡**	**1.14 (1.05 – 1.24)‡**
Vegetable protein	1.13 (0.92 – 1.39)	1.03 (0.83 – 1.28)	1.19 (0.95 – 1.49)	0.262	1.07 (0.98 – 1.16)
Total carbohydrate	1.07 (0.87 – 1.31)	0.92 (0.74 – 1.14)	0.95 (0.75 – 1.20)	0.432	0.94 (0.86 – 1.02)
Mono-disaccharide	**0.80 (0.65 – 0.99)**	0.82 (0.66 – 1.02)	**0.78 (0.61 – 0.99)**	0.067	0.94 (0.86 – 1.03)
Polysaccharide	0.91 (0.74 – 1.12)	0.95 (0.77 – 1.17)	**0.78 (0.62 – 0.99)**	0.076	0.95 (0.87 – 1.03)
Fibre	0.96 (0.77 – 1.18)	1.13 (0.89 – 1.42)	1.14 (0.88 – 1.49)	0.166	1.09 (0.99 – 1.20)
Total fat	1.01 (0.82 – 1.24)	1.15 (0.94 – 1.42)	1.08 (0.87 – 1.34)	0.310	1.02 (0.94 – 1.10)
Saturated fat	0.97 (0.78 – 1.20)	1.07 (0.84 – 1.37)	1.04 (0.76 – 1.42)	0.628	0.97 (0.84 – 1.12)
MUFA	1.15 (0.93 – 1.44)	1.09 (0.85 – 1.38)	1.20 (0.90 – 1.60)	0.349	1.10 (0.97 – 1.24)

Table 2 (*continued*)

	Q2	Q3	Q4	*P* for trend	Continuous (per SD increase)
PUFA	1.06 (0.86 – 1.30)	0.89 (0.71 – 1.10)	0.80 (0.63 – 1.02)	**0.033**	**0.89 (0.81 – 0.99)**
Trans fatty acid	0.95 (0.77 – 1.18)	1.15 (0.91 – 1.45)	1.12 (0.84 – 1.49)	0.232	1.05 (0.93 – 1.18)
Model 4 (Metabolic + log-transformed BMI)					
Total protein	1.13 (0.91 – 1.41)	1.13 (0.91 – 1.42)	0.99 (0.79 – 1.25)	0.933	1.01 (0.93 – 1.09)
Animal protein	**1.31 (1.05 – 1.64)‡**	1.01 (0.80 – 1.27)	1.15 (0.90 – 1.48)	0.725	1.01 (0.92 – 1.10)
Vegetable protein	1.07 (0.86 – 1.33)	0.98 (0.78 – 1.23)	1.12 (0.88 – 1.43)	0.543	1.04 (0.95 – 1.14)
Total carbohydrate	1.15 (0.93 – 1.44)	1.05 (0.83 – 1.33)	1.17 (0.91 – 1.51)	0.357	1.02 (0.93 – 1.11)
Mono-disaccharide	0.87 (0.70 – 1.08)	1.00 (0.79 – 1.27)	0.94 (0.72 – 1.23)	0.940	1.02 (0.92 – 1.13)
Polysaccharide	0.99 (0.80 – 1.23)	1.04 (0.83 – 1.31)	0.85 (0.66 – 1.10)	0.339	1.00 (0.92 – 1.10)
Fibre	0.93 (0.74 – 1.16)	1.09 (0.85 – 1.40)	1.08 (0.81 – 1.42)	0.372	1.06 (0.95 – 1.17)
Total fat	1.06 (0.85 – 1.32)	1.18 (0.95 – 1.47)	1.05 (0.84 – 1.33)	0.470	1.00 (0.92 – 1.09)
Saturated fat	1.02 (0.81 – 1.29)	1.07 (0.82 – 1.39)	1.10 (0.79 – 1.53)	0.537	1.01 (0.86 – 1.18)
MUFA	1.20 (0.95 – 1.51)	1.05 (0.81 – 1.36)	1.14 (0.84 – 1.56)	0.672	1.03 (0.90 – 1.17)
PUFA	1.13 (0.91 – 1.40)	0.99 (0.78 – 1.24)	0.91 (0.70 – 1.18)	0.343	0.93 (0.84 – 1.04)
Trans fatty acid	0.90 (0.72 – 1.14)	1.08 (0.84 – 1.39)	1.04 (0.76 – 1.41)	0.508	1.03 (0.91 – 1.18)

Values are odds ratios with 95% confidence intervals taking quartile 1 as reference. **P*-trend is calculated across quartiles. Bold values indicate $P<0.05$. ‡Indicates significant values using $P < 0.021$ as determined by Sidák. Model 1 (socio-demographic) is adjusted for age, gender, education level and study cohort. Model 2 (lifestyle confounding) is in addition previous model adjusted for past or current smoking, alcohol in E%, physical activity, energy intake and DHDI. Model 3 (metabolic) is in addition to the previous model adjusted for cholesterol, metabolic syndrome and diabetes mellitus. Model 4 (metabolic + log-transformed BMI) is in addition to the previous model adjusted for log-transformed BMI. Abbreviations BMI: body mass index; MUFA: mono-unsaturated fatty acid; PUFA: poly-unsaturated fatty acid; Q: Quartile.

inverse association with NAFLD (*Table 2*). Finally, after correction for log-transformed BMI, none of the associations remained significant. Also, no association between dietary fibre and NAFLD was found. The associated did not differ by BMI (*P* for interaction was *P*=0.08 for total carbohydrate, *P*=0.45 for mono-and disaccharides, *P*=0.16 for polysaccharides, and *P*=0.55 for fibre intake). Comprehensive results of the stratified models are shown in *Supplementary Table 3*. As depicted in *Figure 2A*, only mono and disaccharides were significantly associated with lower odds for NAFLD in overweight individuals. Yet, direction and magnitude of estimates in lean participants were comparable to those of overweight participants (OR 0.72 for both, model 2). After adjustment for metabolic covariates in model 3, the association for mono and disaccharides in overweight dissipated ($OR_{Q4vs.Q1}$ 0.83, 95%CI 0.63–1.10; $P_{for\ trend}$=0.28, *Figure 2B* and *Supplementary Table 3*).

Fat consumption and NAFLD

PUFA consumption was associated with lower odds for NAFLD in the first 3 models, but results diminished after adjustment for log-transformed BMI (*Table 2)*. No effect modification by BMI was observed (*P* for interaction was *P*=0.16 for total fat, *P*=0.20 for saturated fat, *P*=0.21 for MUFAs, *P*=0.54 for PUFAs, and *P*=0.96 for trans fatty acids). *Figure 2A & Figure 2B* show the stratified models 2 and 3, in which PUFA intake was no longer associated with NAFLD (*Supplementary Table 3)*. Saturated fat became associated with lean NAFLD after metabolic adjustment ($OR_{Q4vs.Q1}$ 2.21, 95%CI 1.03–4.72; $P_{for\ trend}$=0.03, *Figure 2B* and *Supplementary Table 3)*.

Substitution Analyses

We did not observe consistent substitution effects when one (sub)type of macronutrient was substituted for another (sub)type of macronutrient (*Supplementary Table 4*).

Sensitivity analyses

First, 3259 participants had complete data on all variables, and 623 had missing data on at least one covariate. Estimates derived from the complete case analyses were more pronounced than the imputed analyses, with all results pointing in the same direction. For example, in the imputed analyses (*Supplementary Table 3)* animal protein had an $OR_{Q4vs.Q1}$ of 1.36 (95%CI 1.05–1.77; $P_{for\ trend}$=0.09) for overweight NAFLD compared to an $OR_{Q4vs.Q1}$ of 1.52 (95%CI 1.14–2.03; $P_{for\ trend}$=0.03) in the complete case analyses *(Supplementary Table 5).* We therefore compared the group with complete cases to the group with missing data. In the latter group (total n=623), physical activity was the variable most often missing before imputation (n=358), followed by smoking status (n=203), and education level (n=50). The participants with missing variables were older (71.1 vs. 69.5), and were less often female (47% vs. 60%). Moreover, they had higher prevalence of diabetes (17.3% vs. 12.4%), and NAFLD (38.4% vs. 33.7%) and had a higher BMI (27.4 vs. 26.8kg/m^2). More detailed information is shown in *Supplementary Table 6*.

Second, we excluded the third cohort from the final analysis in order to avoid possible bias induced by a time lag of 5.5 years (median) between completion of the FFQs and liver imaging. The direction of the results did not change, but significance attenuated as can be seen in *Supplementary Table 7*. However, the results should be interpreted in light of decreased power and the difference in cohort characteristics, such as age (mean age RSI and II was 75.3 vs. 62.0 years in RS III). Third, additional adjustment for coffee as potential confounding covariate did not change the association between macronutrients and NAFLD (data not shown).

Discussion

This is the first large population-based cohort study to examine macronutrient intake using an extensive and externally validated semi-quantitative FFQ in relation to ultrasound-confirmed NAFLD. The results of this cross-sectional analysis, with FFQs preceding ultrasound, imply that specific macronutrients are associated with NAFLD independent of energy intake. Specifically, high animal protein intake was associated with higher prevalence of NAFLD in overweight participants. In addition, we found a trend towards lower prevalence of NAFLD in those with high consumption of mono and disaccharides. However, this association did not hold true after adjustment for log-transformed BMI. We did not observe consistent substitution effects of macronutrient replacement, emphasizing the need of a diverse diet. Recent dietary review papers on NAFLD have advocated implementation of the Mediterranean diet, which is rich in MUFAs, fruits, legumes, and nuts; and low in saturated fat, carbohydrates, and red meat.[258] Although we analysed diet based on macronutrient composition and not on predefined dietary patterns, we found that intake of animal protein was significantly associated with overweight NAFLD independent of socio-demographic, lifestyle, and metabolic traits. Furthermore, we found that the association between animal protein and NAFLD is mainly present in the highest quartile and does not appear to be dose-responsive.

Our findings are in line with previous studies, which showed that NAFLD patients consumed significantly more meat than controls[259] even after adjustment for confounders and energy intake.[57] Moreover, another recent Dutch population study found similar results, showing higher intake of protein from animal sources in individuals with a fatty liver, identified by the Fatty Liver Index (a non-invasive algorithm) rather than liver imaging.[247] In this study however, BMI was not taken into account as a covariate, and macronutrients were not adjusted for dietary quality (e.g. DHDI). Interestingly, a large epidemiological study showed that high red meat intake was associated with all-cause mortality, and in particular with mortality from liver diseases (hazard ratio 2.30, highest vs. lowest quintile).[260] In addition, a study from Israel found that high meat consumption, specifically high red and processed meat consumption were associated with NAFLD and insulin resistance, independent of saturated fat intake and BMI.[261] Yet, two other studies did either not find a difference in protein consumption between patients and controls, or found that controls consumed slightly more protein than patients.[245,246] However, both studies used absolute consumption in grams instead of energy adjusted intake and did not distinguish between animal or vegetable protein. Another recent study suggested a beneficial effect of protein. This was an intervention study in 37 diabetics with mild steatosis (<30% lipid content on MRI), in whom intrahepatic lipid content reduced upon a strict high vegetable or animal protein diet for 6 weeks.[262] Since this study differs from ours in various ways (i.e. our study included N=3882 individuals with a low prevalence of diabetes (13%) in an observational

rather than an interventional study design with an outcome defined by >30% steatosis as set by the detection limits of ultrasonography), direct comparison is difficult.

Contrary to common belief, we did not find a 'harmful' association between carbohydrates and NAFLD. In contrast, participants with high mono and disaccharide intake initially showed lower odds for NAFLD, but this association attenuated after BMI adjustment. Although the general assumption is that fructose intake harms the liver, evidence for this assumption is indeterminate.[263] In most studies, it is difficult to separate the contribution of fructose-containing sugars from that of other dietary factors, such as origin of food item, energy intake, and overall dietary quality.[244] This lack of clarity is supported by experimental studies showing that isocaloric carbohydrate intake was not associated with steatosis, but rather with amount of calories.[264,265] Most studies to date have shown detrimental effects on NAFLD, but focussed only on fructose intake from soft drinks.[266,267] In fact, in this predominantly elderly population median soft drink consumption was less than 1 glass per day (i.e. 44.5% consumers that have a median consumption of 0.36 glasses/day (0.14–0.91)). Indeed, the main food group contributing to mono and disaccharides in this population was actually fruit, and soda was not amongst the top 3 correlated contributors. This may partly explain why we did not observe a negative association.

Our results also do not suggest a "beneficial" role for MUFAs. This is in line with previous studies on macronutrient associations with NAFLD, which either did not find an association with MUFAs or found higher MUFA consumption in the NAFLD-group than in controls.[57,245-247] In contrast, a randomised controlled trial from Italy showed a reduction in liver fat following a MUFA enriched-diet in patients with diabetes.[268] However, this study was small (9 participants/arm) and showed a significant but small decrease in liver fat percentage (2.2% in the MUFA-arm). In addition, MUFA-intake in this study was much higher (~25%) than in our study (10.7%).[268] Dietary fat consumption, in particular saturated fat, remains a widely debated topic[269] and evidence on associations of (subtypes of) fat with incident metabolic disease is heterogeneous and suffer from residual confounding.[270] Our substitution analyses did imply a favourable trend when substituting PUFAs for animal and total protein intake in overweight participants. However, this association was not significant.

There are several possible mechanistic explanations as to how high animal protein intake could be associated with overweight NAFLD. Although we adjusted for overall dietary quality, the association might be explained by other dietary components. One hypothesis is, that constituents, such as nitrate, nitrite, haem iron and their by-products, in both unprocessed and processed meat could act as mediators between dietary intake and cardiovascular and metabolic homeostasis.[260] Haem iron is associated with increased oxidative stress and insulin resistance.[271] Nitrate and nitrite have been associated with endothelial dysfunction and insulin resistance.[272] Moreover, a large prospective cohort study found

that nitrate, nitrite, and haem iron from red meat intake were all associated with higher risk of chronic liver disease.[273]

Another possible mechanism through which animal protein could be associated with NAFLD is low-grade metabolic acidosis induced by a high diet-dependent acid load. The Western diet, characterized by high intake of acidic food items (e.g. animal protein) and low intake of alkali, potassium-rich food items (e.g. vegetables/fruits) increases daily acid load.[274] Recently, diet-dependent acid load has been associated with a higher risk of NAFLD.[259,275] The authors of these studies hypothesized that high dietary acid load might suppress growth hormone secretion and subsequent insulin-like growth factor-1 response, which both have been associated with NAFLD. In addition, some experimental studies have showed that high dietary acid load reduces extracellular pH, and insulin sensitivity and decreases beta cell response.[276] This could lead not only to diabetes, but also to NAFLD, as insulin resistance is the key dysfunction in this disease.[13]

The main strength of this study is the use of a large unselected study population and a robust statistical analysis with correction for a great number of sociodemographic, lifestyle, and metabolic traits, as assessed by well-validated tools. Furthermore, we used widely accepted nutritional epidemiologic methods and performed sensitivity analyses, emphasizing the robustness of our results. Also, we corrected for multiple comparisons using Sidák-corrected alpha-levels, taking into account that dietary exposures inter-correlate. Finally, abdominal ultrasound is a widely used and reliable imaging technique that yields high sensitivity and specificity for moderate and severe steatosis.[50]

Nonetheless, there are some limitations that need to be addressed. First, due to the cross-sectional design of this study, it is not possible to draw conclusions on causality. Although reverse causality is unlikely (participants were not aware of having NAFLD when filling in the FFQs), residual confounding may still remain. In particular, participants with diabetes (13%) might have adapted their eating habits due to this comorbidity. Nonetheless, we corrected for this potential confounder in the analyses of the third, metabolic model. Second, part of our study population completed the FFQ 5.5 years prior to abdominal ultrasound (i.e. RS III). Because dietary data is known to be stable over time,[109] we assumed that dietary habits in this elderly population would be rather constant. This was indeed recently shown in a paper from The Rotterdam Study.[277] Nevertheless, study cohort was added as covariate in all regression models and sensitivity analysis was performed excluding the third cohort from all main analyses. The results of this sensitivity were largely similar albeit no longer statistically significant due to smaller sample size. Third, as with any self-administered questionnaire, data are subject to potential reporter and recall bias. This is reflected in the probable caloric under-reporting in overweight participants.[278] However, the 389-item FFQ used in this study has been extensively validated in previous studies.[98,249] In addition, unreliable FFQs were excluded. Moreover, we adjusted for total energy intake and thereby accounted for extraneous variation in energy intake and potential measurement error.[251]

Fourth, in an attempt to avoid bias due to missing data, we performed multiple imputations on our data. Contrary to our expectation, the complete case analyses showed more pronounced associations with NAFLD. Differences between the complete and imputed cases were marginal, but the imputed group had more frequently metabolic disorders. We therefore hypothesize that this somewhat more unhealthy group could have affected the association through a phenomenon in which a relative contribution of a macronutrient to an "already higher risk group" is less pronounced.[279] Either way, this could have more likely led to underestimation (rather than overestimation) of the effect that we observed. Finally, in terms of generalisability to younger, non-Caucasian cohorts, results have to be interpreted in the context of a different range of consumption of the various macronutrients by this predominant elderly population.

In conclusion, we found an independent association of high animal protein consumption and NAFLD in an overweight, predominantly aged Caucasian population. The results of this large study add to the current evidence on the importance of dietary composition in NAFLD. In particular, it shifts focus from the carbohydrate and fat debate towards the third, previously underexplored macronutrient, protein. The cause-effect relation and mechanistic pathways of this association remain unanswered for which more studies are needed. Ultimately, we need to understand more about the dietary components that put individuals at risk for NAFLD, before we can make any firm dietary recommendations for the prevention and treatment of NAFLD.

Supplementary Files

Supplementary Methods: details on the multiple imputation process

	Multiple imputation
Software used	R version 3.4.1
Imputation method and key settings	Fully conditional specification (package mice version 2.25)*;* maximum iterations: 50
No. of imputed data sets created	10
Analyses variables	total cholesterol; triglycerides; high density lipoprotein cholesterol; body mass index; glucose; weight; physical activity; smoking status; systolic blood pressure; diastolic blood pressure; ethnicity; education level; alcohol consumption; fat intake; waist circumference; kilocalorie intake; Dutch healthy diet index; age; anti-diabetic drugs; lipid-lowering drugs; antihypertensive drugs; fibre intake; study cohort, gender, steatosis, protein intake, carbohydrate intake;
Auxiliary variables	aspartate transaminase; alanine transaminase; homeostasis model assessment of insulin resistance; hip circumference; heart rate; spleen size; calcium intake; creatinine; glomerular filtration rate; vitamin E intake; potassium intake; magnesium intake; phosphorus intake; gamma-glutamyl transferase
Treatment of not normally distributed continuous variables	Predictive mean matching
Treatment of normally distributed variables	Linear regression
Treatment of binary/categorical variables	(Proportional odds) logistic regression
Population	For the imputation we used reliable and completed FFQs. In addition participants had to have less than 30% missing on study variables. Imputed population (n=4.754).

Supplementary Table 1: Imputation Characteristics

	Original Data n=3882	Imputed data*
Demographics		
Age (years)	**69.7 ± 8.8**	no missing data
Female (%)	**58.3**	no missing data
Caucasian (%)	**97.7**	97.6
Education Level (%)		
Low	**48.4**	48.4
Intermediate	**30.3**	30.2
High	**21.3**	21.3
Smoking status (%)		
Never / Past or Current	**36.7 / 63.3**	36.1 / 63.9
Alcohol (units/d)	**0.45 (0.05 – 1.20)**	no missing data
Physical Activity†	**41.3 (15.8 – 78.6)**	40.6 (15.7 – 77.6)
Caloric Intake (kcal/day)	**2031 (1620 – 2515)**	no missing data
Physical examination		
BMI (kg/m^2)	**26.9 (24.5 – 29.7)**	26.9 (24.5 – 29.7)

Supplementary Table 1 (*continued*)

	Original Data n=3882	Imputed data*
Lean	**30.2**	30.2
Overweight	**46.7**	46.7
Obese	**23.0**	23.0
WC (cm)		
Men	**98.2 ± 10.6**	98.2 ± 10.6
Women	**89.1 ± 12.2**	no missing data
Biochemistry		
AST (U/L)	**24 (21 – 28)**	24 (21 – 28)
ALT (U/L)	**18 (15 – 24)**	18 (15 – 24)
GGT (U/L)	**23 (17 – 34)**	23 (17 – 34)
Platelets (*10^9/L)	**262 (223 – 305)**	262 (223 – 305)
HOMA-IR	**2.6 (1.7 – 4.1)**	2.6 (1.7 – 4.1)
Total Cholesterol (mmol/L)	**5.4 ± 1.1**	5.4 ± 1.1
HDL-C (mmol/L)	**1.5 ± 0.4**	1.5 ± 0.4
Triglycerides (mmol/L)	**1.3 (1.0 – 1.7)**	1.3 (1.0 – 1.7)
Comorbidities		
Metabolic Syndrome (%)	**51.9**	51.9
- WC>88cm (♀) or >120cm (♂)	**43.2**	43.2
- Triglycerides >150mg/dL	**46.2**	46.0
- HDL-C <40mg/dL (♂) or 50mg/dL (♀)	**44.8**	44.7
- Blood pressure ≥130/85mmHg	**84.3**	84.3
- FPG>100mg/dL	**41.5**	41.5
Diabetes Mellitus (%)	**13.2**	13.1
Hypertension (%)	**74.0**	74.0
NAFLD (%)	**34.4**	no missing data

*Pooled data based on 10 imputations represent % for categorical variables and for continuous variables mean ± SD or median (P25-P75).†Physical activity in metabolic equivalent task hours/week.

Supplementary Table 2: Absolute macronutrient consumption in grams and energy percent per quartile

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Total Protein				
in grams per Q	74.0 ± 24.6	81.5 ± 25.0	85.0 ± 26.2	86.3 ± 31.0
in energy % per Q	12.4 ± 1.5	14.8 ± 0.47	16.4 ± 0.49	19.5 ± 2.2
Animal protein				
in grams per Q	35.2 ± 14.3	46.3 ± 14.5	51.4 ± 15.7	61.1 ± 23.6
in energy % per Q	5.9 ± 1.3	8.3 ± 0.48	10.1 ± 0.58	13.6 ± 2.6
Vegetable protein				
in grams per Q	26.0 ± 9.9	31.2 ± 10.2	34.3 ± 10.7	41.7 ± 15.8
in energy % per Q	4.7 ± 0.68	5.9 ± 0.23	6.7 ± 0.24	8.1 ± 0.93

Supplementary Table 2 (*continued*)

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Total Fat				
in grams per Q	54.2 ± 20.1	67.8 ± 21.8	80.4 ± 25.6	108.1 ± 57.8
in energy % per Q	24.4 ± 3.0	30.0 ± 1.2	33.9 ± 1.2	41.3 ± 6.7
Saturated fat				
in grams per Q	19.0 ± 6.9	24.5 ± 8.2	29.0 ± 9.6	38.9 ± 19.4
in energy % per Q	8.3 ± 1.1	10.6 ± 0.48	12.3 ± 0.55	15.6 ± 2.6
MUFA fat				
in grams per Q	17.3 ± 6.7	22.5 ± 7.6	27.0 ± 8.0	39.9 ± 24.8
in energy % per Q	7.8 ± 1.1	10.0 ± 0.43	11.5 ± 0.48	14.9 ± 3.5
PUFA fat				
in grams per Q	10.0 ± 4.0	13.5 ± 4.6	16.6 ± 5.3	24.2 ± 14.2
in energy % per Q	4.5 ± 0.65	5.9 ± 0.31	7.0 ± 0.33	9.3 ± 2.3
Trans fatty acid				
in grams per Q	0.74 ± 0.31	1.07 ± 0.36	1.32 ± 0.44	1.92 ± 0.92
in energy % per Q	0.33 ± 0.07	0.46 ± 0.03	0.56 ± 0.03	0.76 ± 0.15
Total Carbohydrate				
in grams per Q	194.3 ± 75.9	233.1 ± 75.9	255.1 ± 79.0	289.8 ± 103.4
in energy % per Q	36.9 ± 5.2	44.1 ± 1.2	48.4 ± 1.3	54.9 ± 3.5
Mono- & disaccharides				
in grams per Q	75.1 ± 34.7	107.9 ± 37.0	134.0 ± 42.6	174.3 ± 64.0
in energy % per Q	14.3 ± 3.2	20.6 ± 1.3	25.1 ± 1.4	32.7 ± 4.4
Polysaccharides				
in grams per Q	91.3 ± 36.4	115.0 ± 37.9	126.6 ± 40.6	148.2 ± 53.6
in energy % per Q	16.8 ± 2.8	21.3 ± 0.85	24.2 ± 0.93	29.4 ± 3.3
Fibre				
in grams per Q	19.5 ± 7.5	25.4 ± 8.7	29.5 ± 9.5	36.0 ± 13.1
in energy % per Q	1.8 ± 0.30	2.4 ± 0.13	2.8 ± 0.14	3.6 ± 0.48

Mean energy intake in grams or energy% (±SD) per quartile.

Supplementary Table 3: Stratified stepwise logistic regression models with macronutrient as independent variables and NAFLD as dependent variable using quartile 1 as reference

	Lean (BMI < 25 kg/m²) n=1174				
	Q2	Q3	Q4	*P* for trend	Continuous (per SD increase)
Model 1 (Sociodemographic)					
Total protein	1.10 (0.69 – 1.77)	0.91 (0.54 – 1.53)	1.08 (0.64 – 1.84)	0.965	0.99 (0.82 – 1.20)
Animal protein	**1.75 (1.09 – 2.81)‡**	0.94 (0.52 – 1.70)	1.33 (0.76 – 2.35)	0.718	1.00 (0.82 – 1.22)
Vegetable protein	1.00 (0.60 – 1.68)	0.85 (0.50 – 1.45)	0.78 (0.45 – 1.36)	0.304	0.92 (0.75 – 1.12)
Total carbohydrate	1.05 (0.64 – 1.74)	0.72 (0.42 – 1.23)	0.66 (0.38 – 1.16)	0.075	0.84 (0.69 – 1.02)
Mono-disaccharide	0.73 (0.44 – 1.21)	0.64 (0.38 – 1.09)	**0.55 (0.31 – 0.98)**	**0.038**	0.82 (0.66 – 1.01)
Polysaccharide	1.13 (0.66 – 1.93)	1.19 (0.69 – 2.04)	0.70 (0.37 – 1.32)	0.354	0.95 (0.76 – 1.17)
Fibre	0.69 (0.41 – 1.17)	0.65 (0.38 – 1.09)	0.68 (0.38 – 1.22)	0.173	0.94 (0.75 – 1.17)
Total fat	0.97 (0.55 – 1.71)	1.42 (0.83 – 2.43)	**1.70 (1.01 – 2.87)**	**0.017‡**	1.16 (0.98 – 1.37)
Saturated fat	1.03 (0.57 – 1.84)	1.22 (0.66 – 2.27)	1.83 (0.89 – 3.77)	0.089	1.16 (0.84 – 1.60)
MUFA	1.29 (0.72 – 2.29)	1.29 (0.68 – 2.43)	1.62 (0.77 – 3.39)	0.246	1.08 (0.82 – 1.41)
PUFA	0.92 (0.54 – 1.57)	0.79 (0.45 – 1.38)	0.85 (0.46 – 1.55)	0.503	0.90 (0.71 – 1.16)
Trans fatty acid	0.77 (0.44 – 1.35)	0.76 (0.42 – 1.37)	1.17 (0.60 – 2.28)	0.716	1.02 (0.75 – 1.39)
Model 2 (Lifestyle Confounding)					
Total protein	1.08 (0.67 – 1.73)	0.89 (0.52 – 1.50)	1.02 (0.58 – 1.79)	0.869	0.98 (0.81 – 1.20)
Animal protein	**1.69 (1.05 – 2.72)**	0.93 (0.51 – 1.69)	1.25 (0.69 – 2.28)	0.862	0.98 (0.79 – 1.21)
Vegetable protein	1.13 (0.66 – 1.91)	1.03 (0.59 – 1.80)	0.98 (0.55 – 1.77)	0.866	1.00 (0.81 – 1.23)
Total carbohydrate	1.19 (0.71 – 2.00)	0.88 (0.49 – 1.55)	0.89 (0.48 – 1.65)	0.503	0.93 (0.74 – 1.15)
Mono-disaccharide	0.83 (0.49 – 1.40)	0.76 (0.43 – 1.32)	0.72 (0.38 – 1.36)	0.297	0.91 (0.71 – 1.15)
Polysaccharide	1.16 (0.68 – 1.98)	1.26 (0.73 – 2.18)	0.78 (0.40 – 1.49)	0.591	0.99 (0.79 – 1.23)
Fibre	0.73 (0.43 – 1.25)	0.70 (0.39 – 1.25)	0.80 (0.41 – 1.57)	0.493	1.04 (0.80 – 1.35)
Total fat	1.01 (0.57 – 1.78)	1.46 (0.84 – 2.54)	1.75 (0.99 – 3.08)	**0.024**	1.15 (0.95 – 1.38)
Saturated fat	1.01 (0.56 – 1.83)	1.21 (0.64 – 2.28)	1.92 (0.91 – 4.06)	0.080	1.18 (0.84 – 1.65)
MUFA	1.23 (0.69 – 2.20)	1.25 (0.66 – 2.36)	1.50 (0.71 – 3.18)	0.334	1.03 (0.78 – 1.36)
PUFA	0.95 (0.56 – 1.64)	0.85 (0.48 – 1.49)	0.96 (0.51 – 1.79)	0.803	0.95 (0.74 – 1.22)
Trans fatty acid	0.75 (0.42 – 1.32)	0.73 (0.40 – 1.33)	1.13 (0.58 – 2.22)	0.782	1.02 (0.75 – 1.39)
Model 3 (Metabolic)					
Total protein	1.08 (0.66 – 1.75)	0.87 (0.51 – 1.49)	0.97 (0.55 – 1.72)	0.734	0.99 (0.81 – 1.20)
Animal protein	1.57 (0.97 – 2.55)	0.90 (0.49 – 1.65)	1.20 (0.66 – 2.20)	0.960	0.98 (0.79 – 1.22)
Vegetable protein	1.22 (0.71 – 2.09)	1.10 (0.62 – 1.94)	1.07 (0.59 – 1.94)	0.940	1.04 (0.84 – 1.28)
Total carbohydrate	1.21 (0.71 – 2.07)	0.96 (0.54 – 1.73)	0.96 (0.51 – 1.80)	0.721	0.95 (0.77 – 1.19)
Mono-disaccharide	0.85 (0.50 – 1.45)	0.81 (0.46 – 1.44)	0.77 (0.41 – 1.48)	0.436	0.94 (0.74 – 1.20)
Polysaccharide	1.06 (0.61 – 1.83)	1.25 (0.72 – 2.18)	0.74 (0.38 – 1.43)	0.560	0.99 (0.79 – 1.24)
Fibre	0.79 (0.45 – 1.36)	0.81 (0.45 – 1.45)	0.89 (0.44 – 1.77)	0.743	1.08 (0.83 – 1.40)
Total fat	1.01 (0.56 – 1.80)	1.41 (0.80 – 2.48)	1.69 (0.95 – 3.02)	**0.038**	1.12 (0.92 – 1.35)
Saturated fat	0.95 (0.52 – 1.72)	1.28 (0.67 – 2.44)	**2.21 (1.03 – 4.72)**	**0.030**	1.26 (0.89 – 1.78)
MUFA	1.20 (0.66 – 2.17)	1.24 (0.65 – 2.38)	1.49 (0.69 – 3.22)	0.342	0.98 (0.73 – 1.30)

Supplementary Table 3 (*continued*)

	Lean (BMI < 25 kg/m²) n=1174				
	Q2	Q3	Q4	*P* for trend	Continuous (per SD increase)
PUFA	1.04 (0.60 – 1.80)	0.88 (0.49 – 1.56)	0.98 (0.52 – 1.84)	0.804	0.96 (0.74 – 1.24)
Trans fatty acid	0.74 (0.41 – 1.31)	0.71 (0.39 – 1.31)	1.10 (0.55 – 2.18)	0.862	0.96 (0.70 – 1.32)
Model 1 (Sociodemographic)					
Total protein	1.17 (0.93 – 1.47)	**1.36 (1.09 – 1.69)‡**	**1.49 (1.19 – 1.85)‡**	**<0.001‡**	**1.16 (1.08 – 1.26)‡**
Animal protein	**1.30 (1.03 – 1.64)**	1.16 (0.93 – 1.45)	**1.62 (1.28 – 2.05)‡**	**<0.001‡**	**1.18 (1.08 – 1.28)‡**
Vegetable protein	1.08 (0.87 – 1.34)	0.98 (0.78 – 1.22)	1.25 (0.99 – 1.58)	0.144	1.08 (0.99 – 1.17)
Total carbohydrate	0.93 (0.75 – 1.15)	**0.78 (0.63 – 0.98)**	0.86 (0.68 – 1.09)	0.095	**0.91 (0.84 – 0.99)**
Mono-disaccharide	**0.73 (0.59 – 0.90)‡**	**0.71 (0.57 – 0.89)‡**	**0.67 (0.52 – 0.85)‡**	**0.001‡**	**0.89 (0.81 – 0.97)‡**
Polysaccharide	1.01 (0.81 – 1.25)	0.97 (0.78 – 1.22)	0.90 (0.71 – 1.15)	0.386	1.00 (0.91 – 1.09)
Fibre	0.85 (0.68 – 1.06)	1.00 (0.80 – 1.26)	0.93 (0.73 – 1.18)	0.910	1.01 (0.92 – 1.10)
Total fat	0.98 (0.79 – 1.22)	1.09 (0.88 – 1.36)	1.10 (0.89 – 1.37)	0.249	1.03 (0.96 – 1.12)
Saturated fat	0.96 (0.76 – 1.21)	0.95 (0.73 – 1.24)	0.86 (0.62 – 1.19)	0.406	0.91 (0.78 – 1.06)
MUFA	1.02 (0.81 – 1.30)	0.96 (0.74 – 1.24)	1.10 (0.80 – 1.50)	0.727	1.09 (0.96 – 1.25)
PUFA	1.02 (0.82 – 1.27)	0.97 (0.77 – 1.22)	0.89 (0.68 – 1.15)	0.351	0.94 (0.84 – 1.04)
Trans fatty acid	0.93 (0.74 – 1.17)	1.16 (0.90 – 1.48)	1.21 (0.89 – 1.65)	0.101	1.12 (0.98 – 1.27)
Model 2 (Lifestyle Confounding)					
Total protein	1.16 (0.92 – 1.46)	**1.32 (1.05 – 1.66)‡**	**1.40 (1.11 – 1.77)‡**	**0.003‡**	**1.14 (1.05 – 1.24)‡**
Animal protein	**1.29 (1.02 – 1.63)**	1.13 (0.90 – 1.43)	**1.54 (1.20 – 1.98)‡**	**0.004‡**	**1.14 (1.05 – 1.24)‡**
Vegetable protein	1.11 (0.89 – 1.38)	1.01 (0.81 – 1.28)	**1.29 (1.01 – 1.66)**	0.094	1.10 (1.00 – 1.20)
Total carbohydrate	0.95 (0.76 – 1.18)	0.84 (0.67 – 1.07)	0.93 (0.72 – 1.21)	0.435	0.94 (0.86 – 1.03)
Mono-disaccharide	**0.76 (0.61 – 0.95)**	**0.76 (0.60 – 0.96)**	**0.72 (0.55 – 0.94)‡**	**0.022**	0.93 (0.84 – 1.02)
Polysaccharide	1.00 (0.80 – 1.25)	0.95 (0.76 – 1.20)	0.86 (0.67 – 1.11)	0.233	0.98 (0.89 – 1.07)
Fibre	0.91 (0.72 – 1.15)	1.10 (0.85 – 1.42)	1.07 (0.81 – 1.41)	0.377	1.06 (0.96 – 1.18)
Total fat	0.96 (0.77 – 1.20)	1.04 (0.83 – 1.30)	0.99 (0.78 – 1.25)	0.896	1.00 (0.92 – 1.09)
Saturated fat	0.93 (0.73 – 1.17)	0.87 (0.67 – 1.14)	0.74 (0.53 – 1.04)	0.094	**0.84 (0.72 – 0.99)**
MUFA	1.04 (0.82 – 1.32)	0.96 (0.74 – 1.24)	1.09 (0.80 – 1.50)	0.775	1.12 (0.98 – 1.28)
PUFA	1.02 (0.82 – 1.27)	0.97 (0.77 – 1.22)	0.86 (0.66 – 1.12)	0.262	0.93 (0.83 – 1.04)
Trans fatty acid	0.92 (0.73 – 1.16)	1.15 (0.89 – 1.48)	1.17 (0.85 – 1.59)	0.161	1.12 (0.98 – 1.27)
Model 3 (Metabolic)					
Total protein	1.14 (0.90 – 1.45)	1.25 (0.99 – 1.59)	1.22 (0.95 – 1.55)	0.091	1.09 (1.00 – 1.18)
Animal protein	**1.31 (1.03 – 1.67)**	1.08 (0.84 – 1.67)	**1.36 (1.05 – 1.77)‡**	0.092	1.09 (1.00 – 1.20)
Vegetable protein	1.11 (0.88 – 1.40)	0.99 (0.78 – 1.26)	1.23 (0.95 – 1.59)	0.252	1.07 (0.97 – 1.18)
Total carbohydrate	1.05 (0.84 – 1.32)	0.93 (0.73 – 1.19)	1.06 (0.81 – 1.39)	0.906	0.97 (0.88 – 1.07)
Mono-disaccharide	0.80 (0.63 – 1.01)	0.85 (0.66 – 1.09)	0.83 (0.63 – 1.10)	0.283	0.98 (0.88 – 1.09)
Polysaccharide	0.93 (0.74 – 1.17)	0.96 (0.75 – 1.22)	0.83 (0.64 – 1.09)	0.246	0.97 (0.88 – 1.07)
Fibre	0.96 (0.76 – 1.23)	1.17 (0.90 – 1.52)	1.16 (0.86 – 1.55)	0.181	1.08 (0.97 – 1.20)
Total fat	1.01 (0.80 – 1.27)	1.10 (0.87 – 1.39)	0.98 (0.77 – 1.25)	0.915	0.99 (0.91 – 1.09)
Saturated fat	1.00 (0.78 – 1.28)	0.99 (0.75 – 1.31)	0.88 (0.62 – 1.26)	0.547	0.93 (0.78 – 1.09)
MUFA	1.13 (0.88 – 1.45)	1.00 (0.76 – 1.31)	1.07 (0.77 – 1.48)	0.958	1.08 (0.94 – 1.24)

Supplementary Table 3 (*continued*)

	Lean (BMI < 25 kg/m²) n=1174				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
PUFA	1.04 (0.83 – 1.31)	0.93 (0.73 – 1.18)	0.83 (0.63 – 1.10)	0.142	0.92 (0.82 – 1.03)
Trans fatty acid	0.94 (0.74 – 1.20)	1.16 (0.89 – 1.51)	1.08 (0.78 – 1.50)	0.332	1.07 (0.94 – 1.23)

Bold values indicate P<0.05. ǂ Indicates significant values using P < 0.021. Model 1 (socio-demographic) is adjusted for age, gender, education level and study cohort. Model 2 (lifestyle confounding) is in addition previous model adjusted for past or current smoking, alcohol in E%, physical activity, energy intake and DHDI. Model 3 (metabolic) is in addition to the previous model adjusted for cholesterol, metabolic syndrome and diabetes mellitus.

Supplementary Table 4: Substitution analyses of macronutrients in the metabolic model

Polysaccharide intake						
Mono-and disaccharides	1.00 (0.98 – 1.02)	0.938	1.01 (0.96 – 1.05)	0.800	1.00 (0.98 – 1.02)	0.824
Total protein	1.00 (0.97 – 1.03)	0.974	1.01 (0.94 – 1.09)	0.780	0.97 (0.94 – 1.00)	0.084
Animal protein	1.00 (0.96 – 1.03)	0.867	1.02 (0.94 – 1.11)	0.635	0.97 (0.94 – 1.01)	0.135
Vegetable protein	1.00 (0.88 – 1.13)	0.995	1.09 (0.82 – 1.45)	0.554	0.98 (0.86 – 1.12)	0.820
Total fat	1.00 (0.98 – 1.02)	0.744	0.99 (0.95 – 1.04)	0.730	0.99 (0.97 – 1.01)	0.600
Saturated fat	0.99 (0.94 – 1.04)	0.658	0.92 (0.82 – 1.04)	0.194	1.02 (0.96 – 1.08)	0.506
MUFA	0.98 (0.95 – 1.03)	0.458	1.02 (0.93 – 1.12)	0.708	0.96 (0.92 – 1.00)	0.067
PUFA	1.03 (0.98 – 1.08)	0.298	1.04 (0.92 – 1.18)	0.493	1.01 (0.96 – 1.07)	0.603
Fibre	0.97 (0.85 – 1.10)	0.619	0.92 (0.67 – 1.27)	0.618	0.93 (0.82 – 1.06)	0.297
Total fat intake						
Total carbohydrates	1.00 (0.99 – 1.02)	0.669	1.02 (0.99 – 1.05)	0.223	1.00 (0.99 – 1.02)	0.712
Mono- di saccharides	1.00 (0.99 – 1.02)	0.696	1.02 (0.99 – 1.05)	0.187	1.00 (0.99 – 1.02)	0.799
Polysaccharides	1.00 (0.98 – 1.02)	0.701	1.01 (0.96 – 1.06)	0.719	1.01 (0.99 – 1.03)	0.604
Total protein	1.00 (0.97 – 1.03)	0.957	1.02 (0.95 – 1.09)	0.657	0.97 (0.94 – 1.01)	0.114
Animal protein	1.00 (0.97 – 1.03)	0.961	1.02 (0.95 – 1.09)	0.652	0.97 (0.94 – 1.01)	0.123
Vegetable protein	1.01 (0.92 – 1.11)	0.820	1.04 (0.83 – 1.30)	0.730	1.00 (0.90 – 1.11)	0.995
Fibre	0.99 (0.88 – 1.11)	0.883	1.02 (0.75 – 1.37)	0.915	0.95 (0.84 – 1.07)	0.370
Saturated fat intake						
MUFA	0.99 (0.91 – 1.07)	0.794	1.10 (0.92 – 1.32)	0.289	0.92 (0.85 – 1.01)	0.066
PUFA	1.04 (0.97 – 1.11)	0.277	1.12 (0.96 – 1.31)	0.150	0.99 (0.92 – 1.06)	0.773
Total carbohydrates	1.01 (0.95 – 1.06)	0.792	1.09 (0.97 – 1.24)	0.150	0.96 (0.91 – 1.02)	0.218
Mono- di saccharides	1.01 (0.95 – 1.07)	0.780	1.11 (0.98 – 1.27)	0.105	0.96 (0.90 – 1.02)	0.179
Polysaccharides	1.01 (0.95 – 1.06)	0.803	1.09 (0.96 – 1.23)	0.188	0.97 (0.91 – 1.02)	0.230
Protein	1.00 (0.94 – 1.08)	0.907	1.11 (0.94 – 1.31)	0.209	**0.93 (0.86 – 1.00)**	**0.041**
Animal protein	1.01 (0.94 – 1.08)	0.839	1.11 (0.95 – 1.31)	0.197	**0.93 (0.86 – 1.00)**	**0.039**
Vegetable protein	0.97 (0.87 – 1.07)	0.522	1.06 (0.82 – 1.38)	0.650	0.91 (0.81 – 1.02)	0.093
Fibre	0.92 (0.81 – 1.05)	0.226	1.03 (0.74 – 1.43)	0.876	**0.83 (0.72 – 0.95)**[‡]	**0.006**[‡]
MUFA intake						
Saturated fat	1.00 (0.93 – 1.08)	0.975	0.92 (0.77 – 1.10)	0.357	1.05 (0.96 – 1.14)	0.291
PUFA	1.04 (0.96 – 1.13)	0.342	1.02 (0.84 – 1.23)	0.834	1.05 (0.96 – 1.15)	0.315
Total carbohydrates	1.01 (0.97 – 1.06)	0.502	1.00 (0.91 – 1.09)	0.929	1.03 (0.99 – 1.08)	0.166
Mono- di saccharides	1.01 (0.97 – 1.06)	0.498	1.00 (0.91 – 1.10)	0.941	1.03 (0.98 – 1.08)	0.206
Polysaccharides	1.01 (0.97 – 1.06)	0.553	0.98 (0.89 – 1.08)	0.700	1.04 (0.99 – 1.08)	0.149
Total protein	1.01 (0.97 – 1.06)	0.640	1.01 (0.91 – 1.12)	0.875	0.99 (0.94 – 1.05)	0.834
Animal protein	1.01 (0.97 – 1.06)	0.613	1.01 (0.91 – 1.12)	0.863	0.99 (0.94 – 1.05)	0.819
Vegetable protein	0.97 (0.88 – 1.07)	0.554	0.96 (0.76 – 1.22)	0.747	0.97 (0.87 – 1.08)	0.620
Fibre	0.93 (0.82 – 1.05)	0.258	0.93 (0.67 – 1.30)	0.686	0.89 (0.78 – 1.01)	0.072
PUFA intake						
Saturated fat	0.96 (0.90 – 1.02)	0.181	0.90 (0.78 – 1.05)	0.183	0.98 (0.92 – 1.06)	0.651
MUFA	0.95 (0.87 – 1.03)	0.209	0.99 (0.82 – 1.19)	0.903	0.92 (0.84 – 1.01)	0.065

Supplementary Table 4 (*continued*)

Polysaccharide intake						
Total carbohydrates	0.97 (0.92 – 1.02)	0.183	0.98 (0.87 – 1.11)	0.745	0.96 (0.91 – 1.02)	0.172
Mono- di saccharides	0.97 (0.92 – 1.02)	0.182	0.97 (0.86 – 1.10)	0.677	0.96 (0.91 – 1.02)	0.191
Polysaccharides	0.96 (0.91 – 1.02)	0.204	0.95 (0.83 – 1.09)	0.479	0.97 (0.91 – 1.03)	0.290
Total protein	0.96 (0.91 – 1.02)	0.199	0.99 (0.86 – 1.14)	0.921	**0.93 (0.88 – 0.99)‡**	**0.014‡**
Animal protein	0.96 (0.91 – 1.02)	0.166	0.98 (0.85 – 1.14)	0.821	**0.93 (0.88 – 0.99)‡**	**0.019‡**
Vegetable protein	0.92 (0.82 – 1.03)	0.151	0.94 (0.70 – 1.26)	0.667	0.91 (0.80 – 1.02)	0.117
Fibre	0.89 (0.77 – 1.02)	0.083	0.92 (0.65 – 1.30)	0.652	**0.83 (0.71 – 0.95)‡**	**0.009‡**

Bold values indicate P<0.05. ‡Indicates significant values using P < 0.021. Substitution model was performed adjusted for age, gender, education level, study cohort, past or current smoking, alcohol in E%, physical activity, energy intake, DHDI, cholesterol, metabolic syndrome, diabetes mellitus and for log-transformed BMI in the overall group.

Supplementary Table 5: Complete case analyses

	Overall n=3259				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Model 4 (Metabolic + log-transformed BMI)					
Total protein	1.11 (0.87 – 1.42)	1.16 (0.91 – 1.48)	1.04 (0.80 – 1.34)	0.741	1.02 (0.93 – 1.12)
Animal protein	1.30 (1.01 – 1.66)	0.99 (0.77 – 1.27)	1.23 (0.94 – 1.61)	0.480	1.02 (0.92 – 1.13)
Vegetable protein	0.95 (0.75 – 1.21)	0.81 (0.63 – 1.04)	0.99 (0.76 – 1.30)	0.633	1.02 (0.93 – 1.13)
Total carbohydrate	1.22 (0.96 – 1.56)	1.10 (0.85 – 1.43)	1.24 (0.94 – 1.64)	0.241	1.04 (0.94 – 1.15)
Mono-disaccharide	0.83 (0.65 – 1.06)	0.96 (0.74 – 1.25)	0.97 (0.72 – 1.30)	0.891	1.04 (0.93 – 1.16)
Polysaccharide	0.97 (0.76 – 1.23)	1.04 (0.82 – 1.34)	0.83 (0.63 – 1.09)	0.320	1.02 (0.92 – 1.12)
Fibre	0.88 (0.68 – 1.13)	1.03 (0.78 – 1.35)	1.00 (0.73 – 1.36)	0.697	1.01 (0.90 – 1.14)
Total fat	1.17 (0.92 – 1.48)	1.15 (0.90 – 1.46)	1.03 (0.79 – 1.33)	0.840	0.98 (0.89 – 1.08)
Saturated fat	1.02 (0.79 – 1.32)	1.07 (0.80 – 1.43)	1.02 (0.71 – 1.48)	0.837	0.98 (0.83 – 1.17)
MUFA	**1.32 (1.02 – 1.70)**	1.15 (0.86 – 1.53)	1.29 (0.92 – 1.83)	0.326	1.05 (0.90 – 1.22)
PUFA	1.06 (0.84 – 1.35)	0.90 (0.70 – 1.16)	0.92 (0.69 – 1.23)	0.342	0.92 (0.81 – 1.03)
Trans fatty acid	0.98 (0.76 – 1.26)	1.07 (0.82 – 1.41)	1.02 (0.72 – 1.43)	0.745	1.01 (0.88 – 1.17)
	Lean (BMI < 25 kg/m²) n=1011				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Model 3 (Metabolic)					
Total protein	1.02 (0.60 – 1.74)	0.97 (0.55 – 1.72)	0.85 (0.45 – 1.61)	0.636	0.98 (0.79 – 1.22)
Animal protein	1.53 (0.90 – 2.59)	0.83 (0.42 – 1.62)	1.13 (0.58 – 2.22)	0.839	0.98 (0.78 – 1.24)
Vegetable protein	1.16 (0.65 – 2.09)	0.89 (0.47 – 1.68)	0.99 (0.52 – 1.90)	0.770	1.03 (0.81 – 1.30)
Total carbohydrate	1.15 (0.64 – 2.08)	0.95 (0.50 – 1.82)	1.01 (0.50 – 2.02)	0.877	0.99 (0.77 – 1.26)
Mono-disaccharide	0.89 (0.49 – 1.61)	0.84 (0.44 – 1.60)	0.88 (0.43 – 1.81)	0.719	0.97 (0.74 – 1.27)
Polysaccharide	1.28 (0.69 – 2.35)	1.49 (0.81 – 2.76)	0.78 (0.36 – 1.65)	0.735	1.00 (0.78 – 1.29)
Fibre	0.67 (0.36 – 1.22)	0.70 (0.36 – 1.34)	0.81 (0.38 – 1.72)	0.614	1.04 (0.78 – 1.39)
Total fat	1.11 (0.59 – 2.09)	1.31 (0.70 – 2.44)	1.77 (0.94 – 3.33)	0.064	1.10 (0.89 – 1.37)
	Lean (BMI < 25 kg/m²) n=1011				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Saturated fat	0.91 (0.47 – 1.75)	1.13 (0.55 – 2.32)	**2.67 (1.16 – 6.19)**	**0.022**	1.34 (0.91 – 1.97)
MUFA	1.17 (0.61 – 2.23)	1.12 (0.55 – 2.30)	1.52 (0.65 – 3.52)	0.388	0.92 (0.67 – 1.26)
PUFA	1.00 (0.55 – 1.83)	0.95 (0.51 – 1.79)	1.12 (0.57 – 2.23)	0.788	1.02 (0.77 – 1.36)
Trans fatty acid	0.77 (0.41 – 1.44)	0.64 (0.32 – 1.26)	1.10 (0.51 – 2.36)	0.976	0.91 (0.63 – 1.31)
	Overweight (BMI ≥ 25 kg/m²) n=2248				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Model 3 (Metabolic)					
Total protein	1.15 (0.89 – 1.50)	1.26 (0.97 – 1.64)	**1.34 (1.02 – 1.76)**	**0.028**	**1.12 (1.02 – 1.23)**
Animal protein	1.29 (0.99 – 1.68)	1.05 (0.80 – 1.37)	**1.52 (1.14 – 2.03)‡**	**0.027**	**1.13 (1.02 – 1.25)**
Vegetable protein	0.98 (0.76 – 1.26)	0.83 (0.64 – 1.08)	1.13 (0.85 – 1.50)	0.735	1.06 (0.95 – 1.17)

Supplementary Table 5 (*continued*)

	Overall n=3259				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Total carbohydrate	1.13 (0.88 – 1.46)	0.99 (0.76 – 1.29)	1.11 (0.83 – 1.48)	0.701	0.99 (0.89 – 1.11)
Mono-disaccharide	0.75 (0.58 – 0.97)	0.78 (0.59 – 1.02)	0.84 (0.62 – 1.14)	0.324	1.00 (0.89 – 1.12)
Polysaccharide	0.89 (0.69 – 1.14)	0.95 (0.73 – 1.23)	0.80 (0.60 – 1.07)	0.222	0.98 (0.88 – 1.09)
Fibre	0.95 (0.73 – 1.25)	1.13 (0.84 – 1.51)	1.10 (0.80 – 1.52)	0.354	1.04 (0.92 – 1.17)
Total fat	1.10 (0.86 – 1.42)	1.09 (0.85 – 1.41)	0.94 (0.72 – 1.23)	0.713	0.97 (0.88 – 1.07)
Saturated fat	1.01 (0.78 – 1.33)	1.01 (0.74 – 1.37)	0.78 (0.53 – 1.16)	0.292	0.90 (0.75 – 1.08)
MUFA	1.26 (0.96 – 1.66)	1.12 (0.83 – 1.51)	1.21 (0.84 – 1.75)	0.511	1.12 (0.95 – 1.32)
PUFA	0.98 (0.76 – 1.26)	0.83 (0.63 – 1.08)	0.82 (0.60 – 1.12)	0.120	0.88 (0.78 – 1.01)
Trans fatty acid	1.02 (0.79 – 1.34)	1.16 (0.87 – 1.55)	1.02 (0.71 – 1.46)	0.678	1.05 (0.90 – 1.23)

Bold values indicate P<0.05. ‡Indicates significant values using P < 0.021. Model 3 (metabolic) is in addition to the previous model adjusted for cholesterol, metabolic syndrome and diabetes mellitus. Model 4 (metabolic + log-transformed BMI) is in addition to the previous model adjusted for log-transformed BMI.

Supplementary Table 6: Characteristics of imputed and complete cases

	Complete cases (n=3259)	Imputed cases (n=623)	*P-value
Age (years)	69.5 ± 8.6	71.1 ± 9.7	<0.001
Female (%)	60.4	47.0	<0.001
Caucasian (%)	97.8	97.0	0.256
RS cohort I / II / III (%)	26.6 / 30.5 / 42.9	32.4 / 30.2 / 37.4	0.006
Education Level (%)			
Low	48.2	49.4	0.287
Intermediate	30.1	31.8	
High	21.7	18.8	
Smoking status (%)			
Never / Past or Current	37.2 / 62.8	33.6 / 66.4	0.151
Alcohol (units/d)	0.45 (0.06 – 1.19)	0.40 (0.03 – 1.21)	0.439
Physical Activity†	41.5 (15.8 – 79.0)	36.3 (18.0 – 73.9)	0.645
Caloric Intake (kcal/day)	2040 (1636 – 2514)	1976 (1541 – 2525)	0.079
BMI (kg/m²)	26.8 (24.4 – 29.5)	27.4 (24.8 – 30.6)	<0.001
Lean	31.0	26.2	0.015
Overweight	69.0	73.8	
ALT (U/L)	18 (15 – 24)	18 (15 – 24)	0.607
HOMA-IR	2.6 (1.7 – 4.0)	2.7 (1.8 – 4.3)	0.099
Total Cholesterol (mmol/L)	5.4 ± 1.1	5.3 ± 1.1	0.016
Metabolic Syndrome (%)	51.3	55.0	0.102
Hypertension (%)	73.6	76.1	0.192
Diabetes Mellitus (%)	12.4	17.3	0.001
NAFLD (%)	33.7	38.4	0.025

Data on imputed cases is original data, not imputed on the present variables. Data represent % for categorical variables and for continuous variables mean ± SD or median (P25 – P75).**P*-value is based on T-test, Wilcoxon rank sum test, Chi-square test or Fisher's exact test. †Physical activity in metabolic equivalent task hours/week.

Supplementary Table 7: Sensitivity analyses using RS-cohort I and II in which FFQs were completed at the same time as all other measurements, amongst them abdominal ultrasound

	RS I and II only n=2252				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Model 4 (Metabolic + log-transformed BMI)					
Total protein	1.08 (0.80 – 1.45)	1.02 (0.76 – 1.37)	0.92 (0.68 – 1.25)	0.537	0.97 (0.88 – 1.07)
Animal protein	1.27 (0.93 – 1.72)	0.99 (0.73 – 1.34)	1.11 (0.80 – 1.53)	0.949	0.97 (0.87 – 1.08)
Vegetable protein	0.91 (0.69 – 1.20)	0.79 (0.59 – 1.06)	1.08 (0.78 – 1.49)	0.951	1.01 (0.89 – 1.13)
Total carbohydrate	**1.35 (1.01 – 1.80)**	1.20 (0.88 – 1.64)	1.18 (0.84 – 1.65)	0.493	1.02 (0.91 – 1.15)
Mono-disaccharide	0.91 (0.68 – 1.23)	1.02 (0.74 – 1.40)	0.91 (0.65 – 1.30)	0.786	1.03 (0.91 – 1.17)
Polysaccharide	0.92 (0.70 – 1.22)	0.90 (0.67 – 1.21)	0.88 (0.63 – 1.24)	0.454	0.99 (0.88 – 1.12)
Fibre	0.94 (0.69 – 1.27)	1.13 (0.81 – 1.56)	1.03 (0.71 – 1.50)	0.624	1.06 (0.92 – 1.21)
Total fat	1.19 (0.89 – 1.59)	**1.42 (1.06 – 1.90)**‡	1.16 (0.86 – 1.57)	0.180	1.03 (0.93 – 1.15)
Saturated fat	1.10 (0.80 – 1.51)	1.24 (0.88 – 1.76)	1.16 (0.75 – 1.79)	0.398	1.04 (0.85 – 1.27)
MUFA	1.34 (0.99 – 1.82)	1.16 (0.82 – 1.64)	1.01 (0.67 – 1.53)	0.850	0.96 (0.81 – 1.13)
PUFA	1.28 (0.97 – 1.70)	1.04 (0.77 – 1.41)	0.99 (0.70 – 1.39)	0.728	1.00 (0.87 – 1.14)
Trans fatty acid	0.97 (0.71 – 1.32)	1.20 (0.87 – 1.67)	1.26 (0.84 – 1.90)	0.158	1.08 (0.91 – 1.28)
	Lean (BMI < 25 kg/m²) in RSI and II n=664				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Model 3 (Metabolic)					
Total protein	1.51 (0.81 – 2.85)	0.84 (0.40 – 1.79)	1.07 (0.50 – 2.32)	0.817	0.95 (0.74 – 1.23)
Animal protein	1.64 (0.85 – 3.17)	0.86 (0.37 – 1.97)	1.20 (0.54 – 2.64)	0.998	0.95 (0.72 – 1.25)
Vegetable protein	0.89 (0.45 – 1.77)	0.77 (0.37 – 1.61)	1.16 (0.53 – 2.52)	0.897	1.02 (0.77 – 1.36)
	Lean (BMI < 25 kg/m²) in RSI and II n=664				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Total carbohydrate	1.00 (0.47 – 2.13)	1.44 (0.67 – 3.10)	1.04 (0.45 – 2.43)	0.729	1.00 (0.75 – 1.35)
Mono-disaccharide	0.68 (0.32 – 1.46)	0.82 (0.37 – 1.81)	0.82 (0.35 – 1.94)	0.794	1.02 (0.74 – 1.40)
Polysaccharide	0.83 (0.41 – 1.65)	0.90 (0.43 – 1.90)	0.69 (0.29 – 1.64)	0.482	0.98 (0.72 – 1.34)
Fibre	0.83 (0.40 – 1.74)	0.99 (0.44 – 2.24)	1.12 (0.43 – 2.91)	0.730	1.16 (0.80 – 1.66)
Total fat	1.15 (0.55 – 2.39)	1.44 (0.70 – 2.98)	1.33 (0.61 – 2.87)	0.386	1.07 (0.84 – 1.37)
Saturated fat	0.87 (0.39 – 1.92)	1.08 (0.45 – 2.60)	2.19 (0.80 – 6.01)	0.119	1.44 (0.91 – 2.26)
MUFA	1.50 (0.72 – 3.14)	1.04 (0.45 – 2.44)	1.05 (0.37 – 3.00)	0.850	0.83 (0.56 – 1.23)
PUFA	0.93 (0.45 – 1.90)	1.03 (0.49 – 2.16)	0.94 (0.40 – 2.23)	0.964	1.03 (0.73 – 1.45)
Trans fatty acid	0.85 (0.40 – 1.83)	0.66 (0.28 – 1.54)	1.07 (0.41 – 2.77)	0.947	0.91 (0.61 – 1.36)

Supplementary Table 7 *(contined)*

	Overweight (BMI ≥ 25 kg/m²) in RSI and II n=1588				
	Q2	Q3	Q4	*P* for trend	Continuous (per SD increase)
Model 3 (Metabolic)					
Total protein	1.03 (0.75 – 1.41)	1.12 (0.82 – 1.53)	1.16 (0.85 – 1.59)	0.282	1.07 (0.96 – 1.18)
Animal protein	1.29 (0.93 – 1.78)	1.07 (0.78 – 1.47)	1.33 (0.95 – 1.85)	0.229	1.07 (0.96 – 1.20)
Vegetable protein	0.99 (0.74 – 1.31)	0.85 (0.63 – 1.16)	1.17 (0.84 – 1.64)	0.675	1.03 (0.91 – 1.16)
Total carbohydrate	1.30 (0.96 – 1.75)	0.96 (0.69 – 1.32)	1.04 (0.73 – 1.47)	0.747	0.97 (0.86 – 1.09)
Mono-disaccharide	0.93 (0.68 – 1.27)	0.88 (0.64 – 1.23)	0.81 (0.57 – 1.16)	0.251	0.98 (0.86 – 1.11)
Polysaccharide	0.89 (0.67 – 1.19)	0.84 (0.62 – 1.15)	0.88 (0.62 – 1.25)	0.388	0.96 (0.85 – 1.08)
Fibre	1.01 (0.73 – 1.38)	1.19 (0.85 – 1.68)	1.08 (0.74 – 1.59)	0.517	1.08 (0.94 – 1.24)
Total fat	1.05 (0.78 – 1.41)	1.33 (0.98 – 1.79)	1.08 (0.79 – 1.47)	0.345	1.02 (0.91 – 1.14)
Saturated fat	1.08 (0.78 – 1.49)	1.20 (0.84 – 1.71)	0.94 (0.60 – 1.47)	0.996	0.93 (0.75 – 1.15)
MUFA	1.23 (0.89 – 1.69)	1.20 (0.84 – 1.71)	1.04 (0.68 – 1.60)	0.885	1.07 (0.90 – 1.27)
PUFA	1.22 (0.91 – 1.64)	0.93 (0.68 – 1.28)	0.85 (0.60 – 1.21)	0.221	0.93 (0.81 – 1.08)
Trans fatty acid	0.97 (0.71 – 1.33)	1.26 (0.90 – 1.77)	1.29 (0.84 – 1.96)	0.124	1.11 (0.93 – 1.33)

Bold values indicate P<0.05. ‡Indicates significant values using P < 0.021. Model 3 (metabolic) is in addition to the previous model adjusted for cholesterol, metabolic syndrome and diabetes mellitus. Model 4 (metabolic + log-transformed BMI) is in addition to the previous model adjusted for log-transformed BMI.