

SUPPLEMENTARY MATERIAL FOR: Human PNPLA3-I148M variant increases hepatic retention of polyunsaturated fatty acids

Panu K. Luukkonen^{1,2,}, Auli Nick^{1,3*}, Maarit Hölttä-Vuori^{1,3}, Christoph Thiele⁴, Elina Isokuortti^{1,2}, Susanna Lallukka-Brück^{1,2}, You Zhou^{1,5,6}, Antti Hakkarainen^{7,8}, Nina Lundbom⁷, Markku Peltonen⁹, Marju Orho-Melander¹⁰, Matej Orešič^{11,12}, Tuulia Hyötyläinen¹³, Leanne Hodson¹⁴, Elina Ikonen^{1,3,**,§}, Hannele Yki-Järvinen^{1,2,**,§}*

¹Minerva Foundation Institute for Medical Research, Helsinki, Finland, ²Department of Medicine, University of Helsinki and Helsinki University Hospital, Helsinki, Finland, ³Faculty of Medicine, Department of Anatomy, University of Helsinki, Helsinki, Finland, ⁴LIMES Institute, Bonn University, Bonn, Germany, ⁵Systems Immunity Research Institute, Cardiff University, Cardiff, UK, ⁶Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff, UK, ⁷Department of Radiology, HUS Medical Imaging Center, Helsinki University Hospital and University of Helsinki, Helsinki, Finland, ⁸Department of Neuroscience and Biomedical Engineering, Aalto University School of Science, Espoo, Finland, ⁹Department of Public Health Solutions, National Institute for Health and Welfare, Helsinki, Finland, ¹⁰Department of Clinical Sciences, Lund University, Malmö, Sweden, ¹¹Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland, ¹²School of Medical Sciences, Örebro University, Örebro, Sweden, ¹³Department of Chemistry, Örebro University, Örebro, Sweden, ¹⁴Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Oxford, UK, *Authors share co-first authorship, **Authors share co-last authorship, § Corresponding Author.

LIPIDOMIC ANALYSIS

Sample preparation and chemicals

All solvents were HPLC grade or LC-MS grade, from Honeywell (Morris Plains, NJ, USA), Fisher Scientific (Waltham, MA, USA) or Sigma-Aldrich (St. Louis, MO, USA). Mass spectrometry grade ammonium acetate and reagent grade formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Samples were extracted in random order as follows: 10 μL of 0.9% NaCl and, 120 μL of CHCl_3 : MeOH (2:1, v/v) containing internal standards ($c=2.5 \mu\text{g mL}^{-1}$) were added to 10 μL of each plasma sample. The standard solution contained the following compounds: 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (PE(17:0/17:0)), N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine (SM(d18:1/17:0)), N-heptadecanoyl-D-erythro-sphingosine (Cer(d18:1/17:0)), 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (PC(17:0/17:0)), 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(17:0)) and 1-palmitoyl-d31-2-oleoyl-sn-glycero-3-phosphocholine (PC(16:0/d31/18:1)), purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), and tripalmitin- Triheptadecanoylglycerol (TG(17:0/17:0/17:0)) Larodan AB, Solna, Sweden). The samples were vortex mixed and incubated on ice for 30 min after which they were centrifuged ($9400 \times g$, 3 min). 60 μL from the lower layer of each sample was then transferred to a glass vial with an insert and 60 μL of CHCl_3 : MeOH (2:1, v/v) was added to each sample. The samples were re-randomized and stored at -80°C until analysis.

Calibration curves using 1-hexadecyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (PC(16:0e/18:1(9Z))), 1-(1Z-octadecenyl)-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (PC(16:0/16:0)), 1-octadecanoyl-sn-glycero-3-phosphocholine (LPC(18:0)), (LPC18:1), 1-2-dioleoyl-sn-glycerol ((DG(18:1/18:1)), (2-aminoethoxy)[(2R)-3-hydroxy-2-[(11Z)-octadec-11-enoyloxy]propoxy]phosphinic acid (LysoPE(18:1)), N-(9Z-octadecenoyl)-sphinganine (Cer

(d18:0/18:1(9Z))), 1-hexadecyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (PE (16:0/18:1)) and 1-octadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phospho-(1'-myo-inositol)n (PI(18:0-20:4)) from Avanti Polar Lipids, Inc., 1-Palmitoyl-2-Hydroxy-sn-Glycero-3-Phosphatidylcholine (LPC(16:0)) from Larodan, and 1,2,3 trihexadecanoalglycerol (TG16:0/16:0/16:0)), 1,2,3 trioctadecanoalglycerol (TG(18:0/18:0/18:0)) and cholest-5-en-3 β -yl octadecanoate (ChoE(18:0)), 3 β -Hydroxy-5-cholestene-3-linoleate (ChoE(18:2)) from Larodan, were prepared prepared to the following concentration levels: 100, 500, 1000, 1500, 2000 and 2500 ng mL⁻¹ (in CHCl₃:MeOH, 2:1, v/v) including 250 ng mL⁻¹ of each internal standard.

UHPLC-QTOFMS analysis

The UHPLC system used was 1290 Infinity system from Agilent Technologies (Santa Clara, CA, USA). The system was equipped with a multisampler (maintained at 10 °C) using 10% DCM in MeOH and ACN:MeOH:IPA:H₂O (1:1:1:1, v/v/v/v) + 0.1% HCOOH as needle wash solutions after each injection for 7.5 s each, a quaternary solvent manager and a column thermostat (maintained at 50 °C). Separations were performed in a ACQUITY UPLC® BEH C18 column (2.1 mm × 100 mm, particle size 1.7 μ m) by Waters Corporation (Milford, MA, USA). The flow rate was 0.4 mL min⁻¹ and the injection volume was 1 μ L. H₂O + 1% NH₄Ac (1M) + 0.1% HCOOH (A) and ACN:IPA (1:1, v/v) + 1% NH₄Ac + 0.1% HCOOH (B) were used as the mobile phases for the gradient elution. The gradient was as follows: from 0 to 2 min 35-80% B, from 2 to 7 min 80-100% B and from 7 to 14 min 100% B. Each run was followed by a 7 minute re-equilibration period under initial conditions (35% B).

The mass spectrometer used was a 6545 quadrupole time of flight (QTOF) instrument from Agilent Technologies (Santa Clara, CA, USA), interfaced with a dual jet stream electrospray (dual ESI) ion source. Nitrogen generated by a nitrogen generator (PEAK Scientific, UK) was used as the nebulizing

gas at a pressure of 21 psi, as the drying gas at a flow rate of 10 L min⁻¹ (at 193 °C) and as the sheath gas at a flow rate of 11 L min⁻¹ (at 379 °C). Pure nitrogen (6.0) from AGA (Munich, Bavaria, Germany.) was used as the collision gas. The capillary voltage and the nozzle voltage were kept at 3643 V and 1500 V, respectively. The reference mass solution including ions at m/z 121.0509 and 922.0098 was prepared according to instructions by Agilent. The acquisition mass range was m/z 100-1700 and the instrument was run using the extended dynamic range with an approximate resolution of 30 000 FWHM measured on the ion at m/z 1521.9715 (which is included in the tune mixture) during calibration of the instrument. MassHunter B.06.01 software (Agilent Technologies, Santa Clara, CA, USA) was used for all data acquisition and MZmine 2 was used for data processing.

MS data processing was performed using open source software MZmine 2.33. The following steps were applied in the processing: 1.) Mass detection with a noise level of 750, 2.) Chromatogram builder with a min time span of 0.08 min, min height of 800 and a m/z tolerance of 0.006 m/z or 10.0 ppm, 3.) Chromatogram deconvolution using the local minimum search algorithm with a 70% chromatographic threshold, 0.05 min minimum RT range, 1% minimum relative height, 800 minimum absolute height, a minimum ration of peak top/edge of 1.2 and a peak duration range of 0.08 - 5.0, 4.), Isotopic peak grouper with a m/z tolerance of 6.0 ppm, RT tolerance of 0.05 min, maximum charge of 2 and with the most intense isotope set as the representative isotope, 5.) Peak list row filter keeping only peak with a minimum of 10% of the samples in a row, 6.) Join aligner with a m/z tolerance of 0.008 or 10.0 ppm and a weight for of 2, a RT tolerance of 0.1 min and a weight of 1 and with no requirement of charge state or ID and no comparison of isotope pattern, 7.) Gap filling using the same RT and m/z range gap filler algorithm with an m/z tolerance of 0.008 or 10 ppm, 8.) Identification of lipids using a custom database search with an m/z tolerance of 0.006 009 m/z or 10.0 ppm and a RT tolerance of 0.1 min, 9.) Normalization using internal standards (PE (17:0/17:0), SM (d18:1/17:0), Cer (d18:1/17:0), LPC (17:0), TG (17:0/17:0/17:0) and PC (16:0/d30/18:1)) for

identified lipids and closest ISTD for the unknown lipids 10). The concentrations were calculated using lipid-class based concentration curves.

Generation of stable A431 cell lines expressing GFP-tagged PNPLA3-148I and PNPLA3-148M proteins

GFP-PNPLA3-148I and GFP-PNPLA3-148M encoding PCR fragments were inserted into the pSH-EFIRE vector (kind gift from Shiqian Li) encoding a puromycin selection cassette, EF1alpha promoter and AAVS1 homology arms. The constructs were then transfected to A431 PNPLA3-ko cells for integration into the AAVS1/Safe Harbor locus, and the cells were cultured under puromycin selection (1 µg/ml).

SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

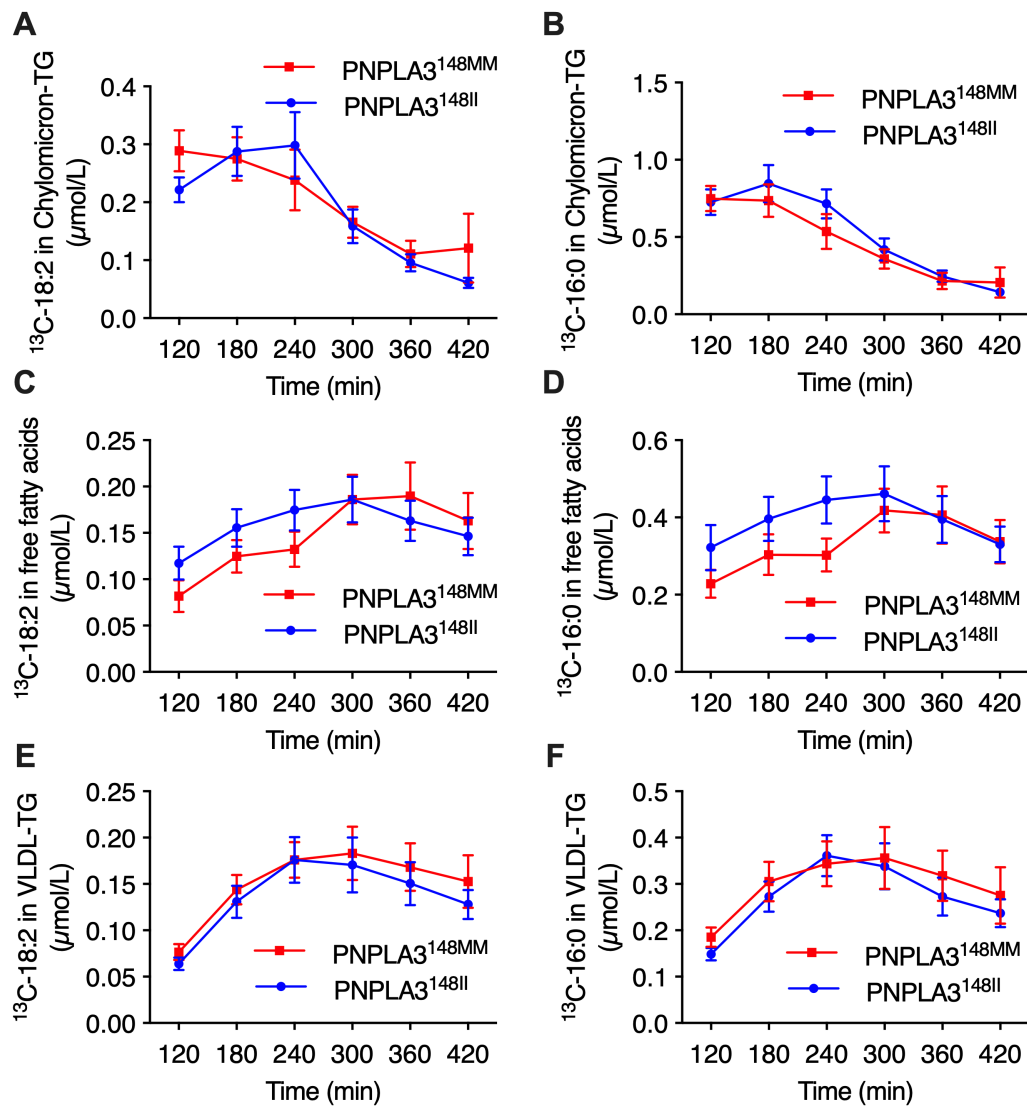


Figure S1. Concentrations of ^{13}C -labeled fatty acids in (A-B) plasma chylomicron-TG, (C-D) free fatty acids and (E-F) VLDL-TG in the PNPLA3^{148MM} and PNPLA3^{148II} groups during the postprandial period. Data are shown as mean \pm SEM. The blue lines and circles denote the PNPLA3^{148II} (n=14) group and the red lines and squares the PNPLA3^{148MM} (n=12) group. There were no significant differences between the groups as determined using 2-way ANOVA.

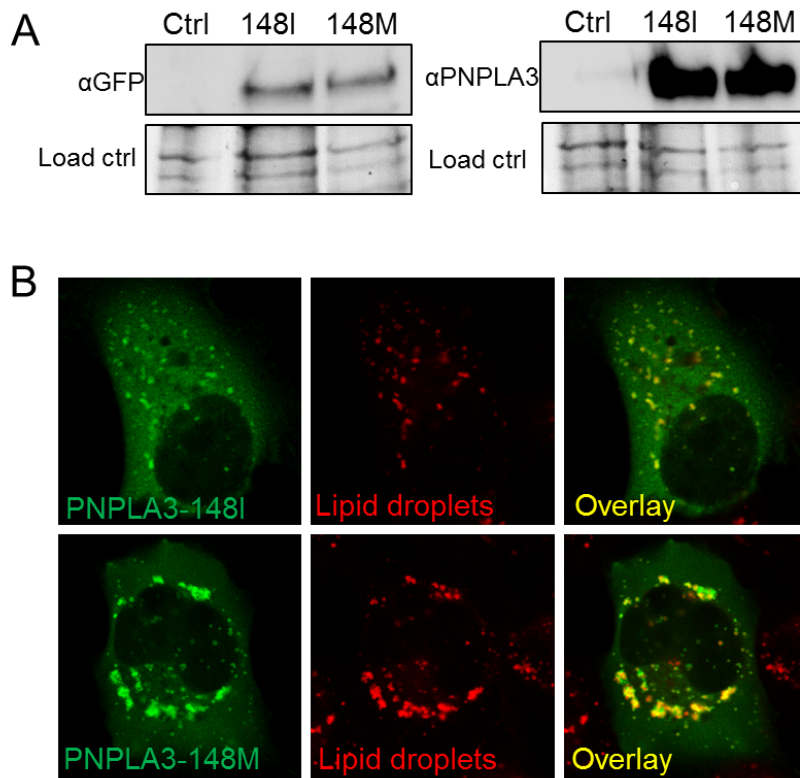


Figure S2. Expression level and lipid droplet association of GFP-tagged PNPLA3-148I and PNPLA3-148M proteins in A431 cells. (A) Western blotting of GFP-tagged PNPLA3-148I and PNPLA3-148M proteins stably expressed on PNPLA3-ko A431 cell background. Cell lysates (30 μ g protein) were immunoblotted using anti-GFP (Abcam ab290) or anti-PNPLA3 (Santa Cruz) antibodies. (B) PNPLA3-ko cells expressing GFP-tagged PNPLA3-148I or PNPLA3-148M proteins were incubated for 50 min with 200 μ M oleic acid, then fixed and stained with the lipid droplet dye LD540. Please note the more prominent localization of PNPLA3-148M on LDs compared to PNPLA3-148I.

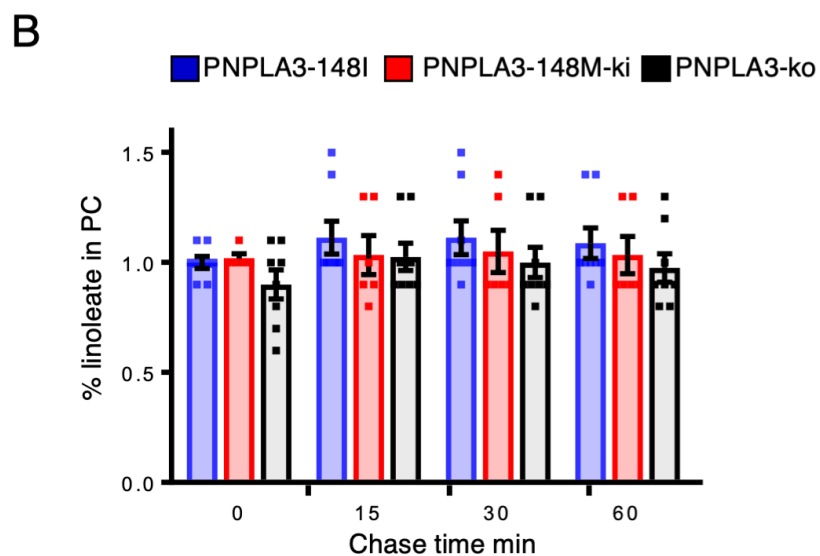
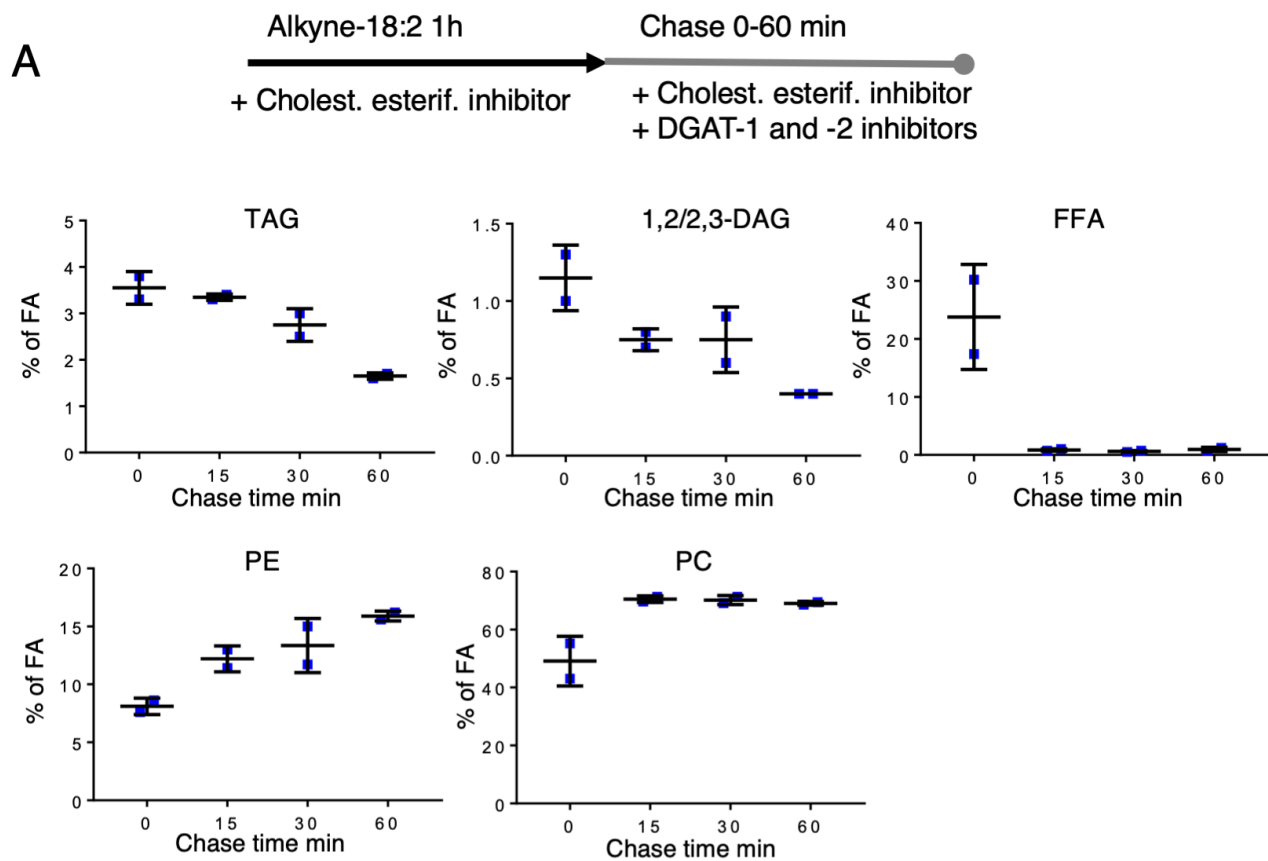


Figure S3. Partitioning of alkyne-labeled linoleate in different lipid species in A431 cells during lipolysis. (A) A431 PNPLA3-148I cells were labeled for 1h min with 100 μ M alkyne-linoleate in the presence of a cholesterol esterification inhibitor. After labeling, cells were either harvested (0 min chase) or further incubated in lipoprotein-deficient medium supplemented with cholesterol

esterification and DGAT inhibitors for the indicated times before collecting. Cells were extracted, click-reacted and analysed by TLC. Bars: % of alkyne-linoleate in indicated lipid species \pm SD, representative experiment in duplicate. (B) Homozygous PNPLA3-148I, PNPLA3-148M-ki and PNPLA3-ko A431 cells were labeled and incubated as in (A). Bars: % of incorporated alkyne-linoleate in PC, normalized to PNPLA3-148I cells at 0 min chase \pm SEM. N=5-8 from 3-4 individual experiments.

Table S1. Physical activity measured using an accelerometer and dietary intake based on food record data of the PNPLA3^{148MM} and PNPLA3^{148II} groups.

| | PNPLA3 ^{148II} | PNPLA3 ^{148MM} |
|------------------------------------|-------------------------|-------------------------|
| <i>Accelerometer data</i> | | |
| Accelerometer wear time (min/day) | 773 (692 – 847) | 799 (728 – 887) |
| Steps (n/day) | 8957 (7220 – 9962) | 8473 (4896 – 11023) |
| Sedentariness (% of wear time) | 58 (57 – 63) | 64 (57 – 70) |
| Light activity (% of wear time) | 34 (29 – 38) | 28 (26 – 40) |
| Moderate activity (% of wear time) | 7 (5 – 8) | 4 (2 – 10) |
| Sitting time (min/day) | 378 (310 – 486) | 389 (323 – 546) |
| Sitting periods >30min (n) | 14 (11 – 19) | 14 (10 – 36) |
| <i>Food record data</i> | | |
| Energy intake (kcal/day) | 2000 (1611 – 2398) | 2033 (1523 – 2220) |
| Protein intake (E%) | 16.6 (12.9 – 21.4) | 17.1 (15.2 – 20.6) |
| Carbohydrate intake (E%) | 41.6 (34.0 – 50.3) | 42.7 (37.3 – 44.7) |
| Fat intake (E%) | 37.4 (32.8 – 45.1) | 37.8 (32.5 – 39.5) |
| Saturated fat intake (E%) | 10.9 (9.7 – 16.0) | 12.1 (10.5 – 14.1) |
| Monounsaturated fat intake (E%) | 12.8 (11.6 – 17.4) | 13.9 (11.2 – 15.2) |
| Polyunsaturated fat intake (E%) | 7.0 (6.5 – 8.2) | 6.7 (5.6 – 8.6) |
| Total fibre intake (g/day) | 20.9 (17.0 – 31.3) | 19.3 (13.4 – 24.5) |

Data are in median (25th-75th percentile). The differences in variables between the groups were compared using Mann Whitney U test. There were no significant differences in any variable. E%, energy percent.