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Replacement of saturated with unsaturated fats had no impact on vascular function but beneficial effects on lipid biomarkers, E-selectin and blood pressure: results from the randomized, controlled Dietary Intervention and VAScular function (DIVAS) study[1,2,3,4]

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32

33 Running title: Dietary fatty acids and cardiovascular risk

34

Abbreviations

36 ABP: ambulatory blood pressure; Ach: acetylcholine; CVD: cardiovascular disease; DBP:

37 diastolic blood pressure; DIVAS: Dietary Intervention and VAScular function; FMD: flow-

38 mediated dilatation; HDL-C: HDL-cholesterol; LDI: laser Doppler imaging; LDL-C: LDL-

39 cholesterol; PP: pulse pressure; SBP: systolic blood pressure; TAG: triacylglycerol; TC:

40 total cholesterol; %TE: percentage of total energy; Δ: change from baseline.

41

Abstract

Background: Public health strategies to lower cardiovascular disease (CVD) risk involve reducing dietary saturated fatty acid (SFA) intake to ≤10% of total energy (%TE). However, the optimal type of replacement fat is unclear.

Objective: We investigated the substitution of 9.5-9.6%TE dietary SFA with either monounsaturated (MUFA) or n-6 polyunsaturated fatty acids (PUFA) on vascular function and other CVD risk factors.

Design: Using a randomized, controlled, single-blind, parallel group dietary intervention, 195 men and women aged 21-60 y with moderate CVD risk (≥50% above the population mean) from the United Kingdom followed one of three 16-wk isoenergetic diets (%TE target compositions, total fat:SFA:MUFA:n-6 PUFA): SFA-rich (36:17:11:4, $n = 65$), MUFA-rich (36:9:19:4, $n = 64$) or n-6 PUFA-rich (36:9:13:10, $n = 66$). The primary outcome measure was flow-mediated dilatation (%FMD); secondary outcome measures included fasting serum lipids, microvascular reactivity, arterial stiffness, ambulatory blood pressure, and markers of insulin resistance, inflammation and endothelial activation.

Results: Replacing SFA with MUFA or n-6 PUFA did not significantly impact on %FMD (primary endpoint) or other measures of vascular reactivity. Of the secondary outcome measures, substitution of SFA with MUFA attenuated the increase in night systolic blood pressure (-4.9 mm Hg, $P = 0.019$) and reduced E-selectin (-7.8%, $P = 0.012$). Replacement with MUFA or n-6 PUFA lowered fasting serum total cholesterol (TC; -8.4% and -9.2%, respectively), low-density lipoprotein cholesterol (-11.3% and -13.6%) and TC to high-density lipoprotein cholesterol ratio (-5.6% and -8.5%) ($P ≤ 0.001$). These

65 changes in low-density lipoprotein cholesterol equate to an estimated 17-20% reduction

66 in CVD mortality.

67 **Conclusions:** Substitution of 9.5-9.6%TE dietary SFA with either MUFA or n-6 PUFA did

68 not impact significantly on %FMD or other measures of vascular function. However, the

69 beneficial effects on serum lipid biomarkers, blood pressure and E-selectin offer a

70 potential public health strategy for CVD risk reduction.

Introduction

Some meta-analyses of observational studies and randomly controlled trials (RCT) have failed to demonstrate significant associations between the intake of SFA and PUFA, and risk of coronary heart disease (CHD) (1, 2). However, these analyses have received criticism for failing to account for the macronutrient which substitutes SFA in the diets, and the presence of trans fatty acids in the PUFA intervention arms. However, a more recent meta-analysis focusing on macronutrient replacement found that replacing SFA with n-6 PUFA, specifically linoleic acid, was associated with a significantly reduced risk of CHD (3). Since observational studies cannot determine cause-and-effect, RCT are necessary to assess the direct impact of SFA-rich diets on CVD risk. Due to the unequivocal link between high SFA intake and raised plasma LDL-cholesterol (LDL-C) (4), reduction of dietary SFA to ≤10% of total energy (%TE) remains a key public health strategy for the prevention of cardiovascular disease (CVD) (5). Although intakes of SFA have fallen, British adults exceed this recommendation at 12.0%TE (6). However, there are no clear dietary guidelines on the optimum macronutrient to replace SFA. Due to the potential detrimental effects of carbohydrates on the metabolic profiles in some population sub-groups (7), substitution of SFA by unsaturated fats has been proposed as an alternative strategy to meet the population target. It is thought that reducing SFA intake by modifying dietary fat composition may reduce cardiovascular events by 14% (8).

Vascular dysfunction, an early marker for atherosclerosis, is characterized by impaired endothelium-dependent vasodilation (9). Prognostic measures of vascular function, such as flow-mediated dilatation (FMD), are strongly associated with increased

94   CVD risk (10, 11). To date, the impact of replacing dietary SFA with MUFA or n-6 PUFA

95   on vascular function, including FMD, remains unclear (12, 13). The effects of SFA

96   substitution on classical CVD risk factors, such as plasma lipids and blood pressure, has

97   been studied previously but this has rarely involved a direct comparison with both MUFA

98   and n-6 PUFA, the latter of which is often confounded by the addition of n-3 PUFA.

99   Currently, insufficient evidence exists to make firm conclusions regarding the optimal

100 class of dietary fat to replace SFA (12, 14, 15). To inform and strengthen the evidence

101 base for public health recommendations, the Dietary Intervention and VAScular function

102 (DIVAS) study evaluated the effects of substituting SFA with MUFA or n-6 PUFA for 16

103 wk on FMD (primary endpoint) in individuals with moderate CVD risk. Secondary

104 outcome measures of this suitably powered RCT included other vascular function

105 measures and classical CVD risk factors.

Subjects and Methods

Subjects

The trial was approved by the West Berkshire Local Research Ethics Committee

(09/H0505/56) and University of Reading Research Ethics Committee (09/40), registered

at www.clinicaltrials.gov (NCT01478958), and conducted according to the Declaration of

Helsinki. Non-smoking men and women aged 21-60 y with moderate CVD risk were

recruited from Reading (United Kingdom; UK) from November 2009 to June 2012 in

three cohorts. The study was completed in October 2012. All participants provided

written informed consent. Details of the study criteria have been previously published in

Weech *et al* (16). Briefly, CVD risk score was determined from fasted measures of serum

total cholesterol (TC), HDL-cholesterol (HDL-C) and glucose, blood pressure, BMI or

waist circumference, and family history of premature myocardial infarction or type 2

diabetes (**Supplemental Table 1** under "Supplemental data" in the online issue). Eligible

participants had a risk score of ≥2 combined points, reflecting a moderate CVD risk

(≥50% above the population mean). Further inclusion criteria included normal blood

biochemistry and not taking dietary supplements or medication for hypertension, raised

lipids or inflammatory disorders (16).

Study design

The DIVAS study was a 16-wk, single-blind, parallel group RCT. Participants were

randomized by study researchers (KV) to one of three intervention diets by minimization

(17), stratifying for gender, age, BMI and CVD risk score. The three isoenergetic

intervention diets (%TE target compositions, SFA:MUFA:n-6 PUFA) were rich in SFA

(17:11:4), MUFA (9:19:4) and n-6 PUFA (9:13:10). Relative to the SFA-rich control diet,

the MUFA- and n-6 PUFA-rich diets replaced 8%TE SFA with unsaturated fatty acids.

130   Since UK dietary guidelines limit n-6 PUFA intake to ≤10%TE (5), SFA was substituted

131   by 6%TE n-6 PUFA and 2%TE MUFA in the n-6 PUFA-rich diet. Intakes of other

132   macronutrients were unchanged allowing total fat to remain at 36%TE for each diet.

133   **Dietary intervention**

134   Full details of the dietary intervention and measures of compliance have been published

135   previously (16). In summary, a flexible food-exchange model was implemented to

136   achieve the target fatty acid intakes in free-living individuals for 16-wk. Participants, who

137   were unaware of the assigned intervention diet, replaced habitually-consumed sources of

138   exchangeable fats with study foods (spread, oils, dairy products and commercially-

139   available snacks) of specific fatty acid composition. Specially-formulated spreads (80%

140   total fat) and oils (Unilever R&D, Vlaardingen, the Netherlands) were used for the MUFA-

141   rich diet (refined olive oil and olive oil/rapeseed oil blended spread) and n-6 PUFA-rich

142   diet (safflower oil and spread). Butter (Wyke Farm, Somerset, UK) was used for the SFA-

143   rich diet. Following the baseline clinical visit, trained nutritionists gave 1:1 verbal and

144   written instructions for manipulating fatty acid intake and were available throughout the

145   study for advice. Every 4-wk, study foods (except dairy products) were provided free of

146   charge. To monitor compliance, 4-d weighed diet diaries (wk 0, 8 and 16), forms

147   recording daily intakes of study foods, and the proportions of plasma phospholipid fatty

148   acids as a short term biomarker of fatty acid intake were analyzed (wk 0 and 16). Body

149   weight, which was to remain constant, was monitored every 4-wk, and changes were

150   addressed.

151   **Clinical visits**

152   Clinical visits took place at the Hugh Sinclair Unit of Human Nutrition, University of

153   Reading, during wk 0 (baseline; V1) and wk 16 (post-intervention; V2). Alcohol and

154 aerobic exercise were avoided 24 h before visits. Participants consumed a provided low-

155 fat meal the evening before visits and fasted for 12 h, only drinking low nitrate water.

156 During visits, participants rested in the supine position for 30 min in a quiet, temperature-

157 controlled environment (22 ± 1 °C) before non-invasive measures of vascular function

158 were conducted under the same conditions. Measurements were performed at the same

159 time of day and by the same trained researcher for both visits. Pre-menopausal women

160 attended during the same phase of their menstrual cycle. Fasted blood samples were

161 also collected.

Assessment of vascular function and 24 h ABP

163 To assess endothelial function, FMD (primary outcome) and laser Doppler imaging (LDI)

164 with iontophoresis were conducted by trained researchers as previously described (18).

165 In brief, FMD assessed endothelial-dependent vasodilation of the macrovasculature

166 using an ATL ultrasound HDI-5000 broadband ultrasound system (Philips Healthcare,

167 Best, the Netherlands) following standard guidelines (19). ECG-gated images collected

168 at 0.25 frames/s using image-grabbing software were analyzed by a single researcher,

169 who was unaware of the intervention allocation, using wall-tracking software (both

170 Medical Imaging Applications-LLC, Coralville, IA). FMD was calculated as the maximum

171 change in post-occlusion brachial artery diameter expressed as a % of the baseline

172 diameter (%FMD). LDI was performed with a LDI2-IR laser Doppler imager (Moor

173 Instruments Ltd., Axminster, UK), using iontophoresis to deliver 1% acetylcholine (Ach)

174 and 1% sodium nitroprusside on the left forearm. Microvascular responses to

175 acetylcholine (endothelium-dependent vasodilation) and sodium nitroprusside

176 (endothelium-independent vasodilation) were determined by the AUC for flux vs. time,

177 measured in arbitrary units.

178   Arterial stiffness of the larger conduit and smaller peripheral vessels was

179 measured in triplicate as detailed elsewhere (20) using carotid-femoral pulse wave

180 velocity (m/s) and radial pulse wave analysis, respectively (SphygmoCor; AtCor Medical,

181 West Ryde, Australia). Pulse wave analysis determined the augmentation index

182 corrected for a heart rate of 75 bpm (%). Digital volume pulse (Pulse Trace PCA2; Micro

183 Medical Ltd., Chatham, UK) determined the stiffness index (m/s) and reflection index (%)

184 as measures of arterial stiffness and vascular tone, respectively (18).

185   Using A/A grade automated oscillometric ambulatory blood pressure (ABP)

186 monitors (A&D Instruments Ltd., Abingdon, UK), ABP and heart rate were measured

187 every 30 min between 07:00-21:59 and every 60 min between 22:00-06:59,

188 approximately 48 h before the clinical visits. Mean 24 h, day and night measurements

189 were calculated using sleep times recorded on participant activity forms. Pulse pressure

190 (PP) was calculated as the difference between systolic (SBP) and diastolic blood

191 pressure (DBP).

192 **Biochemical analysis**

193 Fasted blood samples were centrifuged at 1800 × g for 15 min at 20°C (for serum) and

194 4°C (for plasma), and stored at −80°C. Plasma total nitrites and nitrates were measured

195 with ozone-based chemiluminescence (21). ELISA kits analyzed circulating plasma

196 insulin (Dako Ltd., Ely, UK), von Willebrand factor (Abnova, Taipei City, Taiwan),

197 vascular and intercellular adhesion molecules, E-selectin and P-selectin, with high

198 sensitivity kits for TNF-α and IL-6 (R&D Systems Europe Ltd., Abingdon, UK). C-reactive

199 protein, serum lipids (TC, HDL-C and triacylglycerol (TAG)), glucose and non-esterified

200 fatty acids were quantified using an autoanalyzer (reagents and analyzer:

201 Instrumentation Laboratory Ltd., Warrington, UK; non-esterified fatty acid reagent: Alpha

202 Laboratories, Eastleigh, UK). LDL-C was estimated using the Friedewald formula (22).

203 Insulin resistance was estimated by HOMA-IR, and insulin sensitivity by the original and

204 revised quantitative insulin sensitivity check indices using standard equations (23).

205 Microalbumin was determined in fresh 24 h urine samples, collected before each clinical

206 visit, using a turbidimetric assay (Alpha Laboratories) on the autoanalyzer and corrected

207 for the total volume of urine (mg/24 h) (24). Mean intra- and inter-assay CV were <5% for

208 the automated assays and <10% for other assays. The CVD risk assessment tool used

209 at screening determined CVD risk scores at both clinical visits (16).

210 **Statistical analysis**

211 To detect a 2% inter-group difference in %FMD (primary outcome) using a SD of 2.3,

212 90% power and 5% significance level, $n = 171$ participants were required ($n = 57$ per

213 group), increasing to $n = 228$ for a 25% dropout rate ($n = 76$ per group). Statistical

214 analyses were performed using SPSS version 19.0 (SPSS Inc.). For continuous

215 variables, suitable checks for normality were implemented as appropriate. Differences

216 between diet groups at baseline were assessed using one-way ANOVA or the Kruskal-

217 Wallis test (if non-normally distributed). For discrete data, the Chi-squared test was used.

218 To evaluate the effects of the dietary intervention on the primary (%FMD) and secondary

219 (vascular reactivity and stiffness, serum lipid biomarkers, ABP, indices of insulin

220 resistance, inflammation and endothelial activation) outcome measures, a general linear

221 model using the difference from baseline (Δ; V2-V1) as the dependent variable was

222 implemented, with baseline values of the variable of interest, BMI, age, gender and

223 intervention diet as prognostic variables. The overall effect of diet assessed the

224 replacement of SFA with MUFA and n-6 PUFA, and was subject to post-hoc analysis

225 using Tukey's correction if significant. This adjusted for the three intervention groups, but

226 not for the general approach being applied to the various endpoint variables. When a

227 significant overall 'diet' effect was observed, one-sample t-tests were performed to

228 determine whether the response (Δ) within each dietary arm was different from zero. $P \leq$

229 0.05 was considered significant. Data presented in the text, tables and figure represents

230 the raw mean ± SEM.

Results

Study participation

Of the 202 participants randomized to the intervention, 195 (97%) successfully

completed the study (**Figure 1**). Baseline characteristics of the three diet groups,

referred to as the SFA, MUFA and n-6 PUFA diet groups going forward, are shown in

Table 1. These groups were well-matched for the CVD risk score criteria. No significant

differences in the baseline measures between the three diet groups for %FMD or any of

the secondary outcomes (including measures of compliance) were evident, except for IL-

6 ($P = 0.001$) and TNF-α ($P = 0.026$) concentrations which were higher in the participants

randomised to the SFA relative to the MUFA group.

Compliance

Data for all compliance measures are presented in detail elsewhere (16). In summary,

dietary fatty acid targets were broadly met, with increases of 6.11 ± 0.43 %TE SFA, 6.77

± 0.38 %TE MUFA and 5.48 ± 0.36 %TE n-6 PUFA in the respective diets relative to

baseline intakes (**Supplemental Table 2** under "Supplemental data" in the online issue).

During the intervention, SFA intakes in the SFA (17.6 ± 0.4 %TE), MUFA (8.1 ± 0.2

%TE) and n-6 PUFA (8.0 ± 0.2 %TE) groups corresponded to a larger replacement of

SFA in the MUFA (9.5%TE) and n-6 PUFA (9.6%TE) interventions than anticipated

(8.0%TE) when compared with the SFA diet. Significant overall diet effects for changes

in dietary SFA, MUFA and n-6 PUFA between groups ($P \leq 0.001$) were broadly

supported by changes in the proportions of plasma phospholipid total SFA, MUFA and n-

6 PUFA, which were significant for the total proportions of SFA and MUFA between diet

groups ($P \leq 0.001$) (**Supplemental Table 3** under "Supplemental data" in the online

issue). There were no significant changes in BMI between groups.

Vascular function

For the primary endpoint, %FMD, there was no difference between the groups following

the intervention. Furthermore, additional measures of vascular function (LDI and

reflection index) and arterial stiffness (pulse wave velocity, augmentation index and

stiffness index) were not significantly different between intervention groups (**Table 2**).

24 h ABP

There were significant overall diet effects for mean changes in night SBP ($P = 0.019$) and

night PP ($P = 0.048$) between diet groups. The increase in night SBP observed following

the SFA diet (3.8 ± 1.4 mm Hg) was attenuated by the MUFA diet (-1.1 ± 1.2 mm Hg),

reflecting a mean difference of -4.9 mm Hg when MUFA replaced SFA. Although overall

diet effects were not evident for other ABP parameters, there was a tendency for

increased 24 h DBP (1.5 ± 0.7 mm Hg; $P = 0.074$) following the SFA diet (Table 2).

Plasma markers of endothelial activation and inflammation

There was an overall diet effect for the change in plasma E-selectin between intervention

groups ($P = 0.012$), reducing by 7.8% when MUFA replaced SFA (**Table 3**). No

significant diet effects were evident for other markers of endothelial activation or

inflammation.

Fasting serum lipids, indices of insulin resistance and CVD risk score

The changes in fasting TC, LDL-C, non-HDL-C, and ratios of TC:HDL-C and LDL-

C:HDL-C showed significant differences between diet groups ($P ≤ 0.001$) (**Figure 2**;

Supplemental Table 4 under "Supplemental data" in the online issue). In response to

the SFA diet, there were significant increases in TC (7.7 ± 1.5%), LDL-C (9.8 ± 1.9%)

and TC:HDL-C ratio (4.0 ± 1.4%). Replacing SFA with MUFA or n-6 PUFA attenuated

these increases in TC (-8.4% and -9.2%, respectively), LDL-C (-11.3% and -13.6%) and

279   TC:HDL-C ratio (-5.6% and -8.5%), whereas there were no significant differences

280   between the MUFA and n-6 PUFA groups.

281   At baseline, the mean CVD risk score for all groups was 3.3 ± 0.1 points. There

282   was an overall diet effect for the change in CVD risk scores between groups ($P = 0.003$)

283   (Supplemental Table 4 under "Supplemental data" in the online issue). Within-group

284   analysis revealed the response to the SFA diet increased the CVD risk score (0.46 ±

285   0.14 points; $P ≤ 0.001$). Replacement of SFA with MUFA attenuated this rise (-0.46

286   points; $P = 0.027$), whereas replacement with n-6 PUFA reduced the CVD risk score (-

287   0.60 points; $P = 0.003$).

Discussion

288 The DIVAS study is the first suitably-powered dietary intervention in a free-living

289 population to investigate the replacement of SFA with both MUFA or n-6 PUFA on

290

291 several markers of macro- and microvascular reactivity, novel markers that are strongly

292 related to CVD development (10, 11), and classical CVD risk factors.

293 Few studies have investigated the long-term replacement of SFA with unsaturated

294 fats on %FMD (12, 13). In agreement with Sanders *et al*, who replaced 5.2%TE SFA with

295 MUFA for 24 wk in insulin-resistant adults (25), substituting dietary SFA with either

296 MUFA (9.5%TE) or n-6 PUFA (9.6%TE) for 16 wk did not significantly impact on %FMD.

297 These findings are in contrast with those of Keogh *et al* who observed high intakes of

298 SFA reduce %FMD by approximately 50% compared with high intakes of MUFA or total

299 PUFA in healthy participants (26). However, the unsaturated fatty acid-rich diets may

300 have been confounded by high intakes of almonds (45g/d) or walnuts (35g/d), which as

301 sources of L-arginine and α-linolenic acid may have improved vascular function (27, 28).

302 Furthermore, replacement of SFA had no effect on arterial stiffness, similar to others

303 reporting no change in pulse wave velocity when SFA was replaced with MUFA (25) and

304 total PUFA (26). Sanders *et al* (25) suggest arterial stiffening is a slow, progressive

305 process, so a longer exposure to changes in dietary fat composition may be required to

306 demonstrate a significant finding.

307 Hypertension, an independent CVD risk factor, is closely related to arterial

308 stiffness (29). The small number of RCT investigating SFA substitution with unsaturated

309 fats on blood pressure are inconclusive (12), with many limited by the use of total rather

310 than n-6 PUFA and clinic blood pressure measurements rather than ABP (a superior

311 prognostic tool) (30). The DIVAS study demonstrated that replacing SFA with MUFA

312 improved night SBP, which is reported to be a better predictor of cardiovascular events

313 than clinic SBP or day ambulatory SBP, as previously reported (31, 32). Our findings

314 may reflect the beneficial effects of increased dietary MUFA as well as reduced SFA,

315 suggesting the type of replacement fat is important, since there was no significant impact

316 of the n-6 PUFA diet on night SBP relative to the SFA diet group. Other groups have

317 reported improvements in blood pressure when SFA was replaced with MUFA (33-35)

318 and n-6 PUFA (34), but the absence of a between-treatment washout in the latter study

319 cannot rule out a carryover effect. Relative to baseline, the small reductions in macro-

320 and microvascular reactivity in response to the SFA diet may have contributed to the rise

321 in night SBP, night DBP and 24 h DBP, as previously reported (36). Although other

322 dietary components such as sodium and potassium influence blood pressure (37),

323 intakes of these micronutrients were not different between diet groups. The changes in

324 night SBP observed when MUFA replaced SFA (-4.8 mm Hg) are of public health

325 importance since a 3 mm Hg reduction in SBP has been associated with a 5% reduction

326 in CHD mortality (38). Interestingly, only night ABP measurements were influenced by

327 the intervention. The large range of recorded daily activity levels (data not shown) may

328 have influenced the variability of 24 h and daytime ABP, masking any effects of the diets.

329 High circulating E-selectin concentrations are associated with endothelial

330 activation and atherosclerosis (39). In the current study, E-selectin was significantly

331 reduced when MUFA replaced SFA, similar to other findings (40). Since studies in

332 children have reported positive correlations between circulating E-selectin and blood

333 pressure (41), the reduction in E-selectin may have contributed to the observed decrease

334 in night SBP in the MUFA group. However, since the changes in E-selectin were not

335 paralleled by significant changes in other biomarkers of endothelial activation or

336 inflammation, further investigation is required to confirm this finding. Of note, intakes of

337 10%TE n-6 PUFA (the maximum recommended intake) (5) did not appear to increase

338   inflammation. High intakes of linoleic acid may increase the synthesis of pro-

339   inflammatory eicosanoids (42), although a systematic review reported no effect of linoleic

340   acid on various markers of inflammation (43).

341   Consistent with previous evidence (14, 15), dietary SFA had unfavourable effects

342   on the fasting serum cholesterol profile. Although there is evidence that the replacement

343   of SFA with MUFA beneficially affects the cholesterol profile, the evidence is more limited

344   than replacement with n-6 PUFA (4, 14, 15). Improvements in TC, LDL-C and TC:HDL-C

345   ratio were observed when SFA was replaced with either MUFA and n-6 PUFA. Paralleled

346   by changes in the fasting cholesterol profile, the increase in CVD risk score in the SFA

347   group was attenuated or reduced upon replacement with MUFA and n-6 PUFA,

348   respectively. This is in contrast to data from observational studies that suggest low

349   dietary intakes of SFA and high intakes of n-6 PUFA do not appear to reduce coronary

350   risk (1), although this analysis has been criticized for failing to account for the effects of

351   the macronutrient which substitutes SFA in the diet, and the presence of trans fatty acids

352   in the PUFA intervention arm of studies. Since CVD mortality is linked to increased LDL-

353   C (44), the changes in serum LDL-C observed from replacing SFA with MUFA (-11.3%)

354   and n-6 PUFA (-13.6%) are of public health relevance. Evidence supports a 1%

355   reduction in hard CHD events (myocardial infarction and CHD death) (45) and an

356   estimated 1.5% reduction in CVD risk (46) with every 1% decrease in serum LDL-C. This

357   equates to an estimated 11-14% and 17-20% reduction in CHD events and CVD,

358   respectively, strongly supporting the replacement of SFA with either MUFA or n-6 PUFA

359   to improve the fasting cholesterol profile in adults at moderate CVD risk. Our findings for

360   n-6 PUFA are also in line with a meta-analysis that concluded for every 5%TE increase

361   in linoleic acid intake, the risk of CHD events reduced by 9% (3), both of which support

362   current dietary recommendations.

363   Strengths of the DIVAS study were its large sample size (n = 195) and long

364 duration (16-wk) relative to other studies investigating dietary fatty acid intake on

365 vascular function (13), and effective dietary fat manipulation with minimal impact on other

366 dietary components and total energy intake. In addition, the n-6 PUFA intervention diet

367 was not confounded by an increase in n-3 PUFA. Although the SFA substitution was

368 achieved primarily by exchanging added fats and oils, hazelnut consumption (2.7%TE)

369 was necessary in both unsaturated diets to achieve the target intakes (16), which could

370 be considered a limitation. However, the beneficial effects of hazelnuts on vascular

371 function and the fasting lipid profile are reported for intakes far higher than those in the

372 DIVAS study (18-20%TE) (47). Also, intakes of trans fat and cholesterol were greater in

373 the SFA group, as previously discussed (16), but these remained below the maximum

374 UK and USA recommended intakes of 2%TE (48) and 300 mg/d (45), respectively.

375 Although their impact on outcome measures cannot be ruled out, detrimental effects on

376 CVD risk are reported at intakes greater than those consumed (49). A systematic review

377 and meta-analysis concluded there is no relationship between intake levels of ruminant

378 trans fats up to 4.19%TE and CVD risk factors, including plasma lipids (50).

379   This is the first suitably-powered, RCT investigating the long-term impact of

380 replacing dietary SFA with MUFA or n-6 PUFA on multiple novel and classical CVD risk

381 biomarkers in adults at moderate CVD risk. Although there were no significant

382 differences between diets on our primary endpoint %FMD or other measures of vascular

383 function, substituting SFA with MUFA or n-6 PUFA attenuated the unfavourable effects of

384 SFA on the serum cholesterol profile and improved CVD risk scores. Furthermore,

385 substitution with MUFA reduced night SBP and E-selectin. Therefore, replacing SFA with

386 unsaturated fats offers a potential public health strategy for reducing multiple significant

387 CVD risk biomarkers in those at moderate risk (≥50% above the population mean).

388

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Table 1 Baseline characteristics of participants at moderate risk of cardiovascular disease ($n = 195$)[1]

Characteristic	SFA diet	MUFA diet	n-6 PUFA diet	*P*
N	65	64	66	
Male gender (*n*)	29	27	29	0.960
Age (y)	45 ± 1	43 ± 1	45 ± 1	0.478
BMI (kg/m^2)	26.7 ± 0.5	26.3 ± 0.5	27.0 ± 0.5	0.534
Waist circumference (cm)	92.1 ± 1.6	88.2 ± 1.4	92.1 ± 1.7	0.128
24 h SBP (mm Hg)	121 ± 2	121 ± 1	124 ± 2	0.150
24 h DBP (mm Hg)	75 ± 1	74 ± 1	76 ± 1	0.373
TC (mmol/L)	5.38 ± 0.12	5.43 ± 0.13	5.57 ± 0.16	0.605
HDL-C (mmol/L)	1.45 ± 0.04	1.48 ± 0.05	1.51 ± 0.05	0.650
TC:HDL-C ratio	3.92 ± 0.15	3.85 ± 0.13	3.85 ± 0.14	0.923
LDL-C (mmol/L)	3.67 ± 0.12	3.71 ± 0.12	3.81 ± 0.14	0.731
Triacylglycerol (mmol/L)	1.31 ± 0.10	1.18 ± 0.07	1.26 ± 0.09	0.724
Fasting glucose (mmol/L)	5.09 ± 0.06	5.00 ± 0.06	5.05 ± 0.06	0.558

Family history of premature myocardial infarction or type 2 diabetes[2] [n (%)]	23 (35)	20 (31)	24 (36)	0.810
CVD risk score[3]	3.3 ± 0.2	3.0 ± 0.2	3.4 ± 0.2	0.336

Adapted with permission from Supplemental Table 1 in the Online Supporting Material from Weech *et al* J Nutr (2014; 144:846-55), American Society for Nutrition (16).

[1] Values are mean ± SEM unless stated otherwise. Between-group comparisons derived by ANOVA for continuous variables (and Kruskal-Wallis test for age) and Chi-squared test for discrete variables.

[2] Age of diagnosis was ≤55 y for father/brother and ≤65 y for mother/sister.

[3] A score of ≥2 points indicates a moderate CVD risk (≥50% above the population mean) (16).

CVD: cardiovascular disease; DBP: diastolic blood pressure; HDL-C: HDL-cholesterol; LDL-C: LDL-cholesterol; SBP: systolic blood pressure; TC: total cholesterol.

Table 2 Vascular outcomes and ambulatory blood pressure in participants at moderate risk of cardiovascular disease at baseline (wk 0) and post-intervention (wk 16)[1]

	SFA diet			MUFA diet			n-6 PUFA diet			P^2
P^2	Baseline	Post	Δ	Baseline	Post	Δ	Baseline	Post	Δ	
Endothelial function:										
%FMD	5.41 ± 0.35	5.03 ± 0.34	-0.39 ± 0.24	5.81 ± 0.38	5.74 ± 0.42	-0.07 ± 0.32	5.86 ± 0.39	5.78 ± 0.35	-0.08 ± 0.31	0.238
Pre-occlusion artery diameter (mm)	3.96 ± 0.10	3.98 ± 0.10	0.02 ± 0.04	3.75 ± 0.09	3.81 ± 0.09	0.06 ± 0.03	3.83 ± 0.09	3.84 ± 0.09	0.01 ± 0.03	0.550
LDI-Ach AUC (AU)	1509 ± 122	1285 ± 77	-223 ± 126	1604 ± 109	1554 ± 105	-50 ± 109	1461 ± 105	1440 ± 98	-21 ± 96	0.172
LDI-SNP AUC (AU)	1397 ± 87	1261 ± 74	-137 ± 119	1529 ± 105	1332 ± 92	-197 ± 118	1319 ± 77	1374 ± 80	56 ± 100	0.372
Reflection Index (%)	65.4 ± 1.5	64.0 ± 1.7	-1.4 ± 1.5	60.7 ± 1.9	64.1 ± 1.9	3.4 ± 1.6	63.3 ± 1.8	64.2 ± 1.8	1.0 ± 1.8	0.306
Arterial stiffness:										
Pulse wave velocity (m/s)	6.98 ± 0.15	7.04 ± 0.15	0.06 ± 0.11	6.63 ± 0.15	6.66 ± 0.16	0.03 ± 0.12	6.94 ± 0.15	6.91 ± 0.16	-0.03 ± 0.14	0.581
Augmentation index (%)	16.1 ± 1.5	17.5 ± 2.2	1.4 ± 1.4	13.0 ± 1.7	14.2 ± 1.7	1.2 ± 0.7	15.1 ± 1.5	15.6 ± 1.5	0.5 ± 0.6	0.775
Stiffness index (m/s)	6.84 ± 0.23	6.87 ± 0.23	0.03 ± 0.23	6.47 ± 0.21	6.89 ± 0.24	0.42 ± 0.21	7.13 ± 0.28	7.07 ± 0.26	-0.06 ± 0.26	0.450
Ambulatory blood pressure:										
24 h SBP (mm Hg)	120.7 ± 1.6	122.3 ± 1.7	1.6 ± 1.1	120.6 ± 1.3	119.6 ± 1.3	-1.0 ± 1.0	124.2 ± 1.6	123.8 ± 1.6	-0.4 ± 1.2	0.225
Day SBP (mm Hg)	124.7 ± 1.7	126.1 ± 1.8	1.5 ± 1.1	124.9 ± 1.3	124.0 ± 1.4	-1.0 ± 1.1	128.5 ± 1.7	128.0 ± 1.6	-0.6 ± 1.3	0.381

Night SBP (mm Hg)	105.6 ± 1.8	109.4 ± 1.8**	3.8 ± 1.4[a]	105.8 ± 1.4	104.7 ± 1.1	-1.1 ± 1.2[b]	109.5 ± 1.5	110.0 ± 1.7	0.5 ± 1.3[ab]	0.019
24 h DBP (mm Hg)	74.6 ± 1.1	76.2 ± 1.1	1.5 ± 0.7	73.6 ± 0.8	73.3 ± 0.8	-0.3 ± 0.7	75.6 ± 1.1	74.8 ± 1.1	-0.8 ± 0.8	0.074
Day DBP (mm Hg)	77.6 ± 1.1	79.0 ± 1.2	1.4 ± 0.8	77.2 ± 0.9	76.5 ± 0.9	-0.6 ± 0.9	78.9 ± 1.2	77.6 ± 1.2	-1.3 ± 0.9	0.140
Night DBP (mm Hg)	63.4 ± 1.2	65.9 ± 1.2	2.6 ± 1.0	61.9 ± 0.8	62.7 ± 0.8	0.8 ± 0.7	64.8 ± 1.0	65.1 ± 1.1	0.3 ± 0.9	0.114
24 h PP (mm Hg)	46.0 ± 0.8	46.1 ± 0.8	0.1 ± 0.9	46.9 ± 0.8	46.2 ± 0.9	-0.7 ± 0.7	48.5 ± 1.0	49.0 ± 1.0	0.5 ± 0.7	0.187
Day PP (mm Hg)	47.1 ± 0.9	47.1 ± 0.9	0.0 ± 1.0	47.8 ± 0.9	47.5 ± 1.0	-0.3 ± 0.7	49.6 ± 1.1	50.4 ± 1.1	0.8 ± 0.9	0.230
Night PP (mm Hg)	42.2 ± 0.8	43.4 ± 0.9	1.2 ± 1.0	43.9 ± 1.0	42.1 ± 0.7*	-1.9 ± 1.0	44.8 ± 1.1	44.9 ± 0.9	0.1 ± 0.9	0.048
24 h heart rate (bpm)	70.1 ± 1.1	71.6 ± 1.2	1.5 ± 0.8	71.4 ± 1.0	72.1 ± 1.0	0.7 ± 0.9	70.4 ± 1.2	70.2 ± 1.2	-0.2 ± 0.8	0.306
Day heart rate (bpm)	72.2 ± 1.1	74.2 ± 1.2	2.0 ± 0.9	74.3 ± 1.1	75.0 ± 1.1	0.7 ± 1.0	72.6 ± 1.3	73.0 ± 1.3	0.4 ± 1.0	0.462
Night heart rate (bpm)	62.5 ± 1.2	63.3 ± 1.2	0.8 ± 1.2	62.1 ± 1.0	62.2 ± 1.1	0.1 ± 0.9	63.4 ± 1.2	61.0 ± 1.3	-2.4 ± 1.0	0.051

[1] Values are mean ± SEM, n = 48-62 per diet group. For %FMD (primary outcome), n = 59, 57 and 55 for the SFA, MUFA and n-6 PUFA diets, respectively. No significant between-group differences were identified at baseline (one-way ANOVA or Kruskal-Wallis test for non-normally distributed data). %FMD and pre-occlusion artery diameter, LDI-Ach AUC, LDI-SNP AUC and stiffness index (secondary outcomes) were log transformed for statistical analysis.

[2] Analysis of primary and secondary endpoints: overall between group diet effects for each Δ derived from general linear models with baseline values for the variable of interest, BMI, age, gender and intervention diet as prognostic factors. Post-hoc analyses used Tukey's correction to adjust for multiple testing. Different superscript letters within a row ([a,b])

identify intervention groups significantly different from one another ($P ≤ 0.05$). Where the overall diet effect was

significant, one-sample t-tests determined whether Δ for each dietary arm was different to zero, which were identified as:

*$P ≤ 0.05$, **$P ≤ 0.01$, ***$P ≤ 0.001$.

Ach: acetylcholine; AU: arbitrary units; DBP: diastolic blood pressure; FMD: flow-mediated dilatation; LDI: laser Doppler

imaging; Post: after the intervention; PP: pulse pressure; SBP: systolic blood pressure; SNP: sodium nitroprusside; Δ:

change from baseline.

Table 3 Markers of endothelial activation, inflammation and insulin resistance in participants at moderate risk of cardiovascular disease at baseline (wk 0) and post-intervention (wk 16)[1]

	SFA diet			MUFA diet			n-6 PUFA diet			P^2
P^2	Baseline	Post	Δ	Baseline	Post	Δ	Baseline	Post	Δ	
Circulating biomarkers of endothelial activation and inflammation:										
C-reactive protein (mg/L)	2.68 ± 0.50	2.56 ± 0.46	-0.12 ± 0.50	1.91 ± 0.36	1.87 ± 0.36	-0.04 ± 0.21	2.37 ± 0.42	2.49 ± 0.41	0.12 ± 0.36	0.792
NOx (μmol/L)	29.3 ± 2.6	29.4 ± 2.8	0.1 ± 2.2	25.4 ± 1.8	24.1 ± 1.7	-1.3 ± 1.5	27.4 ± 2.0	25.5 ± 1.8	-1.9 ± 1.3	0.799
VCAM-1 (ng/mL)	666 ± 18	644 ± 17	-22 ± 11	675 ± 25	683 ± 18	8 ± 16	664 ± 21	677 ± 24	13 ± 11	0.077
ICAM-1 (ng/mL)	220 ± 6	222 ± 6	2.2 ± 3.2	215 ± 5	219 ± 5	4.3 ± 3.2	220 ± 7	223 ± 7	3.1 ± 4.2	0.887
IL-6 (pg/mL)	1.85 ± 0.16	1.93 ± 0.22	0.08 ± 0.16	1.19 ± 0.09	1.27 ± 0.12	0.08 ± 0.10	1.69 ± 0.15	1.88 ± 0.19	0.18 ± 0.16	0.533
TNF-α (pg/mL)	1.33 ± 0.11	1.31 ± 0.10	-0.02 ± 0.04	1.03 ± 0.07	1.01 ± 0.05	-0.03 ± 0.03	1.06 ± 0.04	1.07 ± 0.05	0.01 ± 0.02	0.514
E-selectin (ng/mL)	34.7 ± 1.8	35.9 ± 2.1	1.3 ± 1.0[a]	34.7 ± 1.9	32.2 ± 1.6**	-2.4 ± 0.9[b]	35.9 ± 1.8	35.1 ± 1.9	-0.9 ± 0.7[ab]	0.012
P-selectin (ng/mL)	43.2 ± 1.6	44.0 ± 2.0	0.8 ± 1.1	42.3 ± 1.9	41.0 ± 1.7	-1.3 ± 0.9	39.9 ± 1.6	38.0 ± 1.8	-1.9 ± 0.9	0.091
vWF (μU/mL)	953 ± 54	916 ± 56	-36 ± 59	849 ± 44	893 ± 46	43 ± 54	804 ± 42	896 ± 56	92 ± 55	0.796
Microalbumin (mg/24 h)	4.50 ± 1.14	4.27 ± 0.79	-0.23 ± 0.84	2.74 ± 0.35	3.49 ± 0.62	0.75 ± 0.69	5.07 ± 1.04	6.14 ± 1.42	1.06 ± 0.86	0.976
Indices of insulin resistance:										

Glucose (mmol/L)	5.09 ± 0.06	5.15 ± 0.06	0.06 ± 0.04	5.00 ± 0.06	5.06 ± 0.06	0.06 ± 0.03	5.05 ± 0.06	5.08 ± 0.05	0.04 ± 0.05	0.784
Insulin (pmol/L)	30.9 ± 2.2	32.9 ± 2.4	2.0 ± 1.9	29.1 ± 1.9	29.8 ± 2.2	0.7 ± 1.4	30.2 ± 2.5	32.7 ± 2.6	2.4 ± 1.4	0.434
NEFA (µmol/L)	508 ± 17	485 ± 21	-23 ± 23	463 ± 23	457 ± 21	-6 ± 22	474 ± 25	480 ± 23	6 ± 17	0.862
HOMA-IR	1.19 ± 0.09	1.29 ± 0.11	0.10 ± 0.08	1.05 ± 0.07	1.10 ± 0.09	0.05 ± 0.06	1.13 ± 0.11	1.24 ± 0.11	0.10 ± 0.06	0.587
QUICKI	0.39 ± 0.01	0.39 ± 0.01	0.00 ± 0.00	0.39 ± 0.00	0.39 ± 0.01	0.00 ± 0.00	0.39 ± 0.00	0.39 ± 0.01	-0.01 ± 0.00	0.376
rQUICKI	0.45 ± 0.01	0.45 ± 0.01	0.00 ± 0.01	0.46 ± 0.01	0.46 ± 0.01	0.00 ± 0.01	0.46 ± 0.01	0.45 ± 0.01	-0.01 ± 0.01	0.345

[1] Values are mean ± SEM, n = 56-66 per diet group. No significant between-group differences were identified at baseline (one-way ANOVA or Kruskal-Wallis test for non-normally distributed data), except for IL-6 (P = 0.001) and TNF-α (P = 0.026) between the SFA and MUFA groups. C-reactive protein, NOx, IL-6, microalbumin, insulin and rQUICKI (secondary endpoints) were log transformed for statistical analysis.

[2] Analysis of secondary endpoints: overall between group diet effects for each Δ derived from general linear models with baseline values for the variable of interest, BMI, age, gender and intervention diet as prognostic factors. Post-hoc analyses used Tukey's correction to adjust for multiple testing. Different superscript letters within a row ([a,b]) identify intervention groups significantly different from one another ($P \leq 0.05$). Where the overall diet effect was significant, one-sample t-tests determined whether Δ for each dietary arm was different to zero, which were identified as: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

ICAM-1: intercellular cell adhesion molecule-1; NEFA: non-esterified fatty acids; NOx: total nitrites and nitrates; Post: after the intervention; QUICKI: quantitative insulin sensitivity index; rQUICKI: revised quantitative insulin sensitivity index; VCAM-1: vascular cell adhesion molecule-1; vWf = von Willebrand factor; Δ: change from baseline.

Figure 1 Flow of recruitment

Figure 2 Changes from baseline fasting lipid profile when dietary SFA was substituted isoenergetically with MUFA (9.5%TE) or n-6 PUFA (9.6%TE) for 16 wk.

Data shown as mean ± SEM, n = 58-62 per diet group. Overall diet effects, derived by general linear model using the change from baseline as the dependent variable with baseline values of the variable of interest, BMI, age, gender and intervention diet as prognostic variables, were significant for TC, LDL-C and TC:HDL-C ratio ($P ≤ 0.001$). Post-hoc analysis, using Tukey's correction to adjust for multiple testing, identified significant between-group differences (*$P ≤ 0.05$, **$P ≤ 0.01$, ***$P ≤ 0.001$). HDL-C: HDL-cholesterol; LDL-C: LDL-cholesterol; TAG: triacylglycerol; TC: total cholesterol; %TE: percentage of total energy.