Effects of a Mediterranean-inspired diet on blood lipids, vascular function and oxidative stress in healthy subjects

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ABSTRACT

Mediterranean-inspired diets have been shown to decrease cholesterol levels in patients with hypercholesterolaemia, who frequently exhibit endothelial dysfunction. The aims of the present study are to improve endothelial function by dietary intervention in healthy subjects with lipid levels representative of a Western population. Twenty-two healthy subjects (mean total cholesterol, 5.6 mmol/l) were given a Mediterranean-inspired diet rich in ω-3 fatty acids and sterol esters, but low in saturated fat, or an ordinary Swedish diet, for 4 weeks in a randomized cross-over study. The composition of the diets were: in the Swedish diet, 2090 kcal (where 1 kcal = 4.184 kJ; 48 % of energy from carbohydrate, 15 % from protein and 36 % from fat) and 19 g of fibre; in the Mediterranean-inspired diet, 1869 kcal (48 % of energy from carbohydrate, 16 % from protein, 34 % from fat) and 40 g of fibre. After each dietary period, fasting blood lipids, insulin and glucose levels, as well as apo B (apolipoprotein B) and LDL (low-density lipoprotein) particle size, were analysed. Endothelial-dependent and -independent vasodilation was measured invasively by venous occlusion plethysmography, and arterial distensibility was assessed by echocardiography tracking. Fibrinolytic capacity across the forearm, as well as oxidative stress measured through urinary F2-isoprostane, were evaluated. Total, LDL- and apo B-cholesterol and triacylglycerol (triglyceride) concentrations were decreased by 17 %, 22 %, 16 % and 17 % respectively, after the Mediterranean-inspired diet compared with the Swedish diet (P < 0.05 for all). However, no differences in plasma concentrations of insulin and glucose and LDL particle size, endothelial function, arterial distensibility, fibrinolytic capacity or oxidative stress were detected. Treatment for 4 weeks with a Mediterranean-inspired diet decreased blood lipids in healthy individuals with a low-risk profile for cardiovascular disease. This beneficial effect was not mirrored in vascular function or oxidative stress evaluation.

INTRODUCTION

Atherosclerosis remains the leading cause of morbidity and mortality in developed countries. Risk factors for CVD (cardiovascular disease) include elevated LDL (low-density lipoprotein)-cholesterol, low HDL (high-density lipoprotein)-cholesterol, a predominance of small dense LDL particles and high insulin levels, as well as

Key words: diet, endothelial function, fibrinolysis, cholesterol, oxidative stress.

Abbreviations: ACH, acetylcholine; apo B, apolipoprotein B; BMI, body mass index; CVD, cardiovascular disease; \(E_p\), elastic modulus; HDL, high-density lipoprotein; PAI, plasminogen activator inhibitor; LDL, low-density lipoprotein; PGF\(_{2\alpha}\), prostaglandin F\(_{2\alpha}\); SNP, sodium nitroprusside; t-PA, tissue plasminogen activator.

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elevated CRP (C-reactive protein) levels [1]. Moreover, endothelial dysfunction is recognized to be a key event in the initiation and progression of early atherosclerosis [2]. Impairment in bioavailability of locally produced substances such as NO (nitric oxide) and t-PA (tissue plasminogen activator) results in increased adhesion of leucocytes, vasoconstriction, vascular permeability, oxidative stress and production of growth factors, as well as a procoagulant state [3].

Vascular endothelial function may potentially be improved by lifestyle changes, such as a health-promoting change of diet. Dietary intervention trials, aimed at decreasing serum cholesterol, show a favourable lowering of CVD morbidity and mortality rates in hypercholesterolaemic men [4]. Several studies have also documented beneficial effects of low-fat/high-fibre diets on LDL-cholesterol in healthy subjects with normal or moderately elevated cholesterol levels [5]. A Mediterranean diet rich in olive oil, vegetables and fresh fruits has been shown to decrease insulin resistance and improve endothelium-dependent vasodilation in diabetic patients [6]. Intake of ω-3 fatty acids, found in oily fish, at least once a week has been shown to have a protecting effect on sudden death and stroke in both healthy subjects and patients with CVD [7], whereas consumption of a meal high in mono-unsaturated fat was associated with acute impairment of endothelial function when compared with a carbohydrate-rich meal in healthy subjects [8].

The primary aim of the present study was to investigate whether a Mediterranean-inspired diet could beneficially affect vascular function. Secondary aims were to assess the effects on traditional, as well as novel, CVD risk factors. Our specific questions were: does a 4-week dietary intervention rich in fruit, vegetables, fish, plant sterols and slow-release carbohydrates, without a decrease in total fat intake (Mediterranean-inspired diet) in healthy subjects (i) improve endothelium-dependent vasodilation in the forearm; (ii) increase the stimulated local net release of t-PA; (iii) improve $E_p$ (elastic modulus) of the carotid artery; (iv) decrease serum cholesterol and triacylglycerol (triglyceride) levels without changes in LDL particle size; (v) decrease the plasma insulin and glucose concentrations and; (vi) decrease the oxidative stress?

### MATERIALS AND METHODS

**Subjects**

Healthy subjects ($n = 22$; 12 men and 10 women) with total cholesterol and LDL-cholesterol levels between 5–7 mmol/l and 3–4 mmol/l respectively, were recruited into the study (Table 1). These levels were within the limits representing 95% of the Swedish population [9]. All subjects were healthy non-smokers and were not taking any medication. The age ranged from 30–51 years and the mean BMI (body mass index) was 26 kg/m².

<p>| Table 1  | Inclusion characteristics of the subjects |</p>
<table>
<thead>
<tr>
<th>Values are means ± S.E.M.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>43 ± 1</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>12/10</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 ± 0.6</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/l)</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>5.2 ± 0.1</td>
</tr>
</tbody>
</table>

**Study design**

Subjects received an ordinary Swedish diet for 4 weeks and a Mediterranean-inspired diet for 4 weeks in a randomized cross-over design with 4 weeks of washout in between (Figure 1). Blood samples were taken before starting each diet and after the diet period of 4 weeks. In 1997, the Swedish National Food Administration evaluated the eating habits of the Swedish population [10]. Based on the results from this report, a diet was designed to be representative of an ordinary Swedish diet. Before entry, the subjects were subjected to a 7-day food diary. The baseline diet was similar to that seen in the Swedish population. Subjects received 60 % of the daily caloric requirement each week from the study dietician. This included one cooked meal/day with a rotational 7-day menu. All meals were prepared in the research facilities. Food products without an exact composition were analysed to make this clear. Foods containing ω-3 fatty acids were all scrutinized. The composition of the Mediterranean-inspired diet, in contrast with the Swedish diet, contained twice the amount of fibre, 4–9 times more antioxidants, three times the amount of polyunsaturated and ω-3 fatty acids, less than half the amount of saturated fat, almost three times less cholesterol and a 35 % decrease in the glycaemic index. In addition, sterol esters (2 g/day)
were given as an ingredient in margarine. The daily intake of calories, protein, carbohydrate and total amount of fat was intended to be similar in the two diets. Subjects came to the laboratory for individual food consultation, to pick up the food and to assess their weight. Left-over food, if any, was collected and the amount estimated. We attempted to keep the weight of the subjects unchanged. Likewise, the subjects were asked to maintain their physical activity at a similar level during both diet periods (maximum of two physical exercise periods/week). The amount of alcohol was also aimed to be constant in the two diet periods; however, consumption of beer was preferable during the Swedish diet and wine during the Mediterranean-inspired diet. The amount of alcohol intake was restricted to 10 g of alcohol/day and to be consumed evenly throughout the week. Low-fat products were chosen by the subjects themselves for the remaining daily energy intake (40 %). Compliance, as well as the self-selected foods, was assessed by use of three unannounced telephone interviews (24-h recalls) per period. Three 24-h recalls were also performed during the wash-out period. Nutritional composition was calculated by a software package (Dietist®; Aivo, Bromma, Sweden), based on data from the Swedish National Food Administration.

Forearm vascular function, fibrinolytic capacity and elasticity of the carotid artery were measured at the end of each diet period. Blood samples of fasting lipids, insulin and glucose were taken before start as well as after each 4-week diet period. Apo B (apolipoprotein B), LDL particle size and concentrations of F2-isoprostane, as a biomarker of oxidative stress, in morning urine were measured after each 4-week diet period. Blood samples of fasting lipids, insulin and glucose were taken before start as well as after each 4-week diet period. Apo B (apolipoprotein B), LDL particle size and concentrations of F2-isoprostane, as a biomarker of oxidative stress, in morning urine were measured after each 4-week diet period. Apo B (apolipoprotein B), LDL particle size and concentrations of F2-isoprostane, as a biomarker of oxidative stress, in morning urine were measured after each 4-week diet period. Blood and urinary samples, as well as forearm blood flow analyses, were blinded.

**Forearm blood flow study**

Subjects entered the laboratory in the morning after a 12 h fast and were placed in a temperature-controlled room at 22 °C. Throughout the study the subjects rested in a supine position with the arms at the level of the heart. The brachial artery of the non-dominant arm was cannulated with a 20-gauge cannula under local anaesthesia (2 % xylocain; Astra Pharmaceuticals Ltd, Göteborg, Sweden), and an 18-gauge venous cannula was inserted into a subcutaneous vein in the same arm. Acetylcholine (ACH; Miochol, Roskilde, Denmark) was infused at doses of 10, 30 and 60 µg/min (55, 165 and 330 nmol/min; 5 min for each dose) and, after 20 min of saline infusion, sodium nitroprusside (SNP; Abbott Scandinavia AB, Kista, Stockholm) was infused at doses of 0.5, 2.5 and 10 µg/min (1.7, 8.4 and 33.6 nmol/min; 5 min for each dose). Drugs were dissolved in normal saline (0.9 % NaCl; Braun Medical Ltd, Bromma, Sweden) and infused intra-arterially. The total rate of intra-arterial infusions was maintained constant throughout the study at 100 ml/h. All solutions were prepared aseptically from sterile stock solutions on the day of the study.

Before administration of drugs, saline was infused for 30 min, followed by baseline measurement. Forearm blood flow was measured in both arms simultaneously by venous occlusion plethysmography using a dual-channel strain gauge plethysmograph (Elektromedicin AB, Kullavik, Sweden), and calibration was made before each measurement. To exclude the circulation of the hands, wrist cuffs were inflated to 200 mmHg 1 min prior to and during each measurement. Upper arm cuffs were intermittently inflated to 400 mmHg to prevent venous outflow from the forearms and thus obtain plethysmographic recordings.

Blood pressure was recorded intra-arterially in the infused arm immediately after each forearm blood flow measurement. Plethysmographic data were recorded, stored on a computer and analysed using software developed in our Department. For each time point, an average of at least three inflations was used for each arm. All blood flows were analysed by a single investigator with no knowledge of the individuals’ identity or diet period. The ratio of blood flow in the non-infused and infused arm was calculated and expressed as the percentage change from baseline.

**Blood and urine samples**

At baseline and after the highest dose of ACH and SNP, blood samples for t-PA activity were collected simultaneously from the brachial artery and vein into acidified buffered citrate tubes (Biopool Stabilyte, Umeå, Sweden) and kept on ice until centrifuged at 2000 g for 20 min at 4 °C. Plasma was separated and stored at −80 °C until analysed.

Venous blood was collected into citrate tubes for the determination of PAI-1 (plasminogen activator inhibitor-1) antigen (Vacutainer Systems, Plymouth, Devon, U.K.) at baseline only.

Plasma t-PA activity (Chromolize™ t-PA; Biopool, Umeå, Sweden) and PAI-1 antigen concentrations (TintElize PAI-1; Biopool) were analysed using an ELISA.

Fasting total cholesterol, HDL-cholesterol and triacylglycerols were analysed using enzymic methods (Roche Diagnostics, Mannheim, Germany). LDL-cholesterol was calculated using the Friedewald equation. Apo-B was analysed by an immunoturbidimetry method (Orion Diagnostica, Espoo, Finland), and LDL peak particle diameters were determined using gradient-gel electrophoresis as described previously [11]. Fasting serum insulin was analysed by a radioimmunochemical method (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden), and fasting blood glucose was determined by an enzymic method (YSI 2700 turntable 2710, hexokinase). Concentrations of free 8-iso-PGF2α (prostaglandin F2α, a major F2-isoprostane) in morning urine were analysed by
a valid RIA as described by Basu [12]. The levels of urinary 8-iso-PGF₂α were adjusted for creatinine values.

**Net release of t-PA**

Net release or uptake of t-PA activity was calculated according to the formula:

\[ \text{Net release of t-PA} = (C_v - C_a) \times \text{FPF} \]

where \(C_v\) and \(C_a\) denote venous and arterial plasma concentrations of t-PA respectively. FPF is the forearm plasma flow, which was estimated from forearm blood flow (FBF) and haematocrit and corrected for 1% trapped plasma, according to the formula:

\[ \text{FPF} = \frac{\text{FBF} \times (101 - \text{haematocrit})}{100}. \]

**Carotid artery elasticity**

Arterial elasticity was assessed by echo-tracking as described previously by Hansen et al [13]. Briefly, pulsatile diameter changes in the common carotid artery were measured non-invasively using an electronic echo-tracker device (Diamove; Teltec AB, Lund, Sweden).

This instrument is interfaced with a real-time ultrasound scanner (EUV 240; Hitachi Tokyo, Japan) and fitted with a 5 MHz linear array transducer. Examinations were performed with subjects in a supine position. The carotid artery was visualized both in longitudinal and transversal section on the real-time image. The echo-tracker device measures the distance between the posterior and anterior vessel walls. A data-acquisition system containing a PC (Dell Dimension U400c) was used for monitoring the vessel diameter. Arterial blood pressure was measured intra-arterially immediately after each measurement of the pulsatile diameter. The arterial strain, or fractional diameter change, was defined as

\[ \text{Strain} = \frac{(D_{\text{Systolic}} - D_{\text{Diastolic}})}{D_{\text{Diastolic}}} \]

Pressure strain \(E_p\) was defined as

\[ E_p = 133.3 \times \frac{(P_{\text{Systolic}} - P_{\text{Diastolic}})}{(D_{\text{Systolic}} - D_{\text{Diastolic}})/D_{\text{Diastolic}}} \]

where \(P_{\text{Systolic}}\) and \(P_{\text{Diastolic}}\) are systolic and diastolic blood pressure levels in mmHg respectively, and \(D_{\text{Systolic}}\) and \(D_{\text{Diastolic}}\) are diameters in mm. The unit of \(E_p\) is in N/m².

The constant accounts for the conversion from mmHg into N/m². \(E_p\) of the carotid artery was then calculated from at least three consecutive beats. A minimum of four measurements were made and the average of both longitudinal and transversal images was calculated for each subject.

**Statistical analysis**

Results are expressed as means ± S.E.M. Student’s \(t\) test for paired observations was used for normally distributed variables. When the variables showed a non-normal distribution, Wilcoxon signed-rank test for paired comparisons was used. Two-way ANOVA was used for the forearm blood flow data. Statistical significance was defined as a two-sided \(P < 0.05\).

**RESULTS**

Although we aimed to keep the total energy content constant during the intervention diets, a minor difference in the contents was observed during the 24-h recalls (Table 2). This was also reflected in a small, but statistically significant, decrease in body weight (0.7 ± 1.1 kg; \(P < 0.05\)).

Total cholesterol, LDL-cholesterol and apo B levels were decrease by 17 %, 22 % and 16 % respectively, after 4 weeks of the Mediterranean-inspired diet compared with 4 weeks of the Swedish diet \((P < 0.05\) for all; Table 3). There was no difference in LDL particle size after the two study diets. Triacylglycerol concentrations were decreased by 17 % during the Mediterranean-inspired diet compared with the Swedish diet \((P < 0.05; \text{Table 3})\).

Neither fasting insulin nor glucose levels differed between the two study regimens \((P > 0.05; \text{Table 3})\). Blood lipid levels before the start of each diet period did not differ, thus no carry-over effects were observed.

The increase in forearm blood flow with the highest dose of ACH [Swedish diet, 559 % (419–699 %); Mediterranean-inspired diet, 481 % (288–674 %); values are means (95 % confidence interval)] and the highest dose of SNP (Swedish diet, 412 % (349–475 %); and Mediterranean-inspired diet, 479 % (380–578 %); values are means (95 % confidence interval)] did not differ between the two diets \((P > 0.05\) for all; Table 3).
Table 3  Biochemical parameters measured after four weeks of either a Swedish or Mediterranean-inspired diet

<table>
<thead>
<tr>
<th></th>
<th>Swedish diet (n = 22)</th>
<th>Mediterranean-inspired diet (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerols (mmol/l)</td>
<td>1.2 ± 0.1</td>
<td>1.0 ± 0.1*</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.3 ± 0.1</td>
<td>4.4 ± 0.1*</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.5 ± 0.1</td>
<td>2.7 ± 0.1*</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Apo-B (mg/dl)</td>
<td>103 ± 2</td>
<td>86 ± 3*</td>
</tr>
<tr>
<td>LDL size (nm)</td>
<td>26.5 ± 0.1</td>
<td>26.5 ± 0.1</td>
</tr>
<tr>
<td>Fasting insulin (milli-units/l)</td>
<td>6.5 ± 0.8</td>
<td>6.2 ± 0.6</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.8 ± 0.1</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>8-Iso-PGF2α (nmol/mmol of creatinine)</td>
<td>0.26 ± 0.03</td>
<td>0.25 ± 0.02</td>
</tr>
</tbody>
</table>

Figure 2  Forearm blood flow response to ACH and SNP infusion

Values are means ± S.E.M. O, Swedish diet; ●, Mediterranean-inspired diet. Two-way ANOVA was used for statistical analyses. ns, not significant.

There was no difference in $E_p$ measured in the carotid artery when comparing measurements after the two diets. The urinary concentrations of F2-isoprostane showed similar levels after the two study diets.

**DISCUSSION**

Although the present Mediterranean-inspired dietary intervention was effective in decreasing triglycerols, total cholesterol and LDL-cholesterol in healthy low-risk subjects, we did not observe any beneficial effects on endothelial function, endothelium-mediated vasodilation and endothelium-derived net release of t-PA across the forearm between the two diets. Moreover, the plasma concentrations of insulin and glucose, oxidative stress and arterial distensibility were not affected by the diet intervention.

There are several factors that may explain the lack of effect on vascular function by the Mediterranean-inspired diet, in spite of the beneficial influence on lipid profile. Firstly, one may question whether the present study had enough power to detect any difference between the two study diets. The study was designed to detect a 20 % increase in forearm blood flow after ACH with a type I error of 20 %. However, the blood flow measurements in the present study showed higher variability compared with what has been reported previously, implying that the present study had only 80 % power to detect a 40 % improvement in endothelial function.
after the Mediterranean-inspired diet. The magnitude of this effect is in agreement with previous reports in subjects with hypercholesterolaemia following 4 weeks of simvastatin treatment [15]. Thus, although a large effect of the Mediterranean-inspired diet on endothelial function seems unlikely, we cannot exclude minor effects. Secondly, the duration of the intervention may have been too short in order to affect vascular function. Thirdly, the studies that have demonstrated positive effects on vascular function after either drug treatment or dietary intervention have been performed in individuals at high risk for, or with established, CVD [16,17]. In contrast, the present study assessed vascular function in healthy subjects with a low-risk profile. Hence, some pathological conditions, such as hypertension, hypercholesterolaemia, diabetes or smoking, may have to prevail in order to show a positive effect on vascular function induced by a dietary change. Furthermore, our subjects had a normal diet before entering into the study.

The magnitude of the decrease in cholesterol achieved during the Mediterranean-inspired diet intervention was impressive considering that it was achieved in subjects with normal cholesterol values at baseline, and that this decrease was observed after only 4 weeks. These results confirm findings by others, although most previous studies have been performed in subjects with elevated cholesterol levels [18]. It is, however, difficult to compare the present results with other studies, since there is no clear definition of a Mediterranean diet. The common ingredients in a Mediterranean diet are a high content of fresh fruits, vegetables, whole grain cereals and fish, together with decreased amounts of saturated fat. In the present study, sterol esters were used in addition to the Mediterranean diet as an ingredient in the margarine. Sterol esters have been shown to decrease LDL-cholesterol by 10–15 % in subjects with normal cholesterol values [19,20] and, hence, may explain approx. 10 % of the LDL-cholesterol decrease achieved in the present study. Furthermore, decreased saturated fat, high soluble fibre and low dietary cholesterol may have explained 3–5 % each of the total effect [21]. Apart from the LDL-cholesterol concentration, there are several studies indicating that the small dense LDL particles are atherogenic [22]. Individuals with small dense LDL particles have a higher risk for coronary artery disease than subjects with large buoyant LDL particles [23]. Vakkilainen and co-workers [11] found impaired ACH-induced forearm blood flow increases in healthy subjects with small LDL particles compared with subjects with large LDL particles, albeit the subjects had similar concentrations of both total cholesterol and LDL-cholesterol. A low-fat diet could decrease the particle size in susceptible individuals and change the cardiovascular risk unfavourably [24]. However, there are convincing data indicating that a decrease in triacylglycerols could affect the size of the LDL lipoprotein particles by causing a shift towards a larger size [25]. In the present study, with similar fat content in both study arms, LDL particle size remained unaffected during the Mediterranean-inspired diet. This is in agreement with the effects observed when ω-3 fatty acids are supplemented as a lipid-lowering agent in diabetic subjects [26]. Taken together, the present data are positive with regard to the effect on lipid profile and underline the importance of considering an initial diet change to lower LDL-cholesterol before medication is prescribed to healthy individuals. Recent large-scale studies suggest that most patients with established atherosclerotic vascular disease would benefit from statin therapy regardless of their blood cholesterol values [27]. However, convincing data emphasize the importance of a well-balanced diet in addition to drug therapy. In a recent study [28], which partly supports our results, a modified Mediterranean-type diet rich in ω-3 fatty acids efficiently potentiate the cholesterol-lowering effect of simvastatin.

The fibrinolytic factor t-PA and its inhibitor, PAI-1, are important risk factors for atherothrombosis [29]. Both t-PA and PAI-1 are synthesized in, and released from, endothelial cells. The endothelium has a large potential to increase the release of t-PA in response to different stimuli, such as coagulation factors, in order to prevent thrombosis [30]. In smokers and patients with hypertension or chronic renal failure, the capacity for stimulated t-PA release from vascular endothelium is impaired [31,32]. We could not detect any improvement in the magnitude of ACH-induced net release of t-PA across the forearm at the end of the Mediterranean-inspired diet, probably due to an already physiologically intact and responsive vascular function from the start.

Oxidative free radicals have been implicated in the pathogenesis of endothelial dysfunction and atherosclerosis in humans. Although the Mediterranean-inspired diet used in the present study consisted of 4–9 times more antioxidants compared with the Swedish diet, we did not observe any differences in urinary F$_2$-isoprostane concentrations between the two study diets, possibly due to the treatment period being too short, the subjects having a low level of oxidative stress, or an inability to detect a small or moderate treatment effect on oxidative stress.

In summary, in healthy subjects with normal blood lipids, 4 weeks of a Mediterranean-inspired diet, together with margarine containing sterol ester, decreased both triacylglycerols and LDL-cholesterol without affecting the size of LDL particles compared with a typical Swedish diet. Neither vascular function nor biomarkers of oxidative stress differed between the two diet regimens, most probably due to the fact that these subjects demonstrated a normal vascular function prior to the study or that effects are more likely to occur during an extended intervention period.
ACKNOWLEDGMENTS

We are grateful to Malin Karlsson, Gun Bodehed-Berg and Eva Sejby for excellent assistance. We thank Unilever Bestfoods, Risenta, Ridderheims and Zeta for sponsoring the food products. This study was supported by Sahlgrenska University Hospital funds, the Swedish Heart and Lung Foundation, Helsinki University Central Hospital Research Foundation, Emelle Foundation and Geriatrics Research Foundation.

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