

### HUMAN AND CLINICAL NUTRITION

## Substitution of TAG oil with diacylglycerol oil in food items improves the predicted 10 years cardiovascular risk score in healthy, overweight subjects

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#### Abstract

Dietary fat is normally in TAG form, but diacylglycerol (DAG) is a natural component of edible oils. Studies have shown that consumption of DAG results in metabolic characteristics that are distinct from those of TAG, which may be beneficial in preventing and managing obesity. The objective of the present study was to investigate if food items in which part of the TAG oil is replaced with DAG oil combined with high  $\alpha$ -linolenic acid (ALA) content would influence metabolic markers. A 12-week double-blinded randomised controlled parallel-design study was conducted. The participants ( $n$  23) were healthy, overweight men and women, aged 37–67 years, BMI 27–35 kg/m<sup>2</sup>, with waist circumference >94 cm (men) and >88 cm (women). The two groups received 20 g margarine, 11 g mayonnaise and 12 g oil per d, containing either high ALA and *sn*-1,3-DAG or high ALA and TAG. Substitution of TAG oil with DAG oil in food items for 12 weeks led to an improvement of the predicted 10 years cardiovascular risk score in overweight subjects by non-significantly improving markers of health such as total body fat percentage, trunk fat mass, alanine aminotransferase, systolic blood pressure,  $\gamma$ -glutamyl transferase, alkaline phosphatase and total fat-free mass. This may suggest that replacing TAG oil with DAG oil in healthy, overweight individuals may have beneficial metabolic effects.

**Key words:** Diacylglycerol: TAG: Overweight human subjects: Liver markers

Prevention and treatment of obesity and obesity-related diseases are major public health challenges<sup>(1–3)</sup> and both the total amount of fat and the distribution of fat in the body are important determinants of the complications associated with obesity<sup>(4)</sup>. Surplus energy intake is stored as TAG in

adipose tissue either as subcutaneous or visceral fat. Visceral fat depots, located in the trunk surrounding the organs, are recognised to be a more active endocrine organ, and contribute to an increased risk of metabolic complications, than subcutaneous fat depots<sup>(4–7)</sup>. Hence, the endocrine activity of

**Abbreviations:** ALA,  $\alpha$ -linolenic acid; ALAT, alanine aminotransferase; ALP, alkaline phosphatase; ASAT, aspartame aminotransferase; CRP, C-reactive protein; DAG, diacylglycerol; FFM, fat-free mass;  $\gamma$ -GT,  $\gamma$ -glutamyl transferase; HOMA-IR, homeostatic model assessment for insulin resistance; hsCRP, high-sensitivity CRP.

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visceral adipose tissue is an important factor in the development of the metabolic syndrome<sup>(8)</sup>, and central obesity is more strongly associated with increased risk of insulin resistance, the metabolic syndrome and CVD than BMI alone<sup>(9)</sup>. The endocrine activity of adipose tissue includes secretion of a number of adipokines (cytokines and hormones) leading to a pro-inflammatory state, and it has been shown that the reduction of visceral fat effectively prevents cardiovascular events<sup>(10)</sup>. Also, hepatic accumulation of fat is common in obese individuals and is associated with hepatic dysfunction<sup>(11)</sup>. Increased adipose tissue, and in particular visceral adipose tissue, is linked to increased levels of circulating liver function markers, such as alanine aminotransferase (ALAT) and  $\gamma$ -glutamyl transferase ( $\gamma$ -GT)<sup>(12–16)</sup>.

Dietary fat is normally in TAG form, but diacylglycerol (DAG) is a natural component of various edible oils<sup>(17)</sup> and is currently used in small quantities in foods as an emulsifier. Recent studies have shown that high consumption of DAG results in metabolic characteristics that are distinct from those of TAG, and that these characteristics may be beneficial in preventing and managing obesity. Experimental studies in human subjects have shown that DAG (mainly *sn*-1,3-DAG) decreases postprandial TAG compared with TAG controls<sup>(18,19)</sup>. Kamphuis *et al.* showed that meals rich in DAG increase fat oxidation<sup>(20)</sup>. Dietary DAG has also been shown to reduce body fat (total, subcutaneous, visceral and hepatic fat mass) compared with TAG<sup>(21)</sup>, and consumption of DAG oil as part of a reduced-energy diet enhances loss of body weight and fat compared with TAG control oil<sup>(22)</sup>.

Vegetable oils such as flaxseed oil and rapeseed oils are the main dietary sources of the vegetable *n*-3 fatty acid  $\alpha$ -linolenic acid (ALA; 18 : 3). The relation between intake of ALA and CVD has been studied less extensively than the marine *n*-3 fatty acids and the relation is less clear. Some studies, including clinical trials, epidemiological investigations and experimental studies, have shown that intake of ALA has been beneficially associated with CVD risk<sup>(23)</sup> or markers of risk<sup>(24)</sup>. Other studies have found no effect of ALA on CVD risk markers<sup>(25–28)</sup>.

The aim of the present study was to investigate whether intake of food items in which part of the TAG-based oil has been replaced with DAG combined with high ALA content would influence metabolic markers such as C-reactive protein (CRP), blood lipids, body composition, liver function markers and overall cardiovascular risk score in healthy, overweight individuals.

## Experimental methods

### Subjects

Healthy, overweight men and women who were willing to consume margarine, mayonnaise and oil daily for 14 weeks were recruited via advertisements in local newspapers from October 2008 to February 2009. The inclusion criteria were: age 18–70 years, BMI 27–40 kg/m<sup>2</sup> and waist circumference >94 cm for men and >88 cm for women. Exclusion criteria were: type 2 diabetes mellitus, kidney-, liver-, gall bladder-, coronary-, endocrine- or chronic rheumatic disease, malign

cancer during the last 5 years, hypertension ( $\geq 160/100$  mmHg), pregnancy and lactation. Regular use of anti-inflammatory, lipid lowering and anti-hypertensive medications was not permitted. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Regional Committee of Medical Ethics (approval no. 6.2008.1368) and by the Norwegian Social Science Data Services (approval no. 19667). Written informed consent was obtained from all subjects. The study was registered at <http://www.clinicaltrials.gov> (no. NCT01034436).

### Study design

The present study is part of a larger study involving healthy overweight subjects randomised to three different intervention groups: high ALA-DAG (*n* 10), high ALA-TAG (*n* 13) and low ALA-TAG (*n* 10) where the aim of the study was (1) to study the effect of replacing TAG-based food items with DAG-based food items and (2) to study the effect of low *v.* high ALA in TAG-based food items. In the present study, only data relating to the first aim are presented. In total, ninety-six subjects were screened for eligibility (minimum 4 weeks prior to the run-in period). For the present aim, forty-nine subjects were randomised and thirty-nine subjects received allocated interventions. Of the subjects, ten discontinued the intervention (declined to eat products, hospitalisation, lack of time and no explanation) and six had to be excluded from the analyses, leaving twenty-three subjects (*n* 10 and 13) included in the final statistical analyses. A 12-week double-blinded randomised controlled parallel-design study was conducted at Akershus University College from February to June 2009. Subjects were allocated to high ALA-DAG or high ALA-TAG further described in detail later. During a 2-week run-in period prior to the intervention period, all subjects included the same set of test products. The test products in the run-in period consisted of sunflower oil-based margarine (20 g) and mayonnaise (11 g) and sunflower oil (12 g). Fatty acids were given as TAG and the total amount of ALA in the run-in period was 0.15 g per d.

At baseline, subjects were randomly assigned and stratified by sex into the intervention groups receiving products containing either high ALA (8.29 g/d) and *sn*-1,3-DAG or high ALA (8.51 g/d) and TAG (from now on referred to as the high-ALA-DAG group and high-ALA-TAG group, respectively) (Table 1). The test products were 20 g margarine, 11 g mayonnaise and 12 g (13 ml) oil per d. Margarine was the only test product where *sn*-1,3-DAG oil was added. Rapeseed oil was used in mayonnaise as TAG (8.84 g/d), and in the margarine either as *sn*-1,3-DAG (11.2 g/d) and TAG (2.88 g/d) (high-ALA-TAG group) or as TAG only (14.12 g/d) (high-ALA-TAG group) (Table 1). The 12 g oil per d was the identical flaxseed oil in the two groups in order to obtain a standardised and high level of ALA in the diet. The products constituted in total approximately 35 g fat per d (Table 1), corresponding to about 1255.2 kJ/d (300 kcal/d). The food items were delivered at baseline and after 6 weeks of intervention. At 4 weeks prior to the run-in period and during the intervention

**Table 1.** Fatty acid composition in the test products (g per portion\* and g per d)

(g)	High ALA-DAG (n 10)				High ALA-TAG (n 13)			
	Margarine	Mayonnaise	Oil†	Per d	Margarine	Mayonnaise	Oil†	Per d
Total fat	14.08	8.84	12.0	34.92	14.12	8.84	12.00	34.96
DAG	11.20	–	–	11.20	–	–	–	–
TAG	2.88	8.84	12.0	23.72	14.12	8.84	12.00	34.96
SFA	2.80	0.67	0.83	4.30	2.80	0.67	0.83	4.30
MUFA	7.40	5.06	1.80	14.26	7.20	5.06	1.80	14.06
18 : 3n-3 (ALA)	0.78	0.79	6.72	8.29	1.00	0.79	6.72	8.51
20 : 5n-3 (EPA)	0.01	0.01	0.01	0.03	0.01	0.01	0.01	0.03
22 : 6n-3 (DHA)	0.01	0.01	0.01	0.03	0.01	0.01	0.01	0.03
Total n-3	0.80	0.79	6.84	8.43	1.02	0.79	6.84	8.65
18 : 2n-6 (LA)	2.20	1.76	1.80	5.76	2.20	1.76	1.80	5.76
Vitamin E	6.20	1.54	11.32	19.06	3.26	1.54	11.32	16.12

ALA,  $\alpha$ -linolenic acid; DAG, diacylglycerol; LA, linoleic acid.

\* One portion of margarine, mayonnaise and oil equals 20, 11 and 12 g, respectively.

† Flaxseed oil.

period, the subjects were not allowed to use *n*-3 fatty acid supplements and other dietary supplements. At each visit they were reminded by a nutritionist to keep a stable weight. Fasting body weight and body composition measurements were registered at all visits. The subjects were informed not to change the physical activity and dietary habit during the study. The diet was registered at each visit using the SmartDiet questionnaire which is a validated qualitative dietary questionnaire<sup>(29)</sup>.

**Blinding and randomisation.** The present study was double-blinded by the identical appearance of different food items; their contents were identifiable only by ID numbers on the food items. Randomisation was performed by LINK Medical Research AS using Microsoft Excel and its random generator.

**Compliance.** Participants were instructed to bring back food containers (lids of margarine, sachets of mayonnaise and bottles of oil) and compliance was estimated by counting the empty food containers and estimating the leftovers. We estimated compliance by dividing the amount of leftovers by the total amount of food scheduled for the intervention period. The mean compliance for each subject was expressed as a percentage. According to the protocol, subjects with low compliance (<70 %) were excluded from the study. Compliance was estimated to be 90–100 % in both groups.

### Study products

Margarine and mayonnaise were based on commercial products manufactured by Mills DA. Mayonnaise with 80 % fat was based on refined sunflower oil (Aarhus Karlshamn) for the run-in period and refined rapeseed oil (Aarhus Karlshamn) for the high-ALA groups. Mayonnaise was packed in commercial portion sachets of 11 g.

Margarine with 70 % fat was based on sunflower oil (run-in period), refined rapeseed oil (AarhusKarlshamn AB) (high-ALA-TAG group) or rapeseed oil with DAG (Kao

Corporation) (high-ALA-DAG group), produced by Danisco AS. The margarine was packed in commercial lids, 140 g in each.

Refined sunflower oil (AarhusKarlshamn AB) (run-in period) was filled into green, transparent polyethylene terephthalate bottles of 500 ml. Cold pressed flaxseed oil (Norsk Froppresse AS) (high-ALA groups) was fortified with vitamin E acetate from BASF in order to obtain equal amounts of vitamin E for both groups and filled into identical bottles. The participants were told to cover the bottles with aluminum foil and keep the oil refrigerated.

The fatty acid profile and vitamin E in the products were analysed by the GC method in a routine laboratory (Eurofins). Results are shown in Table 1.

### Measurements of body composition

Subjects wore light clothing and no shoes. Two trained persons carried out all measurements. Height was measured by a wall-mounted stadiometer to the nearest 0.1 cm. Weight was measured by the Tanita BC-418 manufactured by Tanita Corporation to the nearest 0.1 kg. To correct for light clothing, 1 kg was subtracted from the measured value. BMI was calculated by the Tanita BC-418. Waist circumference and hip circumference were measured to the nearest mm with a standard, non-stretch tape. Waist circumference was measured at the point midway between the iliac crest and the lower rib margin. Hip circumference was measured at the maximum circumference of the buttocks posterior and the symphysis anterior. All subjects were measured while standing in a relaxed position and with normal respiration. Body composition was estimated by bioelectric impedance analysis (Tanita BC-418), a hand-to-foot system, according to the manufacturer's manual. Information on age, height and body type (all were routinely classified as 'standard' body type) was entered before measuring. Bioelectric impedance analysis measurements were carried out at a frequency of 50 kHz.

### Blood and urine sampling

The day prior to blood sampling, the subjects were told to refrain from alcohol consumption and vigorous physical



activity; venous blood samples were drawn after an overnight fast ( $\geq 12$  h). Serum was obtained from silica gel tubes (Becton Dickinson vacutainer) and kept at room temperature for at least 30 min, until centrifugation (1500 g, 12 min). Serum was kept at room temperature and immediately prepared for subsequent analysis of routine laboratory analysis or aliquoted and stored at  $-80^{\circ}\text{C}$  until further analyses. Plasma was obtained from EDTA tubes (Becton Dickinson vacutainer), immediately placed on ice and centrifuged within 10 min (2000 g,  $4^{\circ}\text{C}$ ). Plasma samples were aliquoted and stored at  $-80^{\circ}\text{C}$  until further analyses. Morning spot urine samples were refrigerated ( $4^{\circ}\text{C}$ ) until aliquoted and stored at  $-80^{\circ}\text{C}$  until further analysis.

### Routine laboratory analysis

Fasting serum high-sensitivity CRP (hsCRP), total cholesterol, LDL-cholesterol, HDL-cholesterol, TAG, glucose, C-peptide, insulin, HbA1c, thyroid-stimulating hormone,  $\gamma$ -GT, alkaline phosphatase (ALP), ALAT and aspartate aminotransferase (ASAT) were measured by standard methods at a routine laboratory (Frst Medical Laboratory). Fasting plasma NEFA were measured by standard methods (Department of Medical Biochemistry, Rikshospitalet University Hospital, Norway).

8-iso-PGF<sub>2 $\alpha$</sub>  and creatinine in morning spot urine samples were determined by Vitas AS. Urinary 8-iso-PGF<sub>2 $\alpha$</sub>  was analysed by liquid chromatography with negative electrospray ionisation coupled to tandem mass spectrometric detection according to Bastani *et al.*<sup>(30)</sup>. Urinary levels of 8-iso-PGF<sub>2 $\alpha$</sub>  are presented as the ratio of 8-iso-PGF<sub>2 $\alpha$</sub>  to creatinine. Urine creatinine was determined by ELISA using the Cayman Chemical Creatinine Assay kit. The ninety-six-well plate format ELISA kit, validated for urine samples, uses colorimetric detection at 495 nm. Assay range: 0–150 mg/l (normal range 250–4000 mg/l).

Plasma  $\alpha$ -tocopherol analysis<sup>(31)</sup> and quantification of fatty acids in plasma lipids<sup>(32)</sup> were performed by Vitas AS.

### Enzyme immunoassay

Serum was stored at  $-80^{\circ}\text{C}$  in multiple aliquots until analysis. Serum leptin, adiponectin, resistin and fetuin-A were measured by enzyme immunoassay by R&D Systems according to the manufacturer's instructions.

### Statistics

Sample size was calculated based on the existing literature primarily on the effects of ALA on hsCRP in dietary intervention studies since this outcome measurement would require the highest number of subjects. The calculations used an expected change in hsCRP from baseline to the end of the study of 20 (SD 0.5) %. The level of significance was set to 5 % (two-sided) and the power to 80 %. A total of seventy-five subjects were required in the main study (twenty-five per arm; three arms). Seventy-five subjects in total were randomised, with forty-nine subjects being randomised to the present two groups. Due to a

high withdrawal rate, a total of twenty-three subjects completed the study. Due to sample size, data were analysed with non-parametric tests and are presented as median (25–75 percentiles). Differences between the randomisation groups were analysed at baseline and after 12 weeks of intervention (baseline adjusted values). The changes between the two groups or within groups after 12 weeks of intervention were analysed by the Mann–Whitney *U* test or the Wilcoxon matched-pair signed-rank test, respectively. Bonferroni correction to adjust for multiple testing was performed (adjusted  $P \leq 0.001$ ). Correlation analysis was performed using Spearman's rank-order correlation. All analyses were performed using SPSS for Windows (version 19.0).

### Results

In the present study, twenty-three healthy, overweight subjects (six women and seventeen men) participated, randomised in two intervention groups. The subjects were 52 (45–62) years, with a median BMI of 30 (28–32) kg/m<sup>2</sup>. At baseline there were no differences in age between the intervention groups.

### Body composition

At baseline there were no significant differences in blood pressure and body composition (total body fat percentage, total body fat mass, trunk fat mass and fat-free mass (FFM)) between the intervention groups. Prior to Bonferroni adjustment, there was a significant difference between the two groups in weight, BMI and waist circumference at baseline ( $P = 0.03$ ,  $0.05$  and  $0.01$ , respectively); however, these differences disappeared after Bonferroni adjustment (Table 2).

Since intake of DAG oil has been associated with weight loss, we investigated the effect on weight change. After 12 weeks of intervention, body weight did not change between or within the two groups. However, there was a non-significant trend towards an increase in weight in the high-ALA-DAG group ( $P = 0.07$ ). We also investigated the effect on body composition. Prior to Bonferroni adjustment, there was a significant decrease in both total body fat percentage and trunk fat mass within the high-ALA-DAG group from baseline to 12 weeks of intervention ( $P = 0.04$  and  $0.05$ , respectively) and this decrease was significantly different from the effect observed in the high-ALA-TAG group ( $P = 0.03$  and  $0.04$ , respectively). There was no significant change within or between the groups in total body fat mass. Concomitantly, there was a significant increase in the amount of FFM within the high-ALA-DAG group ( $P = 0.016$ ) from baseline to the end of the study and this change in FFM was borderline significant ( $P = 0.06$ ) between the two groups (Table 2). All differences disappeared after Bonferroni adjustment.

### Blood pressure

Systolic and diastolic blood pressure was measured at baseline and after 12 weeks of intervention (Table 2). Prior to



**Table 2.** Clinical characteristics and body composition at baseline and after 12 weeks of intervention (Medians and 25th–75th percentiles)

	High ALA-DAG (n 10)					High ALA-TAG (n 13)					
	Baseline		End of study		P*	Baseline		End of study		P†	P‡
	Median	25th–75th percentiles	Median	25th–75th percentiles		Median	25th–75th percentiles	Median	25th–75th percentiles		
Sex (n)											
Male	6					11					
Female	4					2					
Age (years)	57	45–65				52	43–60				
Systolic BP (mmHg)	135	121–144	126	121–136	0.05	136	126–154	141	134–149	0.2	0.05
Diastolic BP (mmHg)	81	77–88	79	76–92	1.0	83	77–89	87	84–91	0.1	0.2
Weight (kg)	86.3	80.2–102.9	87.2	80.9–104.1	0.07	95.9	91.7–106.9	97.5	91.6–106.0	1.0	0.3
BMI (kg/m <sup>2</sup> )	29	28–31	29	28–32	0.2	31	30–33	31	30–33	0.7	0.2
Waist (cm)	99	96–109	99	96–107	0.3	107	102–116	105	103–114	0.5	0.9
Hip (cm)	106	103–110	106	102–109	0.4	108	105–114	111	105–115	0.4	1.0
Waist:hip ratio	0.90	0.90–1.03	0.95	0.90–1.00	0.6	1.00	0.95–1.05	1.00	0.90–1.00	0.2	0.6
Fat %	29.3	23.6–41.3	28.4	23.0–40.1	0.04	31.0	26.8–33.8	31.5	26.3–33.8	0.8	0.03
Total body fat (kg)	28.8	20.5–34.1	29.0	20.2–32.0	0.09	29.1	24.7–37.2	29.9	25.5–37.7	0.5	0.2
Trunk fat (kg)	16.0	12.8–19.1	15.1	12.6–19.0	0.05	18.3	15.3–21.3	17.9	15.8–21.0	0.4	0.04
FFM (kg)	63.8	47.0–69.1	65.1	48.5–71.0	0.02	70.2	60.6–74.8	70.3	60.7–73.9	0.8	0.06
Fat %:weight ratio	0.31	0.26–0.52	0.30	0.26–0.49	0.02	0.30	0.27–0.37	0.28	0.27–0.36	0.5	0.03

ALA, α-linolenic acid; DAG, diacylglycerol; BP, blood pressure; FFM, fat-free mass.

\* Within the high-ALA-DAG group (prior to Bonferroni correction).

† Within the high-ALA-TAG group (prior to Bonferroni correction).

‡ Comparing change from baseline to the end of the study between the high-ALA-DAG and high-ALA-TAG groups (prior to Bonferroni correction).



Bonferroni adjustment, there was a significant decrease in systolic blood pressure within the high-ALA-DAG group ( $P = 0.05$ ) after intervention, and the change in systolic blood pressure was significantly different between the two groups ( $P = 0.05$ ); however, this was non-significant after Bonferroni adjustment. There was no significant difference in diastolic blood pressure after 12 weeks of intervention between or within any of the groups (Table 2).

#### *Serum lipids, C-reactive protein and insulin sensitivity markers*

At baseline, no differences in serum levels of hsCRP, glucose, insulin, C-peptide, HbA1c, total cholesterol, LDL-cholesterol, HDL-cholesterol and TAG between the intervention groups were observed (Table 3). Baseline values of homeostatic model assessment for insulin resistance (HOMA-IR) were significantly different between the two groups ( $P = 0.05$ ). After 12 weeks of intervention, there was no significant difference in the serum hsCRP, glucose, insulin, HOMA-IR, C-peptide, HbA1c, total cholesterol, HDL-cholesterol and TAG between or within the two groups (Table 3). However, after 12 weeks of intervention, there was a non-significant ( $P = 0.06$ ) difference in LDL-cholesterol between the high-ALA groups, with a non-significant decrease in the high-ALA-DAG group (4.0–3.7 mmol/l) and a non-significant increase in the high-ALA-TAG group (3.3–3.6 mmol/l) (Table 3). All significant differences disappeared after Bonferroni adjustment.

#### *Circulating NEFA*

Circulating NEFA have been shown to be elevated in obese subjects; we therefore measured fasting plasma NEFA concentrations. At baseline, there was no difference in the NEFA concentration between the intervention groups (Table 3). The changes in circulating fasting NEFA concentration were non-significantly (after Bonferroni adjustment) different ( $P = 0.02$ ) between the two groups after 12 weeks of intervention, with a non-significant decrease within the high-ALA-DAG group and a non-significant increase within the high-ALA-TAG group (Table 3).

#### *Liver function parameters*

Obesity leads to accumulation of fatty acids in the liver and therefore we measured markers of liver function such as ASAT, ALAT,  $\gamma$ -GT and ALP. At baseline no differences in these markers between the intervention groups were observed (Table 4). However, prior to Bonferroni adjustment, the concentration of ALAT,  $\gamma$ -GT and ALP was significantly reduced within the high-ALA-DAG group ( $P = 0.02$ ,  $0.02$  and  $0.01$ , respectively) and the change in ALAT was significantly different from the high-ALA-TAG group ( $P = 0.02$ ), whereas the change in  $\gamma$ -GT and ALP was not significantly different from the high-ALA-TAG group (Table 4). These differences disappeared after Bonferroni adjustment. No significant differences within or between the groups in the ASAT:ALAT ratio after 12 weeks of intervention were observed (data not shown). The change in ALAT was positively correlated with

the change in trunk fat mass ( $r 0.5$ ;  $P = 0.007$ ) and total body fat mass ( $r 0.5$ ;  $P = 0.03$ ) in the groups calculated together. The change in  $\gamma$ -GT and ALP did not correlate with the change in total body fat mass or trunk fat mass in any of the groups. In addition, we measured the serum concentration of fetuin-A, a suggested marker for hepatic steatosis<sup>(33)</sup>. However, no changes in serum concentration of fetuin-A were found within or between the groups (Table 4).

#### *Adipokines in serum and plasma*

Adipokines are secreted from adipose tissue and since intake of DAG oil has been shown to influence body composition, we measured the circulating hormones leptin, resistin and adiponectin. Despite a non-significant decrease in total body fat percentage and trunk fat mass, no changes between or within any of the groups were observed in any of these hormones after 12 weeks of intervention (Table 5). Also, there were no correlations between the change in hormones and total body fat mass or trunk fat mass.

#### *Oxidative stress markers*

Since ALA has been shown to be rapidly oxidised<sup>(34)</sup>, we measured the oxidative stress markers urinary 8-iso-PGF<sub>2 $\alpha$</sub>  and the antioxidant vitamin E. No significant changes between or within the groups were observed for urinary 8-iso-PGF<sub>2 $\alpha$</sub>  after 12 weeks of intervention (Table 5). Prior to Bonferroni adjustment, the ratio of  $\alpha$ -tocopherol:total lipids was significantly reduced within the high-ALA-DAG group ( $P = 0.02$ ) and the high-ALA-TAG group ( $P = 0.05$ ), with no significant difference between the groups after 12 weeks of intervention (Table 5). These differences disappeared after Bonferroni adjustment.

#### *Fatty acid composition in plasma*

The fatty acid composition in plasma was measured and is shown in Table 6. Prior to Bonferroni adjustment, a significant increase in ALA was observed both within the high-ALA-DAG ( $P = 0.005$ ) and the high-ALA-TAG groups ( $P = 0.002$ ) (Table 6). The change in plasma ALA between the groups was significantly different ( $P = 0.04$ ) after 12 weeks of intervention, with the largest increase observed in the high-ALA-TAG group. Plasma EPA (20 : 5n-3) was significantly increased within both groups ( $P = 0.04$  and  $0.02$ , respectively), but the change was not significantly different between the groups. No increase was observed in plasma DHA (22 : 6n-3) either within or between the two groups. Linoleic acid (18 : 2n-6) was significantly reduced within both groups ( $P = 0.03$  in both groups) after 12 weeks of intervention, but there was no significant difference between them. Arachidonic acid (20 : 4n-6) was not changed within any of the groups, but a significant change between the two groups ( $P = 0.05$ ) was found. Minor changes were observed among the other fatty acids (Table 6). All these differences disappeared after Bonferroni adjustment.



**Table 3.** Plasma lipids and biochemical measurements at baseline and after 12 weeks of intervention (Medians and 25th–75th percentiles)

	High ALA-DAG ( <i>n</i> 10)				High ALA-TAG ( <i>n</i> 13)			
	Baseline		End of study		Baseline		End of study	
	Median	25th–75th percentiles	Median	25th–75th percentiles	Median	25th–75th percentiles	Median	25th–75th percentiles
Total cholesterol (mmol/l)	5.9	5.2–6.2	5.6	4.9–6.0	5.4	5.1–5.8	5.4	5.0–6.1
LDL-cholesterol (mmol/l)	4.0	3.1–4.3	3.7	3.0–4.2	3.3	3.1–3.8	3.6	3.1–4.3
HDL-cholesterol (mmol/l)	1.3	1.2–1.4	1.3	1.1–1.50	1.1	1.1–1.4	1.2	1.0–1.4
TAG (mmol/l)	1.3	1.0–1.7	1.2	1.0–1.5	1.5	1.3–2.0	1.6	1.4–2.1
NEFA ( $\mu$ mol/l)	0.53	0.44–0.60	0.40	0.33–0.54	0.43	0.34–0.58	0.53	0.40–0.63
ApoA-1 (g/l)	1.4	1.3–1.5	1.4	1.3–1.5	1.4	1.3–1.6	1.4	1.3–1.5
ApoB (g/l)	1.0	0.7–1.1	0.9	0.8–1.0	0.9	0.8–1.0	0.9	0.9–1.0
Glucose (mmol/l)	5.5	5.0–5.9	5.5	5.2–5.8	5.8	5.4–6.2	5.5	5.3–6.0
Insulin (pmol/l)	61.5	45.0–81.3	63.0	50.5–79.8	80.0	63.0–109.0	83.0	48.0–116.5
HbA1c (%)	5.6	5.3–5.9	5.7	5.5–5.9	5.5	5.3–6.0	5.7	5.4–5.9
C-peptide (pmol/l)	900	735–1097	883	805–1063	994	792–1270	1021	802–1278
HOMA <sub>1<math>\beta</math></sub>	2.2	1.6–3.0	2.3	1.6–2.8	3.0	2.2–3.8	3.0	1.9–4.4
hsCRP (mg/l)	1.9	1.3–3.1	1.6	1.1–1.9	1.5	0.7–2.8	1.5	0.7–2.6
TSH (mU/l)	1.0	0.9–2.5	1.3	0.8–1.8	1.6	1.1–1.9	1.6	1.3–2.0

ALA,  $\alpha$ -linolenic acid; DAG, diacylglycerol; HOMA<sub>1 $\beta$</sub> , homeostasis model assessment for insulin resistance; hsCRP, high-sensitivity C-reactive protein; TSH, thyroid-stimulating hormone.

\* Within the high-ALA-DAG group (prior to Bonferroni correction).

† Within the high-ALA-TAG group (prior to Bonferroni correction).

‡ Comparing change from baseline to the end of the study between the high-ALA-DAG and high-ALA-TAG groups (prior to Bonferroni correction).

§ HOMA<sub>1 $\beta$</sub>  = (fasting plasma insulin (mU/l)  $\times$  fasting plasma glucose (mmol/l))/22.5, where 1 mU/l = 6.945 pmol/l.



**Table 4.** Plasma liver function markers at baseline and after 12 weeks of intervention (Medians and 25th–75th percentiles)

	High ALA-DAG (n 10)				<i>P</i> *	High ALA-TAG (n 13)				<i>P</i> †	<i>P</i> ‡
	Baseline		End of study			Baseline		End of study			
	Median	25th–75th percentiles	Median	25th–75th percentiles		Median	25th–75th percentiles	Median	25th–75th percentiles		
ASAT (U/l)	25.5	23.0–32.3	25.5	22.8–29.3	0.2	27.0	24.0–28.0	27.0	24.0–31.0	0.3	0.1
ALAT (U/l)	29.0	20.5–37.5	24.5	19.5–31.5	0.02	28.0	22.5–37.0	27.0	22.0–38.0	0.6	0.02
γGT (U/l)	24.0	18.5–32.0	19.0	14.0–28.5	0.02	32.0	22.0–48.5	30.0	21.5–63.0	0.6	0.3
ALP (U/l)	71.0	63.5–75.0	63.5	56.3–69.3	0.01	62.0	57.0–76.0	64.0	53.0–85.5	0.6	0.1
Fetuin-A (μg/ml)	478.2	360.0–592.6	452.2	348.1–514.2	0.6	446.6	368.8–498.6	441.8	326.2–587.6	0.9	0.7

ALA, α-linolenic acid; DAG, diacylglycerol; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; γGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase.

\* Within the high-ALA-DAG group (prior to Bonferroni correction).

† Within the high-ALA-TAG group (prior to Bonferroni correction).

‡ Comparing change from baseline to the end of the study between the high-ALA-DAG and high-ALA-TAG groups (prior to Bonferroni correction).

### Risk of CVD (10-year risk)

A cardiovascular risk profile has been developed based on the results of the Framingham heart study<sup>(35,36)</sup> for use in primary care (<http://www.reynoldsriskscore.org/>). In addition to sex, age, systolic blood pressure, total cholesterol, HDL-cholesterol and hsCRP, the Reynold risk score uses information about smoking and genetic disposure (whether or not a parent had a heart attack before the age of 60 years) to predict CVD risk. In the present study, even though the cardiovascular risk score was relatively low, there was a mean predicted relative risk reduction of 18.5 % (from 6.5 % to 5.3 %,  $P = 0.04$ ) in the high-ALA-DAG group, while the high-ALA-TAG group had a relative risk increase of 13.3 % (from 7.5 % to 8.5 %,  $P = 0.4$ ) after intervention. There was a significant difference in relative risk reduction between the two groups ( $P = 0.001$ ). This significant difference remained valid even after full Bonferroni adjustment.

### Discussion

In the present study, we found that replacing part of the TAG-containing margarine with DAG-containing margarine (20 g) for 12 weeks led to an improved predicted 10 years cardiovascular risk score in healthy, overweight subjects. This reduction in cardiovascular risk may possibly be due to an improved metabolic phenotype appearing as reduced total body fat percentage and reduced trunk fat mass, and increased total FFM, in addition to reduced blood pressure. Additionally, the liver function markers ALAT, γ-GT and ALP were also non-significantly decreased after DAG intake.

The results are in line with other studies that showed a change in body composition after intake of DAG with a reduction of visceral fat mass<sup>(21,37,38)</sup> and total fat mass<sup>(21,22,38)</sup> or total fat percentage<sup>(37)</sup>. The different metabolic effects after intake of DAG compared with those after intake of TAG may be due to their structural differences and not only their fatty acid composition since the energy value, absorptive and digestive properties are similar<sup>(39)</sup>. The main end products of lipase action on *sn*-1,3-DAG are free glycerol and NEFA<sup>(40)</sup>. These end-products of DAG are less readily

re-synthesised into chylomicron TAG because such a synthesis requires a glycerol-3-phosphate intermediate. Therefore, fatty acids from DAG are likely to bypass the lymphatic system and enter the blood via the portal vein<sup>(41,42)</sup> and may hence lead to increased hepatic β-oxidation, which have been reported both in animal<sup>(43–47)</sup> and human studies<sup>(20,48–51)</sup>.

Plasma levels of NEFA have been shown to be elevated in obese subjects<sup>(52)</sup> as a consequence of increased release from the enlarged adipose tissue and reduced clearance<sup>(53)</sup>. NEFA have been shown to inhibit the anti-lipolytic action of insulin, which again will further increase the level of NEFA and subsequently induce insulin resistance<sup>(54,55)</sup>. However, the mechanisms by which obesity and NEFA cause insulin resistance are not clearly understood; nevertheless the reduced release of NEFA into the circulation improves insulin sensitivity<sup>(56)</sup>. In the present study, we found no significant difference in the change in NEFA between the two groups, and no correlation was found between the change in NEFA concentration and the change in insulin resistance markers (insulin, glucose, C-peptide, HbA1c and HOMA-IR) (data not shown).

Despite a non-significant reduction in total body fat percentage and trunk fat mass, total body weight in the high-ALA-DAG group was not reduced; rather a non-significant increase was observed. Inconsistent results have been described regarding the effect on body weight after intervention with dietary DAG, with some finding a reduced body weight<sup>(21,22)</sup> while others find no change in weight<sup>(57,58)</sup>. The lack in weight reduction in the present study may be partly explained by the finding that the amount of FFM was non-significantly increased in the high-ALA-DAG group. The change in FFM in the high-ALA-DAG group corresponds to a 1.3 kg or 2 % increase in FFM, thus balancing the potential positive effect of the reduced trunk fat mass. However, taken together, these small non-significant changes in body composition may suggest an improvement of the metabolic phenotype in healthy, overweight individuals after dietary DAG intervention.

Even though the total trunk fat mass was slightly reduced in the high-ALA-DAG group, hip circumference was not changed after DAG intervention, indicating the potential effect of DAG intake on visceral fat mass in preference to subcutaneous fat mass. Visceral fat and subcutaneous fat are







**Table 6.** Fatty acid composition ( $\mu\text{g/ml}$ ) in plasma at baseline and after 12 weeks of intervention (Medians and 25th–75th percentiles)

	High ALA-DAG ( <i>n</i> 10)						High ALA-TAG ( <i>n</i> 13)						
	Baseline			End of study			Baseline			End of study			
	Median	25th–75th percentiles		Median	25th–75th percentiles	<i>P</i> *	Median	25th–75th percentiles		Median	25th–75th percentiles	<i>P</i> †	<i>P</i> ‡
14 : 0	25.8	21.4–32.4		25.1	20.2–31.7	0.8	33.0	22.8–40.3		33.2	27.1–45.8	0.9	0.7
15 : 0	5.9	5.1–6.7		6.1	4.7–6.6	0.7	6.4	5.3–7.5		6.7	5.3–7.8	0.7	0.6
16 : 0	690.7	607.0–802.4		691.5	570.0–754.1	0.4	741.3	655.6–891.5		789.1	689.2–877.8	0.6	0.2
18 : 0	233.8	196.5–252.4		216.8	199.7–228.9	0.06	250.2	206.1–274.1		253.8	202.8–278.9	0.8	0.1
20 : 0	8.3	6.8–9.3		8.4	6.9–8.9	0.6	8.2	7.4–9.8		9.1	8.1–9.9	0.2	0.1
22 : 0	31.3	23.5–32.6		27.5	23.2–28.7	0.03	24.9	23.1–29.8		25.6	21.0–27.4	0.3	0.1
23 : 0	10.8	9.0–12.6		10.0	9.3–13.1	0.7	9.3	8.9–11.0		10.7	9.0–11.5	0.1	0.2
24 : 0	30.3	23.7–33.1		28.4	22.8–31.0	0.07	26.8	22.9–28.8		26.7	22.0–29.8	0.9	0.06
16 : 1	50.2	45.1–68.1		49.4	42.6–65.7	1.0	57.0	39.7–85.9		69.6	54.0–86.2	0.2	0.2
18 : 1 $\alpha$ –11	7.9	6.7–9.6		9.1	6.7–9.9	1.0	9.9	7.2–11.2		9.7	8.5–12.9	0.7	0.7
18 : 1 $\alpha$ –9	595.8	517.9–790.3		594.5	515.2–723.4	0.6	625.6	500.8–765.8		776.8	681.0–798.9	0.06	0.03
18 : 1 $\alpha$ –11	44.7	41.0–52.7		42.5	41.0–54.5	1.0	46.5	36.9–57.1		52.0	45.0–60.7	0.04	0.04
24 : 1 $n$ –9	41.4	34.9–46.8		39.8	39.2–53.8	0.07	38.0	33.8–44.9		42.2	38.9–46.0	0.1	0.7
18 : 3 $n$ –3	23.0	20.0–32.2		48.6	39.9–62.2	0.005	24.9	21.2–28.3		58.3	49.5–84.9	0.002	0.04
20 : 5 $n$ –3	35.8	24.4–67.8		57.0	38.9–91.5	0.04	28.0	22.7–64.7		56.5	40.5–76.7	0.02	0.8
22 : 5 $n$ –3	18.7	17.0–25.7		20.8	18.9–24.6	0.7	21.1	18.5–24.3		25.9	22.3–29.2	0.007	0.3
22 : 6 $n$ –3	106.3	79.1–136.7		87.8	72.1–110.7	0.1	90.1	76.3–129.1		93.9	69.0–129.6	0.2	0.6
18 : 2 $n$ –6	1128.5	995.7–1223.8		936.8	857.4–1141.0	0.03	1088.0	1018.2–1234.3		984.1	924.3–1202.0	0.03	0.4
18 : 3 $n$ –6	14.2	13.0–18.4		10.8	8.3–15.0	0.09	18.4	14.1–21.1		14.3	9.8–21.3	0.3	0.7
20 : 2 $n$ –6	9.6	8.4–11.4		8.6	6.9–10.1	0.02	10.1	9.0–12.9		10.9	8.8–11.6	0.9	0.1
20 : 3 $n$ –6	51.9	37.7–64.4		39.2	32.1–45.8	0.007	53.3	39.1–64.1		42.1	35.3–56.8	0.1	0.4
20 : 4 $n$ –6	210.7	171.0–230.2		188.7	146.5–209.2	0.06	201.9	168.3–213.1		206.5	164.7–217.1	0.5	0.05
22 : 4 $n$ –6	4.6	4.0–6.3		4.3	3.3–4.9	0.02	5.4	5.1–6.7		5.6	4.4–6.3	0.5	0.2
22 : 5 $n$ –6	12.9	10.1–13.9		12.1	11.0–13.1	0.7	11.7	11.1–13.6		11.8	10.4–14.3	0.9	0.5

ALA,  $\alpha$ -linolenic acid; DAG, diacylglycerol.

\* Within the high-ALA-DAG group (prior to Bonferroni correction).

† Within the high-ALA-TAG group (prior to Bonferroni correction).

‡ Comparing change from baseline to the end of the study between the high-ALA-DAG and high-ALA-TAG groups (prior to Bonferroni correction).



$\beta$ -oxidation may be the reason for reduced fatty liver formation after DAG intake<sup>(47)</sup>. Fetuin-A, a marker of hepatic steatosis<sup>(33)</sup>, was not changed within or between the groups after intervention. However, fetuin-A is a marker of a more established disease (hepatic steatosis) while the other liver function markers we measured (ASAT, ALAT, ALP and  $\gamma$ -GT) reflect a more general hepatic dysfunction. This may partly explain why fetuin-A is not changed during intervention in the present group of healthy, overweight subjects.

The Reynold risk score is a score based on several risk factors of CVD (sex, age, blood pressure, CRP, total cholesterol, HDL-cholesterol, smoking habits and familiar disposal). The risk score gives a CVD prediction for the following 10 years and is intended for use in primary care. In the present study, even though the cardiovascular risk score was relatively low at intervention start, intervention with DAG led to a relative risk reduction of 18.5 % and this reduction was significantly different from the TAG group, supporting the notion of improved health after DAG intake.

The strength of the present study is the randomised controlled design and the high compliance regarding intake of the study products. However, a major weakness in the present study is that the number of participants was considerably reduced due to a high withdrawal rate during the study. In a previous study investigating bioavailability of flaxseed oil, a relatively high withdrawal rate was also observed<sup>(75)</sup>. In the present study, the subjects were randomised and stratified according to sex and age. Due to the high withdrawal rate, however, the final groups had a skewed sex distribution. We therefore recalculated the results based on the male population alone ( $n$  6 and  $n$  11 in the high-ALA-TAG and high-ALA-DAG group, respectively), and the majority of the results still showed the same pattern. Within the high-ALA-DAG group (Wilcoxon matched-pair signed-rank test) a reduction after intervention was found for the following parameters: systolic blood pressure, ASAT,  $\gamma$ -GT, ALP, Reynold risk score and  $\alpha$ -tocopherol/total lipids even though not all the recalculated results (only the males) now reached statistical significance. Differences (non-significant) between the two groups (Mann–Whitney  $U$  test) were still observed for ALAT, fat percentage and Reynold risk score similarly to the whole group. In the present study, power analysis was based on the existing literature on the effect of ALA on hsCRP, based on the assumption that changes in hsCRP would require the largest sample size. However, in addition to hsCRP, the present study aimed to investigate several other metabolic markers after intake of DAG oil in a Norwegian population, and may therefore be considered exploratory. Due to the limited numbers of subjects and the multiple testing, we performed Bonferroni correction. We cannot rule out the possibility that the results regarding some of the other markers would have been different in a larger population. However, body composition, another outcome in the present study, is shown by others to significantly change with approximately ten subjects in each group<sup>(38,76,77)</sup>, which is comparable to our sample size. In order to further study beneficial health effects of DAG, future studies should include larger sample size and individuals with features of the metabolic syndrome which are more at risk of

developing chronic diseases such as fatty liver, CVD and type 2 diabetes mellitus.

In conclusion, the results from the present study show that replacing TAG-based food items with DAG-based food items led to improved predicted 10 years cardiovascular risk score together with a possible beneficial effect on body composition and blood pressure. Even though weight was not reduced, intake of DAG-containing food items seemed to improve the metabolic phenotype in healthy, overweight subjects with small beneficial non-significant changes in total body fat percentage and trunk fat mass, FFM, ALAT,  $\gamma$ -GT and ALP. Altogether, this may potentially suggest beneficial health effects of DAG in healthy overweight subjects.

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