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Determining the mechanisms of dietary turnip rapeseed oil on cholesterol metabolism in men with metabolic syndrome

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ABSTRACT

We have earlier reported the reduction of total cholesterol, low-density lipoprotein (LDL) cholesterol and oxidized LDL caused by short-term modification of diet with cold-pressed turnip rapeseed oil (CPTRO) instead of butter. The aim of this supplementary study was to determine whether the beneficial effects resulted from altered cholesterol metabolism during the intervention.

Thirty-seven men with metabolic syndrome (MetS) completed an open, randomized and balanced crossover study. Subjects' usual diet was supplemented with either 37.5 g of butter or 35 mL of CPTRO for 6–8 weeks. Otherwise normal dietary habits and physical activity were maintained without major variations. Serum non-cholesterol sterols were assayed with gas-liquid chromatography and used as surrogate markers of whole-body cholesterol synthesis and absorption efficiency. Serum proprotein convertase subtilisin/kexin type 9 (PCSK9) concentration was analyzed with Quantikine ELISA Immunoassay. Serum cholesterol synthesis markers and serum cholestanol (absorption marker), all as ratios to cholesterol, did not differ between the periods. Serum campesterol and sitosterol ratios to cholesterol were significantly increased after the administration of CPTRO resulting from the increased intake of 217 mg/day of plant sterols in CPTRO. Serum PCSK9 concentration did not differ between CPTRO and butter periods. The reduction in serum cholesterol by 7.2% after consumption of rapeseed oil could not be explained by changes in cholesterol absorption, synthesis or PCSK9 metabolism in MetS.

TRIAL REGISTRATION NUMBER

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INTRODUCTION

Metabolic syndrome (MetS) is a combination of cardiovascular risk factors such as visceral obesity, hypertension, dyslipidemia and abnormal glucose tolerance or diabetes. Cardiovascular disease and all-cause mortality are increased in men with MetS.¹ A comprehensive meta-analysis of 951 083 patients demonstrated that MetS is associated with a twofold increase in adverse cardiovascular outcomes and a

Significance of this study

What is already known about this subject?

- ▶ Rapeseed oil consumption decreases serum total and low-density lipoprotein cholesterol concentration when compared with butter.
- ▶ Serum non-cholesterol sterols are generally considered valid biomarkers of cholesterol absorption efficiency and cholesterol synthesis.
- ▶ Recent evidence has demonstrated that linoleic acid (n–6 polyunsaturated fatty acid), also present in turnip rapeseed oil, decreased serum proprotein convertase subtilisin/kexin type 9 (PCSK9) concentration.

What are the new findings?

- ▶ Cholesterol absorption efficiency markers campesterol and sitosterol were significantly higher after a period of 6–8 weeks of cold-pressed turnip rapeseed oil (CPTRO) when compared with butter.
- ▶ However, CPTRO contains high amounts of campesterol and sitosterol and there was no difference in the endogenous cholesterol absorption marker cholestanol.
- ▶ CPTRO had no effect in the markers of cholesterol synthesis or PCSK9 concentrations when compared with butter.

How might these results change the focus of research or clinical practice?

- ▶ A diet low in saturated fatty acids and rich in unsaturated fatty acids such as in CPTRO has beneficial effects on serum lipids. Further studies are necessary to determine the mechanisms since the reduction could not be explained by changes in whole-body cholesterol or PCSK9 metabolism.

1.5-fold increase in all-cause mortality.² The increase in overweight and obesity is a global phenomenon and contributes to an increase in subjects with MetS as well. The accumulation of cholesterol in the arterial walls is known to play a key role in the development and progression of atherosclerosis.



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An easily reproducible way to impact populations' cardiovascular risk is implementation of a heart healthy diet. The so-called traditional Mediterranean diet low in saturated fatty acids (SFA) and rich in unsaturated fatty acids derived from olive oil, nuts, fruits, vegetables and fish has been shown to decrease the incidence and mortality of cardiovascular diseases.^{3–5} Olive oil's counterparts in the North-European and Canadian diet are rapeseed oil (canola oil) and turnip rapeseed oil, which also contain large amounts of unsaturated fatty acids, and are the most typical sources of dietary plant-derived n–three polyunsaturated fatty acids (PUFA).

Unsaturated fatty acid consumption has several favorable effects on serum and lipoprotein lipids. We have earlier reported the beneficial effects of dietary intake of cold-pressed turnip rapeseed oil (CPTRO) on low-density lipoprotein (LDL) cholesterol and oxidized LDL concentrations compared with butter in men with MetS.⁶ We found that LDL cholesterol concentration was reduced by 11% and that of oxidized LDL by 15% compared with butter with no effect on high-density lipoprotein (HDL) cholesterol and serum triglyceride concentrations. CPTRO, as rapeseed oils in general, contains on average over 95% of unsaturated fatty acids, of which two-thirds are monounsaturated fatty acids (MUFA) and one-third PUFA.⁶ Regarding the cholesterol-lowering mechanism of rapeseed oil compared with an SFA-based control diet, two of three earlier studies^{7–9} demonstrated that after substitution of rapeseed oil-based diet serum biomarkers of cholesterol absorption diminished⁷ and those of cholesterol synthesis increased.^{7,8} In the third study, however, cholesterol synthesis did not differ from that of the baseline diet in spite of a significant reduction of total and LDL cholesterol.⁹ In a fourth study comparing rapeseed oil and olive oil consumption in subjects with ileostomy, serum cholesterol levels were lower, fecal cholesterol excretion was higher and cholesterol absorption tended to be lower during rapeseed oil than olive oil diet.¹⁰ Thus, three of the four earlier rapeseed oil diet studies suggested that rapeseed oil interfered with cholesterol metabolism. All of these previous studies were performed in non-obese mildly to moderately hypercholesterolemic subjects. Since cholesterol metabolism differs in subjects with MetS from those without MetS,^{11,12} it is possible that rapeseed oil consumption might have a different impact on cholesterol metabolism in MetS

compared with non-obese hypercholesterolemic subjects without MetS. Recent evidence has also demonstrated that linoleic acid (n–6 PUFA), also present in CPTRO, decreased serum proprotein convertase subtilisin/kexin type 9 (PCSK9) concentration suggesting that this may be a possible cholesterol-lowering mechanism of linoleic acid consumption.¹³ PCSK9 is a protease that regulates cholesterol metabolism by binding to hepatic LDL receptors and promoting their degradation. This is a continuation and further elaboration to our earlier study. The aim of this supplementary study was to evaluate whether the reduction in cholesterol concentration by CPTRO in men with MetS results from alterations in cholesterol metabolism assayed with serum non-cholesterol sterols, biomarkers of whole-body cholesterol synthesis and absorption efficiency,^{14–16} and/or from alterations in serum PCSK9 concentrations and to do so by performing further analysis of samples collected in our original trial.

MATERIALS AND METHODS

Subjects, design and diets

Hämeenlinna Metabolic Syndrome research program is a regional study investigating atherosclerotic risk factors in men with MetS.¹⁷ We have earlier described the detailed structure, methods, baseline data and the results of this dietary intervention on LDL cholesterol, oxidized LDL and arterial elasticity.⁶ In brief, 37 men with MetS according to the National Cholesterol Education Program Adult Treatment Panel III criteria¹⁸ and age from 35 to 65 years (mean 53.5) accomplished the clinical phase of a randomized and balanced study with two open dietary interventions in a crossover design.

The subjects were randomly divided into turnip rapeseed oil and butter groups. In the oil period, in addition to their habitual diet the subjects consumed a daily dose of 35 mL CPTRO, and during the butter period they consumed 37.5 g of butter. Both dietary periods lasted from 6 to 8 weeks. After the first period, all subjects had an 8-week wash-out period before changing to the group of the other fat adjunct for another period of 6–8 weeks (figure 1). The subjects were asked to maintain their diet and amount of physical activity otherwise unchanged throughout the study. CPTRO contained 96% of MUFA and PUFA. Butter contained 62% of SFA. The composition of cholesterol, plant sterols,

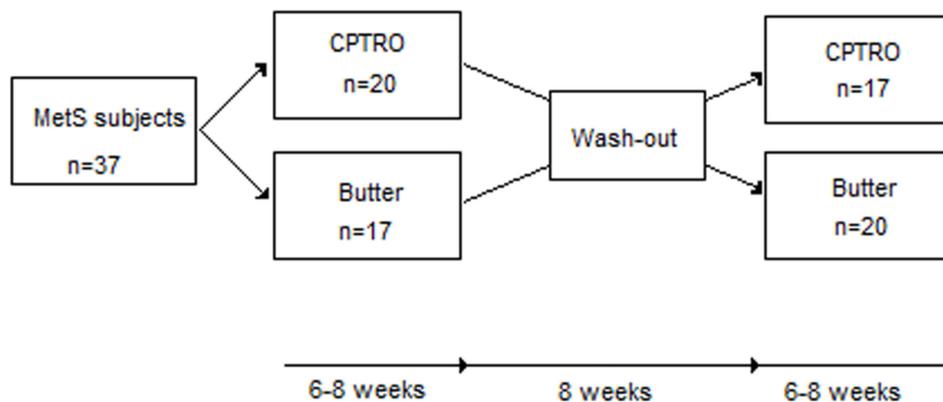


Figure 1 The study design. CPTRO, cold-pressed turnip rapeseed oil; MetS, metabolic syndrome.

Table 1 Cholesterol and non-cholesterol sterol composition in butter and in cold-pressed turnip rapeseed oil

Variables	Butter mg/100 g butter	CPTRO mg/100 g oil
Cholesterol	188.8	3.1
Campesterol	0.5	255.0
Sitosterol	0.2	333.7
Stigmasterol	0.1	1.1
Avenasterol	0.3	25.1
Sitostanol	0.5	4.7
Squalene	6.1	1.2

sitostanol and squalene in butter and CPTR0 is shown in table 1.

The Ethics Committee of the Kanta-Häme Hospital District approved the study protocol and the study followed the ethical principles outlined in the Declaration of Helsinki. Each study subject gave a written informed consent for the study.

Blood sampling and biochemical analysis

Blood samples were collected into 10 mL EDTA tubes, 5 mL lithium-heparin gel tubes and 2 mL sodium-fluoride tubes at the end of both dietary periods after a 12-hour overnight fast and at least 10 min of rest.

Serum PCSK9 concentration was analyzed with the Quantikine ELISA Immunoassay using a monoclonal catcher and a polyclonal detection antibody specific for human PCSK9 (www.rndsystems.com/pdf/DPC900.pdf). According to the manufacturer, the intra-assay and interassay %CVs (coefficient of variability) vary from 4.1% to 6.5%, and the mean serum concentration in 37 apparently healthy subjects was 313 (SD 71.5) ng/mL and the range was 177–460 ng/mL.

Cholesterol and non-cholesterol sterols in serum, butter and CPTR0 were quantified by gas–liquid chromatography (GLC) with a 50 m Ultra 2 capillary column (Agilent 6890N Network GC System, Agilent Technologies) as described previously.¹⁹ Serum non-cholesterol sterols were expressed as the ratio of $100 \times \mu\text{mol}/\text{mmol}$ of cholesterol by adjusting the concentrations with the cholesterol value in the same GLC run. Ratios of serum cholesterol precursors to cholesterol (squalene, cholesterol, desmosterol and lathosterol) reflect whole-body cholesterol synthesis,^{15–16} whereas the ratios of plant sterols (campesterol, sitosterol and avenasterol) and cholestanol reflect cholesterol absorption efficiency.^{14–16} We also calculated the lathosterol–cholestanol ratio, which reflects cholesterol metabolism.¹⁵

Statistical analysis

We made a sample size calculation for the secondary end points now studied. For the effect of 7% (SD of the change=0.14; $\alpha=0.05$, two tailed), we needed 31–32 patients to yield power of 80%. Statistical analysis was carried out with IBM SPSS Statistics V. 23 software. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to test the data for Gaussian distribution. Results are presented as mean \pm SEM for normally distributed data and as median and IQR for not normally distributed results. Comparison of outcome measurements after diets was performed using paired samples t-test or Wilcoxon signed-rank test

for dependent samples, according to the normality of data distribution. p Values <0.05 were considered significant.

RESULTS

After both periods, cholesterol precursors (squalene, cholesterol, desmosterol and lathosterol as ratios to cholesterol) were interrelated (r -values from 0.517 to 0.943, $p < 0.001$ for all). Likewise, the absorption markers cholestanol, campesterol, sitosterol and avenasterol were interrelated after both interventions (r -values from 0.498 to 0.778, $p < 0.002$). The markers of synthesis absorption correlated inversely with each other after both interventions suggesting that cholesterol homeostasis was intact (eg, lathosterol–cholestanol ratio to cholesterol, CPTR0: $r = -0.498$, $p = 0.002$, and butter: $r = -0.692$, $p = 0.000$).

In addition to the different composition of fatty acids in butter and CPTR0, the amount of cholesterol and non-cholesterol sterols were different (table 1). Thus, regarding the advised daily intake of 35 mL CPTR0 and 37.5 mg butter, the intake of cholesterol was 1 mg in CPTR0 versus 71 mg in butter, and those of plant sterols 217 mg in CPTR0 versus 0.6 mg in butter. Serum cholesterol concentration was 0.38 ± 0.01 mmol/L (7.2%) lower after the CPTR0 period compared with the butter period ($p < 0.001$) (table 2).

The ratios of cholesterol synthesis markers to cholesterol did not differ between the CPTR0 and butter periods (table 2). Of the absorption markers, cholestanol and avenasterol ratios to cholesterol were similar after both periods. However, the ratios of campesterol and sitosterol to cholesterol were 39% and 16% higher after the CPTR0 period compared with those of butter ($p < 0.001$ for both). The only interference of statin treatment was that in subjects on statin, the difference in sitosterol ratios to cholesterol was non-significant between the intervention groups. The lathosterol/cholestanol ratios were similar after both periods. The concentrations of oxidized LDL did not correlate with serum non-cholesterol sterols after either treatment. Serum PCSK9 concentrations did not differ between CPTR0 and butter periods (table 2).

DISCUSSION

The main new findings in this supplementary study of our earlier randomized cross-over intervention were that in spite of significant serum cholesterol reduction by 7.2% assayed with GLC dietary period of 6–8 weeks of supplementary CPTR0 did not affect cholesterol absorption efficiency, cholesterol synthesis, or serum PCSK9 concentration when compared with the dietary period of butter in men with MetS. Because of the higher daily plant sterol intake in CPTR0 compared with butter (217 mg vs 1 mg), serum plant sterol ratios to cholesterol increased on average by 21% after the CPTR0 versus butter diets. The result is comparable to earlier rapeseed oil interventions, in which the consumption of rapeseed oil containing 360–388 mg/day of plant sterols increased their serum concentrations by 33%–37%.^{7–10}

The evaluation of absolute whole-body cholesterol metabolism is laborious both for the study participants and the laboratory. Thus, since the serum non-cholesterol sterols have turned out to reliably reflect cholesterol metabolism,^{14–16 20–24} they are generally considered

Table 2 Serum PCSK9 and cholesterol concentrations and non-cholesterol sterol ratios to cholesterol after consumption of butter and CPTRO

Variables	Butter n=37	CPTRO n=37	p Value
PCSK9, ng/mL	214.2 (191.2 to 237.2)	229.3 (197.1 to 229.3)	0.248
Cholesterol, mmol/L	5.30 (4.87 to 5.73)	4.92 (4.50 to 5.33)	<0.001
Markers of cholesterol synthesis			
Squalene*	14.9 (10.5 to 19.2)	13.7 (10.4 to 17.1)	0.792
Cholestanol*	17.0 (12.1 to 22.0)	16.5 (9.8 to 23.2)	0.850
Desmosterol*	95.2 (79.7 to 110.7)	89.0 (78.2 to 99.8)	0.792
Lathosterol*	122.6 (85.3 to 159.8)	111.0 (72.8 to 149.3)	0.645
Markers of cholesterol absorption efficiency			
Cholestanol*	138.3 (128.0 to 148.6)	138.1 (126.4 to 149.7)	0.541
Campesterol*	199.7 (150.3 to 249.1)	277.0 (224.0 to 330.0)	<0.001
Sitosterol*	97.7 (86.3 to 109.2)	113.1 (104.0 to 122.2)	<0.001
Avenasterol*	33.0 (27.7 to 38.2)	35.0 (28.9 to 41.0)	0.734
Marker of cholesterol metabolism			
Lathosterol/cholestanol, µg/ µg	0.76 (0.36 to 1.16)	0.80 (0.61 to 1.00)	0.934

Values shown are median and 95% CI of the median except mean and 95% CI of the mean for total cholesterol.

*100 x µmol/mmol of cholesterol.

CPTRO, cold-pressed turnip rapeseed oil; PCSK9, proprotein convertin subtilisin/kexin 9.

valid biomarkers of cholesterol absorption efficiency and cholesterol synthesis. In this study, cholesterol homeostasis was intact after both dietary periods (cholesterol absorption efficiency and cholesterol synthesis were interrelated) suggesting that the non-cholesterol sterols reflected cholesterol metabolism as expected in this intervention. However, we applied serum cholestanol to cholesterol ratio to evaluate cholesterol absorption efficiency instead of serum plant sterols because of the unbalanced amount of plant sterols in the diets. Under these conditions, it is likely that serum plant sterol levels primarily reflect their increased intake and not cholesterol absorption efficiency. Cholestanol is an endogenous metabolite of cholesterol and its serum concentration is practically diet unrelated so that it serves as a reliable biomarker of cholesterol absorption also under these conditions.

Rapeseed oil consumption decreases serum total and LDL cholesterol concentration,^{6–9} HDL cholesterol concentration either remains unchanged,^{6,8,25} increases,⁷ or decreases,⁹ and serum triglycerides remain unchanged^{6,8,9} or decrease^{7,25} when compared with diets rich in SFA. The cholesterol-lowering mechanisms of rapeseed oil have been studied by evaluating cholesterol metabolism and LDL apoprotein B turnover studies. Thus, rapeseed oil consumption compared with high SFA or olive oil-based diets decreased cholesterol absorption efficiency,^{7,10} increased compensatorily cholesterol synthesis^{7,8} and increased fecal cholesterol excretion¹⁰ in three out of four studies.^{7–10} The inhibition of cholesterol absorption is probably based on the high plant sterol content in rapeseed oil. In the study by Ellegård *et al*,¹⁰ the diets between rapeseed and olive oil were otherwise completely comparable including the cholesterol, fiber and energy contents and the fatty acid profiles except that the mean plant sterol concentration was 584 mg/day in the rapeseed oil diet versus 258 mg/day in the olive oil diet. This difference, about 300 mg/day of plant sterols, is large enough to alter cholesterol metabolism. In a well-controlled dietary intervention, Lin *et al*²⁶ compared serum lipids, non-cholesterol sterols and variables of

cholesterol metabolism during consumption of diets with naturally occurring plant sterols 126 or 449 mg/2000 kcal/day. Serum total, LDL and HDL cholesterol and serum triglycerides were similar between the two diets, but cholesterol absorption efficiency and serum cholestanol to cholesterol ratio were significantly lower during the 449 mg/2000 kcal diet than the 126/2000 kcal diet, and total cholesterol excreted into feces and serum lathosterol to cholesterol ratio were significantly higher. Thus, the higher plant sterol intake interfered with cholesterol absorption efficiency and increased cholesterol synthesis and neutral sterol excretion.

Regarding lipoprotein turnover studies, it is known that diets rich in unsaturated fatty acids, mainly PUFAs, increase LDL receptor activity²⁷ increasing the uptake of cholesterol into tissues, and decrease very low density lipoprotein (VLDL) and LDL production rates^{28,29} followed by diminished circulating cholesterol concentration. It can be assumed that the increased cholesterol synthesis during rapeseed oil diet also upregulates the LDL receptor activity because of their mutual cellular regulatory mechanisms.

Why was cholesterol metabolism unchanged in spite of LDL cholesterol lowering after CPTRO in this study? The similar serum PCSK9 concentrations during both diets confirmed that PCSK9 metabolism was not affected by CPTRO although serum PCSK9 concentration was lower than during a linoleic acid-enriched diet than during the SFA diet.¹³ One possible reason for the unresponsiveness of cholesterol metabolism could be the metabolic profile of cholesterol in the study population. Subjects with MetS in general have low absorption efficiency and high synthesis of cholesterol (low absorption/high synthesis).^{11,12} It is conceivable that the type of the metabolic profile may have an impact on the individual's response to changes in cholesterol absorption or cholesterol synthesis. There is evidence that subjects who have low absorption/high synthesis of cholesterol responded less effectively to cholesterol absorption inhibition with plant sterols than subjects with high absorption/low synthesis.³⁰ In fact, in addition to a smaller cholesterol reduction the changes in serum campesterol

and sitosterol were also more modest in subjects with low absorption/high synthesis than in those with high absorption/low synthesis, a finding which also could explain our negative results. In another study, subjects with high cholesterol synthesis mimicking the metabolic profile in MetS were non-responders to cholesterol absorption inhibition during plant sterol consumption.³¹ There are also controversial results, for example, in a third study no indication was found that individuals could be classified as either preferential statin responders or ezetimibe responders based on their baseline profile of cholesterol metabolism.³² However, the reduced intake of cholesterol and SFA in CPTR0 compared with butter is able to lower LDL cholesterol concentration by upregulating LDL receptor activity and downregulating VLDL and LDL production.^{27–29}

One of the potential limitations of this trial is that the study population included only men with MetS, and thus the findings cannot be generalized to represent the whole population. Second, due to the nature of the dietary intervention only the research personnel performing the laboratory measurements were blinded about the diet periods but not the subjects. Third, this study was an examination of secondary end points from samples collected in an earlier trial with different primary endpoint.

In conclusion, modifying the diet with CPTR0 instead of butter in men with MetS significantly lowered LDL cholesterol concentration, but the reduction could not be explained by changes in whole-body cholesterol or PCSK9 metabolism.

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Competing interests None declared.

Patient consent Obtained.

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