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ORIGINAL CONTRIBUTION

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Effects of dietary maritime pine seed oil on lipoprotein metabolism and atherosclerosis development in mice expressing human apolipoprotein B

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Summary *Background* Conifer seeds are used for food preparation in several countries. *Aim of the study* To assess the lipid-lowering and antiatherogenic properties of maritime pine (*Pinus pinaster*) seed oil. *Methods* The effects of maritime pine oil supplementation (20 % w/w) for 2 weeks were compared to those of coconut and sunflower oil in mice expressing human apolipoprotein B (hApoB). Atherosclerosis lesion development was measured in hApoB mice fed 1.25 % (w/w) cholesterol and 0.05 % (w/w) sodium cholate and either coconut, sunflower or maritime pine oil (20 % w/w) for 8 weeks. *Results* After 2 weeks of dietary treatment, plasma cholesterol ($p < 0.0001$), triglyceride ($p < 0.0003$), phospholipid ($p < 0.0001$) and apolipoprotein B ($p < 0.0001$) levels were lower in mice supplemented with maritime pine oil than in those treated with coconut oil. These effects were accounted for by a lowering of LDL-cholesterol, LDL-phospholipids and LDL-triglycerides, as well as a decrease in HDL-cholesterol and HDL-phospholipids. After 8 weeks of dietary treatment cholesterol and cholate, the mean area of aortic lesions was not statistically different between fat groups. *Conclusions* Feeding maritime pine oil is associated with major changes of lipid and lipoprotein levels in hApoB mice. However, in the long term, maritime pine oil has no preventive effect on cholesterol-induced aortic lesion development in hApoB mice.

Key words apolipoprotein B – atherosclerosis – lipoprotein – maritime pine oil – diet

Introduction

Conifer seeds are used for food preparation in several countries. The oils extracted from some of these seeds have substantial lipid-lowering effects in rodents [1, 2]. Among these oils, Maritime pine (*Pinus pinaster*) oil contains two particular unsaturated fatty acids: all *cis* 5,9,12–18:3 (pinolenic acid) and all *cis* 5,11,14–20:3 (sciadonic acid) [3, 4]. Sciadonic acid resemble eicosapentaenoic acid (all *cis* 5,8,11,14,17–20:5) in that it contains 20 carbon atoms and *cis* double bond in positions Δ5, Δ11 and Δ14 (Fig. 1). However, it lacks double bonds in positions Δ8 and Δ17. An earlier study in rats had shown that dietary maritime pine oil lowers triglycerides, VLDL-triglycerides and VLDL-cholesterol when compared to lard suggesting a potential interest for the treatment of dyslipidemia [5].

Further evaluation of maritime pine oil was carried out using transgenic mice with targeted modifications of lipoprotein metabolism [6, 7]. In mice expressing human apolipoprotein A-I, dietary maritime pine oil decreased HDL-cholesterol and the ability of serum to promote *in vitro* cholesterol efflux [8]. This finding was consistent with a reduction of the initial step of reverse cholesterol transport upon treatment with maritime oil.

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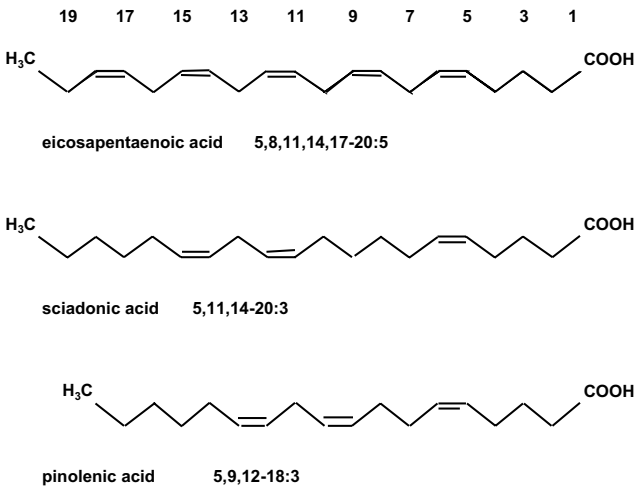

Fig. 1 Schematic representation of eicosapentaenoic, sciadonic and pinolenic fatty acids.

In the apoE deficient mice, replacement of lard by maritime pine oil was associated with a lowering of cholesterol and VLDL-cholesterol but a major increase in triglycerides and VLDL-triglycerides [9, 10]. In spite of these changes, maritime pine oil had no protective effect against atherosclerosis. Further study demonstrated that mice lacking apolipoprotein E were not well-suited to study the properties of maritime pine oil [10].

Altogether these findings led us to use the human apolipoprotein B (hApoB) transgenic mice model to test whether maritime pine seed oil could prevent atherosclerosis. Human apolipoprotein B mice develop a lipid disorder characterized by an elevation of plasma LDL concentrations [11–14]. In contrast to mice lacking apoE, whose hypercholesterolemia is secondary to a defect in VLDL clearance, LDL accumulation in the plasma of hApoB mice results from the stimulation of apolipoprotein B production followed by increased VLDL secretion. Thus, the lipoprotein profile and physiopathological mechanism of the lipid disorder is clearly different between the two transgenic strains.

Materials and methods

Animals

Studies were performed with female human apolipoprotein B mice originally created by Callow and coworkers [13] and raised in C57BL/6J background after multiple backcrosses (Transgenic Alliance, IFFA CREDO, L'Arbresle, France). Before dietary studies, the animals were acclimatized for at least 1 wk under conditions of controlled temperature (20 ± 1 °C) and lighting (dark from 20 00 h – 08 00 h) in a room of low background noise.

Dietary experiment

Before each dietary experiment, a blood sample was drawn for dietary group assignment based on human apolipoprotein B levels. Two to three mice were housed per cage and were given free access to a fat-free semi-purified chow (UAR, Villemoisson sur Orge, France) supplemented with different oils according to the experiment's design (Table 1). Coconut oil was used in the control group to reproduce the Western type diet with a high-saturated fatty acid content. The fat-free semi-purified chow contained 630 g of carbohydrate, 225 g of casein, 60 g of cellulose, 70 g of a salt mixture, and 10 g of vitamins per kg. Weight gain was monitored throughout these studies. At the end of each dietary intervention, mice were food-deprived for 4 h and exsanguinated under diethylether anesthesia by cardiac puncture. Blood samples were mixed with EDTA and kept at 4 °C.

Comparison with coconut oil and sunflower oil. Fifteen mice (5 per diet group) aged between 3 and 4 months were given the fat-free semi-purified chow supplemented with either 20 % (w/w) coconut oil (Sigma-Aldrich Chimie SARL, Saint Quentin Fallavier, France), 20 % (w/w) sunflower oil (Bertin, Lagny le Sec, France) or 20 % (w/w) maritime pine oil (D'A Noste, Vendays-Montalivet, France) for 2 weeks.

Histological study. Thirty-nine mice (13 per diet group) aged between 6 and 9 weeks were fed with the fat-free semi-purified chow supplemented with 1.25 % (w/w) cholesterol and 0.05 % (w/w) sodium cholate plus either 20 % (w/w) coconut oil, 20 % (w/w) sunflower oil

Table 1 Fatty acid composition of the experimental diets

Fatty acids (g/100g)	Coconut oil	Sunflower oil	Maritime pine oil
Saturated			
12:0	46,1	nd	nd
14:0	18,5	nd	nd
16:0	9,2	6,0	3,6
17:0	0,1	0,1	0,1
18:0	2,8	5,2	2,4
Monounsaturated			
16:1	0,1	0,3	0,2
18:1	7,0	20,4	18,1
20:1	0,2	0,8	1,0
Polyunsaturated			
9,12- 18:2	1,7	64,1	55,9
9,12,15- 18:3	nd	0,5	1,3
11,14- 20:2	nd	nd	0,8
Δ5 olefinic acids			
5,9- 18:2	nd	nd	0,7
5,9,12- 18:3	nd	nd	7,1
5,11- 20:2	nd	nd	0,8
5,11,14- 20:3	nd	nd	7,1
Others	14,3	2,6	0,9

Results are expressed in percentage of total fat. *nd* no detected

or 20% (w/w) maritime pine oil for 8 weeks. Female mice were fed cholesterol to initiate and promote atherosclerosis as suggested previously [14].

Lipid measurements and apolipoprotein B measurement

Plasma was separated by centrifugation (630 g) for 20 min at 4 °C. Lipids were determined enzymatically using commercially available kits for triglycerides (Triglycerides GPO-PAP, Boehringer Mannheim, Mannheim, Germany), cholesterol (Cholesterol C System, Boehringer Mannheim) and phospholipids (Phospholipids PAP 150, BioMérieux, Lyon, France). Human apolipoprotein B was measured by immunonephelometry using the BNA system and apoB standards (Behringwerke, Marburg, Germany).

Gel filtration chromatography

Gel filtration chromatography was performed using a Superose 6 HR 10/30 column (Pharmacia, Pharmacia LKB Biotechnology, S-751 82 Uppsala, Sweden). The gel was allowed to equilibrate with phosphate buffered saline ($10\,mmol \cdot L^{-1}$) containing $0.1\ g \cdot L^{-1}$ EDTA and $0.1\,g \cdot L^{-1}$ sodium azide; 200 µL of plasma were eluted with the buffer at room temperature at a flow rate of $0.2\,mL \cdot min^{-1}$. Elution profiles were monitored at 280 nm and recorded with an analog-recorder chart tracing system (Pharmacia, Pharmacia LKB Biotechnology). The effluents were collected in 0.24 ml fractions. Triglycerides, cholesterol and phospholipids were measured in each collected fraction using commercially available enzymatic kits (Triglycerides GPO-PAP, Cholesterol C System, Boehringer Mannheim and Phospholipids PAP 150, BioMérieux).

Histology

The hearts were sectioned just below the atria. Hearts were dipped overnight in OCT liquid (Tissue-Tek; Sakura Finetek U. S. A., Torrance, CA). The following day, the heart was placed in fresh OCT liquid on a cryostat plate (Cryostat 3050; Leïca micro-systèmes S. A., Rueil Malmaison, France) with the apex of the heart facing the plate and frozen at –25 °C. The heart was sectioned perpendicular to the axis of the aorta and working towards the apex of the heart. Each 10 µm section was mounted on gelatinized slides until the disappearance of aortic valve leaflets. Sections were air-dried overnight and rinsed briefly in 60% isopropyl alcohol. Sections were then stained with oil red O, counter stained with Harris hematoxylin and sealed with aquamount Gurrâ. A total of 10 sections was used to quantify atherosclerotic lesions. The first section used for quantification was the one that allows the identification of two leaflets on the apex side of the heart. The next section was the one located 100 µm towards the aorta and so on up to a total of 10 sections. Each section was recorded using a Nikon microscope (Diaphot, Nikon France S.A, Champigny sur Marne, France) and a color video camera (Sony CCD IRIS DXC 107 AP, SONY France, Paris, France). Color images were acquired using a PC fitted with a frame grabbing board (Snappy, Video Snapshot, HCS MISCO, Verrières le Buisson, France). Quantification of atherosclerosis lesion areas was performed using Scion Image software.

Statistical analysis

One-way and two-way ANOVA (SPSS Software release 7.5 for Windows; SPSS Institute Inc., Paris, France) was used to compare the effect of the various oils on lipids and total lesion area. Whenever the difference was statistically significant at the $p < 0.05$ levels Scheffe test was used for post hoc analyses.

Results

Lipid levels

Cholesterol ($p < 0.0001$), triglyceride ($p < 0.0003$), phospholipid ($p < 0.0001$) and apolipoprotein B ($p < 0.0001$) levels were lower in the maritime pine oil group than in the coconut oil group (Table 2). Similarly, cholesterol ($p < 0.0007$), triglyceride ($p < 0.0002$), phospholipid ($p < 0.0003$) and apolipoprotein B ($p < 0.0001$) levels were lower in the sunflower oil group than in the coconut oil group. There were no statistically significant differences in lipid and apolipoprotein B levels between the sunflower oil and the maritime pine oil group.

Table 2 Plasma lipid and human apoB levels in hApoB mice after 2 weeks of supplementation with coconut oil, sunflower oil or maritme pine oil

Oils	Coconut	Sunflower	Maritime pine	p
Cholesterol (g/l)	2.32±0.31	1.45±0.37***	1.16±0.20***	0.0002
Triglycerides (g/l)	2.04±0.24	1.32±0.22***	1.35±0.20***	0.0003
Phospholipids (g/l)	1.75±0.22	1.07±0.23***	0.89±0.19***	0.0001
Human apoB (g/l)	1.69±0.09	1.26±0.09***	1.11±0.16***	0.0001

Values are mean±SD of 5 female mice per dietary group.
One-way ANOVA was used for mean comparisons. Post hoc analysis was performed with Scheffe test: *** p<0.001 comparison to coconut oil group

Gel filtration chromatography

LDL-cholesterol, LDL-triglycerides and LDL-phospholipids were lower in both sunflower and maritime pine oil group than in the coconut oil group (Fig. 2). Similarly, HDL-cholesterol and HDL-phospholipids were lower in the sunflower and maritime pine oil group than in the coconut oil group.

Atherosclerotic lesions

Cholesterol ($p < 0.001$), triglyceride ($p < 0.001$), phospholipid ($p < 0.001$) and apolipoprotein B ($p < 0.01$) levels were lower in the maritime pine oil group than in the coconut oil group (Table 3). Similarly, cholesterol ($p < 0.05$), triglyceride ($p < 0.001$), phospholipid ($p < 0.001$) and apolipoprotein B ($p < 0.05$) levels were lower in the sunflower oil group than in the coconut oil group. In addition, cholesterol ($p < 0.002$) and apolipoprotein B ($p < 0.005$) levels were lower in the maritime pine oil group than in the sunflower oil group. LDL-cholesterol, LDL-triglycerides and LDL-phospholipids were lower in both sunflower and maritime pine oil group than in the coconut group (Fig. 3). However, this reduction appeared to be less marked than in the 2 week experiment without

Table 3 Plasma lipid and human apoB levels in mice hApoB mice after 8 weeks of supplemantation with cholesterol and cholate and either coconut oil, sunflower oil or maritme pine oil

	Coconut	Sunflower	Maritime pine	p
Cholesterol (g/l)	3.48±0.47	3.07±0.25*	2.56±0.27***	0.0001
Triglycerides (g/l)	0.82±0.22	0.45±0.06***	0.44±0.06***	0.0001
Phospholipids (g/l)	3.55±0.48	2.27±0.12***	1.81±0.19***	0.0001
Human apoB (g/l)	1.42±0.28	1.26±0.09*	1.01±0.15**	0.0001

Values are mean±SD of 13 female mice per dietary group.
One-way ANOVA was used for mean comparisons. Post hoc analysis was performed with Scheffe test: * p<0.05; ** p<0.01; *** p<0.001 comparison to coconut oil group

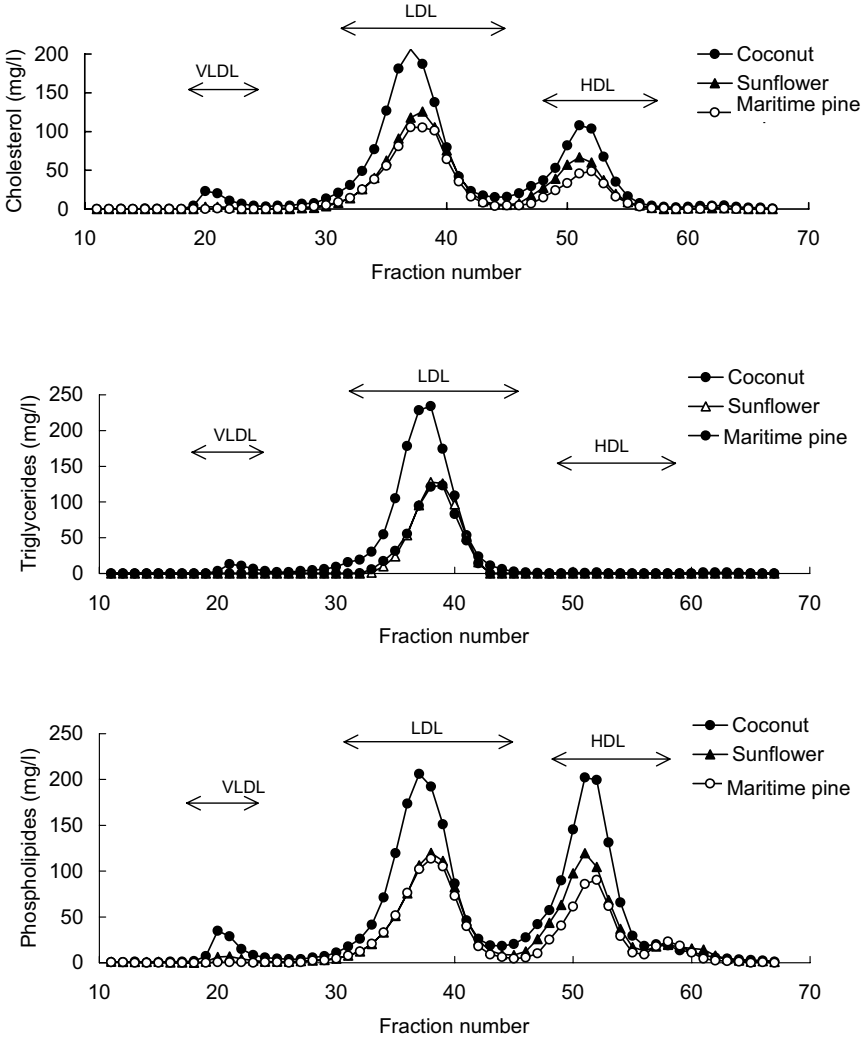

Fig. 2 Plasma cholesterol, triglyceride and phospholipid distribution of mice expressing hApoB supplemented for 2 weeks with 20% (w/w) of either coconut, sunflower or maritime pine oil. Measurements were performed in one pooled sample of 5 mice per dietary group.

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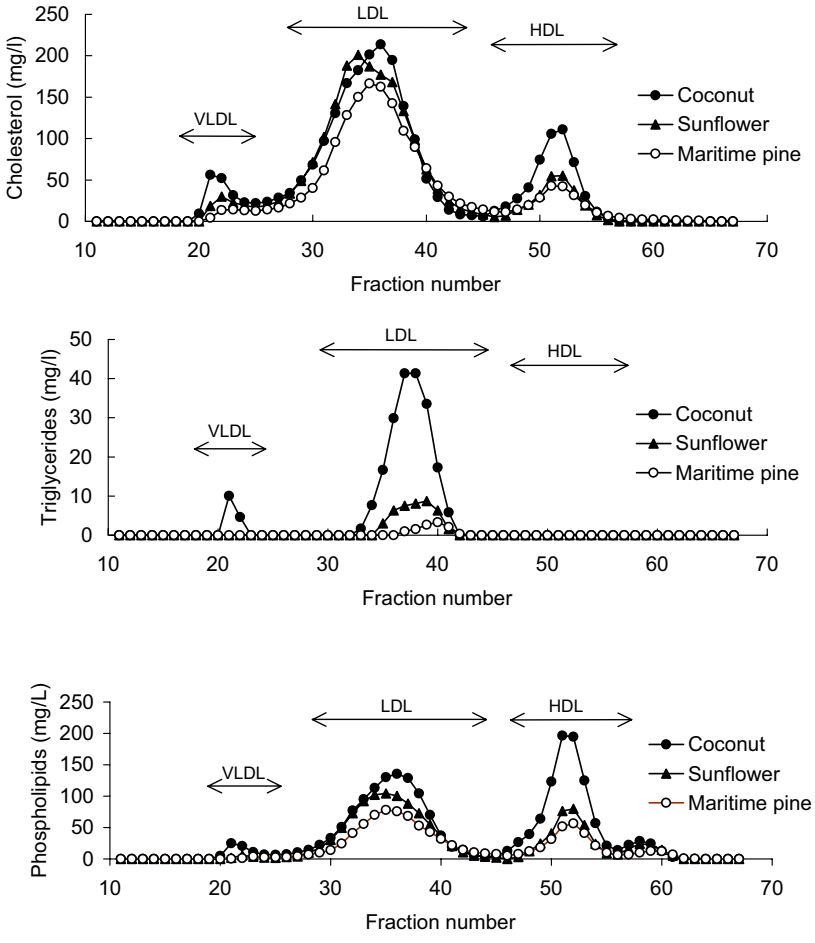

Fig. 3 Plasma cholesterol, triglyceride and phospholipid distribution of mice expressing hApoB supplemented for 8 weeks with cholesterol and cholate and either 20 % (w/w) of either coconut, sunflower or maritime pine oil. Measurements were performed in one pooled sample of 5 mice per dietary group.

cholesterol and cholate. HDL-cholesterol and HDL-phospholipids were lower in the sunflower and maritime pine oil group than in the coconut oil group.

In mice fed with coconut oil, mean lesion area increased progressively from the base of leaflets to reach a peak at about 400 µm downstream and then remained relatively constant (Fig. 4). In the sunflower oil group, the mean lesion area was higher than in the coconut oil group from the leaflet to 1000 µm downstream. In the maritime pine oil group, the mean lesion area was higher than in the coconut or sunflower oil group from about 600 µm to 1000 µm downstream. There was, however, no evidence for any statistically significant difference in the mean affected area among diets (Fig. 5).

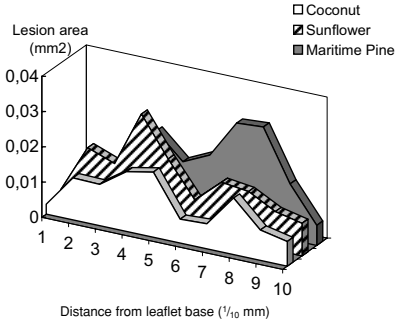

Fig. 4 Aortic lesion distribution in hApoB mice supplemented with cholesterol (1.25 % w/w) and cholate (0.05 % w/w) and 20 % (w/w) of either coconut, sunflower or maritime pine oil. Values are means of 13 mice per dietary group.

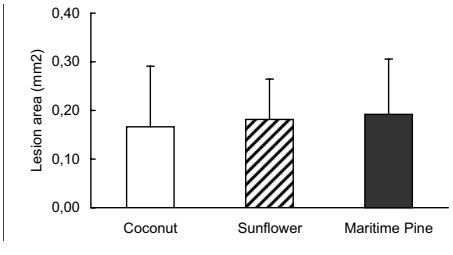

Fig. 5 Mean aortic lesion area of hApoB mice supplemented with cholesterol (1.25 % w/w) and cholate (0.05 % w/w) and 20 % (w/w) of either coconut, sunflower or maritime pine oil. Values are means of the areas under the curves of 13 mice per dietary group shown in Fig. 4.

Discussion

The goal of the present study was to assess the effect of maritime pine oil on plasma lipid levels and atherosclerosis extension in hypercholesterolemic mice whose lipid disorder results from an overproduction of human apoB [12, 14]. The results of the present study showed that consumption of maritime pine oil decreases plasma cholesterol, triglycerides and LDL-cholesterol in hApoB mice indicating a favorable effect on lipid parameters. However, despite these lipid-lowering properties, maritime pine oil could not prevent atherosclerosis induced by cholesterol and cholate feeding.

Maritime pine oil decreased plasma cholesterol, triglycerides, phospholipids and human apolipoprotein B in the hApoB mice. This effect was accounted for by a reduction in LDL and HDL levels. The effect on triglyceride levels was significant (approximately – 35 %) and was accounted for by a reduction in LDL-triglycerides which carry most of plasma triglycerides in this particular mice strain. These later observations are in marked contrast with the findings in apoE deficient mice in which maritime pine oil and fish oil increased triglycerides [9, 10]. Therefore, depending on the genetic defect, the maritime pine oil appears to produce a completely different metabolic effect in mouse triglycerides. Moreover, since dyslipidemia is the consequence of increased secretion of VLDL in the hApoB mice, these findings suggest that maritime pine oil is more efficient in lipid disorders due to lipoprotein overproduction than defective clearance.

Atherosclerosis lesion studies were performed in female rather than male mice because the latter are resistant to lesion development [14]. Moreover, feeding high cholesterol plus cholate was necessary to promote atherosclerosis in a reasonable amount of time. Therefore, in agreement with Linton et al. [14] feeding female hApoB mice induced aortic lesions including foam cells, extracellular matrix expansion and smooth muscle cell proliferation with numerous cholesterol clefts. Supplementation with maritime pine oil had no significant impact on lesion development or lesion distribution as compared to sunflower or coconut oil. This observation is consistent with our previous experiment which showed no benefit of maritime pine oil in the apoE deficient mice [9]. Similarly, Adan and coworkers failed to demonstrate an improvement in aortic lesion formation in apoE deficient mice treated with docosahexaenoic acid [15] suggesting that apoE deficient mice tend to resist to dietary intervention. In contrast, Rudel et al. showed that dietary n-3 and n-6 fatty acids reduce cholesterol accumulation in the aortic wall of transgenic mice combining LDL-receptor deficiency and human apoB expression [16]. A number of reasons could explain the lack of cardioprotection in hApoB mice supplemented with maritime pine oil. First, as suggested by gel filtration chromatography, the effect of the maritime pine oil on plasma lipoproteins might not be sufficient to prevent the formation of atherosclerotic lesions in the cholesterol-fed hApoB mice. Second, sunflower and maritime pine oil appeared to decrease the HDL fraction. The latter effect, which comes along with a reduction of cholesterol efflux capacities [8] might reduce the benefit of LDL reduction on lesion formation. Third, the addition of cholesterol and cholate to the diet could override any beneficial effect of the maritime pine oil diet. Finally, maritime pine oil may lack some beneficial effect on biochemical pathways that are important for atherosclerosis development, such as thrombosis, inflammation or vasomotricity.

In conclusion, this study shows that maritime pine oil supplementation is associated with major changes in lipid and lipoprotein levels in hApoB mice. However, maritime pine oil treatment does not prevent aortic lesion formation induced by cholesterol and cholate feeding. Therefore, further studies under different physiological conditions are necessary to assess the potential benefits of maritime pine oil and to recommend the use of this oil in human lipid disorders.

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