

Effects of emulsified policosanols with different chain lengths on cholesterol metabolism in heterozygous LDL receptor-deficient mice^S

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Abstract Policosanol is a mixture of long-chain primary aliphatic saturated alcohols. Previous studies in humans and animals have shown that these compounds improved lipoprotein profiles. However, more-recent placebo-controlled studies could not confirm these promising effects. Octacosanol (C28), the main component of sugarcane-derived policosanols, is assumed to be the bioactive component. This has, however, never been tested in an in vivo study that compared individual policosanols side by side. Here we present that neither the individual policosanols (C24, C26, C28, or C30) nor the natural policosanols mixture (all 30 mg/100 g diet) lowered serum cholesterol concentrations in LDL receptor knock-out (LDLr^{+/-}) mice. Moreover, there was no effect on gene expression profiles of LDLr, ABCA1, HMG-CoA synthase 1, and apolipoprotein A-I (apoA-I) in hepatic and small intestinal tissue of female LDLr^{+/-} mice after the 7 week intervention period. Finally, none of the individual policosanols or their respective long-chain fatty acids or aldehydes affected de novo apoA-I protein production in vitro in HepG2 and CaCo-2 cells. Therefore, we conclude that the evaluated individual policosanols, as well as the natural policosanols mixture, have no potential for reducing coronary heart disease risk through effects on serum lipoprotein concentrations.—Dullens, S. P. J., R. P. Mensink, M. C. E. Bragt, A. K. Kies, and J. Plat. **Effects of emulsified policosanols with different chain lengths on cholesterol metabolism in heterozygous LDL receptor-deficient mice.** *J. Lipid Res.* 2008. 49: 790–796.

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Policosanols are a natural mixture of long-chain primary aliphatic saturated alcohols that is isolated not only from sugarcane wax (*Saccharum officinarum* L.) but also from wheat germ, rice bran, or beeswax (1–3). The policosanols derived from sugarcane may be an effective food

ingredient or a dietary supplement to lower serum LDL-cholesterol (LDL-C) and total cholesterol (TC) concentrations in humans. More than 50 human and animal studies have demonstrated strong reductions in serum TC and LDL-C ranging between 17% to 21%, and 21% to 29%, respectively (as reviewed in Ref. 4). In addition to lowering the atherogenic LDL-C fraction, the policosanols also increased HDL-C concentrations by 8% to 15% (4). These promising effects on serum lipoprotein profiles, however, could not be confirmed in six more-recent well-controlled double-blinded human intervention studies from Europe and the USA (5–10). One possible reason for these discrepancies might be a difference in composition between the originally used sugarcane-derived policosanols mixture and the mixtures used in the later studies (11). Also, very long chain fatty acids and aldehydes with different chain lengths are present in policosanols mixtures, although in minor amounts (12). Altogether, this implies that the composition of policosanols mixtures can vary considerably, which might relate to the discrepancy in outcomes between studies. Because octacosanol (C28) is the main component of the sugarcane-derived policosanols mixture, this compound is generally assumed to be the bioactive component of the policosanols mixtures (12). This latter assumption has, however, never been tested in a study that compared individual policosanols components in vivo side by side.

We evaluated the potential LDL-C-lowering effects of the individual policosanols components in heterozygous LDL receptor knock-out mice (LDLr^{+/-}). For this, we carried out two independent studies, in which the diets were enriched with 30 mg per 100 g diet purified and emulsified tetracosanol (C24), hexacosanol (C26), octacosanol (C28), or triacontanol (C30). This policosanols diet was provided to the animals to reach a dose of

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approximately 50 mg/kg/day, which equals the doses used in previous animal studies that demonstrated effective effects of a natural policosanols mixture on cholesterol metabolism (13–15). Effects of these policosanols-enriched diets were compared with that of a diet containing an emulsion of the natural policosanols mixture Lesstanol⁶⁰ that has been reported to effectively lower LDL-C in humans (11), or with a control (empty emulsions). Because LDLr^{+/-} mice, as most rodent models, are not a suitable model for studying HDL metabolism, we evaluated these effects in vitro in hepatic (HepG2 cells) and in small intestinal (fully differentiated CaCo-2 cells) human cell models. Individual policosanols varying in chain length (C24 to C30) and their respective long-chain fatty acids (tetracosanoic acid, hexacosanoic acid, octacosanoic acid, and melissic acid) and aldehydes (tetracosanal, hexacosanal, octacosanal, and triacontanal) were tested on de novo apolipoprotein A-I (apoA-I) production and on expression levels of a panel of cholesterol metabolism-related genes.

EXPERIMENTAL PROCEDURES

Test products

Tetracosanol, hexacosanol, octacosanol, triacontanol, tetracosanoic acid, hexacosanoic acid, octacosanoic acid, and melissic acid were purchased from Sigma (St. Louis, MO), and all had a purity of >95%, as determined by gas chromatography (GC). The natural policosanols mixture Lesstanol⁶⁰ (a purity of >97% as determined by GC) was obtained from Garuda International, Inc. (Lemon Cove, CA). Tetracosanal, hexacosanal, octacosanal, and triacontanal were prepared by DSM Pharmaceutical Products (Geleen, The Netherlands). The purity of each of the four aldehyde products used was >95%, as determined using ¹H NMR analysis.

Animals and diets

Experiments involving the use of animals were performed according to protocols approved by the Maastricht University Health Sciences Animal Welfare Committee and in accordance with the guidelines established by the Dutch Council on Animal Care. LDLr^{+/-} mice were obtained by breeding LDLr^{-/-} male mice (16) with C57BL/6 female mice (Charles River Laboratories, Inc., Wilmington, MA). Seven and eight week-old female and male LDLr^{+/-} mice were used for the dietary interventions. Animals had ad libitum access to semi-synthetic diets.

The feed (pellets) contained 37 percent of energy (en%) fat with a Western-like fatty acid profile (26% palm oil, 10% coconut

oil, 23% soy oil, 13% olive oil, and 28% cacao oil), 45 en% carbohydrates (36 en% sucrose, and 9 en% corn starch), and 19 en% protein (casein), 0.25 g cholesterol, 0.25 g vitamin mixture, 0.25 g cholate, and 4.85 g mineral mixture/100 g diet (Arie Blok Diervoeding, Woerden, The Netherlands). This Western-like control diet was enriched with 0.03 g of the individual policosanols or the natural policosanols mixture Lesstanol⁶⁰ per 100 g diet, which is comparable to amounts used in previous animal studies (1, 15). In this manner, mice consumed about 50 mg policosanols per kg per day. Lesstanol⁶⁰ contained 2.6% tetracosanol (C24), 6.5% hexacosanol (C26), 61.2% octacosanol (C28), and 16.8% triacontanol (C30). Other policosanols were present in smaller amounts. The total alcohol content was 97.4%. To ensure a homogenous distribution of policosanols in the diets, they were incorporated into emulsions. These emulsions were prepared by DSM Nutritional Products (Kaiseraugst, Switzerland) and contained 3% (individual) policosanols, 1% Tween 20, 70% glycerol, and 26% water. This emulsification step resulted in a strong reduction of the policosanols particle size, from 30 μm or larger, toward a mean particle size of 399 nm, ensuring a better distribution of the policosanols within the diets (Fig. 1). Next, these policosanols emulsions were incorporated into the mouse diets, first by mixing the policosanols emulsions into the fat phase, followed by blending this phase with the solid components to obtain a well-homogenized product. The control diet was prepared by mixing an empty emulsion into the feed, i.e., an emulsion without addition of a policosanols.

Experimental procedures of the animal studies

In the first intervention study, the effects of policosanols-enriched diets, i.e., hexacosanol (C26), octacosanol (C28), triacontanol (C30), or Lesstanol⁶⁰, on serum cholesterol concentrations were compared with the control diet. After a two week run-in period, in which animals received the control diet, 48 female and 39 male mice were randomly allocated to one of the five intervention groups, as indicated in Fig. 2 (three mice per cage). For the next 7 weeks, one intervention group continued to use the control diet, whereas the other groups received one of the individual policosanols- or Lesstanol⁶⁰-enriched diets. Animals were weighed in weeks 2, 3, 5, 7, and 9 (Fig. 2). At these time points, blood was sampled by orbital puncture under anesthesia (1–2% isofluran) for analysis of serum TC concentrations. At the end of week 9, animals were euthanized under anesthesia (1–2% isofluran) by an aortic puncture. Blood was sampled to determine lipoprotein profiles, and hepatic and small intestinal tissue (starting approximately 2 cm distal from the stomach) was collected for gene expression analysis. Isolated tissues were first washed with cold phosphate buffered saline (PBS), then snap frozen in liquid nitrogen and stored at -80°C until analysis.

The aim of the second intervention study in LDLr^{+/-} mice was to confirm the results obtained from study 1. In contrast to the

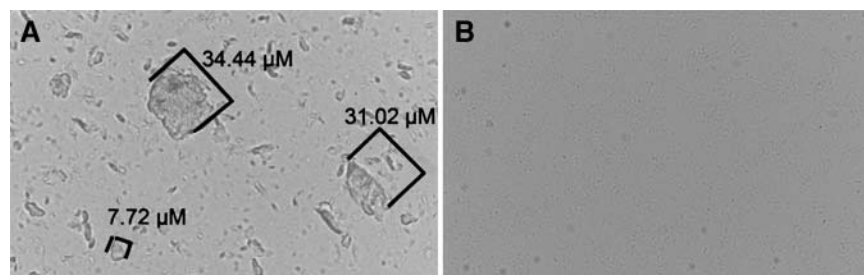


Fig. 1. Policosanols solutions before (A) and after (B) emulsification.

Design Study 1

Control diet n=87 (48 female, 39 male)	Control diet (n=17; 9 female, 8 male)				
	Control diet + Hexacosanol (n=18; 10 female, 8 male)				
	Control diet + Octacosanol (n=18; 10 female, 8 male)				
	Control diet + Triacosanol (n=18; 10 female, 8 male)				
	Control diet + Lesstanol (n=16; 9 female, 7 male)				
0	2	3	5	7	9
Weeks					

Design Study 2

Control diet n=60 30 female, 30 male)	Control diet (n=12; 6 female, 6 male)				
	Control diet + Tetracosanol (n=12; 6 female, 6 male)				
	Control diet + Hexacosanol (n=12; 6 female, 6 male)				
	Control diet + Octacosanol (n=12; 6 female, 6 male)				
	Control diet + Lesstanol (n=12; 6 female, 6 male)				
0	2	3	5	7	9
Weeks					

Fig. 2. Design of the animal studies. In study 1, 48 female and 39 male heterozygous LDLr^{+/-} mice were included, whereas 30 female and 30 male heterozygous LDLr^{+/-} mice were used in study 2. Policosanol diets were prepared using policosanol emulsions to obtain a well-homogenized product. Targeted policosanol intake was 50 mg/kg/day. Policosanol emulsions were added to the Western-like control diets (empty emulsion) containing 17.2 g/100 g (or 37 en%) fat and 0.25 g/100 g cholesterol. During the run-in period of 2 weeks, animals received the Western-like control diet. The Western-like fat blend consisted of 26% palm oil, 10% coconut oil, 23% soy oil, 13% olive oil, and 28% cacao oil. Mice were weighed and blood was sampled during the indicated weeks, and mice were euthanized at the end of week 9.

first study, triacosanol (C30) was replaced with tetracosanol (C24) because promising results on serum cholesterol concentrations in humans were described for grain sorghum wax, which contains a high proportion of tetracosanol (17). After a new breeding program, 30 female and 30 male LDLr^{+/-} mice were enrolled in a 2 week run-in period and received the control diet, containing an empty emulsion. Next, they were randomly allocated to one of five intervention groups. Again, one group (control) continued on this control diet, whereas the other intervention groups received tetracosanol-, hexacosanol-, octacosanol-, or Lesstanol⁶⁰-enriched diets. Diets were prepared as described above. Blood sampling, weighing, and other measurements were performed as indicated for study 1.

Measurements of serum lipoproteins and lipoprotein profiles

After blood sampling at weeks 2, 3, 5, 7, and 9, serum TC concentrations were directly measured enzymatically with a commercially available kit (CHOD/PAP method; Roche Diagnostics, Basel, Switzerland). At the end of week 9, serum lipoprotein profiles were determined by means of fast-protein liquid chromatography (FPLC), as described previously (18). Briefly, 50 μ l of serum (pooled mice sera from six mice out of the same diet group) was loaded on a Superose-6PC 3.2/30 column (Amersham Biosciences, Piscataway, NJ) using PBS-EDTA as a mobile phase and automatically fractionated into 36 portions of 50 μ l. TC concentrations were measured in all fractions enzymatically, as described above.

Gene expression in mice

By using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA), total RNA was isolated from liver and small intestine samples. Treatment of total RNA with RNase-free DNase (QIAGEN Benelux B.V., Venlo, The Netherlands) removed possible traces of contaminating DNA. Next, cDNA was synthesized, and real-time quantitative PCR reactions were performed as previously described (19). Taqman MGB probes (FAMTM dye-labeled) from Applied Biosystems (Foster City, CA) were used to determine mice gene expression profiles. Taqman gene expression assays of apoA-I (Mm00437569_m1), LDLr (Mm00440169_m1), ABCA1 (Mm00442646_m1), HMG-CoA synthase 1 (Mm00524111_m1), and housekeeping gene cyclophilin A (Mm02342430_g1) were

used. mRNA expression was expressed as comparative threshold cycle values as previously described (20).

Cell lines and experimental procedures

Human hepatoma cells (HepG2 cells; Mabtech, Nacka Strand, Sweden) were maintained in minimal essential medium containing 2 mM L-glutamine (Invitrogen Life Technologies), supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) nonessential amino acids, and 1% (v/v) penicillin/streptomycin, at 37°C in a humidified atmosphere of 5% CO₂/95% O₂ (v/v). Experimental procedures were performed when approximately 90% confluent monolayers were reached. For this, 500,000 cells were seeded per well in 6-well tissue culture plates. Policosanol emulsions containing purified C24, C26, C28, or C30 policosanols were added to the cells, in a dose range of 0.5, 5, or 50 μ g/ml. These concentrations were in line with those used in earlier effective in vitro studies (21). In line with the animal studies, Lesstanol⁶⁰ was also included, and the empty emulsion (without policosanols) was used as control. In addition, 10 μ M 9-*cis* retinoid acid (RA) was included as positive control, because it elevates de novo apoA-I production (22). Furthermore, the effects of fatty acids and aldehydes with chain length C24, C26, C28, or C30, incorporated into the same emulsions, were evaluated. After 3 h incubation, cell lysates were collected and used for RNA isolation, followed by gene expression analysis of the genes HMG-CoA synthase 1, LDLr, apoB, ABCA1, ACAT1, and apoA-I, essentially as described above. Human probe sets of apoA-I (Hs00163641_m1), LDLr (Hs00181192_m1), apoB (Hs00181142_m1), ABCA1 (Hs00194045_m1), ACAT-1 (Hs00608002_m1), HMG-CoA synthase 1 (Hs00266810_m1), and the housekeeping gene cyclophilin A (Hs99999904_m1) were used. To measure human apoA-I protein concentrations in culture medium of the HepG2 cells, these experiments were prolonged for 48 h. ApoA-I was measured with an apoA-I sandwich ELISA (Mabtech; Nacka Strand, Sweden). In addition to the hepatic cell model, fully differentiated CaCo-2 cells, a model for the small intestine, were also used to evaluate effects of 0.5, 5, or 50 μ g/ml Lesstanol⁶⁰ (as emulsions) on de novo apoA-I production.

Statistics

Data are presented as means \pm SEM. Differences between mean values in the different intervention groups (either from the

mice or from the cell studies) were tested by Kruskal-Wallis or Mann-Whitney U test.

RESULTS

Animal studies

In study 1, no significant differences between treatments were observed on absolute serum TC concentrations in week 9 ($P = 0.67$) and on changes in serum TC concentrations (change between weeks 2 and 9: $P = 0.82$) in female LDLr^{+/-} mice (Fig. 3). However, significant effects on serum TC concentrations were observed in week 3 with Lesstanol⁶⁰ ($P = 0.03$), in week 5 with Lesstanol⁶⁰ ($P = 0.02$), and in week 7 with triacontanol ($P = 0.04$), compared with control diet. Next, no changes in serum lipoprotein profiles were observed between the control diet and the C26-, C28-, or C30-policosanols formulations, or Lesstanol⁶⁰ (Fig. 4). See supplementary figure for comparable data of serum TC concentrations in male LDLr^{+/-} mice. Serum lipoprotein profiles in male LDLr^{+/-} mice were comparable to those in female LDLr^{+/-} mice (data not shown). Body weight development of the mice was not differently affected by treatment.

In study 2, there were no significant differences in the absolute TC concentrations at the end of week 9 ($P = 0.23$) or in changes in serum TC concentrations during the study ($P = 0.43$) between the different policosanols formulations (C24, C26, or C28), Lesstanol⁶⁰, and the control diet (Fig. 3). However, significant differences

in serum TC concentrations were only observed in tetracosanol-treated female LDLr^{+/-} mice in week 7 ($P = 0.04$), compared with the control group. As in study 1, no effects on serum lipoprotein profiles were observed (Fig. 4). Comparable results were observed in male LDLr^{+/-} mice, as shown in the supplementary figure (data of serum TC concentrations). Serum lipoprotein profiles in male LDLr^{+/-} mice were comparable to those in female LDLr^{+/-} mice (data not shown). In addition, body weight development of the mice was also not significantly affected by treatment.

Effects of policosanols-enriched Western-like diet on hepatic and small intestinal gene expression in female LDLr^{+/-} mice

Gene expression profiles of LDLr, ABCA1, HMG-CoA synthase 1, and apoA-I were determined in isolated hepatic and small intestinal tissue of female LDLr^{+/-} mice of study 2. As shown in Table 1, hepatic and small intestinal gene expression levels of LDLr, ABCA1, HMG-CoA synthase 1, and apoA-I were not affected by a 7 week dietary intervention with the different policosanols emulsions as compared with the control diet.

Effects of policosanols emulsions on de novo apoA-I production in HepG2 and differentiated CaCo-2 cells

After an incubation period of 48 h with policosanols, policosanols, or policosanoic acid emulsions (C24, C26, C28, or C30; 0.5, 5, or 50 µg/ml), or with Lesstanol⁶⁰ (0.5, 5, or 50 µg/ml), no differences in apoA-I concentra-

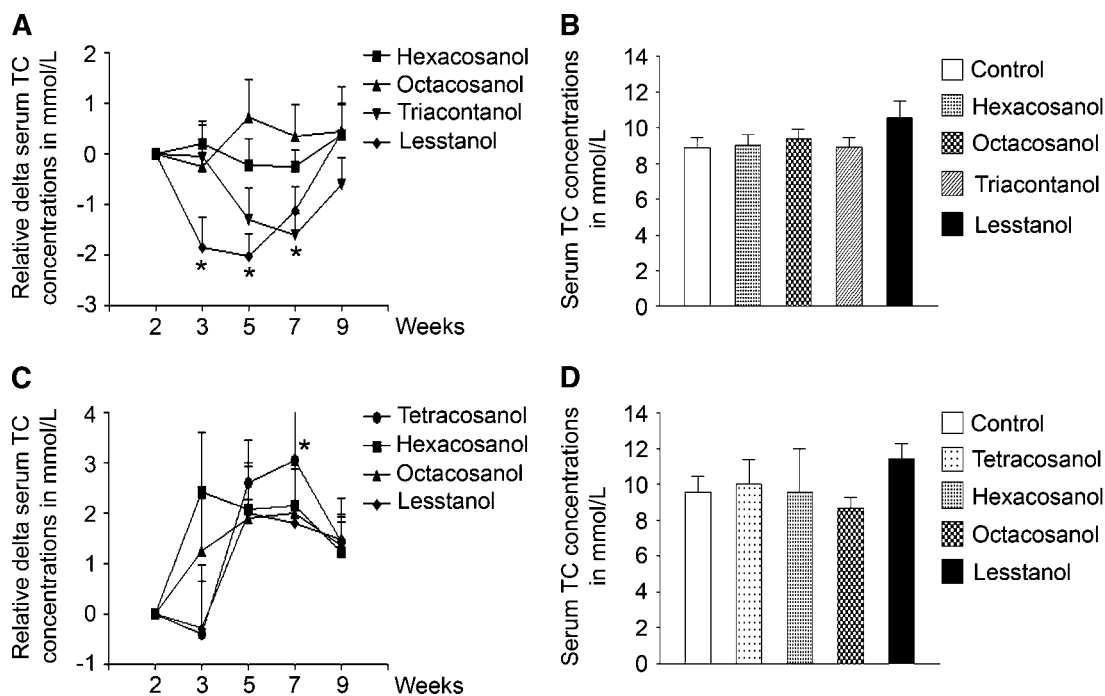


Fig. 3. Serum TC concentrations in female heterozygous LDLr^{+/-} mice as affected by different policosanols. A: Serum total cholesterol (TC) concentrations relative to the control treatment of study 1. B: Serum TC concentrations at the end of week 9 of study 1. C: Serum TC concentrations relative to the control treatment of study 2. D: Serum TC concentrations at the end of week 9 of study 2. Values are means ± SEM (N = 48 in study 1; N = 30 in study 2). * $P < 0.05$. See supplementary figure for data in male heterozygous LDLr^{+/-} mice.

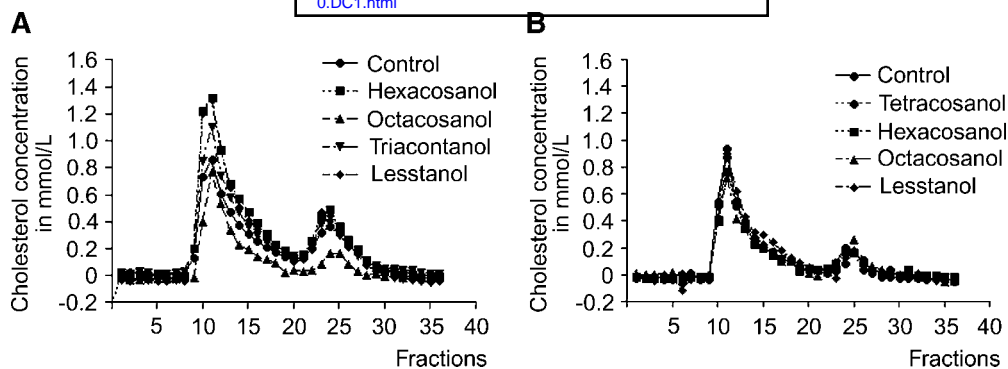


Fig. 4. Serum lipoprotein profile of female heterozygous LDLR^{+/-} mice after a 9 week dietary intervention with policosanols-enriched diets. Study 1 evaluated the effects of policosanols C26-, C28-, C30-, or Lesstanol⁶⁰-enriched diets, versus a control diet (A). Effects of C24-, C26-, C28-, or Lesstanol⁶⁰-enriched diets, versus a control diet were evaluated in study 2 (B) (N = 48 in study 1; N = 30 in study 2).

tions in cell culture medium were observed, compared with control in HepG2 cells. In **Fig. 5**, results for the 5 µg/ml treatments are shown, but results for the other concentrations were similar. De novo apoA-I production was, however, increased by the positive control, i.e., 10 µM RA (+23%, *P* = 0.03). Moderate apoA-I-lowering effects were observed for C24 and C26 policosanols, policosanals, and policosanoic acids, but these effects were not significant. The effect of Lesstanol⁶⁰ on de novo apoA-I production in fully differentiated CaCo-2 cells was comparable to that in HepG2 cells (data not shown).

Gene expression profiles of cholesterol-modulating genes in HepG2 cells

In line with the absence of an effect on apoA-I protein secretion by HepG2 cells, Lesstanol⁶⁰ did not affect apoA-I mRNA transcription, as compared with the control. Also, the gene expression of ACAT1 was not changed. Gene expressions of ABCA1, HMG-CoA synthase 1, LDLr, and apoB were, however, dose-dependently elevated by Lesstanol⁶⁰, with 5 µg/ml giving the maximal response (**Table 2**). An effect on gene expression of ABCA1 and HMG-CoA synthase 1 could also be shown for some of

the individual policosanols or their respective metabolites (**Table 2**). These data shed light on the functional component within Lesstanol⁶⁰ responsible for the observed effect on elevated ABCA1 and HMG-CoA synthase 1 gene expression. Although concentrations of the individual policosanols applied to the cells were not identical to those used in the Lesstanol⁶⁰ mixture, ABCA1 mRNA-elevating effects (comparative threshold cycle of 2.52 for 5 µg/ml Lesstanol⁶⁰) were most likely ascribed to C28 policosanols, policosanals, and policosanoic acids, whereas the elevated HMG-CoA synthase 1 mRNA levels (comparative threshold cycle of 1.80 for 5 µg/ml) were mostly associated with C30 policosanols, policosanals, and policosanoic acids (data not shown). Moreover, gene expression levels of LDLr and apoB were moderately elevated as compared with the control (comparative threshold cycle of values of 1.52 and 1.56 for 5 µg/ml, respectively).

DISCUSSION

In the past two decades, an impressive amount of data from various animal and human intervention studies has

TABLE 1. Effects of different policosanols formulations on hepatic or small intestinal gene expression in female heterozygous LDLR^{+/-} mice

Treatment	ABCA1	HMG-CoA Synthase 1	LDLr	ApoA-I
Liver				
Control	1.00 ± 0.14	1.00 ± 0.10	1.00 ± 0.17	1.00 ± 0.07
C24	0.98 ± 0.15	0.83 ± 0.19	1.39 ± 0.17	0.99 ± 0.24
C26	0.99 ± 0.16	0.96 ± 0.06	1.13 ± 0.22	0.99 ± 0.13
C28	0.80 ± 0.19	0.81 ± 0.11	1.13 ± 0.29	0.91 ± 0.19
Lesstanol ⁶⁰	0.79 ± 0.25	1.06 ± 0.19	0.66 ± 0.11	0.90 ± 0.13
Small intestine				
Control	1.00 ± 0.10	1.00 ± 0.14	1.00 ± 0.24	1.00 ± 0.09
C24	1.20 ± 0.18	1.07 ± 0.09	0.97 ± 0.13	1.10 ± 0.24
C26	1.07 ± 0.36	1.17 ± 0.14	0.90 ± 0.29	1.31 ± 0.17
C28	1.01 ± 0.31	1.02 ± 0.08	1.28 ± 0.13	1.06 ± 0.09
Lesstanol ⁶⁰	0.84 ± 0.24	1.05 ± 0.17	1.08 ± 0.22	0.89 ± 0.20

ApoA-I, apolipoprotein A-I; LDLR^{+/-}, LDL receptor knock-out. Results are expressed as means ± SEM of the comparative threshold cycle values in which the control group is set at 1. (N = 30 in total or N = 6 animals in each diet group.)

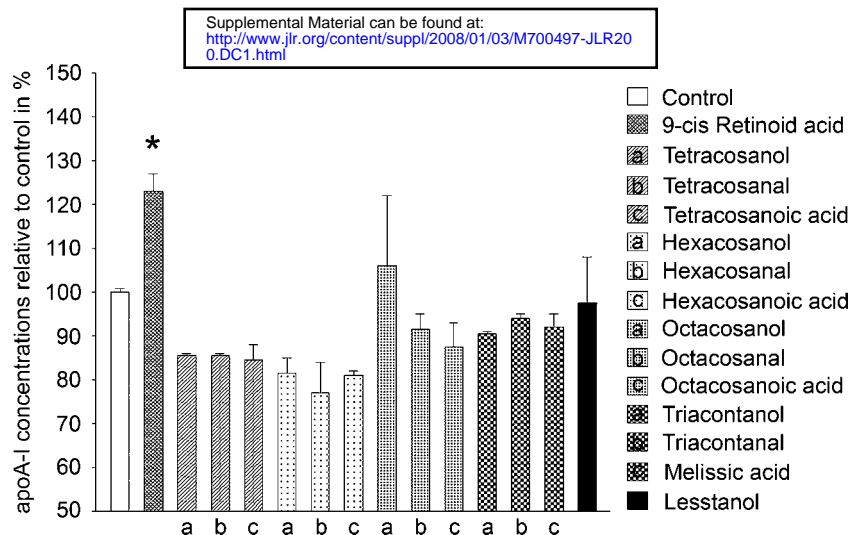


Fig. 5. Effects of 5 $\mu\text{g}/\text{ml}$ policosanols, policosanols, or policosanoic acid with chain lengths of C24, C26, C28, or C30 on de novo apolipoprotein A-I production in HepG2 cells. a = policosanols, b = policosanols, c = policosanoic acid. Values are means \pm SEM, as percentage compared with the control. * $P < 0.05$ vs. control. $N = 8$.

suggested that policosanols have strong LDL-C-lowering and HDL-C-elevating effects (4). However, all of these studies have used a natural mixture composed of policosanols varying in chain length. Also, other constituents, such as very long chain fatty acids and aldehydes, were present. Therefore, the bioactive component remained to be identified. We tested, therefore, the effects of the individual policosanols in heterozygous $\text{LDLR}^{+/-}$ mice. None of the evaluated individual policosanols, including the natural policosanols mixture Lesstanol⁶⁰, however, effectively lowered serum TC concentrations or changed the serum lipoprotein profiles. Also, human hepatic or small intestinal cell lines did not suggest that these compounds affected cholesterol metabolism. These results are in line with more-recent human intervention studies in non-Cuban populations (6–8, 23) and in hamsters (24).

In addition to lowering serum TC and LDL-C concentrations, policosanols have also been suggested to elevate serum HDL-C concentrations (4). An increase in de novo apoA-I production is one of the potential mechanisms by which serum HDL-C concentrations may be elevated. We recently showed that synthetic ligands, as well as specific dietary fatty acids, are able to elevate de novo apoA-I production in human hepatic (HepG2) cells and small intestinal cell lines (fully differentiated CaCo-2 cells) (23). However, in the same cell models, no effects of either one of the policosanols on de novo apoA-I production were

observed, whereas retinoid X receptor agonist 9-*cis* retinoid acid increased apoA-I concentrations in culture medium. This indicates that if policosanols do elevate HDL-C concentrations, this is not regulated at the level of de novo apoA-I production.

ABCA1 gene expression was strongly elevated in HepG2 cells treated with Lesstanol⁶⁰. ABCA1 plays an important role in regulating serum HDL-C concentrations. In contrast to the cell experiments, however, none of the policosanols were able to change ABCA1 gene expression in the liver or the small intestine in the in vivo experiments in $\text{LDLR}^{+/-}$ mice. In addition to differences between the models used, this may also be related to the fact that the bioavailability of these components from the gastrointestinal tract into the circulation in the animals is extremely low (1). Generally, absorption of nutrients with long-chain carbon backbones and a high degree of saturation is low (25–27). Therefore, policosanols concentrations reaching the liver in vivo are most likely lower than those used in cell culture studies. Therefore, earlier observations in rat hepatoma cells (21), showing that both triacontanol and the natural policosanols mixture, but not hexacosanol and octacosanol, lowered cholesterol biosynthesis by activating AMP-kinase but without reducing HMG-CoA reductase enzyme levels, might be of less importance for the in vivo situation. However, the finding that the inhibiting effect of triacontanol on cholesterol biosynthesis in vitro in rat hepatoma cells is most likely localized above the rate-

TABLE 2. Comparative threshold cycle values of different policosanols formulations compared with control diet in HepG2 cells

Policosanols Formulation	ABCA1	HMG-CoA Synthase 1	LDLR	apoB	ACAT1	apoA-I
Control	1.00 \pm 0.17	1.00 \pm 0.00	1.00 \pm 0.00	1.00 \pm 0.02	1.00 \pm 0.03	1.00 \pm 0.06
Lesstanol ⁶⁰ 0.5 $\mu\text{g}/\text{ml}$	2.41 \pm 0.00	1.83 \pm 0.08	1.38 \pm 0.01	1.27 \pm 0.04	1.13 \pm 0.10	1.27 \pm 0.02
Lesstanol ⁶⁰ 5 $\mu\text{g}/\text{ml}$	2.52 \pm 0.26	1.80 \pm 0.01	1.52 \pm 0.05	1.56 \pm 0.05	0.98 \pm 0.02	1.21 \pm 0.03
Lesstanol ⁶⁰ 50 $\mu\text{g}/\text{ml}$	1.92 \pm 0.08	1.69 \pm 0.04	1.31 \pm 0.06	1.58 \pm 0.02	0.91 \pm 0.03	1.21 \pm 0.07

Results are expressed as means \pm SEM of the comparative threshold cycle values, in which the control is set at 1. $N = 8$.

limiting HMG-CoA reductase step (21) is in line with our finding that HMG-CoA synthase mRNA expression was not lowered by triacontanol, triacontanal, or melissic acid in HepG2 cells.

In conclusion, in line with recent observations in human intervention studies (1, 6–8, 10), we were not able to show a reduction in serum TC or an improvement in lipoprotein profiles for any of the evaluated policosanols, nor for the natural policosanols mix Lesstanol⁶⁰ in heterozygous LDLr^{+/-} mice. Furthermore, we could not demonstrate that policosanols or one of its metabolites elevated de novo apoA-I production, one of the key players in HDL metabolism, in HepG2 or in fully differentiated CaCo-2 cells. Therefore, we conclude that the evaluated individual policosanols, as well as the natural policosanols mixture Lesstanol⁶⁰, have no potential in reducing coronary heart disease risk through effects on serum lipoprotein concentrations. **FF**

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