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*Original Contribution*

# DARK CHOCOLATE CONSUMPTION INCREASES HDL CHOLESTEROL CONCENTRATION AND CHOCOLATE FATTY ACIDS MAY INHIBIT LIPID PEROXIDATION IN HEALTHY HUMANS

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**Abstract**—Cocoa powder is rich in polyphenols and, thus, may contribute to the reduction of lipid peroxidation. Our aim was to study the effects of long-term ingestion of chocolate, with differing amounts of polyphenols, on serum lipids and lipid peroxidation ex vivo and in vivo. We conducted a 3 week clinical supplementation trial of 45 nonsmoking, healthy volunteers. Participants consumed 75 g daily of either white chocolate (white chocolate, WC group), dark chocolate (dark chocolate, DC group), or dark chocolate enriched with cocoa polyphenols (high-polyphenol chocolate, HPC group). In the DC and HPC groups, an increase in serum HDL cholesterol was observed (11.4% and 13.7%, respectively), whereas in the WC group there was a small decrease (−2.9%, $p < 0.001$). The concentration of serum LDL diene conjugates, a marker of lipid peroxidation in vivo, decreased 11.9% in all three study groups. No changes were seen in the total antioxidant capacity of plasma, in the oxidation susceptibility of serum lipids or VLDL + LDL, or in the concentration of plasma $F_2$-isoprostanes or hydroxy fatty acids. Cocoa polyphenols may increase the concentration of HDL cholesterol, whereas chocolate fatty acids may modify the fatty acid composition of LDL and make it more resistant to oxidative damage. 

**Keywords**—Chocolate, Cocoa polyphenols, Lipid peroxidation, Fatty acids, Serum lipids, HDL, Free radicals

## INTRODUCTION

Chocolate contains a variety of different compounds such as saturated fat, polyphenols, sterols, di- and triterpenes, aliphatic alcohols, and methylxanthines [1]. Cocoa, the main ingredient of chocolate, is rich in polyphenols, particularly in flavan-3-ols such as epicatechins, catechins, and procyanidins [1–6]. Polyphenols are widely distributed in vegetables, fruits, and beverages such as tea and are consumed daily by most people [2]. Evidence from epidemiological studies suggests that a high intake of dietary flavonoids, a subgroup of polyphenols, may reduce the risk of coronary heart disease (CHD) [7,8]. The antioxidant properties of flavonoids may partially account for the protective effect [9]. The oxidative modification of LDL plays an important role in atherogenesis [10–12], and agents that are able to prevent LDL oxidation in the arterial wall might delay the onset of atherosclerosis [13].

Cocoa powder and chocolate have been shown to have antioxidant potential and to inhibit LDL oxidation in vitro [14–17]. Previous studies have also shown that ingestion of a single bolus of cocoa or chocolate may increase the antioxidant capacity of plasma [17–20], decrease the formation of plasma 2-thiobarbituric acid–reactive substances (TBARS) [18,19], and inhibit LDL oxidation ex vivo [14]. Recent evidence suggests that long-term consumption of cocoa polyphenols also increases the antioxidative capacity of plasma and inhibits LDL oxidation ex vivo [20–22]. However,

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studies dealing with the effects of long-term consumption of chocolate on lipid peroxidation in vivo are scarce [22].

Because of its high saturated fat content, chocolate is often postulated to have a hypercholesterolemic effect. However, clinical trials have shown that chocolate consumption has neutral effects on serum total and LDL cholesterol [23]. This is probably due to the high content of stearic acid (~30% of fatty acids), which is considered to be neutral with respect to total and LDL cholesterol. Consumption of cocoa or dark chocolate may actually have a beneficial effect on serum lipids. In a recent study, the consumption of cocoa with dark chocolate increased the serum concentration of HDL cholesterol by 4% [20].

Chocolate consumption may have a favorable effect on lipid peroxidation ex vivo and on the serum concentration of HDL, but few long-term studies of the effects of chocolate consumption on lipid peroxidation in vivo have been published. Also, previous studies have not answered the question of whether the changes in lipid peroxidation and the concentration of HDL are due to the fatty acids of chocolate or to the cocoa mass itself. We conducted a clinical trial in which we studied the effects of long-term consumption of chocolates on serum lipids and lipid peroxidation ex vivo and in vivo. To differentiate between the effects of polyphenols and fatty acids of chocolate in inducing lipid peroxidation, the chocolates administered were identical in their fatty acid content but differed in the amount of polyphenols they contained.

## MATERIALS AND METHODS

### *Subjects*

Forty-five nonsmoking volunteer men ($n$ = 12) and women ($n$ = 33) with a mean age of 26 years (range of 19–49 years) were recruited from the Kuopio area in eastern Finland. The study was advertised in a local university student newspaper and via e-mail at the University of Kuopio. Potential participants were screened in an interview for the following inclusion criteria: (i) no severe obesity (BMI < 32 kg/m$^2$); (ii) no regular use of any drugs or supplements with antioxidative (β-carotene, vitamins C or E) or lipid-lowering properties; (iii) no chronic diseases such as diabetes, CHD, or other major illness; and (iv) willingness to consume 75 g of study chocolate daily for 3 weeks. Informed consent was obtained in writing from all participants after they had read a description of the experimental procedures. The study protocol was approved by the Research Ethics Committee, Hospital District of Northern Savo.

### *Study design*

The study was a 3 week clinical supplementation study; to ensure compliance, the study was not randomized. The subjects chose whether they would prefer to consume 75 g of white chocolate (white chocolate group, WC group), dark chocolate (dark chocolate group, DC group), or dark chocolate enriched with cocoa polyphenols (high-polyphenol chocolate, HPC group) daily. Of the 45 volunteers, 5 men and 10 women participated in the WC group, 3 men and 12 women in the DC group, and 4 men and 11 women in the HPC group. The nutrient content of the study chocolates is presented in Table 1. The daily intake of chocolate polyphenols of the WC group was less than 1 mg, of the DC group was 274 mg, and of the HPC group was 418 mg.

The chocolates administered in this study were divided into 21 one-day servings, and the subjects were instructed to consume the daily amount in three portions. Subjects were advised that the use of tea, red wine, cocoa, and chocolate other than the study chocolate be discontinued 1 week prior to the study and that this restriction should be maintained throughout the study period. Subjects were also advised to avoid the use of alcohol and analgesics for 3 days and vigorous physical activity for 1 day before visits.

A 4 day food record was collected before intervention and during the last week of the trial period to control possible confounding factors and to check compliance with the dietary instructions. The instructions for the food records were given, checked with the subjects, and analyzed by a nutritionist using Nutrica software (version 2.5; Social Insurance Institution, Helsinki, Finland).

Blood samples were drawn with Venoject vacuum tubes (Terumo, Tokyo, Japan) after an overnight fast (10 h). All measurements were taken at the baseline and after

Table 1. The Nutrient Content of Study Chocolates

| Nutrient/100 g chocolate | WC | DC | HPC |
|---|---|---|---|
| Energy (kcal) | 560 | 515 | 560 |
| Protein (g) | 7.6 | 6.0 | 7.1 |
| Carbohydrates (g) | 53.0 | 46.0 | 53.4 |
| Fat (g) | 35.0 | 33.0 | 35.7 |
| Myristic acid (14:0) (g) | 1.0 | 0.4 | 0.5 |
| Palmitic acid (16:0) (g) | 8.1 | 6.6 | 6.9 |
| Stearic acid (18:0) (g) | 8.0 | 7.0 | 7.5 |
| Oleic acid (18:1n-9) (g) | 8.8 | 7.6 | 8.0 |
| Linoleic acid (18:2n-6) (g) | 1.2 | 0.9 | 1.0 |
| Linolenic acid (18:3n-3) (g) | 0.1 | 0.1 | 0.1 |
| Total amount of catechins (mg) | 0.3 | 365.5 | 556.8 |
| Catechins (mg) | 0.0 | 25.2 | 99.2 |
| Epicatechins (mg) | 0.0 | 151.5 | 227.0 |
| Gallocatechins (mg) | 0.3 | 23.1 | 16.4 |
| Epigallocatechins (mg) | 0.0 | 3.2 | 11.9 |
| Catechin gallates (mg) | 0.0 | 7.7 | 9.4 |
| Epicatechin gallates (mg) | 0.0 | 0.5 | 0.6 |
| Epigallocatehin gallates (mg) | 0.0 | 46.2 | 43.7 |
| Procyanidins (mg) | 0.0 | 108.1 | 148.6 |

DC = dark chocolate; HPC = cocoa polyphenol–enriched dark chocolate; WC = white chocolate.

the 3 week supplementation period. The protocol of the study visits was as follows: during the first visit before the trial, subjects were given information about the study, and, if the consent form was signed, more specific instructions were given; during the following study visit, a fasting blood sample was drawn, the 4 day food record was checked in consultation with the nutritionist, instructions concerning the study were repeated, and the study chocolates were given; and, at the end of the 3 week study, a fasting blood sample was drawn and the 4 day food record was checked with the nutritionist.

*Serum lipids and lipoproteins*

Serum cholesterol (Konelab, Espoo, Finland) and triglycerides (Roche Diagnostics, Mannheim, Germany) were determined using enzymatic colorimetric tests. The serum concentration of HDL cholesterol was measured on supernatant after magnesium chloride dextran sulphate precipitation. Serum LDL cholesterol was determined by a direct cholesterol measurement (Konelab).

*Serum and LDL fatty acids*

Serum and LDL fatty acids were analyzed after extraction using chloroform-methanol and methylation with sulphuric acid–methanol. The methylated fatty acids were analyzed by a gas chromatograph (HP 5890; Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector and an NB-351 capillary column (HNU-Nordion, Helsinki, Finland) [24]. Serum LDL was isolated by precipitation with buffered heparin. The precipitate was resuspended in phosphate-buffered saline (PBS) [25]. The concentration of cholesterol was determined, and the rest of the suspension was used for measuring LDL-conjugated diene and LDL fatty acids. The fatty acids of serum and LDL are presented as percentages of the total amount of fatty acids analyzed.

*Safety measurements*

Serum aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), γ-glutamyltransferase (γ-GT) activity, and creatinine were measured using a Clinical Chemistry Analyzer (Konelab).

*Plasma total peroxyl radical–trapping potential*

Plasma total peroxyl radical–trapping potential (TRAP) was determined as previously described [26], with a modification [27].

*Resistance of serum lipids to oxidation*

The resistance of serum lipids to oxidation was measured as previously described [27]. Briefly, serum was diluted to a concentration of 0.67% in 0.02 mol/l PBS (pH 7.4). Oxidation was initiated by the addition of 100 μl of 1 mmol/l $CuCl_2$ to 2 ml of diluted, prewarmed (30°C) serum. The formation of conjugated dienes was observed by monitoring the change in the absorbance of 234 nm at 30°C on a Beckman DU-640i spectrophotometer (Beckman Instruments, Fullerton, CA, USA) equipped with a six-position automatic sample changer. The change in absorbance was recorded every 5 min for 4 h. The time required from the start to reach the maximal rate of the reaction (lag time) was determined.

*Serum LDL conjugated dienes*

The amount of conjugated dienes was assessed as previously described [28]. Lipids from the heparin-precipitated LDL were extracted with chloroform-methanol (3:1), evaporated under nitrogen, and dissolved in cyclohexane, and the amount of conjugated dienes was measured by spectrophotometer at 234 and 300 nm. Absorbance at 300 nm was subtracted from that at 234 nm. The conjugated diene concentration was expressed in relation to cholesterol concentration in LDL.

*Plasma hydroxy fatty acids*

Plasma $C_{18}$ hydroxy fatty acids were measured using a gas chromatograph/mass spectrometer (Agilent Technologies, Espoo, Finland) [29]. Plasma fatty acids and fatty acid hydroperoxides were stabilized by hydrogenation using platinum as a catalyst, saponified, esterified by diazomethane, and, finally, to separate hydroxy fatty acids from fatty acids, extracted by solid-phase minicolumns. Prior to the analysis, hydroxy groups were methylated with tetramethylammonium hydroxide. Concentrations of different (methoxy) monohydroxy fatty acid (OHFA) methyl esters were determined by electron impact mass spectrometer. $C_{17}$ and $C_{19}$ OHFAs were used as internal standards.

*Plasma $F_2$-isoprostanes*

A deuterated prostaglandin $F_{2\alpha}$ internal standard was added to the plasma, and $F_2$-isoprostanes were extracted using $C_{18}$ and silica minicolumns. The compounds were converted to pentafluorobenzyl ester trimethylsilyl ether derivates and analyzed by a gas chromatographic/mass spectrometric assay (Agilent Technologies) [30].

*Catechin and procyanidin content of the study chocolates*

Chocolate contains a variety of different polyphenols of which catechins have been studied most intensively in connection with the possible health effects of chocolate. Catechins occur in nature as aglycones but can polymerize into oligomeric structures. In chocolate, catechins occur mainly as monomers [3,4,31] and in oligomeric forms such as di-, tri, and tetramers [32]. Catechins are labile and reactive compounds that are easily decomposed at high temperature and in highly acidic or basic conditions; therefore, we tested different extraction conditions. The

starting point was a previously published method [33], in which catechins were extracted from the matrix in 1 h with 70–90% methanol (MeOH) at room temperature or in 30 min with 90% MeOH at 90°C, depending on the type of the sample. We compared the extraction conditions using different chocolates, cocoa powders or extracts, and pure compounds. Catechins included in the tests were (+)-catechin, (−)-catechingallate, (−)-gallocatechin, (−)-epicatechin, (−)-epicatechingallate, (−)-epigallocatechin, and (−)-epigallocatechingallate.

Test temperatures ranged from room temperature to 90°C and extraction times varied from 30 min to 2 h. Tested extraction solvents were 30–100% methanol or acetonitrile with 0 to 1 M hydrochloric acid (HCl). Catechins are more stable in mild acidic conditions and, therefore, the effects of HCl were tested [31]. The results of the different combinations of these variables were compared, and the best conditions to extract catechins present in chocolate and cocoa powder or extract were found to be 2 h extraction at 50°C with 50% MeOH containing 0.1 M HCl. Under these conditions, pure compounds were not decomposed and the highest values for the amount of quantified catechins in the sample extracts were obtained. Crucial for the extraction efficiency of catechins in chocolate was the temperature. Extraction efficiency increased significantly when the temperature was high enough to melt the chocolate chips in the vial. Detailed results of the comparison are not shown.

Catechin analyses were carried out using high-performance liquid chromatography (HPLC) with a coulometric electrode array detector (ESA Inc., Chelmsford, MA., USA). The detector consists of eight measuring electrode pairs divided into two separate cells. Detection potentials can be set separately for each electrode pair. Potentials ranged from 100 to 700 mV and quantification signals for each catechin were obtained from channel 5 with potential 520 mV. The analytical column was Inertsil C18, 150 × 3 mm, packed with 3 μm end-capped particles (GL Sciences, Tokyo, Japan), and the precolumn was 10 × 3 mm, packed with a similar material of 5 μm particles without end-capping. The mobile phase consisted of two eluents: (i) 50 mM phosphate buffer (pH 2.3), MeOH 90:10 (v/v); and (ii) 50 mM phosphate buffer (pH 2.3), MeOH: ACN 40:40:20 (v/v/v). The analytes were separated with gradient elution in 40 min. The flow rate was 0.3 ml/min and the mobile phase contained 20% eluent B at the beginning.

Three different procyanidin dimers were analyzed using the same HPLC method as for catechin monomers. Available dimers were epicatechin-(4β → 8)-catechin (B1), epicatechin-(4β → 8)-epicatechin (B2), and catechin-(4α → 8)-epicatechin (B4), of which B2 in particular is abundant in chocolate.

An amount of 10–30 mg of chocolate chips, scraped from the piece of chocolate, was weighed into the extraction vials; 2.5 ml of 50% MeOH containing 0.1 M HCl was added and the samples were incubated for 2 h at 50°C. Then, the samples were centrifuged for 10 min at 2400 rpm, supernatants were drawn into volumetric flasks, and the flasks were filled with 50% MeOH. The samples were diluted with the mobile phase before the HPLC run, and seven catechins and three procyanidins were measured. The samples were analyzed in triplicate. White chocolate contained no significant amounts of catechins. Dark chocolate contained a total of 2.6 mg/g of catechin monomers, of which epicatechin was the major compound, representing almost 60% of total catechins. Dark chocolate with additional cocoa extract contained a total of 4.1 mg/g of catechin monomers. The cocoa extract increased the amount of epicatechin and especially the amount of catechin (4-fold) in the chocolate (Table 1). In the dark chocolate used in the study, procyanidin B2 was 1.1 mg/g (108.1 mg/100 g), and in the cocoa polyphenol–enriched chocolate, it was 1.5 mg/g (148.6 mg/100 g). Traces of procyanidin B4 were detected in the cocoa polyphenol–enriched chocolate.

### *Statistics*

The results are expressed as means (± standard deviations). Differences between baseline and end-point values within the pooled groups were tested by paired *t*-test. Means were compared across the study groups by the analysis of variance (ANOVA), and post hoc Tukey's test was used whenever a statistically significant heterogeneity between groups was shown by the ANOVA. Differences with $p$ values of 0.05 or less were considered significant. Simple correlation and stepwise linear regression analysis were used to estimate the contribution of changes in the formation of conjugated dienes. SPSS software (version 10.0; SPSS, Inc., Chicago, IL, USA) was used for the statistical analyses.

## RESULTS

All 45 recruited volunteers completed the study, and no adverse effects were reported by the study subjects or found in the results of safety analyses ASAT, ALAT, or γ-GT (Table 4). Mean weight decreased during the study in the WC group (−1.1 ± 2.7 kg) and increased slightly in the DC (0.4 ± 0.7 kg) and the HPC groups (0.8 ± 0.9 kg). During the study, the total energy intake and the proportions of fat and saturated fat in the diet increased, whereas the proportions of protein and carbohydrates decreased. These calculations of nutrition intake included the study chocolates. The intake of nutrients did not differ among the groups during the study (Table 2).

Table 2. Estimated Nutrient Intake at Baseline and at the End of the Supplementation Period Based on 4 Day Food Recording

| | WC ($n$ = 15) | | DC ($n$ = 15) | | HPC ($n$ = 15) | | |
|---|---|---|---|---|---|---|---|
| Nutrient intake/day | Baseline | Change | Baseline | Change | Baseline | Change | $p$ |
| Energy (MJ/d)* | 7.9 ± 1.4 | 1.8 ± 1.2 | 7.9 ± 1.6 | 1.3 ± 1.5 | 8.1 ± 1.5 | 1.9 ± 2.6 | 0.666 |
| Protein (E%)* | 15.3 ± 2.7 | −2.3 ± 2.1 | 15.2 ± 2.8 | −2.0 ± 2.0 | 16.1 ± 2.5 | −2.5 ± 1.9 | 0.792 |
| Carbohydrates (E%)* | 52.8 ± 4.3 | −4.1 ± 4.3 | 51.7 ± 7.3 | −5.1 ± 5.4 | 51.5 ± 9.4 | −3.9 ± 5.8 | 0.781 |
| Total fat (E%)[a,*] | 30.5 ± 4.4 | 5.9 ± 4.2 | 32.8 ± 6.0 | 5.3 ± 4.8 | 31.4 ± 7.3 | 6.0 ± 6.6 | 0.924 |
| SAFAs (E%)* | 12.2 ± 2.4 | 3.6 ± 2.5 | 13.9 ± 3.2 | 2.1 ± 2.4 | 11.9 ± 2.4 | 2.8 ± 2.6 | 0.304 |
| MUFAs (E%) | 10.1 ± 1.6 | 0.6 ± 1.7 | 10.4 ± 2.2 | 0.5 ± 2.0 | 11.0 ± 3.5 | −0.6 ± 3.0 | 0.305 |
| PUFAs (E%)[†] | 5.3 ± 1.7 | −0.9 ± 1.8 | 4.7 ± 1.3 | −0.6 ± 1.2 | 5.4 ± 2.0 | −0.9 ± 2.2 | 0.850 |
| Fiber (g)* | 19.9 ± 4.9 | 3.8 ± 4.2 | 24.4 ± 11.0 | 2.5 ± 5.7 | 23.5 ± 10.0 | 3.4 ± 5.8 | 0.787 |
| Vitamin E (mg) | 8.6 ± 2.2 | 0.3 ± 2.8 | 8.7 ± 3.3 | −0.4 ± 1.9 | 9.3 ± 3.6 | −0.9 ± 3.3 | 0.509 |
| Vitamin C (mg) | 116 ± 79 | 13.4 ± 72.1 | 143 ± 63 | −42.7 ± 76.9 | 154 ± 122 | −8.0 ± 120.8 | 0.257 |
| Beta-carotene (μg) | 3136 ± 1985 | −1109 ± 2731 | 2381 ± 1494 | 143 ± 1995 | 3069 ± 2082 | −407 ± 1566 | 0.290 |
| Folate (μg) | 247 ± 73 | 0.4 ± 62.7 | 272 ± 70 | −35.4 ± 70.6 | 265 ± 94 | −1.7 ± 79.5 | 0.312 |

Data expressed as means ± SD; $p$ for the differences in changes between the groups (one-way ANOVA); DC = dark chocolate; HPC = cocoa polyphenol–enriched dark chocolate; WC = white chocolate.

[a] Percentage of total daily energy intake.

* Significant differences between baseline and end-point values within the pooled groups, $p < 0.001$ (paired $t$-test).

[†] Significant differences between baseline and end-point values within the pooled groups, $p < 0.05$ (paired $t$-test).

Overall, the compliance with the nutritional instructions and restrictions given was good, as none of the subjects reported consumption of the restricted foods (tea, red wine, cocoa, and chocolate other than the study chocolate).

The supplementation period increased the proportions of serum stearic acid (18:0) and linoleic acid (18:2n-6), while the proportions of serum myristic acid (14:0), palmitic acid (16:0), and α-linolenic acid (18:2n-3) decreased (Table 3). The change in the proportion of myristic acid in the serum was significantly different between the WC and HPC groups, whereas, for the other fatty acids of serum, no differences between study groups were observed.

In the LDL cholesterol, the proportion of stearic acid increased and the proportions of myristic acid, palmitic acid, α-linolenic acid, and arachidonic acid decreased (Table 3). The change in the proportion of myristic acid in the LDL was significantly different between the DC and HPC groups, and the change in the proportion of arachidonic acid was statistically different between the WC and HPC groups.

Table 3. Percentage Proportion of Individual Fatty Acids in Total Fatty Acid Content of Serum and LDL Before and After Supplementation

| | WC ($n$ = 15) | | DC ($n$ = 15) | | HPC ($n$ = 15) | | |
|---|---|---|---|---|---|---|---|
| Fatty acid | Baseline | Change | Baseline | Change | Baseline | Change | $p$ |
| Serum fatty acids | | | | | | | |
| Myristic acid (14:0)[†] | 1.8 ± 0.7 | −0.4 ± 0.7 | 1.7 ± 0.5 | −0.4 ± 0.4 | 1.4 ± 0.3 | −0.0 ± 0.4 | 0.037 |
| Palmitic acid (16:0)[†] | 26.2 ± 1.4 | −0.9 ± 1.3 | 25.4 ± 2.6 | −0.8 ± 1.1 | 25.0 ± 1.1 | 0.0 ± 1.4 | 0.103 |
| Stearic acid (18:0)[†] | 6.5 ± 0.5 | 0.8 ± 0.6 | 6.7 ± 0.7 | 0.7 ± 0.8 | 6.6 ± 0.6 | 0.8 ± 0.5 | 0.631 |
| Oleic acid (18:1n-9) | 22.7 ± 2.0 | 0.0 ± 1.9 | 22.2 ± 2.3 | −0.5 ± 2.1 | 21.8 ± 2.3 | 0.0 ± 2.1 | 0.762 |
| Linoleic acid (18:2n-6)[†] | 25.5 ± 2.1 | 1.3 ± 2.0 | 27.0 ± 4.0 | 1.5 ± 2.2 | 27.7 ± 2.3 | 0.3 ± 2.8 | 0.307 |
| Alpha-linolenic acid (18:2n-3)* | 1.1 ± 0.4 | −0.1 ± 0.3 | 1.1 ± 0.3 | −0.2 ± 0.3 | 1.0 ± 0.2 | −0.1 ± 0.3 | 0.550 |
| Arachidonic acid (20:4n-6) | 5.9 ± 1.1 | −0.0 ± 0.7 | 5.5 ± 0.9 | −0.0 ± 0.4 | 5.7 ± 1.1 | −0.5 ± 0.6 | 0.061 |
| LDL fatty acids | | | | | | | |
| Myristic acid (14:0)[†] | 2.1 ± 0.7 | −0.4 ± 0.7 | 2.4 ± 1.1 | −0.6 ± 0.6 | 1.9 ± 0.7 | 0.1 ± 0.4 | 0.016 |
| Palmitic acid (16:0)* | 24.5 ± 1.4 | −0.8 ± 1.9 | 24.3 ± 2.3 | −0.9 ± 1.8 | 23.0 ± 1.3 | 0.3 ± 1.7 | 0.156 |
| Stearic acid (18:0)[†] | 4.9 ± 0.6 | 0.7 ± 0.6 | 5.0 ± 0.6 | 0.6 ± 0.5 | 4.9 ± 0.6 | 0.8 ± 0.7 | 0.735 |
| Oleic acid (18:1n-9) | 24.9 ± 2.2 | −0.2 ± 2.0 | 24.0 ± 3.0 | −0.1 ± 2.4 | 23.5 ± 2.2 | 0.6 ± 2.0 | 0.551 |
| Linoleic acid (18:2n-6) | 27.5 ± 2.7 | 1.4 ± 2.9 | 28.3 ± 4.4 | 1.8 ± 2.6 | 30.2 ± 2.6 | −0.4 ± 3.2 | 0.105 |
| Alpha-linolenic acid (18:2n-3)* | 1.1 ± 0.3 | −0.1 ± 0.3 | 1.2 ± 0.3 | −0.1 ± 0.3 | 1.2 ± 0.3 | −0.1 ± 0.4 | 0.921 |
| Arachidonic acid (20:4n-6)[†] | 5.0 ± 0.9 | 0.0 ± 0.3 | 4.6 ± 0.9 | −0.1 ± 0.3 | 4.9 ± 1.1 | −0.1 ± 0.4 | 0.034 |

Data expressed as means ± SD; $p$ for the differences in changes between the groups (one-way ANOVA); fatty acids of serum and LDL are presented as percentage proportion of total amount of fatty acids analyzed; DC = dark chocolate; HPC = cocoa polyphenol–enriched dark chocolate; WC = white chocolate.

* Significant differences between baseline and end-point values within the pooled groups, $p < 0.05$ (paired $t$-test).

[†] Significant differences between baseline and end-point values within the pooled groups, $p < 0.01$ (paired $t$-test).

Within the DC and HPC groups, increases in serum HDL cholesterol concentration were observed (11.4% and 13.7%, respectively), while a slight decrease was seen in the WC group (−2.9%). In the post hoc analysis, the change differed significantly between the WC and DC groups ($p = 0.001$) and the WC and HPC groups ($p < 0.001$). The ratio of LDL/HDL cholesterol increased in the WC group (0.17 ± 0.42) and decreased in the DC and HPC groups (−0.19 ± 0.33 and −0.20 ± 0.36, respectively). The change in the LDL/HDL ratio was significantly different between the WC and DC groups ($p = 0.031$) and the WC and HPC groups ($p = 0.024$) in the post hoc analysis. The consumption of the study chocolates did not significantly alter serum total or LDL cholesterol or triglyceride concentrations, and no differences were found among the study groups (Table 4).

The consumption of chocolates decreased lipid peroxidation as measured by the formation of conjugated dienes. The amount of conjugated dienes decreased significantly in all three study groups by a mean 11.9% ($p < 0.001$) with no differences among the study groups. To assess the main determinants of the change in conjugated dienes, all three groups were combined for stepwise linear regression analysis. The change in the oleic acid concentration of LDL explained 56.0% of the change in the conjugated dienes. The changes in the other fatty acids of LDL made no significant contribution. No significant differences in changes among the study groups were found with respect to the concentration of plasma TRAP or the other markers of lipid peroxidation, i.e., oxidation susceptibility of serum lipids, plasma hydroxy fatty acids, and $F_2$-isoprostanes.

## DISCUSSION

The concentration of serum HDL cholesterol and the oxidative modification of LDL play important roles in the pathogenesis of atherosclerosis [10–12,34]. Previous studies have suggested that consumption of cocoa or chocolate may have beneficial effects on both of these factors in humans. Consumption of cocoa and dark chocolate have increased the concentration of HDL cholesterol [20] and plasma antioxidant capacity [17–20], decreased the formation of lipid oxidation products (TBARS) [18,19], and inhibited the oxidation of LDL ex vivo [14,20–22]. The main objective of this clinical trial was to study the effects of long-term consumption of chocolate on serum lipids and their oxidation ex vivo and in vivo. In contrast with previous studies, the chocolates administered differed in the amounts of cocoa but not in the amounts and profiles of the fatty acids. The purpose of this design was to differentiate the effects of polyphenols from those of fatty acids.

In the present study, the long-term ingestion of chocolate unexpectedly affected the fatty acid content of serum and LDL. Two saturated fatty acids (palmitic and stearic acids) and one monounsaturated fatty acid (oleic acid) accounted for over 90% of the total fatty acids in the study chocolates (Table 1). Interestingly, only the proportion of stearic acid increased, while the

Table 4. Body Mass Index, Concentrations of Serum ASAT, ALAT, γ-GT, Creatinine, Serum Lipoproteins, and Oxidation Kinetics Before and After Consumption of Study Chocolates for 3 Weeks

| | WC ($n = 15$) | | DC ($n = 15$) | | HPC ($n = 15$) | | |
|---|---|---|---|---|---|---|---|
| Parameter | Baseline | Change | Baseline | Change | Baseline | Change | $p$ |
| BMI (kg/m$^2$) | 22.3 ± 2.3 | −0.4 ± 1.0 | 21.5 ± 2.9 | 0.1 ± 0.2 | 24.1 ± 3.5 | 0.3 ± 0.3 | 0.012 |
| Serum ASAT (U/l) | 23 ± 7 | −2 ± 7 | 22 ± 8 | 2 ± 12 | 20 ± 6 | 4 ± 9 | 0.230 |
| Serum ALAT (U/l) | 24 ± 13 | −1 ± 15 | 17 ± 8 | −2 ± 7 | 16 ± 9 | 4 ± 18 | 0.478 |
| Serum γ-GT (U/l) | 15 ± 7 | 0 ± 5 | 14 ± 6 | −0 ± 3 | 18 ± 11 | 1 ± 7 | 0.741 |
| Serum creatinine (μmol/l) | 85 ± 11 | 0 ± 9 | 79 ± 7 | 1 ± 6 | 84 ± 11 | 2 ± 7 | 0.842 |
| Serum total cholesterol (mmol/l) | 5.21 ± 0.72 | −0.02 ± 0.51 | 4.74 ± 0.90 | 0.08 ± 0.49 | 4.99 ± 1.01 | 0.12 ± 0.47 | 0.710 |
| Serum LDL cholesterol (mmol/l) | 2.80 ± 0.57 | 0.17 ± 0.60 | 2.57 ± 0.68 | 0.00 ± 0.37 | 2.82 ± 0.62 | 0.00 ± 0.39 | 0.627 |
| Serum HDL cholesterol (mmol/l) | 1.49 ± 0.32 | −0.00 ± 0.14 | 1.41 ± 0.38 | 0.14 ± 0.15 | 1.38 ± 0.29 | 0.18 ± 0.12 | <0.001 |
| Serum LDL/HDL cholesterol ratio | 1.98 ± 0.66 | 0.17 ± 0.42 | 1.96 ± 0.89 | −0.19 ± 0.33 | 2.08 ± 0.53 | −0.20 ± 0.36 | 0.013 |
| Serum triglyceride (mmol/l) | 1.45 ± 0.74 | −0.15 ± 0.59 | 1.12 ± 0.55 | −0.21 ± 0.46 | 0.95 ± 0.35 | 0.00 ± 0.49 | 0.336 |
| Plasma TRAP (μmol/l) | 1057 ± 206 | 22 ± 134 | 973 ± 176 | 85.1 ± 250 | 1155 ± 170 | 91.5 ± 229 | 0.657 |
| Serum lipid oxidation resistance (lag time, min) | 118 ± 43 | 6 ± 31 (14) | 122 ± 35 | 4 ± 32 (14) | 160 ± 61 | −3 ± 28 (14) | 0.711 |
| Serum LDL conjugated dienes (μmol/mmol chol)* | 16.3 ± 3.1 | −4.0 ± 6.9 | 16.7 ± 2.6 | −5.9 ± 7.6 | 15.5 ± 2.7 | −4.6 ± 5.7 | 0.496 |
| Plasma $F_2$-isoprostanes (pg/ml) | 43.4 ± 13.8 | −2.5 ± 7.9 | 48.7 ± 22.0 | −5.3 ± 15.9 | 45.4 ± 11.2 | −0.9 ± 8.1 | 0.554 |
| Plasma hydroxy fatty acids (μmol/l) | 1.02 ± 0.47 | −0.02 ± 0.41 | 1.04 ± 0.48 | −0.05 ± 0.48 | 1.08 ± 0.46 | −0.04 ± 0.32 | 0.987 |

Data expressed as means ± SD; $p$ for the differences in changes between the groups (one-way ANOVA); number of subjects in parentheses except where otherwise indicated; DC = dark chocolate; HPC = cocoa polyphenol–enriched dark chocolate; WC = white chocolate.

* Significant differences between baseline and end-point values within the pooled groups, $p < 0.001$ (paired $t$-test).

proportion of palmitic acid decreased in both serum and LDL.

Palmitic acid is a precursor of other long-chain saturated and unsaturated fatty acids, mainly stearic and oleic acids. Moreover, oleic acid is used in chain elongation reactions as the precursor of other monounsaturated fatty acids. To the best of our knowledge, there are no previous studies reporting the effects of chocolate consumption on the fatty acid content of serum or LDL. However, we speculate that increase in the intake of saturated and monounsaturated fat stimulated the chain elongation reactions of monounsaturated fatty acids. Monounsaturated fatty acids other than oleic acid were not measured in the serum and LDL. On the other hand, it is widely known that there is an inverse relationship between hepatic lipogenesis and the concentration of serum free fatty acids [35]. Thus, increased dietary intake can suppress the hepatic synthesis of fatty acids, which might lead to the present observation that chocolate fatty acids do not necessarily elevate the corresponding plasma concentrations in a long run.

The main finding of this study was that the concentration of HDL cholesterol increased in both groups receiving chocolate that contained cocoa mass. The increase in HDL cholesterol was 11% after the consumption of dark chocolate and 14% after the consumption of dark chocolate enriched with cocoa polyphenols, whereas no effect was observed after the consumption of white chocolate. The ratio of LDL/HDL also changed in a similar manner. Because the fatty acid content in the study chocolates was identical, the compounds in the cocoa mass were responsible for the increase in HDL cholesterol. Cocoa contains a variety of different compound such as polyphenols (flavan-3-ols, flavonols), sterols, di- and triterpenes, aliphatic alcohols, and methylxanthines [1]. It is difficult to determine precisely which compounds present in cocoa would affect the concentration of HDL. It is likely, however, that compounds other than cocoa polyphenols are responsible for the increase, because the results from previous flavonoid supplementation studies lend no support to the view that ingestion of polyphenols would increase the concentration of HDL.

Our data concerning the HDL are supported by a recently reported, long-term, crossover study. Wan and colleagues [20] found that after daily consumption of 22 g of cocoa powder and 16 g of dark chocolate for 4 weeks, the concentration of HDL cholesterol was 4% higher compared to the control diet. The higher amount ingested (75 g or the equivalent of two candy bars) in the present study might explain the greater increase in the HDL cholesterol (11–14%).

A high concentration of HDL cholesterol has been shown to decrease the risk of cardiovascular diseases [36]. The concentration of HDL cholesterol can usually be increased by 10–15% by changing lifestyle behavior, but this strategy is not suitable for everyone, as, for example, vigorous exercise or moderate alcohol consumption are usually needed to significantly increase the HDL [34]. Consequently, the aim of future studies should be to identify the HDL-increasing compound in cocoa.

The second finding of this study was that the consumption of chocolate inhibited significantly the oxidation of LDL in vivo, as measured in the formation of conjugated dienes. It seems that this result was real since there were no methodological shifts in the measurement of conjugated dienes between the study visits. This was confirmed by the use of the same, well-characterized control sample in all the batches measured. Also, owing to the relatively short intervention period, the possibility of seasonal variation can be largely excluded. The decrease in LDL peroxidation in all three study groups indicated the likelihood of this effect being due to the fatty acids in chocolate. It has previously been reported that, compared to polyunsaturated fatty acids, monounsaturated fatty acids inhibit lipid peroxidation [37–39]. A high consumption of saturated or monounsaturated fat in the form of chocolate may modify the lipid content of LDL to make it more resistant to oxidation by increasing the amount of monounsaturated and saturated fats, and by decreasing the amount of polyunsaturated fatty acids.

Although we found that chocolate consumption had an effect on the LDL conjugated dienes in vivo, no effect was detected on the other markers of lipid peroxidation. The formation of conjugated dienes is the first step in the process leading to the formation of oxidized fatty acids [40]. It is possible that the first antioxidative effects can be assessed merely by measuring diene concentration in vivo. Thus, the measurement of conjugated dienes in vivo might be more sensitive compared to the other measurements of lipid peroxidation.

Our results lend no support to the hypothesis that cocoa polyphenols would inhibit lipid peroxidation in humans, which partially conflicts with previous findings. A single bolus of cocoa or chocolate has been shown to increase the plasma antioxidant capacity [17–20], decrease the formation of lipid oxidation products (TBARS) [18,19], and inhibit the oxidation of LDL ex vivo [14], at least for a few hours. Also, in long-term studies, the consumption of cocoa and chocolate have increased the antioxidative capacity of plasma and inhibited lipid peroxidation ex vivo [20–22]. The beneficial effects have been suggested to be due to polyphenolic compounds in cocoa, even though, in these studies, the study supplements or controlled diets differed in both polyphenol and fatty acid contents. In these studies, either there was no control group [14,22] or

vanilla milk chips (nutritional composition not presented) [18], bread [19], sugar [21], or an average American diet [20] was used as a control for cocoa or chocolate administration. Therefore, it is possible that monounsaturated and saturated fats, which occur in high amounts in chocolate and cocoa, have been responsible for the inhibition of lipid peroxidation.

Also, these previous studies used ex vivo methodology and mainly lacked in vivo methods such as $F_2$-isoprostanes, which are considered to be among the most reliable markers of lipid peroxidation in the human body [41]. To date, the formation of isoprostanes has only been measured in two studies, and in these studies consumption of a single bolus or long-term consumption of chocolate had no effect on the formation of isoprostanes [19,22].

We cannot, however, exclude the possibility that cocoa polyphenols would inhibit lipid peroxidation in people with increased oxidative stress. It has been suggested that, in younger people, the stage of lipid peroxidation is lower compared to older subjects [42]. In the present study, the subjects' mean age was only 26, and it can be difficult to attenuate lipid peroxidation that is already at a low level [41]. The study subjects were also eating a relatively healthy diet (see Table 2), which may have made it even more difficult to detect the inhibition of lipid peroxidation. Furthermore, blood samples were drawn after 10 h of fasting; since the biological half-lives of epicatechin and catechin, and possibly also the antioxidant effects in the blood, are relatively short, short-term effects on lipid peroxidation may not have been detected [17–19].

One drawback of our study was that the study design was not the best possible one for the purpose of studying the effects of chocolate fatty acids on lipid peroxidation. The study design should have included, for example, differing amounts of fatty acids, but in similar proportions of the total fat content of the chocolates, and a control group without chocolate fatty acids. Thus, we cannot totally exclude the possibility that the inhibition of lipid peroxidation might have been due to factors other than fatty acids in chocolate.

Although our results suggest that chocolate consumption for 3 weeks causes no weight increase nor has any detrimental effects on serum lipids in healthy subjects, and in fact may have beneficial effects on HDL cholesterol and lipid peroxidation, our results should be interpreted cautiously. Chocolate has a high fat and energy content and daily consumption of large amounts of chocolate may increase weight over the long term. The result in hypercholesterolemic and hypertriglyceridemic subjects may also be different. The effects of chocolate consumption on other CVD risk factors, such as endothelial function and thrombogenic factors, should also be studied. Also, as chocolate consumption may increase the proportion of fat in the diet, it consequently decreases the proportion of carbohydrates and the amount of several nutrients and, thus, may affect detrimentally the quality of the diet.

To summarize, the present study showed favorable effects of cocoa on HDL cholesterol and of chocolate on one of the markers of lipid peroxidation; but, cocoa polyphenols did not inhibit lipid peroxidation in young and healthy subjects. Further studies are needed to clarify our findings and to identify the HDL cholesterol–increasing compounds in cocoa.

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## ABBREVIATIONS

ALAT — alanine aminotransferase
ASAT — aspartate aminotransferase
CHD — coronary heart disease
DC — dark chocolate
γ-GT — γ-glutamyltransferase
HCl — hydrochloric acid
HPC — high polyphenol chocolate
MeOH — methanol
PBS — phosphate-buffered saline
TRAP — total peroxyl radical–trapping potential
WC — white chocolate