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ORIGINAL CONTRIBUTION

Intake of whole apples or clear apple juice has contrasting effects on plasma lipids in healthy volunteers

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Abstract
Purpose Fruit consumption is associated with a decreased risk of CVD in cohort studies and is therefore endorsed by health authorities as part of the '5 or more a day' campaigns. A glass of fruit juice is generally counted as one serving. Fruit may cause protection by affecting common risk factors of CVD.
Methods Apples are among the most commonly consumed fruits and were chosen for a comprehensive 5 × 4 weeks dietary crossover study to assess the effects of whole apples (550 g/day), apple pomace (22 g/day), clear and cloudy apple juices (500 ml/day), or no supplement on lipoproteins and blood pressure in a group of 23 healthy volunteers.
Results The intervention significantly affected serum total and LDL-cholesterol. Trends towards a lower serum LDL-concentration were observed after whole apple (6.7 %), pomace (7.9 %) and cloudy juice (2.2 %) intake. On the other hand, LDL-cholesterol concentrations increased by 6.9 % with clear juice compared to whole apples and pomace. There was no effect on HDL-cholesterol, TAG, weight, waist-to-hip ratio, blood pressure, inflammation (hs-CRP), composition of the gut microbiota or markers of glucose metabolism (insulin, IGF1 and IGFBP3).
Conclusions Apples are rich in polyphenols and pectin, two potentially bioactive constituents; however, these constituents segregate differently during processing into juice products and clear juice is free of pectin and other cell wall components. We conclude that the fibre component is necessary for the cholesterol-lowering effect of apples in healthy humans and that clear apple juice may not be a suitable surrogate for the whole fruit in nutritional recommendations.

Keywords Apples · Pomace · Clear juice · Blood lipids · CVD · ISAFRUIT

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Introduction

In observational studies, a high intake of fruit is associated with reduced incidence of CVD [1–3]. However, it is not clear whether specific fruits or specific compounds present in fruits are responsible for the observed effects. Fruits are

rich in polyphenols and a good source of soluble as well as insoluble dietary fibre, and the benefits of fruit consumption are often linked to these compounds [4–6]. Especially, a diet rich in soluble fibre protects against CVD [7–11], most likely by lowering serum total cholesterol (TC) and LDL-cholesterol concentrations. This has been reported in numerous human studies investigating the effects of the soluble fibre pectin on lipid metabolism [12–19]. Besides affecting cholesterol concentrations, fibre may also affect other CVD risk factors, including fasting triacylglycerol (TAG) concentrations [20], insulin resistance [21, 22], waist-to-hip ratio (WHR) [22], BMI [23], hypertension [24] and high-sensitivity C-reactive protein (hs-CRP) concentration [25]; the latter suggesting a possible anti-inflammatory effect [9].

Polyphenols are strong antioxidants in vitro, but there are limited effects of polyphenols on plasma antioxidant activity in vivo, probably due to the low bioavailability of most polyphenols. However, several studies report increased activity of the antioxidant enzymes, glutathione peroxidase [26, 27], glutathione reductase and superoxide dismutase [28], after supplementation with different fruits and vegetables rich in polyphenols. Increasing the consumption of fruits might decrease oxidative stress through induction of antioxidant enzymes which could potentially lead to a reduced risk of CVD. We have previously shown that a common polymorphism of glutathione peroxidase type 1 decreases GPX1 activity [29] and others have shown that this polymorphism is associated with an increased risk of CVD [30–33].

Apples are a good source of both polyphenols and the soluble fibre pectin. They are the most commonly eaten fruits in Europe and account for approximately 35 % of the total fruit intake in Denmark [34]. The consumption of apples and apple polyphenols may be related to decreases in risk factors for CVD [35–37]. Few human intervention studies have been concerned with the effect of apples and processed apple products on lipid metabolism. In general, there is some indication that apple and apple fibre have cholesterol-lowering effects in these studies but the evidence is inconsistent. Most of the published trials are conducted in small samples of heterogeneous populations and with limited control. Due to differences in study designs, in apple varieties and in baseline cholesterol concentrations of study participants, it is difficult to compare the existing studies or to determine the individual contribution of apple components to the observed health effects. No human study has investigated the effects of whole apples in comparison with commonly consumed apple juices produced from the same apples in a dietary-controlled crossover study, and no study has so far reported on the effect of apple pomace. Apple pomace is a by-product in juice production and due to its content of apple pectin, apple pomace might be an attractive ingredient in functional food products.

In a previous rat study, we observed changes in lipoproteins and in the intestinal microbiota of rats fed whole apples, pomace or apple pectin [38], and we were interested in finding out whether similar concomitant effects could be observed in humans. The present study was initiated to investigate the effect of a four-week consumption of whole apples and processed apple fractions on plasma lipids, excretion of bile acids, blood pressure, and other CVD risk factors along with antioxidant defence, inflammatory markers, gut microbiota composition and markers of glucose metabolism in healthy subjects. It was further designed to clarify whether specific compounds in the fruit, for example, the polyphenol or/and the fibre fractions are necessary for the effects. This is important in order to evaluate whether processed fruit products such as clear juices or pomaces are reasonable surrogates for whole fruit.

Subjects and methods

Subjects

Thirty-four healthy men and women, 18–69 years of age, were recruited by advertisements in local newspapers, on Internet sites and at universities in the Copenhagen area. Exclusion criteria were smoking, obesity (BMI $>$ 30 kg/m^2), family history of chronic diseases, use of any medication, heavy physical exercise (>10 h/week), pregnancy, breast-feeding, birch pollen allergy, hypersensitivity towards para-aminobenzoic acid, use of vitamin/mineral supplements 2 weeks prior to entry into the study and donation of blood less than 3 months before the study. The subjects received oral and written information concerning the study before they gave their written consent. Twenty-three subjects completed all five periods, corresponding to 68 % of the recruited participants. Four subjects never showed up at study start, five subjects disliked the restricted diet and dropped out, one subject had to stop after the first period because his job situation changed, and one subject was excluded from the study after the first 4 periods due to a cancer diagnosis. The research protocol was approved by the Scientific Ethics Committee of the municipalities of Copenhagen and Frederiksberg (01-309947). The first participants were enrolled in October 2006 and the last participant finished in November 2007.

Study design

The study was performed as a randomised, single-blinded, 5 $\times$ 4 weeks crossover study. The five periods consisted of a control period where only the restricted diet was allowed,

and four periods where the restricted diet was supplemented with one of four apple products (whole fresh apples, apple pomace, cloudy apple juice or clear apple juice). Subjects were on a restricted diet (Table 1) from at least 1 week before the start of the first intervention period and throughout the entire study, only interrupted by holiday periods. The study was interrupted for 3 weeks during Christmas and for 5 weeks during summer holidays, where subjects were allowed to eat their usual diet. Two weeks prior to study restart, subjects returned to the restricted diet. The restricted diet was low in polyphenols and pectin. Only few specific fruits and vegetables low in these components were allowed in limited amounts. Otherwise, no other fruits (fresh, frozen, dried or conserved) were allowed, nor were any form of tea, cocoa, red wine, black and dark chocolate, liquorice and wine gum. To avoid vitamin C deficiency, the participants were instructed to eat around 100 g pineapple or one well-peeled orange daily. On the first day of each period and once a week during the 4 weeks, subjects were supplied with apples or apple products to be consumed during the following week. They were asked to keep daily records of apple/apple product consumption, any deviations from the restricted diet and well-being during the study. Apples and apple products were coded with different colours and given in random order. The code was not broken until all analyses were completed and the results were analysed statistically. Blood samples and 24-h faecal samples were collected before and at the end of each period.

Apples and processed apple products

Fresh apples and processed apple products (clear juice, cloudy juice and air-dried pomace) derived from the same harvest of apples (variety 'Shampion') from Poland (2006) were supplied from the Department of Storage and Processing, Research Institute of Pomology and Floriculture, Skierniewice, Poland. Based on the results on polyphenol and fibre content of apples from the harvest 2005, the amounts of apples and apple products to be consumed daily by the participants were calculated. These calculations resulted in subjects supplementing the restricted diet with on average 550 g fresh apple/day, 22 mg apple pomace/day or 500 ml cloudy or clear apple juice/day. Apples and apple products were stored in Poland and continuously delivered throughout the study to the Department of Human Nutrition in Denmark, where fresh apples and apple juices were stored in the dark at 3 °C, and apple pomace was stored in the freezer at −20 °C. Whole apples, apple pomace, cloudy and clear apple juice used in the present study were later analysed for dietary fibre, sugar and polyphenols. The variation in apple composition during storage was generally below 10 % but reached 16 % for acids. The daily dietary intakes of total- and water-soluble pectin, fructose and total sugar, and major polyphenols were calculated based on the results of these analyses (Table 2).

Content of total pectin and soluble pectin was determined according to the standard method No. 26 of the International Federation of fruit juice producers (IFU) (www.ifu-fruitjuice.com). Content of catechin, epicatechin, procyanidin dimers, phloretin xyloglucoside, phloridzin, chlorogenic acid, p-coumarylquinic acid, quercetin glycosides and quercetin was determined by HPLC according to Tsao et al. [39] and Markowski et al. [40].

Anthropometric and blood pressure measurements

All measurements were taken in the morning on fasting subjects, once in the beginning (day 1) and once on the last day of each period (day 28). Wearing only underwear, subjects were weighed, waist and hip circumferences were measured three times using a Seca 200-circumference measuring tape (Seca, Hamburg, Germany) and the average of the three measurements was recorded. Heart rate (HR), diastolic (DBP) and systolic (SBP) blood pressures were taken on fasting subjects in supine position in the morning. Measurements were taken in quadruplicate using a digital blood pressure monitor, A & D Medical UA-787 (Kivex A/S, Hørsholm, Denmark) after 10 min supine resting, and the average of the four measurements was used.

Blood sampling and sample handling

Blood samples were drawn following 12 h of fasting on day one and on the two last days in each period. Subjects were informed not to drink alcohol or perform hard physical activity 48 h prior to blood sampling. All blood samples were taken in the morning and immediately after measurement of HR and blood pressure. Blood samples were collected in 10-ml EDTA-coated tubes (Becton–Dickinson, Plymouth, UK) and plasma was isolated by centrifugation (20 min, 2,200*g*, 4 °C) for the analyses of lipid profile, plasma lipid resistance to oxidation, alanine aminotransferase activity (ALAT) and antioxidant activity. Erythrocytes were lysed by adding an equal volume of MilliQ water and used for measuring antioxidant enzyme activities. Insulin, insulin-like growth factor 1 (IGF1), IGF binding protein 3 (IGF1BP3) and hs-CRP measurements were determined on serum from blood drawn in plain tubes (Becton–Dickinson, Plymouth, UK), left at room temperature for 45 min and centrifuged for 20 min at 2,200*g*. All samples were stored at −80 °C until analysis. When performing the analyses, all samples from each participant were analysed in the same batch and in random order.

Table 1 Restricted diet to be followed during the entire study to minimise the intake of polyphenols and pectin

	Allowed ad libitum	Allowed with restrictions	Forbidden
Fruit	Pineapple	One orange or two tangerines per day (well peeled)	All other fruits—fresh, frozen, dried or conserved
	Watermelon		
	Lemon and lime juice for cooking		
Vegetables	Cucumber	Potato (100–120 g/day)	All other vegetables—fresh, frozen, dried or conserved
	Corn	Carrot (100–120 g/day)	
	Green salad (except spinach and rocket)		
	Mushrooms		
	Canned bamboo shoots		
Beverages	Coffee		Tea/herbal tea
	Soda		Cocoa
	Milk and dairy products without fruit		All juice (except pineapple)
	Beer		Soft drinks with fruit
	White wine		Red wine
	Champagne		
	Vodka		
	Gin		
	White rum		
	Pineapple juice		
Others	Candy	Nuts (max. one handful per day)	Marmalade and jam
	White chocolate	Use of spices should be limited	Stewed fruit
	Selected crisps, biscuits and cakes		Black and dark chocolate
	Meat		Potato chips
	Fish		Licorice
	Egg		Jelly
	Dairy products		
	Bread		
	Grain		
	Pasta		
	Rice		

Faecal sampling and sample handling

Study participants were asked to collect 24-h faecal samples on 2 days before the beginning (days -2 and -1) and on 2 days in the end of each period (days 27 and 28). Samples were collected in pre-weighed plastic pots and kept cold in a cool bag until delivered at the Department of Human Nutrition (University of Copenhagen, Denmark) for further handling. At the department, faecal samples were weighed, added an equal volume of double-distilled water and homogenised using an immersion blender (Braun MR 4050 M HC, Germany). For a random set of eight volunteers, 2 ml homogenate for the analysis of faecal bile acids was transferred to a cryotube and stored at −80 °C until analysed. For the microbiota analysis, two sample aliquots were frozen down, one with 50 % glycerol in ratio 1:1 and kept at −80 °C for the DGGE analysis, and one homogenised sample at −20 °C for the qPCR analysis.

DNA extraction from faecal samples

DNA was extracted from both the sample kept at −80 °C, and the one kept at −20 °C. DNA was extracted from 2 ml of the samples using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) with a bead-beater step in advance, as previously described [41] and stored in 200 µl autoclaved water at −20 °C until use.

Table 2 Calculated daily dietary intake of sucrose, total sugar, major polyphenols and total and water-soluble fibre based on the chemical analyses of apples and processed apple products

	Apples	Apple pomace	Cloudy apple juice	Clear apple juice
Fructose (g/day)	33	1.6	36	40
Total sugar (g/day)	51	2.6	59	63
Catechin	5.1	0.8	4.4	2.3
Epicatechin	60	9	27	19
Procyanidin dimers	81	7	55	35
Phloretin xyloglucoside	6.2	1.7	5.4	7.5
Phloridzin	6.6	8.3	6.1	8.7
Chlorogenic acid	36	1	40	27
p-coumaroylquinic acid	4	0.1	3.2	3
Quercetin glycosides	41	46	4	4.2
Quercetin	0	0.2	0	0.4
Total polyphenols (mg/day)	239	75	145	108
Water-soluble pectin (g/day)	0.69	0.44	0.42	0.02
Total pectin (g/day)	2.87	2.12	0.47	0.03

Denaturing gradient gel electrophoresis (DGGE) of faecal samples

Aliquots (10 µl) of purified DNA from the −80 °C samples were applied to the following to give a 50 µl PCR mixture: 20 µl of PRIME MasterMix (2.5×) (VWR and Bie and Bertnsen, Herlev, Denmark) and 40 pmol of each of the primers. The following primers were used: HDA1-GC/HDA2 targeting 16S rRNA genes from all bacteria [42], g-bifid F/g-bifid R-GC targeting 16S rRNA genes of genus *Bifidobacterium* [43, 44], and Erec 688F/Erec 841R-GC targeting 16S rRNA genes of *Clostridium* clusters XI and XIVa [45]. All primers were purchased from DNA Technology (Aarhus, Denmark). The universal primers HDA1-GC/HDA2 were used in a touchdown PCR as recently described [38]. The *Bifidobacterium* and *Clostridium* primers were used in the following PCR: initial denaturation at 94 °C for 5 min, amplification carried out using 45 cycles including denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s and extension at 68 °C for 1 min. The programme was ended with a final extension at 68 °C for 7 min.

All PCRs were run on a PTC-240 DNA Engine Tetrad 2 Cycler (MJ Research, Bio-Rad Laboratories, Copenhagen, Denmark), and the products were verified by gel electrophoresis before proceeding to DGGE analysis. DGGE was carried out as previously described [46] using a DCode Universal Mutation Detection System instrument and gradient former model 475 according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, California). The gels were made with denaturing gradients ranging from 25 to 65 % for the analysis of 16S rRNA fragments amplified with the universal primers, 40 to 70 % for analysing fragments amplified with the *Bifidobacterium* primers and 35 to 70 % for fragments amplified with the *Clostridium* primers. Finally, identification, excision, cloning and sequencing of differing bands were performed as previously described [38]. Based on this identification, a set of primers for quantitative PCR was selected (Table 3).

Quantitative PCR (qPCR) analysis of faecal samples

Real-time qPCR was performed on an ABI Prism 7900 HT from Applied Biosystems. The amplification reactions were carried out in a total volume of 20 µl containing 10 µl (2× PerfeCTA^{TM} SYBR$^{®}$ Green SuperMix, ROX from Invitrogen, Copenhagen, Denmark), primers (each at 200 nM concentration), 2 µl template DNA and USB-H2O (USB EUROPEgmbh, Staufen, Germany) purified for PCR. The amplification programme consisted of one cycle at 50 °C for 2 min; one cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s and 60 °C for 1 min; and finally one cycle of melting curve analysis for amplicon specificity at 95 °C for 15 s, 60 °C for 20 s and increasing ramp rate by 2 % until 95° for 15 s. The programme was efficient and consistent for all primers used.

All qPCR results were calculated relatively as ratios of species DNA levels to Eubacterial/all bacterial (from the highly conserved V2/V3 16S rRNA region) expression levels in order to correct data for differences in total DNA concentration between individual samples. DNA levels were approximated as 2^{-Ct}, where Ct is the threshold cycle calculated by the ABI software as the PCR cycle, where amplifications signal exceeds the selected threshold value, also set by the software. In the selected region of the PCR curve, the PCR amplification efficiencies were all approximately 100 %. All samples were calculated as means of duplicate determinations.

Plasma ALAT activity

Plasma ALAT activity was determined on a Hitachi 912 analyser (Boehringer Mannheim, Germany) using a commercially available kit (catalogue no. 10851132) from Roche Diagnostics (Mannheim, Germany). Intra- and inter-day variations were 2.6 and 11.4 %, respectively.

Serum hs-CRP, insulin, IGF1 and IGFBP3

Serum hs-CRP concentration was determined on a Pentra 400 analyser (Horiba ABX, Montpellier, France) using a

Table 3 Primers used in the qPCR analysis of the gut microbiota

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
Bifidobacteria (all)	CGCGTCYGGTGTGAAAG	CCCCACATCCAGCATCCA	244 [70]
B.adolescentis	CTCCAGTTGGATGCATGTC	CGAAGGCTTGCTCCCAGT	279 [43]
B.bifidum	CCACATGATCGCATGTGATTG	CCGAAGGCTTGCTCCCAAA	278 [43]
B.breve			
B.pseudocatenulatum	CGGATGCTCCGACTCCT	CGAAGGCTTGCTCCCGAT	289 [43]
Clostridium clusters XI and XVIa	GCGTAGATATTAGGAGGAAC	TGCGTTWGCKRCGGCACCG	211 [45]
Bacteroides spp	CGGCGAAAGTCGGACTAATA	ACGGAGTTAGCCGATGCTTA	360 [38]
Eubacteria (all bacteria)	ACTCCTACGGGAGGCAGCAGT	GTATTACCGCGGCTGCTGGCAC	200 [42]

commercially available kit (catalogue no. A11A01611, Horiba ABX, Montpellier, France). Intra- and inter-day variations were 3.1 and 3.5 %, respectively. Insulin, IGF1 and IGFBP3 concentrations in serum were determined on an Immulite 1,000 analyser (Diagnostic Products Corporation, Los Angeles, CA, USA) using commercially available kits from Siemens (Diagnostic Products Corporation, Los Angeles, CA, USA, catalogue no. LKIN5, LKGF5 and LKGB1, respectively). Intra- and inter-day variations were 4.1 and 4.6 % for insulin, 5.3 and 6.5 % for IGF1, and 7.6 and 9.9 % for IGFBP3, respectively.

Blood lipids

Plasma TC, triacylglycerides (TAG), HDL- and LDL-cholesterol concentrations were determined using commercially available kits from Roche Diagnostics (Basel, Switzerland, catalogue no. A11A01634, A11A01640, A11A01638 and A11A01636, respectively). Analyses were performed on a Pentra 400 (Horiba ABX, Montpellier, France), and intra- and inter-day variations were 1.3 and 1.3 % for TC, 3.5 and 5.4 % for TAG, 1.9 and 2.5 % for LDL-cholesterol, and 2.1 and 2.1 % for HDL-cholesterol, respectively.

Faecal bile acids and pH

The concentration of bile acids in faeces samples was measured by LC–MS/MS. Briefly, each fresh sample of >50 g faeces was weighed, homogenised with one volume (w/v) of water to a slurry, pH was measured in the homogenate and 0.3 g sample was aliquoted. The aliquots were added with a mix of d_4 and d_5 bile acids as internal standards and extracted three times with ethanol. The eluate was diluted with water and concentrated on an Oasis HLB LP 96-well plate (60 mg) (Waters, Milford, MA). The acetonitrile eluate was evaporated to dryness and redissolved in 15 % acetonitrile, 30 % methanol, 0.1 % formic acid (80 % mobile phase A). Samples and standards were analysed on an Acquity UPLC with a triple quadrupole detector (Waters), operated in MRM mode with a gradient from phase A to B (100 % acetonitrile) over 5 min. Between run CV % for the internal standard ($n = 240$) was 13.5 %. The individual bile acids (CA: cholic acid, CDCA: chenodeoxycholic acid, DCA: deoxycholic acid, HDCA: hyodeoxycholic acid, LCA: lithocholic acid, UDCA: ursodeoxycholic acid) were quantified using QuanLynx version 4.1 (Waters) based on the deuterated internal standards and the external calibrants. Based on the analytical results for the individual primary and secondary bile acids, these were summed for each sample.

FRAP, TEAC and ORAC analyses of antioxidant activity and plasma lipid oxidation

Antioxidant activity in plasma was determined as the ferric reducing ability of plasma (FRAP) as described by Benzie and Strain [47], as Trolox equivalent antioxidant capacity (TEAC) using the commercially available total antioxidant status kit Randox NX2332 (Lovmand Diagnostics, Malling, Denmark) and as oxygen radical absorbance capacity (ORAC) as described by Ou et al. [48], using fluorescein as the probe. FRAP and TEAC analyses were performed on a Cobas Mira S (Triolab, Brøndby, Denmark) and ORAC on a Cary fluorescence spectrophotometer (VARIAN Inc, Palo Alto, CA, USA) in a subset of the first 14 volunteers. Plasma lipid resistance to oxidation was determined according to Mayer et al. [51]. The time-dependent decrease in fluorescence intensity was followed using a Wallac 1420 multilabel counter (PerkinElmer, Life Sciences, Allerød, Denmark). The fluorescent marker (2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine, Cat.No. D476) was purchased from Invitrogen (Taastrup, Denmark). The inter-day variation in lag time for a control plasma sample was 7.4 %.

Erythrocyte antioxidant enzyme activities

Activities of the antioxidant enzymes glutathione peroxidase (GPX), glutathione reductase (GR) and catalase

(CAT) were spectrophotometrically assayed in erythrocyte lysates [49] on a Roche/Hitachi 912 Analyser (Roche Diagnostics A/S, Hvidovre, Denmark) and SOD activity using a Cobas Mira S analyser (Triolab, Brøndby, Denmark) according to Wheeler et al. [50]. All enzyme activities were related to the amount of haemoglobin (Hb) in the blood sample. Hb content and SOD activity were determined using commercially available kits (Randox, Ardmore, UK, cat. no. HG 980 and cat. no. SD 125). Samples from each subject were analysed in the same batch in random order to decrease variation, and a control sample was analysed for every 15th sample. Intra-day coefficients of variation were 4.3, 2.7, 9.2 and 12.3 % for GPX, GR, SOD and CAT, respectively.

Statistical analysis

All data are presented as mean ± SD. Statistical analyses were performed using SAS (SAS Enterprise Guide 3, SAS Institute, Cary, NC). Treatment effects were analysed using PROC MIXED with treatment (placebo, fresh apples, apple pomace, cloudy apple juice or clear apple juice) and period (5 periods) as fixed factors, subject as random factor and baseline measurements as covariates. Use of antibiotics (yes or no), sex (female or male), age (under or above the median) and their interactions with treatment were included in the model. Data that could not meet the criteria of variance homogeneity (Levenes test) and normal distribution (determined by residual plot examination and Shapiro–Wilks test) even after log transformation were analysed by a nonparametric test (Friedman). The qPCR data were analysed by Student's *t* test. For all tests, a P value less than 0.05 was considered statistically significant.

Results

Study participants and compliance

Baseline characteristics for the study participants are presented in Table 4. Eighteen participants reported minor deviations from the restricted diet and from the intervention diets. Deviations typically consisted of drinking a glass of red wine, adding one or two teaspoons of spices to the diet, or eating a few pieces of chocolate or fruit slices. In addition, three subjects consumed alcohol within less than 48 h before blood sampling, two at one occasion and one twice. Two subjects were on a penicillin treatment for 8 days, and one took flu treatment (tamiflu) for 4 days of the study. None of these deviations gave us reason to exclude the subjects from the analyses, and we included the use of antibiotics in the statistical analyses. However, exclusion of these individuals did not change any conclusion from this study.

Lipid profile and other clinical markers, faecal pH and bile acid concentrations

Both TC and LDL-C concentrations were significantly affected ($P = 0.0096$ and $P = 0.024$) by the interventions. The responses in TC and LDL-C concentration after consumption of apples (−5.6 and −6.7 %), apple pomace (−3.7 and −7.9 %) and cloudy apple juice (−1.4 and −2.2 %) showed the same trend (Fig. 1). Consumption of whole apples gave the largest decrease in TC concentration, which was borderline statistically significant compared to control ($P = 0.066$) and to cloudy apple juice ($P = 0.064$). LDL-C concentration did not decrease significantly after apple consumption compared to the control period ($P = 0.12$). TC and LDL-C concentrations were significantly increased in periods where clear apple juice was consumed, compared to periods with whole apple or apple pomace ($P = 0.0006$ and $P = 0.0074$, and $P = 0.005$ and $P = 0.004$, respectively), but not placebo ($P = 0.113$ and $P = 0.227$). The increase in TC and LDL-C concentrations after clear apple juice consumption corresponds to 5 and 6.9 %, respectively. There was a significant treatment–gender interaction ($P = 0.026$) on LDL-C concentrations. After stratifying according to gender, the largest effects were observed in women ($P = 0.0019$), where LDL-C concentrations were significantly increased after intervention with clear juice compared to placebo ($P = 0.023$), apple ($P = 0.0032$) and apple pomace ($P = 0.0003$) (Table 5). Cloudy apple juice also differed significantly compared to whole apple ($P = 0.034$) and apple pomace ($P = 0.0048$) in the women. In men, the effect of clear juice on LDL-C concentrations was not statistically significant ($P = 0.099$) (Table 5).

Changes in TC concentrations correlated inversely with the calculated intake of pectin (Fig. 2).

We found no effect of any of the intervention products on body weight, WHR, HR, SBP, DBP, plasma ALAT activity, serum hs-CRP, insulin, IGF1 and IGFBP3 concentrations ($P > 0.05$ for all) (Table 6). Likewise, plasma TAG, HDL-C concentration, TC/HDL-C ratio, concentration and 24-h excretion of total-, primary- and secondary bile acids, and individual bile acids (CA, CDCA, UDCA, HDCA, DCA, LCA) in faecal samples were unaffected by the treatments ($P > 0.05$ for all, Table 5), except for 24-h excretion of LCA. In the statistical model, both treatment ($P = 0.0017$) and its interaction with age (treatment × age, $P = 0.0393$) were statistically significant. However, due to the small sample size, we did not stratify according to age. Compared to control, we found a statistically significant decrease in LCA excretion with apple pomace

Table 4 Baseline characteristics of the 23 study participants (9 men and 14 women)

	Mean ± SD		Mean ± SD
Age (years)	36.2 ± 17.9	WHR	0.82 ± 0.10
BMI (kg/m^2)	22.3 ± 2.59	HR (bpm)	67.2 ± 9.5
TC (mmol/L)	4.67 ± 1.17	SBP (mmHg)	122 ± 15.5
LDL-C (mmol/L)	2.60 ± 0.96	DBP (mmHg)	76.2 ± 11.3
HDL-C (mmol/L)	1.37 ± 0.32	Insulin (pmol/L)	40.6 ± 28.2
TC/HDL-C	3.50 ± 0.92	hs-CRP (mg/L)	0.80 ± 0.80
TAG (mmol/L)	0.81 ± 0.37	IGF1 (ng/mL)	222 ± 89
pH	6.86 ± 0.42	IGFBP3 (μg/mL)	4.19 ± 0.68
CA (μmol/L)[a]	1.06 ± 1.29	ALAT (U/L)	19.8 ± 9.0
CDCA (μmol/L)[a]	6.15 ± 9.25	TEAC (mmol/L)	1.12 ± 0.12
DCA (μmol/L)[a]	78.5 ± 69.5	FRAP (mmol/L)	827 ± 189
LCA (μmol/L)[a]	58.9 ± 30.6	ORAC (mmol/L)[b]	8,845 ± 2,635
UDCA (μmol/L)[a]	2.19 ± 15.2	LIPIDOX (min)	246 ± 51
HDCA (μmol/L)[a]	20.5 ± 18.6	GPX (U/g Hb)	66.8 ± 22.8
PBA excretion (mg/24 h)[a]	2.18 ± 2.96	GR (U/g Hb)	8.13 ± 2.57
SBA excretion (mg/24 h)[a]	135 ± 86.7	SOD (U/g Hb)	953 ± 302

Data are presented as mean ± SD

WHR waist-to-hip ratio, *TC* total cholesterol, *TAG* triacylglycerides, *CA* cholic acid, *CDCA* chenodeoxycholic acid, *DCA* deoxycholic acid, *LCA* lithocholic acid, *UDCA* ursodeoxycholic acid, *HDCA* hyodeoxycholic acid, *PBA* primary bile acids, *SBA* secondary bile acids, *HR* heart rate, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *hs-CRP* high-sensitivity C-reactive protein, *IGF1* insulin-like growth factor 1, *IGFBP3* IGF binding protein 3, *ALAT* alanine aminotransferase, *TEAC* trolox equivalent antioxidant capacity, *FRAP* ferric reducing ability of plasma, *ORAC* oxygen radical absorbance capacity, *LIPIDOX* plasma lipid resistance to oxidation, *GR* glutathione reductase, *GPX* glutathione peroxidase, *SOD* superoxide dismutase

[a] $n = 8$ (3 men and 5 women)

[b] $n = 14$ (6 men and 8 women)

($P = 0.027$) and cloudy apple juice ($P = 0.0092$), and a borderline statistically significant decrease after intake of apples (0.077). Clear apple juice, on the other hand, increased LCA excretion ($P = 0.0004$) and differed significantly from apples, apple pomace and cloudy apple juice ($P = 0.0057$, $P = 0.0013$ and $P = 0.0004$, respectively). Dietary intervention tended to affect pH measured in faeces ($P = 0.085$) (Table 5).

Antioxidant activity in plasma

Plasma antioxidant activity was determined using three different methods. Both FRAP ($P = 0.0029$) and ORAC ($P = 0.025$) were significantly affected by the interventions, whereas we found no effect on TEAC of any of the dietary interventions ($P = 0.555$) (Table 6). Apples ($P = 0.020$) significantly increased FRAP values compared to control, and a similar tendency towards increased antioxidant activity was observed with cloudy apple juice ($P = 0.076$), while the effect of clear apple juice and apple pomace was in the opposite direction, but non-significant ($P = 0.115$ and $P = 0.228$, respectively). However, apple pomace resulted in a significant decrease in FRAP compared to whole apples ($P = 0.0006$), cloudy apple juice ($P = 0.003$) and clear apple juice ($P = 0.006$). Antioxidant activity determined as ORAC was significantly decreased following apple consumption compared to periods where cloudy and clear apple juices were consumed ($P = 0.004$ and $P = 0.011$, respectively).

For both FRAP and TEAC, men had significantly higher values than women ($P = 0.0002$ and $P = 0.0002$, for FRAP and TEAC, respectively), while it was the contrary for ORAC ($P = 0.047$) (data not shown), where the activity was significantly higher in women compared to men ($P = 0.047$). All three markers of antioxidant activity were significantly correlated. The strongest positive correlation was seen between FRAP and TEAC ($r_{\text{teac-frap}} = 0.693$), while ORAC correlated negatively with both of these markers ($r_{\text{teac-orac}} = -0.374$ and $r_{\text{frap-orac}} = -0.325$).

Antioxidant enzyme activities in erythrocytes and plasma lipid resistance to oxidation

The interventions significantly affected GPX1 activity ($P = 0.0085$). Apple pomace significantly increased erythrocyte GPX1 activity compared to placebo ($P = 0.031$) and clear and cloudy apple juices ($P = 0.002$ and $P = 0.005$, respectively), but not whole apples ($P = 0.39$) (Table 6). Clear and cloudy apple juices significantly decreased GPX1 activity compared to apples ($P = 0.023$ and $P = 0.046$, respectively). We saw no effect of any of the treatments on erythrocyte GR ($P = 0.379$), SOD ($P = 0.315$) or CAT ($P = 0.703$) enzyme activity (Table 6).

We found a statistically significant interaction between the use of antibiotics and plasma lipid resistance to oxidation ($P < 0.0001$) with antibiotics increasing the resistance. The use of antibiotics was therefore included in the statistical model as a covariate. With this change to the model, the overall effect of the dietary intervention on plasma lipid resistance to oxidation did not reach statistical significance ($P = 0.0619$) (Table 6).

Gut microbiota analysis

DGGE with universal primers HDA1/HDA2-GC targeting 16S rRNA genes in all bacteria revealed no discernible changes on the DGGE gel (data not shown).

When running DGGE with primers specific for *Bifidobacterium*, a few prominent bands were observed on the gel. Gel bands corresponding to *Bifidobacterium*

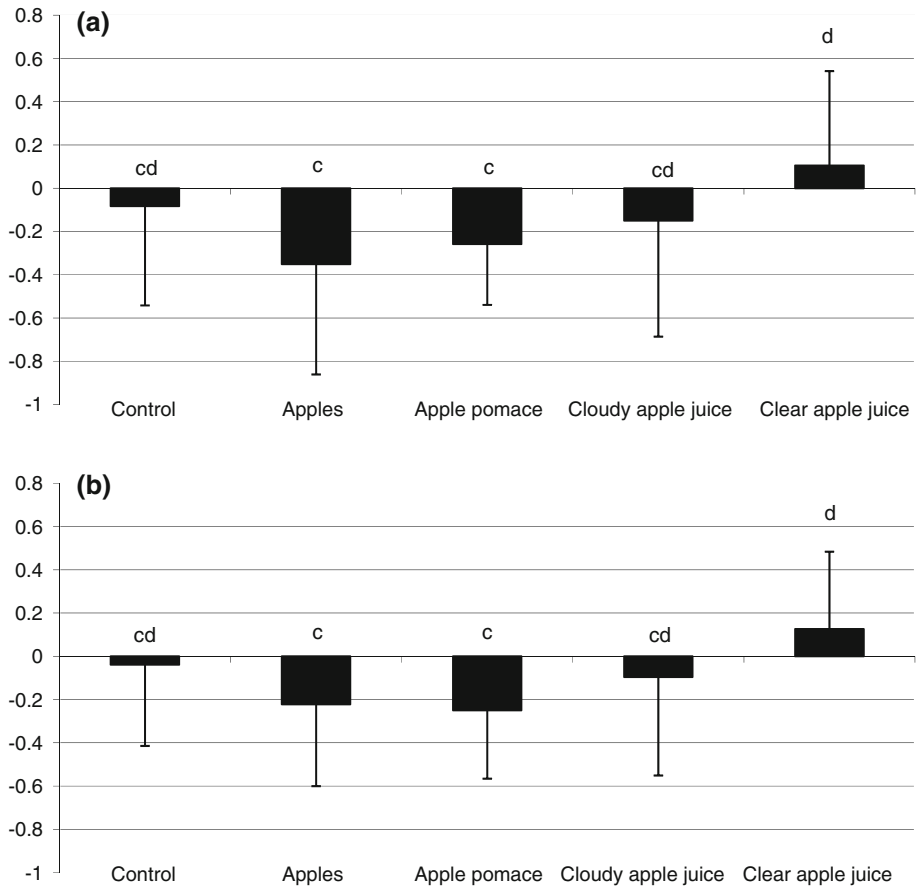

Fig. 1 Changes in (**a**) plasma TC and (**b**) plasma LDL-C after 4 weeks on a restricted diet supplemented with apples or processed apple products. Data are presented as mean ± SD, $n = 23$. Groups not sharing a common symbol (**c**, **d**) differ significantly at $P < 0.05$ (PROC MIXED). *TC* total cholesterol, *LDL-C* LDL-cholesterol

adolescentis and *Bifidobacterium pseudocatenulatum* were stronger, while a band corresponding to *Bifidobacterium bifidum* was fainter after the pomace diet (data not shown). These observations were however not found in all subjects. DGGE with primers targeting *Clostridium* clusters XI and XIVa generated seven predominant bands. However, the sequences generated by the primers were too short to assign a species designation to the cloned fragments (data not shown). None of these DGGE findings could be verified by qPCR, which showed no effect of any diet for any of the primers tested. Moreover, no changes were found for a specific Bacteroides primer.

Discussion

This intervention study in healthy volunteers is, to our knowledge, the first study that has included fresh apples, apple pomace, cloudy and clear apple juices produced from the same harvest of apples into one study, allowing a better comparison of possible effects on blood lipids, bile acid excretion, glucose metabolism, blood pressure, antioxidant defence and other markers related to risk of CVD. In studies where whole fresh apples were given to overweight or hypercholesterolemic individuals, decreased serum cholesterol concentrations were reported after consumption of 3–6 apples a day [52–54]. Only one study has been performed in normocholesterolemic subjects and showed no effect on cholesterol concentrations [55]. The effects of dried apples on glucose and lipid metabolism are inconsistent [56, 57], and no evidence of a beneficial effect of clear apple juice on lipid profile has so far been provided. On the contrary, decreased HDL-C concentration [58] and non-significant increases in TAG concentration [55, 59] were reported.

Our results show that apples and apple products differ significantly in their effects on blood lipids. We found a borderline statistically significant decrease in total serum cholesterol concentration after consumption of whole fresh apple. Apple pomace and cloudy apple juice had similar yet not as pronounced effects on TC concentrations, whereas consumption of clear apple juice had a significantly adverse effect on blood lipids. Both TC and LDL-C concentrations were significantly increased after daily consumption of 500 ml clear apple juice for 4 weeks compared to comparable amounts of apple and apple pomace. The main difference between clear apple juice and the other products is that clear apple juice lacks water-soluble pectin and solid cell wall components. This suggests that a cell wall-related fibre component, rather than

Table 5 Changes in plasma lipid profile, excretion of faecal bile acids and faecal pH after 4 weeks on a restricted diet supplemented with apples or processed apple products

	Control $n = 23$ Mean ± SD	Apples $n = 23$ Mean ± SD	Apple pomace $n = 23$ Mean ± SD	Cloudy apple juice $n = 23$ Mean ± SD	Clear apple juice $i = 23$ Mean ± SD
LDL-C (mmol/L)					
Men ($n = 9$)	0.14 ± 0.23	−0.15 ± 0.51	−0.83 ± 0.20	−0.22 ± 0.63	0.05 ± 0.44
Women ($n = 14$)	-0.15^{ac} ± 0.41	-0.27^{c} ± 0.28	-0.36^{c} ± 0.34	-0.02^{ab} ± 0.30	0.18^{b} ± 0.30
TAG (mmol/L)	−0.02 ± 0.26	−0.06 ± 0.38	−0.09 ± 0.35	0.01 ± 0.36	0.03 ± 0.34
HDL-C (mmol/L)	−0.03 ± 0.12	−0.08 ± 0.13	−0.0004 ± 0.12	0.003 ± 0.12	−0.011 ± 0.14
TC/HDL-C ratio	0.03 ± 0.28	−0.03 ± 0.34	−0.14 ± 0.30	−0.15 ± 0.47	0.11 ± 0.32
pH	0.003 ± 0.38	−0.23 ± 0.31	−0.15 ± 0.44	0.034 ± 0.57	−0.13 ± 0.45
Bile acid concentration					
CA (μmol/L)[d]	1.03 ± 4.71	−1.48 ± 5.66	−1.03 ± 5.59	−0.76 ± 1.39	0.23 ± 0.93
CDCA (μmol/L)[d]	2.92 ± 8.27	−2.54 ± 7.82	0.08 ± 2.39	−0.09 ± 4.15	0.32 ± 1.39
DCA (μmol/L)[d]	48.8 ± 92.4	−4.46 ± 39.8	−1.83 ± 82.1	−33.5 ± 95.3	54.6 ± 180
LCA (μmol/L)[d]	20.5 ± 46.3	−0.11 ± 34.1	11.6 ± 36.0	−15.1 ± 75.2	35.6 ± 53.0
UDCA (μmol/L)[d]	2.33 ± 4.23	−0.19 ± 0.62	0.27 ± 1.71	−1.19 ± 1.40	0.25 ± 1.42
HDCA (μmol/L)[d]	10.9 ± 24.2	0.79 ± 6.46	3.46 ± 15.9	−7.66 ± 16.5	10.3 ± 35.1
Total bile acid excretion					
CA (mg/24 h)[d]	1.75 ± 5.92	−1.44 ± 4.63	−1.03 ± 4.97	−0.72 ± 1.18	0.28 ± 0.44
CDCA (mg/24 h)[d]	2.67 ± 7.50	−1.94 ± 5.74	−0.88 ± 3.73	−0.64 ± 2.63	0.44 ± 0.70
DCA (mg/24 h)[d]	45.1 ± 66.4	−1.09 ± 42.7	−5.99 ± 70.5	−46.5 ± 95.8	51.3 ± 77.4
LCA (mg/24 h)[d]	23.4^{ac} ± 39.6	8.57^{c} ± 34.1	2.99^{b} ± 27.4	-10.3^{b} ± 36.7	34.5^{a} ± 20.2
UDCA (mg/24 h)[d]	0.67 ± 3.72	0.03 ± 0.87	−0.13 ± 2.55	0.19 ± 1.80	0.38 ± 0.61
HDCA (mg/24 h)[d]	10.0 ± 17.3	0.31 ± 8.05	1.55 ± 14.1	−9.38 ± 19.4	9.26 ± 15.5

Data are presented as mean ± SD

LDL-C LDL-cholesterol, *TAG* triacylglycerides, *HDL-C* HDL-cholesterol, *TC* total cholesterol, *CA* cholic acid, *CDCA* chenodeoxycholic acid, *DCA* deoxycholic acid, *LCA* lithocholic acid, *UDCA* ursodeoxycholic acid, *HDCA* hyodeoxycholic acid

[d] $n = 8$ (3 men and 5 women). Groups not sharing a common symbol (a, b, c) differ significantly at $P < 0.05$ (PROC MIXED)

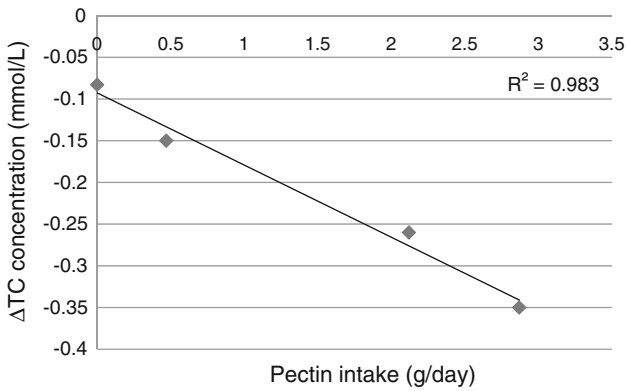

Fig. 2 Changes in plasma cholesterol concentrations as a function of pectin intake from apples and apple products. Data from control and clear apple juice periods are pooled due to the low content of pectin in the clear apple juice ($n = 23$)

the polyphenol fraction, may be the factor mainly responsible for the observed differences between clear juice and whole apples or pomace. Our result supports that cholesterol concentrations decrease with increasing content of water-soluble pectin. Mee and Gee [58] have previously reported that the addition of fibre to clear apple juice may reverse the adverse effect of the clear juice on blood lipid concentrations, indicating a hypocholesterolemic effect of cloudy apple juice. We observed no changes in TAG or HDL-C concentrations or TC/HDL-C ratio with any of the treatments. This is in line with previously published studies, showing that water-soluble fibre, especially pectin, effectively lowers total- and LDL-C concentrations without affecting HDL-C or TAG concentrations [11, 60]. An increase in HDL-C has been reported only after intervention with apples for several months [11, 53].

Apples are rich in polyphenols, and in spite of only a small fraction of these being actually absorbed, two recently published studies by Nagasako-Akazome et al. [61, 62] reported cholesterol-lowering effects after 4 weeks supplementation with an apple polyphenol extract. The studies indicate that the polyphenol fraction may also be partially responsible for the observed effects of apples in some studies. However, part of the polyphenols is retained in the clear juices, indicating some inconsistency regarding

Table 6 Changes in body composition, weight, blood pressure, plasma risk markers, plasma antioxidant activity, erythrocytes GPX, GR, CAT and SOD antioxidant enzyme activities, and plasma lipid resistance to oxidation after 4-week intervention with apples or processed apple products

		Control Mean ± SD	Apples Mean ± SD	Apple pomace Mean ± SD	Cloudy apple juice Mean ± SD	Clear apple juice Mean ± SD
Weight (kg)	23	−0.28 ± 1.0	−0.13 ± 1.08	−0.06 ± 0.83	−0.55 ± 1.32	0.23 ± 0.66
WHR	23	−0.006 ± 0.03	−0.006 ± 0.03	−0.009 ± 0.02	0.002 ± 0.02	−0.001 ± 0.02
HR (bpm)	23	1.85 ± 5.58	−0.27 ± 8.51	0.34 ± 8.38	1.70 ± 6.33	0.17 ± 5.88
SBP (mmHg)	23	2.73 ± 6.1	−1.20 ± 6.6	0.91 ± 4.9	1.42 ± 5.8	−0.24 ± 6.8
DBP (mmHg)	23	1.67 ± 5.5	−1.29 ± 4.9	−0.17 ± 6.7	0.99 ± 5.02	−0.95 ± 3.7
ALAT (U/L)	23	0.16 ± 5.4	0.43 ± 7.2	−1.02 ± 5.1	−1.36 ± 4.2	−0.95 ± 4.7
hs-CRP (mg/L)	23	0.10 ± 1.6	−0.22 ± 0.9	−0.09 ± 1.0	0.14 ± 0.8	0.28 ± 1.2
Insulin (pmol/L)	23	−0.68 ± 20.5	−3.07 ± 17.3	−0.44 ± 10.7	7.47 ± 10.3	3.36 ± 10.6
IGF1 (ng/mL)	23	4.07 ± 41.5	−9.57 ± 39.3	−3.09 ± 30.7	−8.61 ± 31.8	3.35 ± 32.2
IGFBP3 (μg/mL)	23	0.08 ± 0.26	−0.01 ± 0.47	−0.04 ± 0.41	0.14 ± 0.40	0.07 ± 0.39
TEAC (mmol/L)	23	0.002 ± 0.08	−0.021 ± 0.09	0.011 ± 0.07	0.006 ± 0.08	−0.019 ± 0.12
FRAP (mmol/L)	23	-22.0^{ac} ± 76.4	11.2^{b} ± 80.0	-50.8^{c} ± 133	9.48^{ab} ± 59.0	-17.5^{ab} ± 136
ORAC (mmol/L)[d]	14	-945^{ab} ± 2,851	-84.9^{a} ± 1,547	-338^{abc} ± 2,577	951^{c} ± 3,049	555^{bc} ± 2,205
GPX (U/g Hb)	23	-0.69^{ab} ± 2.01	-0.48^{ac} ± 2.49	1.14^{c} ± 2.61	-1.34^{b} ± 6.10	-1.97^{b} ± 5.28
GR (U/g Hb)	23	−0.46 ± 1.82	0.06 ± 1.58	0.20 ± 0.94	0.01 ± 1.59	0.26 ± 1.61
SOD (U/g Hb)	23	54.6 ± 180	20.3 ± 217	41.1 ± 198	28.8 ± 163	−25.3 ± 117
CAT (kU/g Hb)	23	0.13 ± 0.49	0.04 ± 0.50	0.06 ± 0.47	0.01 ± 0.43	−0.04 ± 0.28
LIPIDOX (min)	23	−12.2 ± 29.1	−7.70 ± 26.3	−16.1 ± 27.3	−6.20 ± 18.0	−10.1 ± 40.2

Data are presented as mean ± SD

WHR waist-to-hip ratio, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *ALAT* alanine aminotransferase, *hs-CRP* high-sensitivity C-reactive protein, *IGF1* insulin-like growth factor 1, *IGFBP3* IGF binding protein 3, *TEAC* trolox equivalent antioxidant capacity, *FRAP* ferric reducing ability of plasma, *ORAC* oxygen radical absorbance capacity, *Hb* haemoglobin, *GPX* glutathione peroxidase, *GR* glutathione reductase, *SOD* superoxide dismutase, *LIPIDOX* plasma lipid resistance to oxidation

[d] $n = 14$ (6 men and 8 women). Groups not sharing a common symbol (a, b, c) differ significantly at $P < 0.05$. Differences were tested with PROC MIXED or Friedman

their effect in this matrix. Considering the intakes of polyphenols during the treatments with whole apples, pomace, clear and cloudy juices (Table 2), our results do not support a cholesterol-lowering effect of apple polyphenols in this study, at least not at daily intakes corresponding to concentrations found in 500 ml of clear apple juice. It is possible that apple polyphenols may regulate lipid metabolism at intakes that are considerably higher: the effective daily dose used in the two studies by Nagasako-Akazome et al. [61, 62] was 600 mg of undefined apple polyphenols. The daily doses in our study varied from 75 to 239 mg polyphenols.

We found no effect of apples or processed apple products on HR, blood pressure or anthropometric measures, such as body weight and WHR. It has been reported that an increased intake of fruits, and of apples in particular, can decrease body weight in subjects with a BMI >25 kg/m^2 [63]. Our study participants were normal weight and a study period of only 4 weeks may be too short to observe such effects. Likewise, glucose metabolism, hs-CRP concentrations and ALAT were unaffected by any treatment. Water-soluble fibre can reduce the rise in blood glucose following a meal, as well as the insulin response, and the lack of effect on glucose metabolism may be due to the chosen measuring time after 12 h of fasting. It is possible that we could have observed an effect on glucose metabolism if we had taken postprandial blood samples [63]. Soluble fibres have been suggested to decrease carbohydrate absorption by increasing viscosity and slowing down digestion.

Our study suggests that one mechanism by which apples and apple products could reduce the risk of CVD might be through an improved serum lipid profile, and that the fibre fraction seems to be necessary for the observed effects. The mechanism could be linked to either reduced cholesterol synthesis in the liver or to reduced cholesterol or fat absorption from the diet, due to the gelling capacity of pectin, leading to an increased rate of serum cholesterol clearance. Apple pectin has a high degree of esterification and its high content of branched side chains is responsible for the good gelling properties of apple pectin. These gel-forming apple fibres bind cholesterol and bile acids,

thereby increasing their excretion and decreasing cholesterol and fat absorption [64, 65]. This increased excretion stimulates the de novo synthesis of bile acids in the liver and results in a net reduction in the concentration of TC, the precursor of bile acids [66]. This binding may also slow down the absorption of dietary sugar and decrease LDL synthesis in the liver. In this study, we observed no effect on total or primary bile acid excretion. Of the investigated faecal bile acids, only total excretion of LCA was affected. Apple pomace and cloudy apple juice resulted in decreased excretion of LCA, and the effect of whole apple was in the same direction, although only borderline statistically significant. Since the faecal LCA concentration was not significantly affected, this result is most probably due to a combined effect of a slightly reduced LCA concentration and a slightly increased faecal bulk; especially, the latter factor is well known to be highly variable [67]. Faecal LCA is a product of the gut microbiota. A decreased excretion of this toxic secondary bile acid may be seen as evidence for a decreased overall production resulting from a beneficial change in the functionality of the microbiota. We also found a tendency towards decreased pH in faeces after intake of fresh apples and apple pomace, the two products with highest pectin concentrations. The decrease may be caused by increased synthesis of SCFA by the gut microbiota. We previously reported that the consumption of apple pectin in rats increases the population of butyrate-producing *Clostridiaceae* in the rat gut [38]. Although the DGGE profiles from some of the subjects in the fresh apple and the pomace groups showed differences corresponding to specific markers of butyrate-producing bifidobacteria, the changes were not consistent enough to be verified by qPCR. Hence, consistent findings in the composition of the microbiota similar to the changes found primarily by DGGE in the previously performed animal experiment [38] could not be found in the human study; however, some evidence points to a possible change in the microbiota functionality. The individual differences in bacterial composition seem to outweigh any common effects of the dietary interventions used. Overall, our data with apple and apple products on the microbiota indicate changes but further studies in a larger population, or over longer periods of time, may be needed in order to observe consistent changes.

Several studies have shown that intervention with food rich in dietary polyphenols can affect both enzymatic and non-enzymatic markers of oxidative status [26, 27, 68]. In the present study, two of the investigated markers for antioxidant activity (FRAP and ORAC) were significantly affected by the intervention. Fresh apple significantly increased FRAP while the effect on ORAC was in the opposite direction. The reason for this is not clear. There was no effect on TEAC. Uric acid is the main contributor to FRAP [69] (62 %) compared to 7 and 19 % for ORAC and TEAC, respectively. If the effects on FRAP are mainly due to the effects on uric acid concentrations, this might explain the observed differences in markers of antioxidant activity reported here; however, we have not measured uric acid. The long-term depletion of fruit and vegetables may have affected the ORAC result, although this would not be expected in a balanced design like ours. We have previously shown that a daily intake of 600 g of fruits and vegetables increases erythrocyte GPX activity [26]. The effect could not be explained by the known vitamins and minerals present. This suggests that other compounds in fruits and vegetables are responsible for the observed effect. None of the other antioxidant enzymes were affected in that study. In the present study, we find similar results. GPX was the only antioxidant enzyme modulated by the dietary intervention. However, the effect was only observed in the period with apple pomace. The mechanism is unclear and needs further investigation. Plasma lipid susceptibility to oxidation was not affected by apple or apple products. It has previously been reported that apple consumption inhibits lipid oxidation [51]. However, the protective effect peaked after only 3 h and returned to baseline within 24 h. This might explain the lack of effect of the apples in our study where sampling took place after minimum 12 h of fasting.

In conclusion, the present study shows that supplementing healthy normal-weight subjects with whole fresh apple for 4 weeks may decrease serum TC concentrations without any effect observed on body weight, body composition, blood pressure, TAG, HDL-C concentrations, hs-CRP, gut microbiota composition or glucose metabolism. The effects of apple pomace and cloudy apple juice pointed in the beneficial direction in terms of plasma lipids, but these effects were also not statistically significant. An effect on LCA excretion was observed for these products and indicative for whole apple indicating a beneficial change in the gut functionality. This effect was supported by minor changes in faecal pH. Clear apple juice, which is free of cell wall components, showed adverse effects on TC and LDL-C concentrations and no effects on the gut functionality. This significant difference between clear apple juice and the other apple products might be explained by the lack of soluble as well as insoluble fibres in clear apple juice. Our study does not support the substitution of clear apple juices for whole fruits as a means of reaching the goal of 5 or more daily servings of fruit and vegetables.

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Conflict of interest All authors declare that they have no conflict of interest.

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