**Effects of chocolate supplementation on metabolic and cardiovascular parameters in ApoE3L mice fed a high-cholesterol atherogenic diet**

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**Scope:** Dietary intake of cocoa and/or chocolate has been suggested to exhibit protective cardiovascular effects although this is still controversial. The aim of this study was to investigate the effects of chocolate supplementation on metabolic and cardiovascular parameters.

**Methods and results:** Four groups of ApoE*3Leiden mice were exposed to the following diet regimens. Group 1: cholesterol-free control diet (CO). Group 2: high-dose (1.0% w/w) control cholesterol (CC). Group 3: CC supplemented chocolate A (CCA) and Group 4: CC supplemented chocolate B (CCB). Both chocolates differed in polyphenol and fiber content, CCA had a relatively high-polyphenol and low-fiber content compared to CCB. Mice fed a high-cholesterol diet showed increased plasma-cholesterol and developed atherosclerosis. Both chocolate treatments, particularly CCA, further increased plasma-cholesterol and increased atherosclerotic plaque formation. Moreover, compared to mice fed a high-cholesterol diet, both chocolate-treated groups displayed increased liver injury. Mice on high-cholesterol diet had elevated plasma levels of sVCAM-1, sE-selectin and SAA, which was further increased in the CCB group. Similar effects were observed for renal inflammation markers.

**Conclusion:** The two chocolate preparations showed unfavorable, but different effects on cardiometabolic health in E3L mice, which dissimilarities may be related to differences in chocolate composition. We conclude that discrepancies reported on the effects of chocolate on cardiometabolic health may at least partly be due to differences in chocolate composition.

**Keywords:**
Atherosclerosis / Cardiovascular disease / Inflammation / Liver injury / Renal inflammation

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metabolic factors have been linked to the development of CVD and atherosclerosis but especially elevated plasma lipids have been extensively postulated as key predictors [3, 4].

Most of these cardiometabolic disorders can be averted if necessary precautions are taken. Following a healthy dietary lifestyle can play a potent role in controlling the existing and preventing the occurrence of impending chronic diseases [5–7]. From epidemiological studies it is evident that regular intake of plant-derived products is inversely correlated with CVDs [8]. In particular, the intake of polyphenols, a subgroup of flavonoids commonly known as antioxidants, has been linked to improved cardiovascular health [9–11]. Cocoa and chocolate are a rich source of polyphenols, which are known to exhibit protective cardiovascular effects by improving endothelial function [12, 13].

However, there is conflicting data from human observational and intervention studies with regards to the effect of chocolate on cardiometabolic health. In some, but not all cases beneficial effects were observed [14] and these are attributed to polyphenols that are present in the diet [15]. Yet, it is difficult to make strong conclusions about the beneficial effects of chocolate with human intervention studies because of discrepancies in dietary habits and genetic and ethnic disparity. Similarly, studies in experimental animal models suggest a potential beneficial effect of cocoa on metabolic and vascular health. A study in Kurosawa and Kusanagi hypercholesterolemic rabbits showed that cacao liquor polyphenols decreased atherosclerotic plaque formation and foam cell formation upon cholesterol treatment [16]. Similarly, Loke et al., reported that specific dietary polyphenols attenuate atherosclerosis by alleviating inflammation and endothelial dysfunction in apolipoprotein E-knockout mice [17]. Also, Oskabe and Yasuda have reported that cacao procyandin reduced plasma cholesterol levels in cholesterol-fed rats [18, 19]. However, data on the beneficial effects of chocolate in their natural context on metabolic and vascular health are still limited.

To investigate the effects of chocolate supplementation in experimental models, it is essential that the model chosen mimic the human situation as closely as possible. The APOE3*Leiden (E3L) is an established humanized mouse model to study CVD and atherosclerosis because these mice develop hyperlipidemia and hypercholesterolemia [20]. E3L mice have an attenuated clearance of apoB-containing lipoproteins and consequently display a lipoprotein profile comparable to that of patients with dysbetalipoproteinemia [20]. Unlike many other models of CVD, E3L mice respond positively to lipid-lowering drugs such as, statins [21, 22] and dietary compounds including cafestol [23], fish oil [24], fenofibrates [25], and polyphenols [26].

We previously showed that feeding E3L mice a diet supplemented with high cholesterol (1% w/w), induces atherosclerosis, systemic inflammation and inflammation in liver and kidney [4]. We now used this model to investigate whether dietary supplementation of chocolates beneficially affects metabolic risk factors and disease endpoints in a setting of diet-induced atherosclerosis. To this end, we have compared these parameters in mice fed a control, cholesterol rich (1% w/w) atherogenic diet with mice fed the same diet supplemented with chocolate. Bearing in mind that the composition of commercially available chocolates differs from one type of chocolate to another, in particular with respect to the cocoa content [16], we used two different types of chocolates preparations differing in polyphenol and fiber content. Chocolate A had a relatively high-polyphenol and low-fiber content compared to chocolate B. In this study, our main goal was to compare the effects of the two chocolates that differed in their composition and evaluate their effects on metabolic and cardiovascular parameters in their natural context. The bioavailability of phenolic compounds from cacao and chocolate is very complex and several papers have dealt with this subject in recent years [27, 28]. Since the beneficial effect of the cocoa component is attributed to the polyphenol (flavonoid) content [12, 13] and polyphenols have a variable bioavailability [29, 30], we have assessed plasma polyphenol content, focusing on cocoa-relevant polyphenols, using MS.

2 Materials and methods

2.1 Animals and diets

Animal experiments were was performed according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH) and were approved by an independent institutional ethical committee on animal care and experimentation (DEC, Zeist, The Netherlands, DEC number 2975). Female ApoE*3-Leiden transgenic (E3L) mice (n = 76) were obtained from TNO-Metabolic Health Research, Gaubius Laboratory, Leiden, The Netherlands. Mice (12–14 weeks old) were group housed (three to five mice per cage) with a 12-h light/dark cycle and had access to water and diet ad libitum. Prior to their respective diet treatments, all animals remained on maintenance chow (Sniff R/M diet V1530, Uden, The Netherlands).

2.2 Study design

At the start of the study, the mice were divided into four groups of 19 animals each, matched for plasma cholesterol. Group 1 received a cholesterol free Western-type diet (CO; rodent diet T, Hope Farms, Woerden, The Netherlands), provided as Supporting Information Table 4. Group 2 was fed the same CO, supplemented with a high dose (1.0% w/w) of cholesterol (CC; control cholesterol). Group 3 received a control cholesterol diet supplemented with chocolate A (CCA; control cholesterol chocolate A; ACTICOA chocolate; Article: CHD-Q65ACTICOA-000; Barry Callebaut, Belgium) and Group 4 received a control cholesterol diet supplemented
with chocolate B (CCB; control cholesterol chocolate B; Article: CHD-L108018B01-000; Barry Callebaut, Belgium). The chocolate dosage used in this study is considered relevant for the human situation. The dosage in mice was aligned according to a dosage applicable in humans, assuming an intake of 70 g chocolate/person/day (= 0.875 g/kg/day). Because mice have a tenfold higher metabolic rate, i.e. 8.75 g/kg/day, the chocolate concentrations were in both cases 8.75% w/w of the diet.

Both CCA and CCB are high in polyphenol content but differ in the polyphenol concentration and fiber content. For the CCA and CCB diets, cocoa butter was compensated based on the chocolate content of CCA and CCB and thus comparable between all the groups. All the major components present in both chocolates are same and the total amount of fats in all the diets was kept constant and the diets did not significantly differ in energy content. Both chocolate diets were prepared by homogenously mixing liquid chocolate (chocolate melted in a water bath at 55°C for 3 h), liquid cocoa butter (melted on a stove for approximately 1 h), cholesterol-free basic diet without cocoa butter, and cholesterol mixed with agar. Diets were stored at −20°C until use. The gross energy content of the diets was determined by assessing 0.5 g of diet, in duplicate, in a bomb calorimeter (calorimeter C7000, IKA, Staufen, Germany). The composition of chocolates used in this study is provided in Supporting Information Table 1.

Body weight (individually) and food intake (at cage level) were monitored every 4 weeks and blood samples were taken by tail vein incision after 4 h of fasting at t = 0 and weeks 4, 8, 12, 16, and 20. At sacrifice, spot urine was collected from individual animals. After 20 weeks of the experimental diet feeding, mice were fasted for 4 h and sacrificed by CO2 and organs were isolated and weighed. Adipose tissues, aortas, livers (median lobe), and kidneys (left) were formalin fixed and embedded in paraffin; livers (sinister and caudate lobes) and kidneys (right) were snap frozen in liquid N2 and stored at −80°C until further use.

2.3 Analysis of plasma lipids, lipoprotein profiles, and plasma inflammation markers

Total plasma cholesterol and triglyceride levels were measured after 4 h of fasting, using kits number 11489437 and 11488872 (Roche Diagnostics, Almere, The Netherlands), respectively. For lipoprotein profiles, pooled plasma was fractionated using an ÄKTA FPLC system (Pharmacia, Roosendaal, The Netherlands) [31]. Plasma ALAT (alanine transaminase) levels were determined spectrophotometrically using a Reflotron system (Roche Diagnostics). The plasma levels of E-selectin, VCAM-1 (all R&D Systems Europe, Ltd., Abington, UK), insulin (Alpco, Tilburg, The Netherlands), and SAA (Biosource, Nivelles, Belgium) at sacrifice were determined by ELISA as reported [4].

2.4 Plasma polyphenol metabolites extraction and quantification

The extraction of the chocolate metabolites at sacrifice from serum (nine random serum samples from the same dietary group pooled as three samples, n = 3) samples in the fed state was carried out by using OASIS HLB µElution Plates 30 µm (Waters, Milford, USA) following the method described by Serra et al. (2011) with some modifications [32]. First, the wells were sequentially conditioned by using 250 µL of methanol and 250 µL of miliQ water/acetic acid (99.2:0.2, v/v). For the extraction of the metabolites, 600 µL of a mixture of serum and phosphoric acid 4% containing catechol as internal standard were loaded onto the plate. After that, the clean-up of the plates was sequentially done with 200 µL of miliQ water and 200 µL of miliQ water/acetic acid (99.2:0.2, v/v) to eliminate any interference that the sample might contain. Finally, the elution of the retained metabolites was done with 2 × 50 µL of acetone/miliQ water/acetic acid (70:29:5:0.5, v/v/v).

Chocolate metabolites in the serum samples were quantified and identified by using an HPLC-MS/MS system consisting of an Agilent HPLC 1200 Series (Agilent Technologies, Palo Alto, USA). A Zorbax SB-Aq column (3.5 µm, 150 mm × 2.1 mm i.d.) equipped with a pre-column Zorbax SB-C18 (3.5 µm, 15 mm × 2.1 mm i.d.) (Agilent Technologies) was used to carry on the analysis. The HPLC was coupled to a triple quadrupole mass spectrometer 6410 (Agilent Technologies). Data acquisition was carried out using Masshunter software.

Analyses of the serum metabolites were carried out in electrospray (ESI) in the negative mode. The selected reaction monitoring (SRM) transitions and the individual fragmentor voltage and collision energy for each compound were evaluated by infusing 10 mg/L of each compound to obtain the best instrumental conditions. Two transitions were acquired for each compound, one for quantification and a second for confirmation purposes. The selected SRM transitions and the instrumental parameters for each compound are shown in Supporting Information Table 2A and B.

Quality parameters of the analytical method (linearity, recovery, accuracy, reproducibility, LOD, and LOQ) were evaluated to confirm the reliability of the method. These parameters were determined by spiking basal plasma with 14 commercially standards at known concentrations. Results confirm the suitability of the method to study the phenolic metabolite composition of plasma samples (data not shown).

2.5 Histological analysis of atherosclerosis

Serial cross-sections (5 µm thick) were taken throughout the entire aortic root area for histological analysis of atherosclerosis. Briefly, paraffin-embedded aortic cross-sections were stained with hematoxylin-phloxine-saffron and atherosclerotic lesion area was analyzed blindly in four cross-sections of
each specimen (at intervals of 50 μm). Cell-D software (Olympus Soft Imagine Solutions GmbH) was used for morphometric computer-assisted quantification of lesion number, lesion area, and lesion severity according to the classification of the American Heart Association as established [21].

2.6 Renal RNA extraction and gene expression analysis

Total RNA was extracted from thirty 5 μm thin cryosections from kidney using RNasey Mini plus Kit (Qiagen, Westburg, Leusden, The Netherlands) according to the manufacturer’s instructions. Integrity of RNA was determined by Agarose gel electrophoresis. RNA quantity (OD-260) and quality (OD-260/OD-280) were determined using an ND-1,000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Total RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). To detect the expression of selected target genes Assays-On-DemandTM gene expression primer/probe sets (ABI Systems, Foster City, CA, USA) were used. Endogenous B-actin (assay Mm01205647_g1) was used as a housekeeping gene along with the following probes: CD68 (assay IDMM00839636_g1), monocyte chemotactic protein-1 (MCP-1; assay IDMM00441242_m1), VCAM-1 (assay IDMM00449197_m1), and E-selectin (assay IDMM00441278_m1). Real-time PCR was performed in duplicate and the obtained threshold cycle (CT) values were averaged. Relative mRNA levels were calculated as 2-ΔCT, in which ΔCT is CT gene of interest—CT housekeeping gene.

2.7 Kidney function measured by albuminuria

To assess renal function, the microalbumin levels were measured in mouse urine using a commercially available ELISA kit (Mouse Albumin ELISA Quantitation set, Bethyl Laboratories, Montgomery, Texas, USA) according to the manufacturer’s instructions.

2.8 Renal histology and immunohistochemistry

For light microscopy, 3 μm renal paraffin sections were stained with periodic acid Schiff’s as reported [33]. Immunohistochemical staining for macrophages was performed on acetone-fixed 5 μm cryosections using an anti-rabbit peroxidase-based Envision®+ system (DakoCytomation, Carpinteria, CA, USA). Briefly, sections were incubated for 60 min with 5 μg/mL rat-anti-mouse CD68 (clone FA11, Serotech, Puchheim, Germany), followed by a 30-min incubation with 10 μg/mL unlabeled rabbit-anti-rat secondary antibody (Vector Laboratories, Burlingame, CA, USA). After detection of peroxidase activity with 3-amino-9-ethylcarbazole, sections were counterstained with Mayer’s hematoxylin.

2.9 Liver aconitase and citrate synthase activity

Liver was ground in liquid nitrogen and further homogenized using a disposable pestle in a microtube containing ice cooled 50 mM Tris-HCl, pH 7.4. All handling was done on ice until measurement. Protein concentrations were measured using the DC Protein Assay (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer’s instructions. Maximal aconitase activity was determined in fourfold by monitoring the formation of NADPH as described [34]. Absorbance at 340 nm was measured during 1 h at 37°C in a microplate reader (BioTek, Winooski, USA) and the maximal aconitase activity was calculated from the slope of the linear part of the curve. Citrate synthase was measured in triplicate in a 96-well plates using the Citrate Synthase assay kit (Sigma-Aldrich, St. Louis, USA) according to the manufacturer’s instructions. Absorption was measured for 1 h at 25°C in a microplate reader (BioTek, Winooski, USA) and the maximal citrate synthase activity was calculated from the slope of the linear part of the curve.

2.10 Statistical analysis

Data were analyzed with Graphpad prism (Graphpad software 5.0, San Diego CA, USA) and SPSS 17.0 for Windows. Differences between groups at one specific time point were analyzed with one-way ANOVA followed by LSD posthoc analysis. Changes over time were statistically analyzed with one-way ANOVA (repeated measures for within subject samples) and LSD posthoc, unless stated otherwise. p < 0.05 was considered significant. Results are shown as means ± SEM, unless stated otherwise.

3 Results

3.1 Body weight, fat tissue masses, and food intake

The average food intake expressed as kcal/24 h did not differ significantly between the groups (Fig. 1A). Body weight at baseline (week 0) was 21.6 ± 1.15 g on average of all groups together. All the groups showed a similar body weight gain throughout the experimental period reaching 23.4 ± 2.2 g in CO group, 23.9 ± 1.1 g in CC group, 23.8 ± 1.6 g in CCA group, and 23.5 ± 1.2 g in CCB group at the end of the experimental period of 20 weeks (Fig. 1B). Similarly, all the groups showed comparable visceral, gonadal, and subcutaneous fat tissue weights (data not shown).

3.2 Bioavailability of plasma polyphenol metabolites

The analysis of the phenolic compounds in the plasma of CO-, CC-, CCA-, and CCB-treated mice revealed the occurrence of phenolic acids such as 3,4-dihydroxyphenylacetyl
ticit, protocatechuic, hippuric, p-hydroxyphenylacetic, 3-hydroxyphenylacetic, vanillic, p-coumaric, ferulic, and benzoic acids. Besides, some flavonoids were also identified such as catechin, epicatechin, and procyanidin B2. Additionally, both chocolate (CCA and CCB) treated groups revealed the presence of methyl and sulphate derivatives of (epi)catechin including methyl catechin glucuronide, methyl epicatechin glucuronide, epicatechin glucuronide, catechin sulphate, methyl epicatechin-O-sulphate, epicatechin sulphate, and 3-O-methyl epicatechin. These metabolites are specific of cocoa consumption and therefore, were absent in both CO- and CC-treated groups. The SRM transitions and the instrumental parameters for each plasma metabolite are listed in Supporting Information Table 2. The individual concentrations of these polyphenols and their corresponding metabolites are listed in the Supporting Information Table 3. Comparing the concentrations of these plasma metabolites levels between the two chocolates, values were almost ten times higher in CCA compared to CCB. The principal difference in the phenolic fraction of the two chocolates is in the flavonoids (catechins, epicatechins, and procyanidins), which are considered to constitute the beneficial compounds of cocoa/chocolate [29, 30]. This result is consistent with the different phenolic content of both chocolates used in the study.

### 3.3 Plasma lipids and atherosclerosis

Fasting plasma triglyceride levels did not significantly differ between the groups at all-time points measured (Fig. 2A). At the beginning of the study \( (t = 0) \) total plasma cholesterol levels were similar in all the groups (CC; 2.82 ± 0.39 mM, CCA; 2.80 ± 0.27 mM, CCB; 2.81 ± 0.39 mM, and CO; 2.66 ± 0.33 mM). Mice on CC, CCA, and CCB displayed an increase in plasma cholesterol levels over the 20-wk diet intervention period. Compared to CO fed mice (4.98 ±
Figure 2. Effect of chocolates on plasma cholesterol and lipoprotein profiles at week 20. A) Plasma cholesterol levels of the CC, CCA, and CCB groups were significantly increased compared to CO group, p < 0.001. B) Lipoprotein profiles showed significantly increased VLDL cholesterol levels in CCA and LDL cholesterol levels in CCB, compared to CC at week 20, p < 0.01. The ratio between HDL cholesterol and LDL+VLDL cholesterol of the area under curve (AUC) was higher compared to both chocolate groups (CCA and CCB) (Fig. 2D). A refined morphological analysis of atherosclerotic lesions displayed no signs of atherosclerotic lesions in CO group. Total lesion areas of the other three groups (CC; 102072 ± 61882 µm², CCA; 163907 ± 61567 µm² and CCB; 124372 ± 76837 µm²) were significantly higher compared to CO whereas lesion area of the chocolate groups (CCA versus CCB) did not differ significantly from each other. Furthermore, CCA group showed a significantly increased lesion area compared to CC, while CCB showed no difference compared to CC or CCA (Fig. 3A).

Differences in the lesion severity in the aortic root were assessed according to the classification of the American Heart Association. In the CCA group, more severe lesions of type IV to V (56%) were observed compared to the CC group (Fig. 3B).

3.4 Systemic inflammatory markers

Plasma levels of the vascular inflammatory markers E-selectin and VCAM-1 and of liver-derived SAA in CC group were significantly increased compared to the CO group after 20 weeks, p < 0.01, p < 0.001, p < 0.001, respectively). CCB caused a more pronounced and significant increase in all markers (E-selectin, VCAM-1, and SAA) measured, compared to CC group, p < 0.001, p < 0.01, p < 0.05, respectively. Furthermore, CCB displayed considerably higher E-selectin and VCAM-1 levels in plasma compared to CCA (p < 0.05), while CCA showed no difference compared to CC after 20 weeks (Fig. 4).

3.5 Liver function

At sacrifice, livers were significantly heavier in the CC group (1.40 ± 0.26 g), when compared to the CO group (1.07 ± 0.14 g) (p < 0.001). Treatment with both chocolates further increased liver weights when compared to both CO and CC. However, when compared to CCB, liver weights in the CCA group were significantly lower (1.62 ± 0.34 g versus 1.89 ± 0.25 g, p < 0.05, Fig. 5A). Similarly, in the CC group, a
significant increase in alanine transaminase (ALAT) levels in plasma when compared to CO was observed. Both chocolate treatments further increased plasma ALAT levels compared to both CO and CC, which was most pronounced in the CCB group (Fig. 5B).

Mitochondrial metabolic function in the liver was measured by determining citrate synthase and aconitase activities. All the three cholesterol treated groups (CC, CCA, and CCB) displayed a decreased citrate synthase and aconitase activity (normalized to total protein) compared to CO ($p < 0.01$, $p < 0.001$, $p < 0.001$, respectively) (Fig. 5C and 5D). No change was observed in the aconitase activity/citrate synthase activity ratio between any of the groups (Fig. 5E).

3.6 Renal inflammation, structure, and function

Renal mRNA expression levels of endothelial activation (VCAM-1 and E-selectin) and inflammation (MCP-1, CD68) markers were upregulated with CCB feeding when compared to both CO and CC. Mice fed the CCA diet displayed increased expression of renal inflammatory genes (MCP-1 and CD68) when compared to CO but not when compared to CC. However, CCA showed less increase in expression of VCAM-1, E-selectin, and CD68 when compared to CCB (Fig. 6).

Kidney weight and urinary albumin levels were similar in all groups (Supporting Information Fig. 1A and B). Consistent with this, analysis of periodic acid Schiff’s stained renal sections showed no overt renal abnormalities in all groups (Supporting Information Fig. 1C).

4 Discussion

In recent decades, an increasing number of experimental and observational studies, both in humans and animals, have suggested potential beneficial effects of chocolate on cardiovascular and metabolic parameters [12, 35, 36]. In the current study, we employed a humanized CVD-prone mouse model, the ApoE*3 Leiden (E3L), to determine whether chocolate supplementation is able to improve cardiometabolic risk factors induced by an atherogenic high-cholesterol diet. The high-cholesterol diet was supplemented with two different types of chocolate and various parameters were analyzed and compared including plasma cholesterol levels, atherosclerotic plaque formation, liver and kidney function, and systemic inflammatory responses. Overall, the results from our study showed that dietary chocolate supplementation did not reduce the adverse effects of an atherogenic high-cholesterol diet and in some cases even aggravated these effects.

Hypercholesterolemia and oxidation of lipids are critical steps in the pathogenesis of atherosclerosis [4, 37]. Previous studies, both in humans and animal models, investigating chocolate or cacao powder supplementation have suggested a
protective effect on atherosclerosis development attributed to hypocholesterolemic and/or anti-oxidative properties of chocolate components [17, 38, 39]. Our study does not corroborate these findings since chocolate supplementation was found to increase plasma total cholesterol levels and, in the case of CCA, aggravated atherosclerotic plaque formation. In spontaneously hypercholesterolemic rabbits receiving cocoa liquor extract elevated plasma cholesterol have been reported as well although the cocoa liquor supplementation did reduce atherosclerosis development [16]. Moreover, studies in golden Syrian hamsters have shown that the cardiometabolic effects of cacao powder supplementation may be dose dependent: a high-dose cacao powder (1% Hershey’s cocoa powder) significantly reduced plasma cholesterol levels but failed to reduce atherosclerotic plaque formation whereas a low-dose cacao powder (0.1% Hershey’s powder) did significantly reduce atherosclerosis development [40]. In the same context, Desch et al. reported possible long-term side effects of chocolate intake including increased energy, fat, and sugar content probably leading to adverse metabolic effects. These authors suggested determining the optimal dose of cacao or chocolate before considering it as a treatment for cardiovascular health [41]. This could be one of the reasons behind the differences observed in plasma cholesterol levels and plaque formation between the two chocolates used in our study.

It has been reported that parameters for liver damage (elevated plasma ALAT levels) and lack of anti-oxidative capacity are closely associated [42]. Chocolate is one of the major sources of bioactive antioxidant compounds [38] and both chocolate and cocoa have been reported to be associated with reduced LDL oxidation and platelet aggregation [38]. In our study, we observed that in both chocolate-fed groups, plasma ALAT levels were increased compared to mice fed high cholesterol suggesting that the possible anti-oxidative properties of chocolate polyphenols are insufficient to prevent these adverse effects [43, 44]. Moreover, in the liver, the ratio of aconitase activity over citrate synthase activity, which is considered a marker for oxidative stress, was unchanged indicating that dietary chocolate supplementation did not reduce the level of oxidative stress upon high-cholesterol feeding. This is in line with the current notion that chocolates may have specific physiological effects such as amelioration of atherogenesis and dampening systemic inflammation, rather than having a general anti-oxidant effect [30].

Inflammation plays a crucial role in atherogenesis. Leukocyte binding to endothelial cells and their subsequent infiltration into subendothelial spaces is mediated by various adhesion molecules including E-selectin and vascular cell adhesion molecule-1 [45]. Previously, we have shown that elevated plasma E-selectin, VCAM-1, and SAA levels are associated with atherosclerosis development in E3L mice [4]. In the present study, dietary treatment with either chocolate preparation did not reduce the plasma levels of sVCAM-1, sE-selectin and SAA levels in plasma when compared to mice fed a high-cholesterol diet. In fact, in mice fed CCB the levels of these soluble markers were increased when compared to mice fed the atherogenic diet alone suggesting an increased inflammatory response. Similar effects were observed in the kidney showing significantly upregulated E-selectin and VCAM-1 mRNA levels in the CCB group. These diet-induced renal effects were diminished in the CCA chocolate group, which displayed similar levels of expression as the atherogenic CC group. In addition, enhanced expression of MCP-1 mRNA in conjunction with increased expression of the macrophage marker CD68 was observed in CCB chocolate treated group, while CCA displayed similar levels as CC group. This shows that the overall components and composition of chocolate A is partially able to reduce these adverse effects of the chocolate supplementation, whereas, chocolate B aggravated these renal effects.
In the present study, we observed differential effects of the two chocolates on systemic and organ-specific inflammatory parameters. Both CCA and CCB show no effect or unfavorable effects on plasma parameters, atherosclerosis, and/or kidney status when compared with CC. These unfavorable effects cannot be easily explained because the chocolate preparations differ in many components and we can only speculate. Supplementation with chocolate A (CCA group) appeared to be less unfavorable than chocolate B (CCB group) with respect to plasma and renal inflammatory parameters. This may be related to the higher circulating polyphenol concentrations present in the CCA group (see Supporting Information Table 3), which would be in line with reported anti-inflammatory effects of cocoa polyphenols [46]. However, CCA was unsuccessful in reducing the adverse effects caused by the atherogenic diet (CC) itself and mice in this group developed more severe atherosclerosis compared to the CC group. This may be due to the higher plasma cholesterol levels observed in the CCA group. In contrast, the reduced atherosclerosis development observed in the CCB group may be attributed to a cholesterol-lowering effect of fibers of which the content was higher in the CCB group. Interestingly, in a meta-analysis, dietary fiber intake has been associated with lower cholesterol levels [47].

In all, the present study shows that two chocolate preparations differing in the content of polyphenols, fiber, and others, both unfavorably affected cardiovascular and metabolic health, albeit to a different extent. Together with previous findings demonstrating that different dietary doses of chocolates can also exert dissimilar effects on CVD outcome [40,41], we conclude that discrepancies reported on the effects of chocolate on cardiometabolic health may at least partly be due to differences in chocolate composition and quantity consumed.

The authors' responsibilities were as follows: Conceived and designed the experiments: T.K., R.K., P.Y.W., G.K.Y., P.H., J.K., and J.V.G. Performed the experiments: G.K.Y., P.Y.W., A.B., M.S., J.K., R.K., T.K., and P.H. Analyzed the data: G.K.Y., P.Y.W., A.B., M.S., J.K., R.K., T.K., and P.H. Wrote the paper: G.K.Y., M.S., J.K., T.K., and P.H. All authors read and approved the final manuscript.

The authors have declared no conflict of interest.

5 References

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