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The effect of cocoa flavanols on endothelial function in premenopausal women using combined oral contraception: a randomized controlled trial

Dissertation

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Diese Arbeit widme ich meiner Familie.

Zusammenfassung

Zahlreiche epidemiologische Studien legen nahe, dass flavanolhaltige Lebensmittel wie Schokolade, Tee oder Rotwein positive Auswirkungen auf das kardiovaskuläre Risiko haben. Die bisherige Datenlage zeigt, dass Kakaoflavaole (CF) die Endothelfunktion verbessern können. Die meisten Studien wurden mit Männern oder Frauen nach der Menopause durchgeführt. Studien die die Auswirkungen kombinierter oraler Kontrazeptiva (COC) auf die Endothelfunktion bei prämenopausalen Frauen untersuchen sind begrenzt, zeigen jedoch allgemein ein erhöhtes kardiovaskuläres Risiko bei COC-Anwendern im Vergleich zu Nicht-Anwendern. Derzeit ist nicht bekannt ob COC die positiven Auswirkungen von CF auf das Gefäßendothel beeinflussen können.

Das Ziel unserer Arbeit war, die Wirkung von CF auf die Endothelfunktion bei prämenopausalen Frauen während des hormonellen Verhütungszyklus zu untersuchen. In einer randomisiert, placebo-kontrollierten Doppelblindstudie im Parallel-Design wurde die flussvermittelte Vasodilatation der Arteria brachialis (FMD) bei 24 gesunden Frauen gemessen. Die Untersuchungszeitpunkte entsprachen dem Einnahmezyklus der oralen Kontrazeptiva (pillenfreien Phase 5-7 Tag; Pillenphase 26 bis 28 Tag). Die Messungen wurden zu Studienbeginn (0h) und zwei Stunden nach dem Verzehr eines Testgetränks durchgeführt.

In einer zweiten Studie untersuchten wir gezielt verschiedene COC und deren Einfluss auf die CF Wirkung. Insgesamt 62 Frauen mit 5 verschiedenen Typen von COC wurden randomisiert und der CF- oder der Kontrollgruppe zugeordnet.

Unsere Ergebnisse zeigten, dass CF die FMD im Vergleich zur Kontrolle (p-Haupteffekt-Intervention < 0,001) in der pillenfreien Phase (2h: 2.0±0.4%; 1 Mo: 1.9±0.7%; 1 Mo, 2h: 3.3±0.7%) und aktiven Pillenphase (2h: 1.4±0.5%; 1 Mo: 1.1±0.6%; 1 Mo, 2h: 2.1±0.7%) des Zyklus signifikant erhöhte. Allerdings waren die Ergebnisse während der aktiven Pillenphase im Vergleich zur pillenfreien Phase signifikant niedriger (p-Interaktion [Pille x Intervention] = 0,019). CF hatte keinen Einfluss auf Blutdruck und Blutfette. Es gab auch keinen signifikanten Unterschied zwischen den Flavanolmetaboliten zu den verschiedenen Zeitpunkten im OC-Zyklus.

In der zweiten Studie wurden die Ergebnisse im Hinblick auf akute Effekte bestätigt und zeigten, dass CF sowohl in der aktiven Pillenphase als auch in der pillenfreien Phase nach 2h, im Vergleich zur Kontrolle, die FMD signifikant erhöhte (*p*-Haupteffekt-Intervention < 0,001). Auch hier waren die Effekte in der aktiven Pillenphase (1.5±0.2%) niedriger als in der pillenfreien Phase (2.0±0.2%; *p* Interaktion [Pille x Intervention] = 0,038). Die Effekte unterschieden sich jedoch nicht zwischen den 5 verschiedenen Pillentypen p-Interaktion [Pillentyp x Intervention] = 0,720). Unsere Daten belegen, dass CF die Endothelfunktion signifikant verbessern kann, der Effekt während der Pillenphase im Vergleich zur pillenfreien Phase jedoch abgeschwächt ist. Bei einer so grossen Population von Frauen, die orale Kontrazeptiva einnehmen, wirken sich diese Erkenntnisse weltweit auf Millionen von Frauen aus. Diese Studie liefert wichtige Informationen für zukünftige Ernährungsempfehlungen zur Einnahme von Flavanolen und weist auch auf neue Mechanismen hin, durch die OC die kardiovaskuläre Gesundheit beeinflussen kann.

Summary

Epidemiological studies suggest that the consumption of foods containing flavanol, such as chocolate, tea or red wine, is associated with favorable effects in reducing cardiovascular risk. Accumulating data from clinical intervention trials support this and show that cocoa flavanols CF can improve endothelial function, decrease blood pressure and cholesterol. However, most of the studies were conducted in men or postmenopausal women. Studies investigating the effects of combined oral contraceptives (COC) on endothelial function in premenopausal women are scarce but have shown generally an increased cardiovascular risk in COC users as compare to non-users. At present it is unknown whether synthetic sex hormones such as COC might influence the vascular effects exerted by CF. Here, we investigated the effect of CF on vascular function in healthy women taking different COC during the different phases of the oral contraceptive (OC) cycle. In a 2-month double-masked, parallel-group randomized controlled trial, 24 healthy premenopausal female subjects taking COC were randomly allocated to either a CF drink (410 mg) or flavanol-free Control drink. During the first month, acute responses to a single dose of CF or Control were tested during 2 study visits. During the pill-free phase (days 5-7) and the third week of the active pill phase (days 26-28), FMD and BP were measured at baseline before and at 2 h after 1 dose. After the second visit, the participants ingested either CF or Control twice daily until and during the next cycle. FMD and BP measurements were then taken again before (chronic) and at 2 h after the first dose of the day (acute-on-chronic) during the next pill-free phase (days 5-7) and the third week of the next active pill phase (days 26-28). In addition to FMD and BP, blood samples were collected and assessed for structurally related (-)-epicatechin metabolites analysis in plasma HPLC-FLD-ECD and lipids. In a second study, we investigated if differences existed between different COC in terms of attenuation of acute CF-related FMD improvements. A total of 62 women with 5 different types of COC were randomly allocated to either CF or Control. We tested the acute responses to a single dose of CF or control on FMD and BP and measurements were performed at baseline before and at 2 h after 1 dose during the pill-free phase (days 5-7) and the third week of the active pill phase (days 26-28).

The results showed that CF significantly increased FMD as compared to Control (*p* main effect intervention < 0.001) in the pill-free (2 h: 2.0±0.4%; 1 mo: 1.9±0.7%; 1 mo, 2 h: 3.3±0.7%) and active pill phase (2 h: 1.4±0.5%; 1 mo: 1.1±0.6%; 1 mo, 2 h: 2.1±0.7%) of the OC cycle. However, the responses were significantly lower during the active pill phase as compared to the pill-free phase (p interaction [pill x intervention] = 0.019). CF did not affect blood pressure and blood lipids. There was also no significant difference between CF metabolites at different times in the OC cycle. In the second study, the results in terms of acute responses were confirmed and showed that CF significantly increased FMD at 2 h in both the active pill and pill-free phase as compared to control (p main effect intervention < 0.001). Again, the responses were lower in the active pill phase (1.5±0.2%) as compared to pill-free phase (2.0±0.2%; *p* interaction [pill x intervention] = 0.038). However, the responses did not differ between the 5 different OCs (p interaction [pill-type x intervention] = 0.720).

Our data support that in women taking combined OC, CF can improve endothelial function, but the response is attenuated during the active pill phase as compared to the pill-free phase. This study provides important information for future dietary recommendations on flavanol intake and also points to new mechanisms by which OC may affect cardiovascular health. With such a large population of females currently taking oral contraceptives, these findings impact millions of premenopausal women worldwide.

List of abbreviations

ACE	Angiotensin converting enzyme
AMPK	Adenosinemonophosphate-activated protein kinase
AT	Angiotensin-receptor
Akt - Protein kinase B	Serine/Threonine-specific protein kinase
BMI	Body-Mass-Index
BP	Blood pressure
CRP	C-reactive protein
COC	Combined oral contraception
CF	Cocoa flavanols
CVD	Cardiovascular disease
DRSP	Drospirenone
DNG	Diengost
EC	(-) Epicatechin
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinases
EE	Ethinylestradiol
eNOS	Endothelial nitric oxide synthase
FMD	Flow mediated dilation
GOT	Glutamate oxaloacetate transaminase
GPT	Glutamate pyruvate transaminase
GIT	Gastrointestinal tract
GPER	G-protein-coupled receptor
HRT	Hormone replacement therapy
HUVEC	Human umbilical vein endothelial cells

HPLC-FLD-ECD	High-performance liquid chromatography with fluorescence and electrochemical detection
HDL	High density lipoprotein
LNG	Levonorgestrel
LDL	Low density lipoprotein
MPA	Medroxyprogesterone acetate
NO	Nitric oxide
OH	Hydroxyl
OC	Oral contraceptive
PI3K	Phosphoinositid-3-kinase
PC	Procyanidin
PR	Progesterone receptor
PKA	Protein kinase A
ROS	Reactive oxygen species
RAAS	Renin-angiotensin-aldosterone-system
SREM	Structurally related epicatechin metabolites
Src	Non-receptor tyrosine kinase
VSMC	Vascular smooth muscle cells
VEGF	Vascular endothelial growth factor
VTE	Venous-thromb-embolism
γVLs	γ-valerolactones
γGT	Gamma gltamyl-transferase
RCT	Randomized Controlled Trial

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1 Introduction

1.1 Women and cardiovascular disease

While often declared as a "men's disease", cardiovascular disease (CVD) is the number one killer among the female population (Fig. 1) [1-4] and still the leading cause of death globally whereas raised blood pressure is the leading risk factor [5, 6]. A woman's lifetime risk of developing CVD increases extensively after menopause [7-9], at the age of 50 the estimated CVD risk is about 40% [10]. Depending on the number of specific risk factors (e.g. hypertension, diabetes mellitus, smoking) the incidence can be even higher. On the contrary, heart diseases as well as comorbid conditions, such as hypertension, are conspicuously less during the premenopausal period compared to men of same age [11, 12]. Nevertheless, risk increases over time in both genders whereas an initial onset of CVD appears about 10 years later in women [13-15]. Even though commonly established risk factors are the same in both men and women, some of them can be slightly more attributed to one or the other gender.

One reason for this is the fact that there are gender-specific differences in the prevalence and significance of the individual factors [12, 16]. On one hand, systolic arterial hypertension, smoking, diabetes mellitus, triglyceride, high-density lipoprotein levels and menopause as a unique risk factor, are more relevant in women. On the other hand, hypertension, total cholesterol and low-density lipoprotein have a greater influence in men. Not only the combination of multiple risk factors is more frequently present among the female population, but also a less rigorous drug therapy is performed [16-19]. Overall, women have more time to develop cardiovascular events due to a higher life expectancy compared to men [20, 21].

Lower prevalence rates in younger age groups indicate that women during their fertile age seem to have cardiovascular protective properties. Thereby the risk of CVD in women is often underestimated. Consequently, substantial studies demonstrate that reduced levels of ovarian hormones correlate with a major risk of CVD [22-25]. An explanation for this might be the beneficial effects on the circulatory system exerted by ovarian steroid hormones, in particular, estrogen [26-28]. Estrogen has several modifiable effects on metabolic factors, such as lipids, inflammatory markers, the coagulant and vascular system as well as its influence on endothelial dysfunction and visceral adiposity.

With the onset of menopause, however, decreased synthesis of natural estrogen is associated with a development of systemic hypertension, impaired glucose tolerance, abnormal lipid profile and insulin resistance [29, 30]. These parameters tend to increase the progression and incidence of cardiovascular disorders [31-33]. Therefore, it can be assumed that menopause itself is an independent predictor for CVD.

Furthermore, women are relatively rare in presenting typical symptoms and classical clinical signs. Left-sided chest pain is less frequently in women than in men [34, 35]. Women more often suffer from abdominal pain, dizziness, shortness of breath, frequent indigestion, unusual fatigue and nausea [34, 36]. Therefore, the myocardial infarction is often misinterpreted as abdominal or back pain. Finally, this is often a cause for women to delay seeking emergency care and lifesaving interventions [37]. Exposure to behavioural risk factors such as unhealthy diet, physical inactivity, tobacco use and harmful use of alcohol are the most important factors of cardiovascular diseases women must face when they are at an older age. Unfortunately, many of these risk factors are increasing in prevalence and severity, especially in young women [38-41].

Nonetheless, according to the WHO, the burden remains high considering that CVD accounts for 46% of older women's deaths globally [2]. This underscores the enormity of CVD as a global health issue.

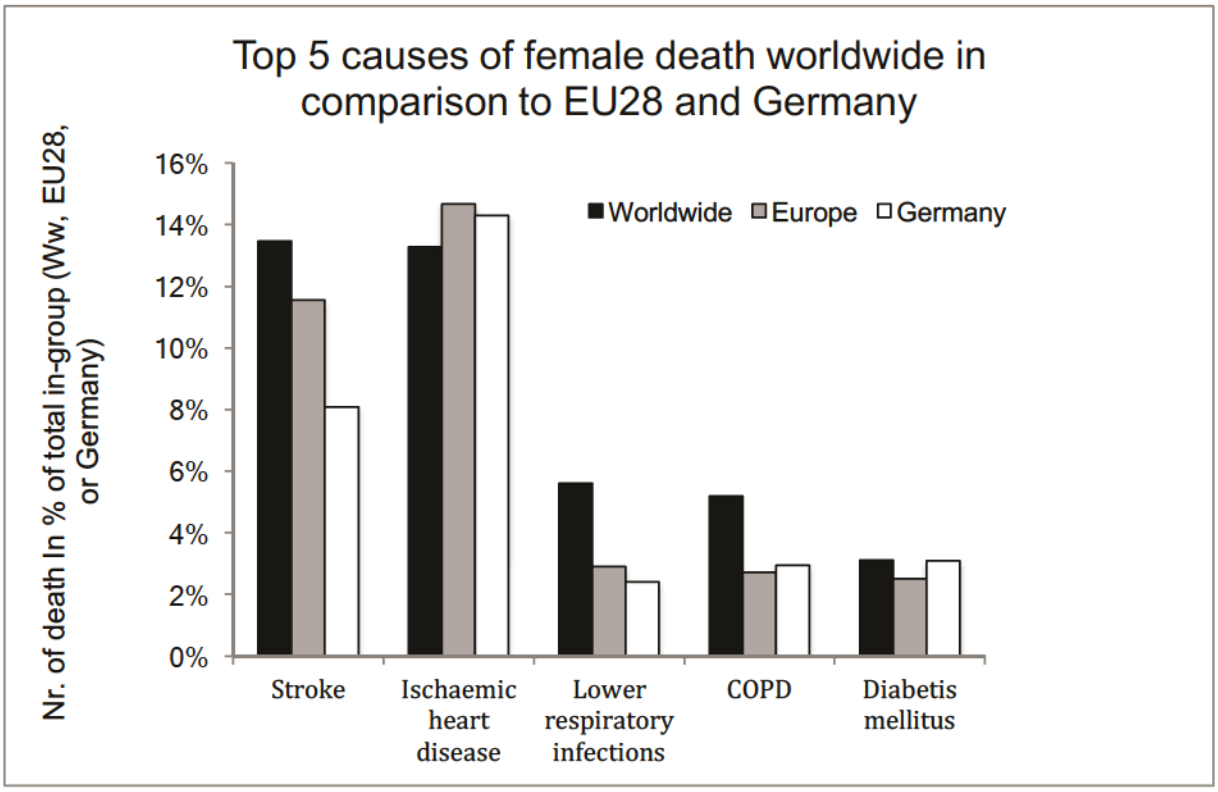

Figure 1: Cardiovascular disease (CVD) and other major causes of death among the female population - modified from GLOBAL HEALTH ESTIMATES 2014 SUMMARY TABLES, May 2014, World Health Organization, Geneva, Switzerland [42]. COPD = Chronic obstructive pulmonary disease

1.2 Polyphenols – potential benefits for human health

Worldwide, more people die from the effects of CVD than from any other cause [6]. With the world population getting older, the incidence of major adverse cardiovascular events will only increase. It has been estimated that by 2030 nearly 23 million people will die from cardiovascular disorders [43]. Diet, as one of the most important lifestyle factors, it can greatly affect the incidence of CVD [44, 45]. In recent years, several observational studies and nutritional intervention trials have indicated cardioprotective effects of diets rich in fruits and vegetables [46-50]. Significant evidence suggests that a large group of natural plant compounds, called polyphenols, may be at least partially responsible for the health benefits of fruits and vegetables [45, 51]. Polyphenols are classified as secondary metabolites since they are not formed and consumed in the primary metabolism of higher plants. These metabolites are involved in many important plant processes such as protection against bacterial infections, control of the plant hormonal balance, use as a signal substance and defence against ultraviolet radiation [52-57].

The most important food sources of polyphenols are vegetables and fruits, green and black tea, red wine, coffee, nuts, olives and olive oil, and some herbs and spices, as well as chocolate and cocoa products [58].

Although polyphenols are an heterogeneous group, they have a common characteristic. They are characterized by the presence of more than one phenol structural units. The basic chemical structure, the phenol, is represented in Fig. 2A. By containing an aromatic ring structure to which hydroxyl (OH) groups are condensed, the number of rings and the positions of the OH- groups can vary. Due to this diversity there are more than 8000 phenolic compounds classified into several sub-groups [59, 60]. Phenolic acids, stilbenes, lignans and flavonoids reflect the main classes (Fig. 2B) [61]. Phenolic acids are non-flavonoid phenolic compounds with just one phenolic ring which can be further divided into two main types, benzoic acid (A) and cinnamic acid (B) derivatives, while flavonoids are the most abundant studied group so far. More than 4000 varieties of flavonoids have been identified, many of which are responsible for bitterness, astringency and the natural colours of plant foods [62, 63].

In general, flavonoids possess three OH-groups, two of them are on ring A at positions five and seven, and the other one is located on ring B at position three. In the subclasses, flavanones, flavones, flavanols and anthocyanidins, the B ring is linked to position two of the basic framework, and the iso-form is in position three. Individual differences within each group arise from the considerable variation which is introduced by distinct chemical substitutions, specially hydroxylation and glycosylation. Usually, flavonoids (in higher plants) are glycosylated with glucose or rhamnose but can also be linked with arabinose, galactose or xylose [64]. Flavan-3-ols are the only subgroup that are not present as glycosides. Concerning the other flavonoids, there is no double bond between C2 and C3, and no C4 carbonyl group in Ring C of flavanols.

One of the major groups of nutritional interest are the flavanols, also termed flavan-3-ols [65]. Cocoa (Theobroma cacao), tea (Camellia sinensis), grape wine (Vitis vinifera) and apples are their major dietary sources [66-69].

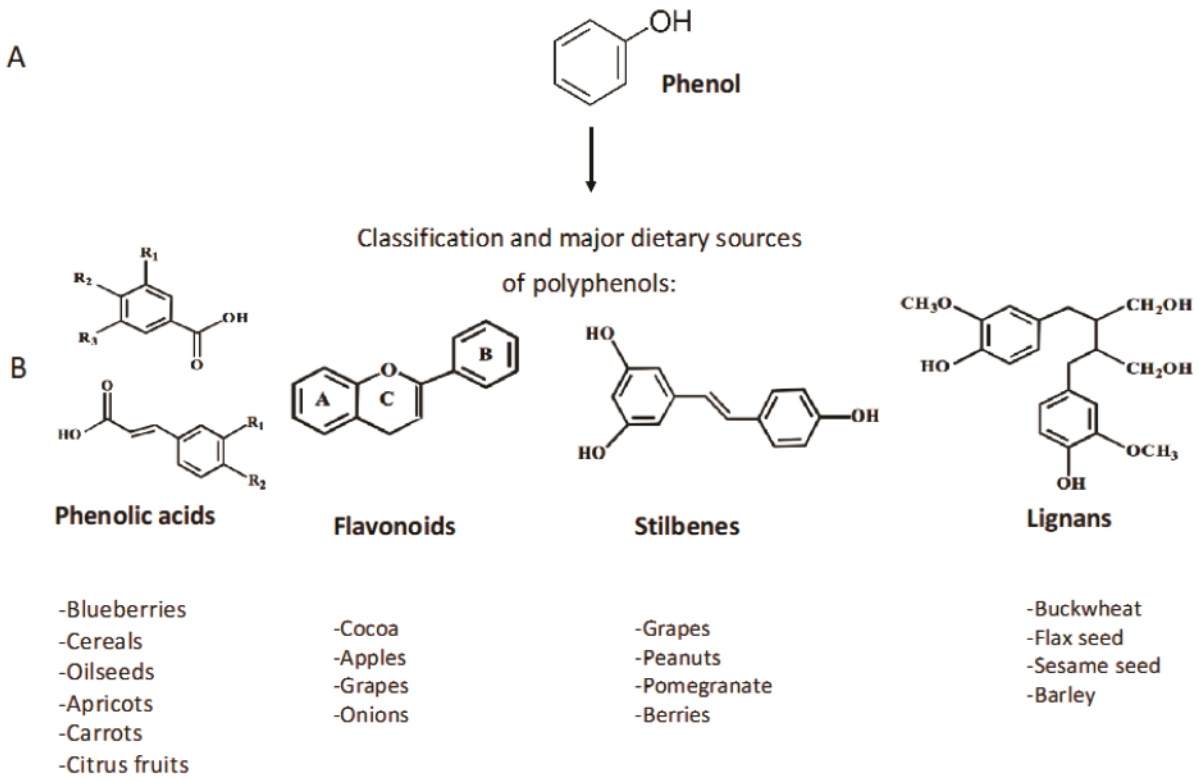

Figure 2: Main polyphenolic structures and occurrence

1.3 Cocoa and its health benefits

For thousands of years, cocoa has been a popular food which is said to have beneficial health effects [70]. In the area of vascular health and chronic disease prevention, several clinical trials have reported that cocoa has positive effects on vascular function. Particularly strong and consistent effects have been shown in endothelial function and blood pressure [71-75]. Seeds of cocoa (*Theobroma cacao*) contain valuable polyphenols. The most-abundant and important flavonoids in cocoa and chocolate are the monomeric flavan-3-ols, (+)–catechin, and (-)– epicatechin (~37%) [76], the procyanidins (oligomeric and polymeric derivatives of flavanols - a distinction is made between A-type (doubly linked) and B-type (singly linked) oligocatechins) (~58%), and to a lesser extent, anthocyanins (~4%). [77-79]

The chemical structure of selected cocoa polyphenols is given in Fig. 3.

(-)-Epicatechin (EC) is the main representative of the flavanol monomers [80], with ca. 35% of the total phenolic amount in unprocessed raw cocoa beans [81]. Moreover, it has been shown that EC is involved in the positive vascular effects associated with the consumption of flavanol-rich cocoa in humans [78, 82]. Ultimately, the manufacturing process determines how much flavanols actually remain in the final cocoa product [83].

The amount of cocoa flavanols (CF) can vary from 10% to 100% throughout the different manufacturing processes [80, 84] as well as depending from the origin and post-harvest handling of cocoa beans [85].

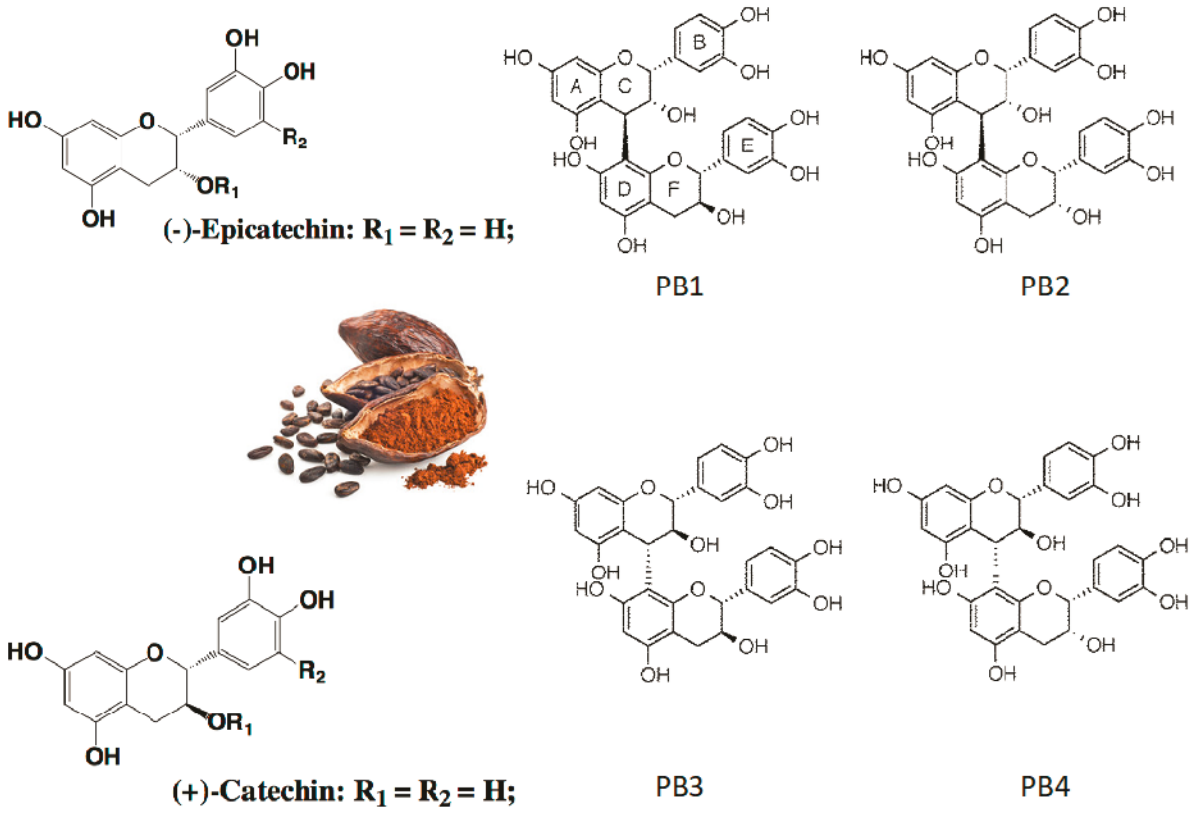

Figure 3: Chemical structure of representative monomeric cocoa flavanols (left side) – and selected oligomers with four most common B- type proanthocyanidins: PB_1, PB_2, PB_3 and PB_4, related to C_4-C_8 chemical bonding.

1.3.1 Bioavailability of cocoa flavanols - Absorption, Distribution, Metabolism and Excretion

The potential of cocoa flavanols to exert biological activity in vivo depends on their biotransformation and conjugation during absorption into the systemic circulation, their distribution, metabolism, and finally excretion (ADME). Furthermore, it has to be taken into consideration that the bioavailability of polyphenols can be influenced by several external and internal factors. These factors may be related to the chemical structure, food matrix, food processing, the environment, interaction with other nutrient compounds, the dose applied, as well as various physical aspects of individuals such as health status, gender and age, and individual genetic polymorphisms in relevant microbial enzyme activity in the gastrointestinal tract (GIT) [86, 87].

When cocoa is consumed, the monomeric flavan-3-ols and polymeric procyanidins are modified and converted into various metabolic products by the intestinal-flora. However, cocoa flavanols are stable during gastric transit and after consumption they are detectable in metabolized and conjugated forms in human plasma and urine.

Both dimers and monomers could be confirmed in plasma within 30-60 minutes [88] and the peak concentration was determined 2-3 hours after ingestion [89-94].

After their intact transit through the acidic milieu of the stomach, monomeric cocoa flavanols (catechin and epicatechin) are indeed absorbed relatively well but only to some extent in the small intestine. About 45% of (−)-EC is neither absorbed nor metabolized in the upper GIT [95] but makes their way into the large intestine where its further metabolized by the colonic microbiota leading to small gut microbial metabolites. As flavan-3-ols are generally not glycosylated, after a direct diffusion through the enterocyte membrane, conjugation takes place, where they are extensively metabolized into SREM (structurally related epicatechin metabolites) via methylation, glucuronidation and sulphation through the actions of uridine-5′-diphosphate glucuronosyltransferases (UGT), catechol-O- methyltransferases (COMT) and sulfotransferases (SULT) [96, 97], as depicted in Fig. 4.

The most relevant metabolites, being (−)-epicatechin-3′-β-D-glucuronide, (−)-epicatechin-3′-sulfate, 3′-*O*-methyl-(−)-epicatechin 5/7 sulfates, are then transported to the liver via the portal vein where further phase II metabolism may occur [92, 97-99]. These circulating metabolites are chemically different from the aglycone forms which are originally present in foods.

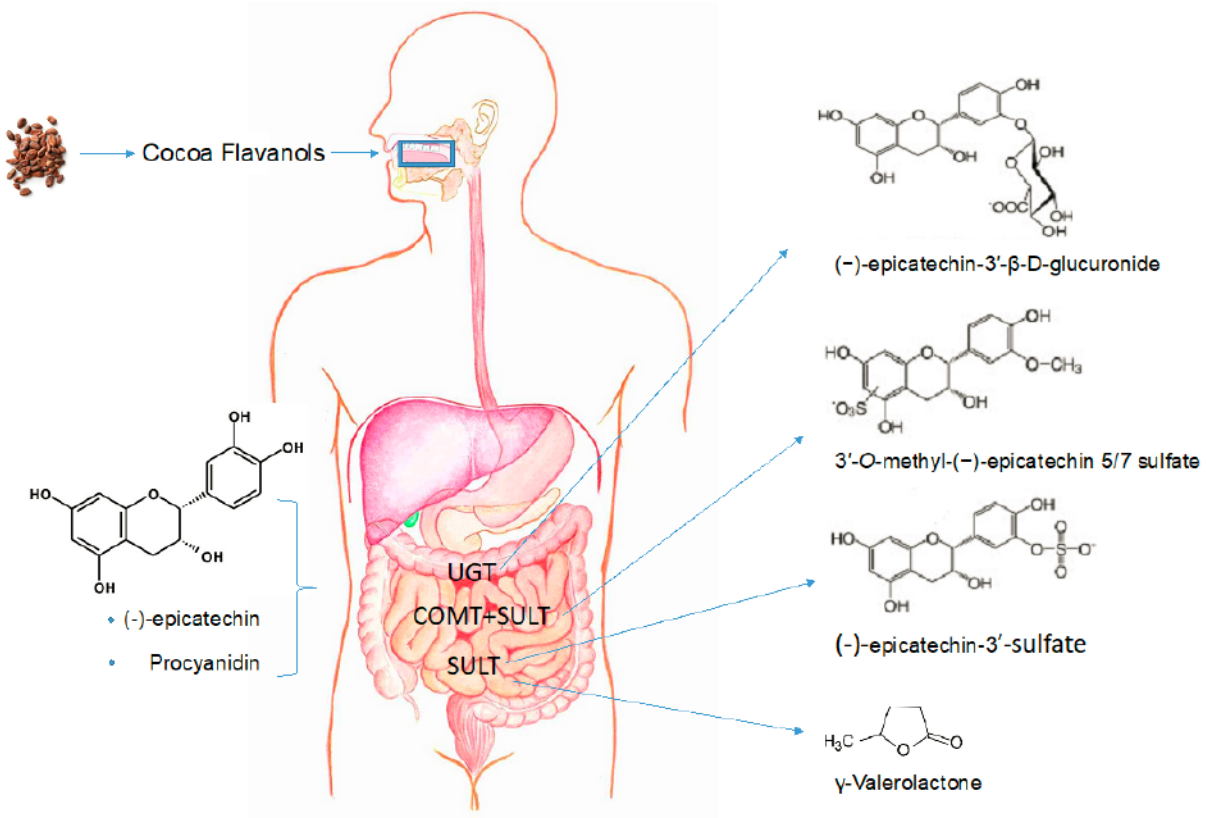

Figure 4: The structurally related (−)-epicatechin and gut microbial metabolites after the consumption of diet-relevant amounts of (−)-epicatechin and procyanidin oligomers from cocoa in humans. uridine-5′- diphosphate glucuronosyltransferases (UGT), catechol-O- methyltransferases (COMT) and sulfotransferases (SULT).

On the one hand this is due to flavanol chemistry and on the other hand because of their structural isomerism and stereoisomerism [100]. Additionally, plasma levels of (+) and (−) forms of catechin were found to be different after cocoa beverage consumption [101, 102]. Once in the circulatory system, plasma concentrations of cocoa flavanols are usually in the nanomolar or low micromolar range.

Regarding flavanol monomers, it is of great importance that the role of the gut microbiome in the metabolism of EC in humans has only recently been recognized.

Ottaviani et al. [103] identified novel aspects of the human gut flora in EC catabolism based on the oral intake of radiolabeled flavanol monomer ^{14}C-EC.

This study provides very strong evidence that most ECs are very well absorbed in different sections of the intestinal tract, following by an extensive biotransformation with more than 20 metabolites in plasma and urine.

Oligomeric procyanidins (PC) remain unaffected and are not absorbed in the small intestinal lumen [104]. Although there is controversy regarding the stability of PCs in

the acidic environment of the stomach, recent work suggests that orally administered PCs remain stable during stomach transit and thus are available for further absorption or metabolism [105]. To date, only dimers have been identified in human plasma [90]. Others larger than tetramers generally pass through the small intestine (duodenum, jejunum, ileum), along with unabsorbed monomeric contents, to the large intestine [106], where they are subjected to catabolic activities of the gut microbiota [107, 108].

Altogether, it is estimated that only 5-10% of polyphenols can be absorbed in the small intestine. The other 90-95% are further transported to the colon [109], where they are degraded into many metabolites, including phenolic acids and γ-valerolactones (γVL) [106, 107, 110, 111] and eventually others not known yet. But currently there is no evidence that these flavanol metabolites, derived from the gut microbiota, have any biological activity.

While γVL and valeric acids have been identified previously as colon-derived catabolites of EC and PCs in humans [112] and [113], γVL is not only considered as one of the most abundant and characteristic metabolites [114] but also anti-inflammatory and antiproliferative activities have been reported [115] and [116].

Results from Ottaviani et al. [103] and Urpi-Sada et al. [117] indicated that γVL could be a potential biomarker of the regular consumption of cocoa and other flavanol-rich foods.

Recently, Rodriguez-Mateos et al. [118] demonstrated that PCs are degraded by the colon-microbiota in to γVL but do not lead to chronic vascular effects. This implies that γVL do not exert chronic effects. However, beyond the impact of vascular health, the authors did not exclude the possibility of health benefits exerted by PCs and γVLs.

Until now, not enough data from human studies have been provided concerning the distribution as well as the cellular uptake. Summing up it can be said that various scientific studies, including cell culture experiments [119] as well as experiments in rats [120], showed that absorbed flavan-3-ols are extensively distributed and can be found in skin fibroblasts and endothelial cells as well as in lymphatic organs (thymus, spleen and mesenteric lymphatic nodes) and also in the liver and the testes. The biological half-life of free and metabolized EC in the blood is $t1/2 \approx 2-4$ h.

Excretion of cocoa flavanols is via urine or the bile. However, intestinal perfusion studies have reported that only a relatively moderate elimination of (−)-EC is partially

executed via the bile [95]. The urinary excretion of the total EC occurs within 24 hours after cocoa or chocolate consumption and reaching not more than 30% of the initial (−)-EC.

The main metabolites in urine are (methylated and non-methylated) sulfoglucuronides and sulfates [91, 92]. Finally, the unabsorbed flavanols will be excreted via faeces. Fig.4 summarizes the absorption, metabolism, distribution and elimination of the monomeric EC and the oligomeric PCs in the human body.

1.3.2 Improvement of cardiovascular risk markers by cocoa flavanols

In recent years, not only several meta-analysis, dietary intervention studies and critical reviews have confirmed beneficial effects of CF on surrogate markers of cardiovascular risk (*e.g.* blood pressure lowering effects) [121-123], but also molecular targets (e.g. iNOS, arginase, angiotensin converting enzyme) have been identified in *in vitro* studies, *in vivo* animal studies and human intervention trials [124-127].

Interestingly, the beneficial effects of cocoa on the cardiovascular system may be supported by observations linked with the Kuna Indians, an indigenous ethnicity in Panama. High blood pressure and heart disease are largely unknown among the Kuna Indians. Comparing to other Panamanians they do have very low blood pressure and a reduced frequency of stroke and myocardial infarction. Hollenberg et al. [128] named the high cocoa consumption of the island Indians as a probable reason for their healthy vessels. The Kuna daily drink three to four cups of dark bitter cocoa. However, as soon as they migrate to mainland, the cocoa consumption is reduced to four cups a week and incidences of hypertension and age-related rise in blood pressure are noticeable [129]. This indicates that the Kuna are not protected by genes but by certain substances in the cocoa.

Intervention trials with cocoa and cocoa products have included various human cohorts since then: high-risk and low-risk individuals, normotensive (young, old, overweight, hypercholesterolemic), pre-hypertensive, and hypertensive with impaired glucose tolerance but detailed analysis of women and in particular on oral contraceptives is unknown.

In general, these studies show that high-flavanol/procyanidin interventions induce an acute and lasting increase or restoration of endothelium-dependent vasodilation [72, 130-135].

Meta-analysis from Ried et al. and Hooper et al. also emphasize the beneficial effects on the human cardiovascular system. Ried et al. observed an acute but moderate blood-pressure lowering effect (2mmHg), which was recently confirmed by a cochrane review [136].

Hooper et al. [122] additionally demonstrated consistent improvements in endothelial function obtained via flow-mediated vasodilation (FMD). Moreover, there are several epidemiological studies which confirmed those findings.

The Iowa Women's Health Study [137] as well as the Zutphen Elderly Study [73] could subsequently show a reduction in CVD after choclate consumption.

Through the analysis of a Dutch population (Zutphen Study), Buijsse et al. [73] had shown that people who consume a daily average of four grams of cocoa not only have lower blood pressure but also a reduced risk of dying of CVD.

Based on the analysis of the British EPIC Norfolk observational study, Kwok et al. [138] evaluated the habitual choclate consumption and cardiovascular outcomes in more than 20.000 men and women. In this large epidemiological study, consuming more chocolate, up to 100 grams per day, was associated with a lower risk of cardiovascular disease and stroke.

Altogether, none of these studies emphasized the magnitude between the effects and the amount of cocoa and chocolate products as well as detailed analysis of women and in particular on oral contraceptives.

1.3.3 Endothelium dependent effects of flavanols on cardiovascular health

The endothelium, which plays a pivotal role in the action of CFs on CVD, is a thin layer of endothelial cells lining the inside of blood vessels. It separates the intraluminal blood compartment from all tissues and organs of the body and also fulfils a variety of physiological functions.

In addition to the important barrier function, it regulates the vascular tone, the adhesiveness to leukocytes and platelets and the proliferation of smooth muscle cells by the release of vasoactive substances [139, 140].

In healthy individuals, the endothelium has a non-thrombogenic surface and therefore it prevents adhesion and aggregation of blood cells on the vessel wall. Through its

secretory performance, the endothelium is actively involved in maintaining the functional and structural integrity of the arterial wall. Thus, it is engaged in inflammatory processes, haemostasis and in the regulation of angiogenesis [141].

The most important endothelial factor is nitric oxide (NO), produced by the endothelial nitric oxide synthase (eNOS) from the precursor L-arginine [142-144]. Besides a number of circulating and locally released substances such as acetylcholine, physicochemical stimuli such as pulsatile stretching of the vessel wall and shear stress are essential for NO formation [145, 146] (Fig. 5).

NO is considered to be a vasoprotective regulator which maintains vascular homeostasis. But an excessive production of NO can cause cell damage [147]. Due to its small size and lipophilicity, the bioactive molecule can cross biological membranes in a short time to reach the smooth muscle cells and perform various local functions [148].

It can mediate vasodilation, it has antiproliferative [149], antioxidant, anti-inflammatory as well as antithrombotic and antiatherogenic properties [150, 151]. It is generally acknowledged that pathological states such as hypertension, atherosclerosis, and ischemic disease among others [152] lead to endothelial dysfunction. The central established process in this pathogenesis is reflected by a diminished supply and/or loss of endothelial nitric oxide [153, 154].

Therefore, preserving NO production must be tightly regulated because a disturbed endothelial function is an important prediction for cardiovascular events [155, 156].

This is the reason why numerous studies have investigated the possibility of reversing endothelial dysfunction by regulating the release of nitric oxide from the endothelium.

To elucidate the effects of cocoa flavanols on endothelial NO bioactivity, Heiss et al. [74] concluded that the ingestion of flavanol-rich cocoa augments the biological activity of NO in individuals with endothelial dysfunction. Plasma concentrations of NO were strongly correlated with (FMD), which itself portrays the extent to which blood vessels dilate in response to nitric oxide. Furthermore, long-term studies with fixed dosage schedules up to 6 weeks showed that a sustained increase in vasodilation can be measured (FMD). This effect (acute and chronic) can be additionally increased by further application [157, 158]. Although accumulating data from literature indicate that cocoa flavanols positively affect vascular function in healthy subjects as well as in

various pathological conditions, not all the involved potential mechanisms have been fully understood yet. Some evidence suggests that the observed effects in recent studies may occur via endothelial nitric oxide synthase (eNOS) activation [159-161], reduction of reactive oxygen species (ROS) [74, 162], angiotensin-converting enzyme inhibition (ACE) [125], and/or endothelin 1 antagonism [163, 164] . (Fig. 5)

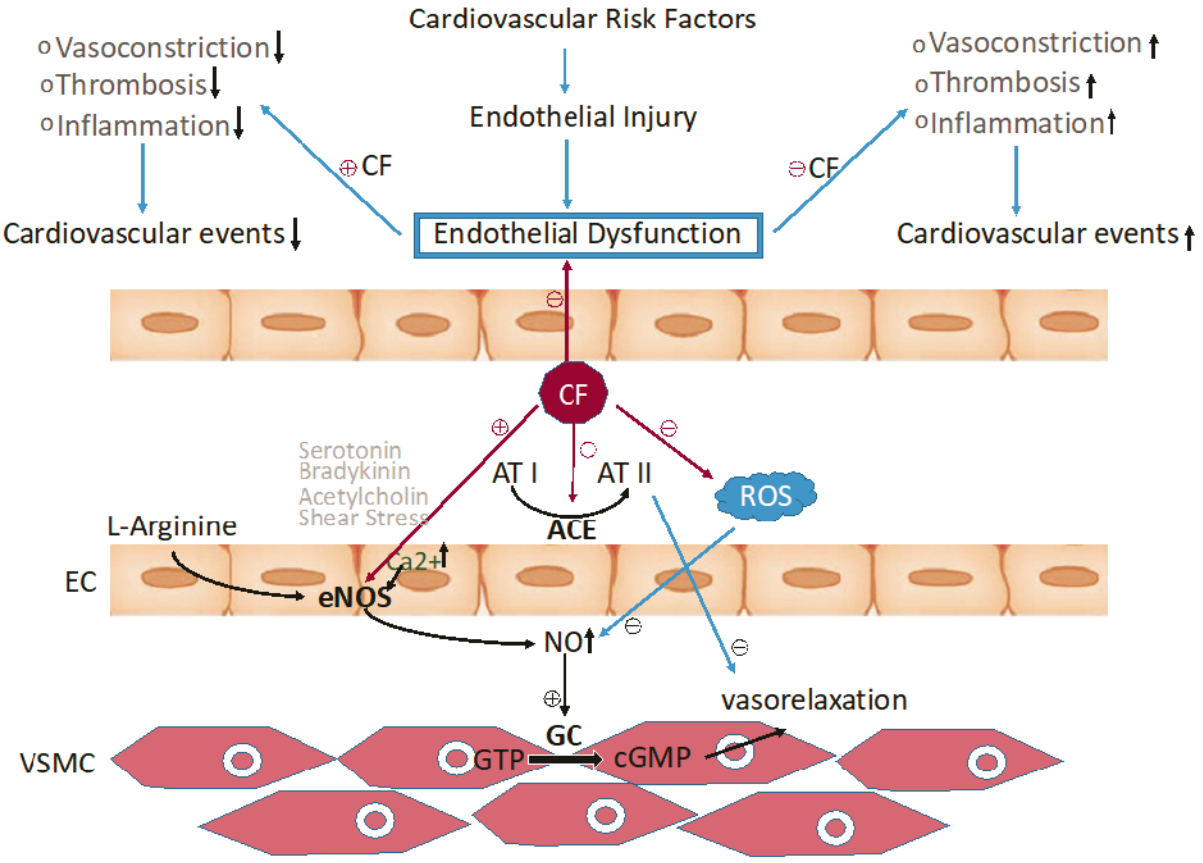

Figure 5: Effects of cocoa flavanols with messenger molecules promoting endothelial homeostasis - endothelial dysfunction is a result of their imbalance

EC: Endotheilail Cells; VSMC: Vascular Smooth Muscle Cells; eNOS: endothelial Nitric Oxide Synthase; NO: Nitric Oxide; CF: Cocoa Flavanol; GTP: Guanosine-5'-triphosphate; GC: Guanylate Cyclase; cGMP: cyclic Guanosine Monophosphate; ACE; Angiotensin Converting Enzyme; AT I: Angiotensin I; AT II: Angiotensin II; ROS: Reactive Oxygen Species

1.4 Combined oral contraceptives – "The Pill"

In Germany, six to seven million women of reproductive age are taking combined oral contraception [165] whereas about 100 million women worldwide use it [166]. This marks the top of the ranking of all pregnancy prevention methods.

Oral contraceptives are drugs used to prevent ovulation by influencing the natural regulatory mechanisms of the female hormonal balance. Thus, hormonal contraceptives contain either a synthetic estrogen (mainly ethinylestradiol) and a

synthetic gestagen or consist of only one progestogen (minipill, e.g., desogestrel, norethisterone acetate). The body's own progesterone is often reffered to as gestagen, while artificial gestagens often reffered to as progestins or progestogen. Ethinylestradiol (EE) is the estrogen used in almost all preparations whereas they are combined with one of a variety of synthetic progesterones.

Products with a modified estrogen component (estrogen-valerate, micronized estrogen) more closely resemble endogenous molecules. Usually, combined oral contraceptives (COCs) are taken once a day for 21 days (conventional regimen, 21/7), followed by a seven-day break (pill-free interval) [167]. Based on time of the introduction, hormonal contraceptives can be classified into "Generations", as depicted in Table 1.

While the first generation of the pill still contained very large amounts of estrogen and the gestagen norethisterone, only preparations with significantly reduced estrogen levels followed. Second-generation pills are combined with the gestagen levonorgestrel.

The most recent birth control pills contain newly developed gestagens, such as gestodene or desogestrel in third-generation pills and drospirenone and cyproterone (fourth generation). Moreover, synthetic progesterones used in oral contraceptives (OCs) have both progestogenic and androgenic activity.

While first generation progestins are no longer used, second and third generation progestins are androgenic (the latter with less androgenic activity than the former) and fourth generation progestins have anti-androgenic activity.

Furthermore, they are categorized as monophasic, biphasic, or triphasic depending on whether the level of hormones stays the same or changes during the first three weeks of the menstrual cycle. Monophasic OCs (single-phase preparations) contain constant daily doses of both estrogen and progestin while in biphasic and triphasic preparations estrogen and / or gestagen dosages gradually change and are adapted to the female cycle [168].

Table 2 refers to the most commonly used oral contraceptives in Germany. The table is sorted by the progesterone component due to the fact that only the type of progesterone changes while estrogen-type remains the same.

Table 1: Classification of progestins

Progestin	Generation	Classification	Brand Name
used in this study		Related to / derivative	used in this study
Levonorgestrel	2	Testosterone / 19 nortestosterone*	Leios®, Levina®, Minisiston®, Evaluna20®, Femigyne® Swingo20®, WEC®, Liana®, Miranova®, Microgynon®
Dienogest	3	Testosterone / 19 nortestosterone*	Maxim®, Velafee®, Aristelle®, Mayra®
Desogestrel	3	Testosterone / 19 nortestosterone*	Cedia20®, Lamuna20®
Chloromadinone-acetate	4	Progesterone / 17α-Hydroxyprogesterone**	Chariva®, Belara®, Minette®, Enriqa® Chloee®, Bellissima®, Beatrice®, Verana®
Drospirenone	4	Spironolactone / 17α-spironolactone	Maitalon20®, Aida®, Yasminelle®, Yasmin®, Layaisa®

*19 nortestosterone - gonane / estrane derivatives

** 17α-Hydroxyprogesterone - pregnane derivatives

Table 2: Overview of the most prescribed combined oral contraception pills on the german market with low-dose estrogen and gestagen in 2014 (without reimports) - an orientation on the different progestins in the pills.

Progestin	Packs per thousand
Dienogest	2027
Desogestrel	743
Levonorgestrel	677
Chlormadinon	445
Drospirenon	341

Modified from IMS, 2015 [169]

1.5 Need and hypothesis

Epidemiological and clinical evidence suggest that cocoa flavanol can improve surrogate markers of cardiovascular risk in a variety of populations. However, most of the studies have been conducted in men or postmenopausal women with or without cardiovascular risk factors.

Although a large population of premenopausal women worldwide using combined oral contraceptives (COC), studies investigating their effects on endothelial function are scarce but have shown generally an increased cardiovascular risk in COC users as compare to non-users.

Currently, detailed analysis of women and in particular on oral contraceptives is missing. Whether cocoa flavanol consumption can improve endothelial function in healthy, premenopausal women and whether synthetic sex hormones such as COC might influence the vascular effects exerted by CF is unknown.

Available studies on endothelial function in premenopausal women have shown an increased risk in developing cardiovascular diseases, such as venous and arterial thromboembolic events, which are associated with combined oral contraceptive use. However, the studies are scarce and inconsistently.

The aim of this work is to investigate the extent to which cocoa flavanols can exert their positive influence on the vascular endothelium in premenopausal women when synthetic hormones are taken (combined oral contraceptives).

2 Materials and Methods

2.1 Study design

In a 2-month double-masked, parallel-group randomized controlled trial, 24 healthy premenopausal female subjects taking COC were randomly allocated to either a CF drink (410 mg) or nutrient-adjusted flavanol-free Control drink, comparing the effect on endothelial function. The trial was conducted at the Division of Cardiology, Pulmonology and Vascular Medicine of Duesseldorf University in Duesseldorf, Germany. The study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures involving human subjects were approved by the Research Ethics Boards of the Heinrich-Heine-University Düsseldorf (Ref. 3870). All participants gave their signed consent. All examinations were conducted in a quiet and darkened temperature controlled room between 7:00 am and 13:00 pm. Additionally, a food frequency questionnaire was completed as well as an international physical activity questionnaire at their first visit. All incidents, missed flavanol intakes, the last menstruation day, additional drugs and vitamins were recorded in an intervention day form. On the examination day, volunteers arrived fasted, for at least 12 hours, with a 24 h low polyphenol diet at the clinic. Female subjects rested for 15 minutes before vascular measurements and blood samples were taken. The primary endpoint was endothelial function, measured by flow-mediated vasodilation (FMD). Secondary endpoints were blood pressure and general blood parameters in plasma. Tertiary endpoints were plasma flavanol metabolites. During the first month, acute responses to a single dose of CF or Control were tested during two study visits. During the pill-free phase (days 5-7) and the third week of the active pill phase (days 26-28). FMD and blood pressure (BP) were measured at baseline before and at 2h after one dose. Subsequently, beverages were provided in anonymous packets, the content being not distinguishable. Shortly before consumption, subjects were instructed to empty and mix the content of one sachet in 500 ml, low nitrate water. After the second visit, the participants ingested either CF or Control twice daily until and during the next cycle. FMD and BP measurements were then taken again before (chronic) and at 2h after the first dose of the day (acute-on-chronic) during the next pill-free phase (days 5-7) and the third week of the next active pill phase (days 26-28).

In addition to FMD and BP, blood samples were collected and assessed for structurally related (-)-epicatechin metabolites (SREM) analysis in plasma with high-performance

liquid chromatography with fluorescence and electrochemical detection (HPLC-FLD-ECD) and blood lipids. (Fig. 6A)

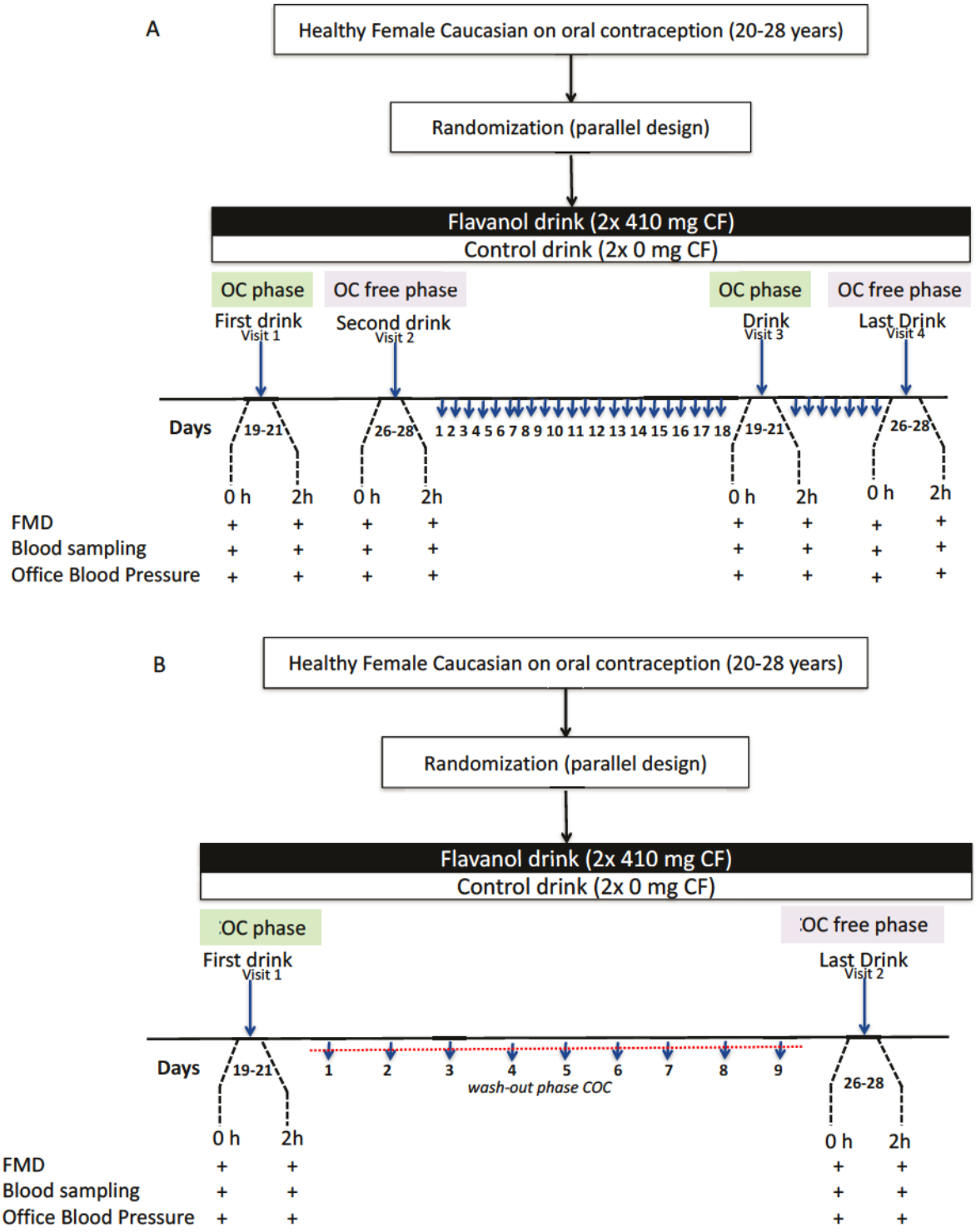

Figure 6: Study design A – CHRONIC and B – ACUTE study population

CF=Cocoa Flavanol; OC= Oral Contraception

In a second study (ACUTE STUDY), we investigated if differences existed between different active ingredients of COC in terms of attenuation of acute CF-related FMD

improvements. A total of 62 women with 5 different types of COC were randomly allocated to either CF or Control. We tested the acute responses (2h) to a single dose of CF or Control on FMD and BP. Measurements were performed at baseline before and at 2 h after one dose during the pill-free phase (days 5-7) and the third week of the active pill phase (days 26-28). (Fig. 6B)

2.2 Study population

We assessed eligibility for all healthy premenopausal caucasian women between 18 and 30 years of age currently taking combined oral contraceptive pills (COCP). (Table 5 and 7) The following inclusion criteria was used to screen potential participants: a general good health assessed by the health and lifestyle questionnaire and physical examination, normal blood pressure (defined as SBP <140 mmHg and DBP <90 mmHg), normal range of blood parameters (such as blood cell counts, glucose, blood lipids, liver enzymes, and C-reactive protein, see Appendix 1), absence of CVD (e.g., heart disease, stroke, hypertension, suffering from any illness that may affect the ability of the blood to clot), no current or previous medication and at least one year of using one of the five more common COCP in Germany, including Dienogest, Desogestrel, Levonorgestrel, Chlormadinon acetate and Drospirenon (Table 3). This type of oral contraception involves one week were no pills are taken (days 22-28) and an active pill phase (days 1-21). Exclusion criteria were chronic heart failure, heart rhythms other than sinus, atrial fibrillation, acute coronary syndrome, liver failure, recent or current inflammatory condition, C-reactive protein levels greater than 0,5 mg/dL, arterial hypertension, allergies to cocoa or dairy products, postmenopausal state, current use of medication, and anaemia.

Volunteers taking probiotics, prebiotics or synbiotics within a 2-month period prior to the study were also excluded.

2.3 Intervention drinks

Both interventions used standardized low-calorie fruit-flavored test substances (provided by Mars, Inc.). All beverages (agglomerated powders) were mixed in 500 ml of low-nitrate water and served to the subjects. Each of the opaque sachets (7g, equals on serving) was labelled with a secure code to enable a double-blind study design. Each flavanol drink contained a total of 410 mg of cocoa flavanols (CF) per serving , with 64 mg of (-)-epicatechin, the main monomeric flavanol. Total flavanol

intake per day was 820 mg CF during the chronic interval. The flavanol-poor and -rich cocoa extracts were equal in taste and appearance, as well as in the content of macro- and micronutrients, calorie content, theobromine and caffeine. They differed only in their flavanol content. Compositional details about the macro- and micronutrient matched and isocaloric test drinks are provided in Table 3. Both treatments were mixed in low-nitrate (10mg/l) water out of 500 ml. (Vio Still, 0,5 l, Apollinaris).

Table 3: Composition of cocoa flavanol - and control drink

	FLAVANOL	CONTROL
Total cocoa flavanols (mg)	410	ND
Monomers (mg)	73	ND
(-)-Epicatechin (mg)	64	ND
(+)-Epicatechin (mg)	7	ND
(-)-Catechin (mg)	2	ND
(+)-Catechin (mg)	ND	ND
Dimers-Decamers (mg)	377	ND
Theobromine (mg)	44	46
Caffeine (mg)	10	6
Carbohydrates (g)	6	6
Fat (g)	0	0
Protein (g)	0.1	0.1
Sodium (mg)	3	3
Potassium (mg)	95	85
Energy (kcal)	25	25
Na (mg)	3	3
K (mg)	95	85

ND = Not Detectable

2.4 Study outcomes

2.4.1 Flow-mediated vasodilation

Endothelium-mediated vasodilation was assessed as flow-mediated dilation (FMD) using established procedures in our laboratory [170]. Briefly, images were captured by ultrasound (Vivid-I, GE Healthcare, USA), before and after inflating a blood pressure cuff for 5 minutes on the right forearm to a value of 50 mmHg above systole. The brachial artery was imaged longitudinally proximal to the antecubital fossa. Images were captured through the length of the measurement to evaluate vessel wall dilation over the cuff and release period. Time points were set as follows: 20s, 40s, 60s, 80s.

To achieve higher accuracy, the image site was initially marked on the upper arm using a permanent marker.

In the final analysis, ultrasound captured images were blinded analysed and assessed with an automated analysis software (Brachial Analyzer for Research, Medical Imaging Applications- llc). The maximal diameter obtained after the deflation of the cuff was determined as the peak diameter. The determination of the diameters was carried out manually, end-diastolic and coincidentally with the R-spike of the ECG. This was performed in order to reduce the error inflow of heart-cycle-induced lumen fluctuations of the brachial artery. Each image sequence consisted of 3-4 cardiac cycles. An example of an ultrasound image is shown in Fig. 7.

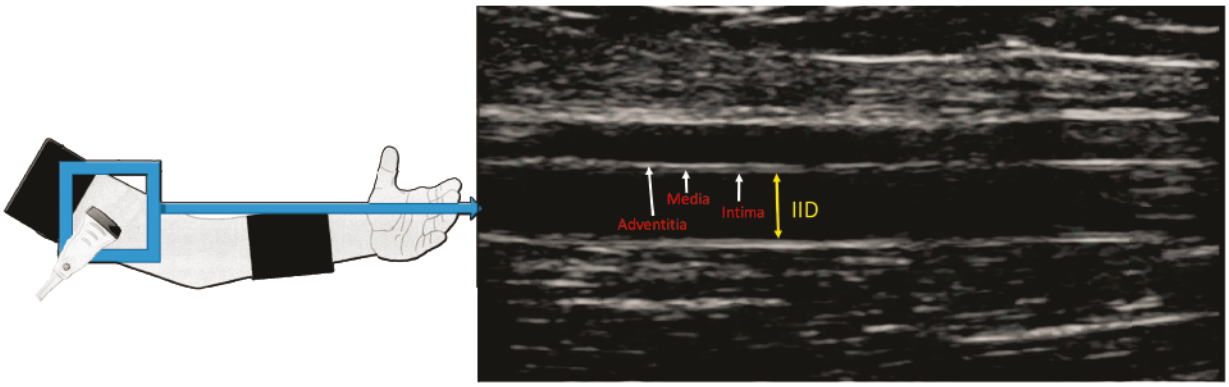

Figure 7: Image of the brachial artery lumen - captured during FMD. IID was defined as the distance from the intima-lumen interface of the near wall to the lumen-intima interface of the far wall; IID = Intima-Intima-Diameter

2.4.2 Blood pressure measurements

After a previous rest period of 15 min, in a sitting position with the arm at heart level, blood pressure was measured non-invasively, with fully automatic digitized sphygmomanometer (BOSO Medicus PC2, Bosch+Sohn, Germany), at the upper left arm. All measurements were performed under the same condition, comfortably seated with the back supported and without constrictive clothing. In addition, care was taken that the legs were not crossed while measurements were taken. The first measurement of three consecutive measurements was discarded and the last two were used for further analysis.

2.4.3 Blood collection and sample processing

After five minutes of rest, blood samples were collected in fasting subjects from a peripheral vein. To avoid hemolysis, attention was paid to a slow blood sampling process and a rapid transfer into the prepared vacuum tubes. Clinical blood parameters (small blood count, creatinine, uric acid, total cholesterol, HDL and LDL cholesterol, triglycerides, glucose, CRP, GOT, GPT, γ-GT, HbA1c, differential leukocyte count, thrombocytes) were immediately processed under standard conditions by the Central Institute for Clinical Chemistry and Laboratory Diagnostics at the Heinrich Heine University Hospital in Duesseldorf. An additional EDTA vacuum tube was collected to determine plasma cocoa flavanol levels. Plasma was obtained by whole-blood centrifugation (800 rcf, 10 min, 4 C), portioned into several 1.2 mL aliquots and spiked with 6µl ascorbic acid. Subsequently, samples were frozen immediately at -80 C until analysis.

2.4.4 Quantification of flavanols and their metabolites by HPLC-FLD-ECD

Flavanol monomers (epicatechin) as well as their structurally related metabolites ((-) -epicatechin-3'-D-glucuronides (E3'G), (-) epicatechin 3 'sulfates (E3'S), 3′-*O*-methyl-(−)-epicatechin-5-sulfate (3'ME5S), and 3'-O-methyl-(-)epicatechin 7 sulfates (3'ME7S) were quantified in plasma of volunteers using high-performance liquid chromatography with fluorescence and electrochemical detection (HPLC-FLD-ECD), and with authentic standards as previously described [97]. The chemical analysis was performed at Reading University.

2.5 Statistical methods

The characteristics of the study population are expressed as mean (standard deviation). Responses to treatments were calculated as changes in respective parameters (e.g. FMD) as compared to 0 h baseline values on first study visit. The primary test for an effect in the RCT was a repeated measurements ANOVA comparing the changes in FMD and blood pressure values at different time points (between subject factor intervention [CF vs Control]) taking into account the within-subject factors timepoints of responses with regards to timing of ingestions (2 h vs 1 mon vs 1 mon/2h, i.e. ‘acute’, ‘chronic’, ‘acute-on-chronic’) and timepoint within oral contraceptive cycle (‘active pill’ vs ‘pill free’). Note that for lipids we only compared baseline and 1 mon at difference cycle stages as change at 2 h would not be expected.

Mean values of results are presented as mean (standard error of the mean) and differences between responses are presented as mean with 95% confidence interval. Differences (CF vs Control, Table 5) are estimated marginal means based on RM ANOVA. To analyze if there were differences between types of pills in the second acute study, we performed again a repeated measurements ANOVA comparing the changes in FMD values at different time points (between subject factor intervention [CF vs Control]) and 'type of pill' as a covariate taking into account the within-subject factor timepoint within oral contraceptive cycle ('active pill' vs 'pill free'). Data not normally distributed are presented as median (interquartile range) and group differences are compared with Mann-Whitney-U tests. Analyses were computed with SPSS 26 (IBM). Correlations were Pearson's r.

3 Results

3.1 Baseline characteristics of the chronic study population

A total of 30 healthy young premenopausal women using different types of combined oral contraceptives were recruited. After we confirmed the assessment for eligibility, 24 subjects were enrolled and randomized to the FLAVANOL or CONTROL intervention group. Fig. 8 reflects the consort flow diagram of the progress through the phases of the parallel randomized trial of both intervention groups.

The average age in the flavanol- (n=12) or control-group (n=12) was 24±2 years and 23±2 years with a body mass index (BMI) of 21±3 and 20±2, respectively.

Demographic and anthropometric parameters were all in normal limits and baseline blood values were in the range for healthy female individuals (Table 4B). Reference values according to our central laboratory is depicted in the Appendix.

At the beginning of the study, clinical and laboratory parameters listed in Table 4A were collected to estimate cardiovascular risk. Baseline parameters indicated no group differences (*all* $p > 0.05$) but statistically significant differences were detectable among users of COCs between the pill-free compared to the active pill phase. (Table 4A)

Total cholesterol ($p = 0.013$) was found to be higher during the pill-free interval as compared to the active pill phase. (Table 4A)

However, neither in comparison of both intervention groups nor within each intervention group significant differences occurred.

We also tested whether the proportions of people who were within the normal reference ranges for adults differed between the pill phases or intervention groups (Tabel 4B). No significant differences were detected at the beginning of the study between the control and flavanol group and all subjects were at normal laboratory reference ranges. None of the participants had clinical signs or symptoms of CVD or were receiving pharmacologic treatment. Smoking was strictly prohibited throughout the study. Furthermore, not a single adverse event was reported and all provided test drinks were well tolerated.

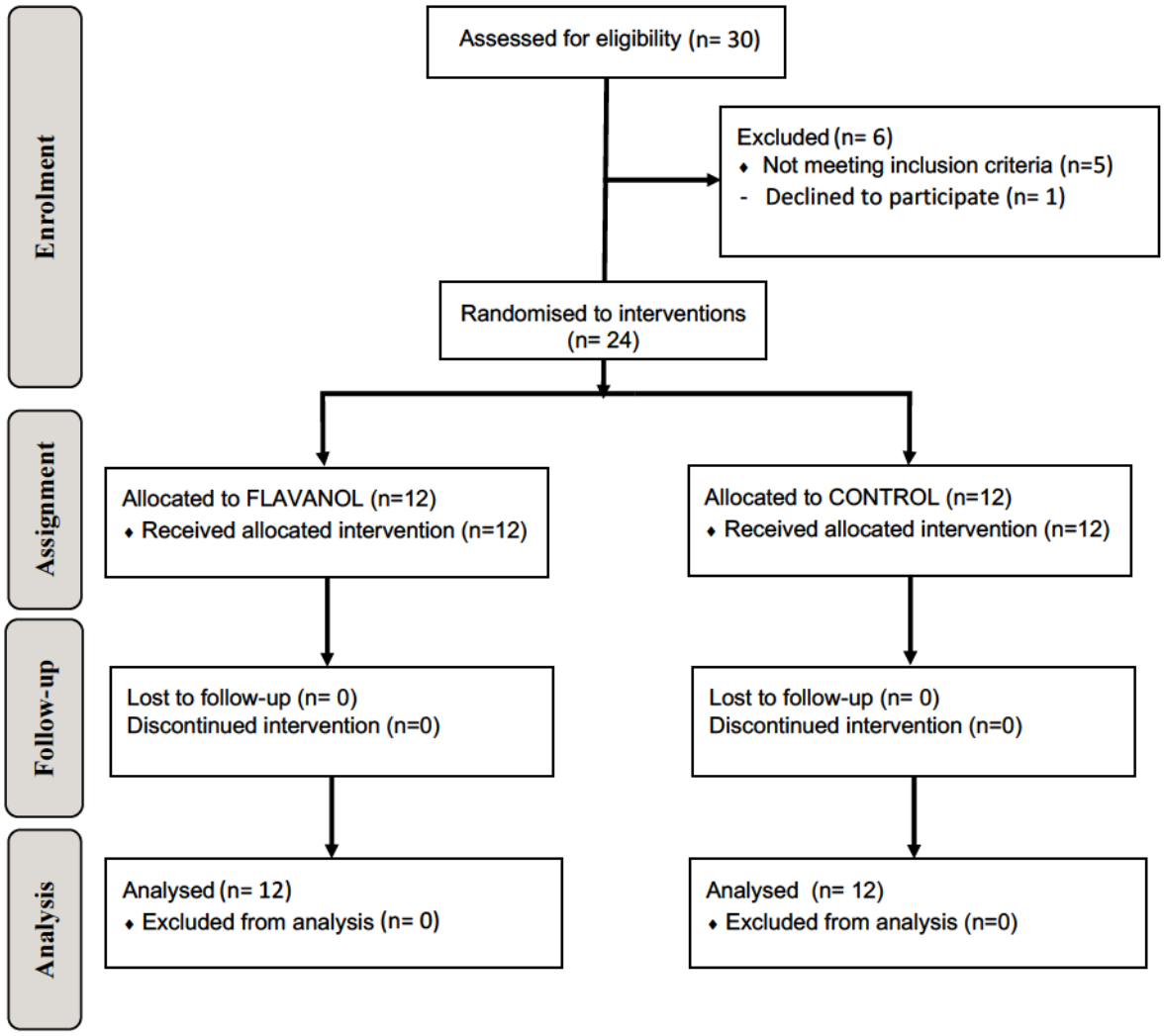

Figure 8: Flow diagram for the CHRONIC Study

Table 4: Baseline characteristics of the chronic study population

	Control			Cocoa flavanol			p		
A	N	Pill-free phase (days 5–7)	Active pill phase (days 26–28)	N	Pill-free phase (days 5–7)	Active pill phase (days 26–28)	Intervention	Pill	Pill × Intervention
FMD (%)	12	7.87 (0.87)	7.50 (0.93)	11	7.99 (1.13)	8.06 (1.18)	0.402	0.364	0.190
SBP (mmHg)	12	113.08 (10.89)	113.25 (11.24)	12	110.67 (14.51)	112.83 (6.55)	0.735	0.548	0.606
DBP (mmHg)	12	72.03 (8.46)	71.75 (8.18)	12	72.08 (8.51)	71.61 (7.27)	0.989	0.796	0.946
Heart Rate	12	71.39 (11.26)	73.78 (10.93)	12	75.28 (8.82)	73.97 (9.22)	0.598	0.735	0.255
Total cholesterol (mg/dl)	12	199.08 (40.37)	185.50 (42.27)	12	200.50 (29.36)	187.67 (29.17)	0.898	0.013	0.939
LDL cholesterol (mg/dl)	7	106.71 (14.58)	101.57 (14.71)	8	88.13 (33.55)	79.75 (23.25)	0.108	0.051	0.617
HDL cholesterol (mg/dl)	7	109.00 (31.06)	105.43 (24.91)	7	122.86 (28.42)	119.57 (21.77)	0.306	0.563	0.981
Triglycerides (mg/dl)	12	85.33 (33.17)	103.08 (31.73)	12	92.17 (50.62)	100.67 (29.02)	0.873	0.055	0.482
Glucose (mg/dl)	12	85.25 (5.15)	86.50 (6.37)	11	80.82 (6.49)	82.27 (6.75)	0.072	0.282	0.934
Hb (g/dl)	12	13.50 (1.28)	13.49 (0.98)	12	13.24 (1.10)	13.51 (0.94)	0.772	0.427	0.399
CRP [mg/dl]	12	0.33 (0.28)	0.29 (0.19)	12	0.62 (0.76)	0.33 (0.27)	0.198	0.230	0.337
Leukocytes (x1000/µl)	12	5.32 (1.90)	5.78 (1.71)	12	5.64 (1.45)	6.28 (1.38)	0.483	0.102	0.790
Thrombocytes (x1000/µl)	12	270.08 (51.40)	272.58 (45.96)	12	293.58 (54.35)	295.83 (50.77)	0.255	0.665	0.982

B	Control			Cocoa flavanol (CF)			p Control vs. CF	
	Pill-free phase (days 5–7)	Active pill phase (days 26–28)	p	Pill-free phase (days 5–7)	Active pill phase (days 26–28)	p	Pill-free phase (days 5–7)	Active pill phase (days 26–28)
Total Cholesterol ≤ 200 mg/dl	50% (6/12)	58% (7/12)	>0.999	58% (7/12)	75% (9/12)	0.500	>0.999	0.667
HDL ≥ 35 mg/dl	100% (7/7)	100% (7/7)	>0.999	100% (8/8)	100% (8/8)	>0.999	>0.999	>0.999
LDL ≤ 160 mg/dl	86% (6/7)	100% (7/7)	>0.999	86% (6/7)	100% (7/7)	>0.999	>0.999	>0.999
Triglycerides ≤ 150 mg/dl	100% (12/12)	92% (11/12)	>0.999	75% (9/12)	100% (12/12)	0.250	0.217	>0.999
Glucose ≤ 100 mg/dl	100% (12/12)	100% (12/12)	>0.999	100% (11/11)	100% (11/11)	>0.999	>0.999	>0.999
Hb 11.9–14.6 g/d	75% (9/12)	75% (9/12)	>0.999	83% (10/12)	92% (11/12)	>0.999	>0.999	0.590
CRP ≤ 0.5 mg/dl	92% (11/12)	83% (10/12)	>0.999	75% (9/12)	92% (11/12)	0.625	0.590	>0.999
Leukocytes 4.5–12.7 x1000/µl	58% (7/12)	75% (9/12)	0.625	75% (9/12)	100% (12/12)	0.250	0.667	0.217
Thrombocytes 173–390 x1000/µl	100% (12/12)	100% (12/12)	>0.999	92% (11/12)	92% (11/12)	>0.999	>0.999	>0.999

Mean values and standard deviations of selected clinical parameters before the start of respective intervention during active pill and pill-free cycle phase. SBP (systolic blood pressure), DBP (diastolic blood pressure), FMD (flow mediated dilation), HDL (high density lipoprotein), LDL (low density lipoprotein), Hb (hemoglobin), CRP (C-reactive protein). P values refer to 2-way repeated measures ANOVA with 1 within-subject factors (pill phase) and 1 between subject factor (intervention).

A – relevant clinical- and blood parameters at the beginning of the study. Comparison of baseline measurements in the different intervention groups at different pill phases.

B – reflects the the proportions of subjects within the normal laboratory reference ranges, expressed as % and absolute values.

3.2 Influence of oral contraception on the vascular effects mediated by cocoa flavanols

3.2.1 CF increases flow mediated vasodilation during oral contraceptive cycle but the effect is attenuated by OC

A main effect of the intervention and an interaction between intervention and pill was observed (Table 5). Basal FMD values in both intervention groups were not significantly different at the start of the intervention with regard to the pill and pill free phase (Table 4).

Compared to the control-group, the flavanol-rich test drink led to a significant increase in FMD two hours post-ingestion, in both pill- and pill free-phases. Although baseline FMD measurements were similar, the effects were significant lower in the active pill phase compared to the pill-free phase. (Fig. 9)

Difference change in FMD between CF and control from baseline at 2h was 2 % (CI 1.1, 2.9) and 1.4 % (CI 0.3,2.6), at 1 month 1.9 %(CI 0.5,3.3) and 1.1 % (CI -0.1, 2.3) and acute after one month of bi-daily consumption of the intervention drinks 3.3 % (CI 1.9, 4.7) and 2.1 % (CI 0.7, 3.5), pill-free and pill cycle respectively.

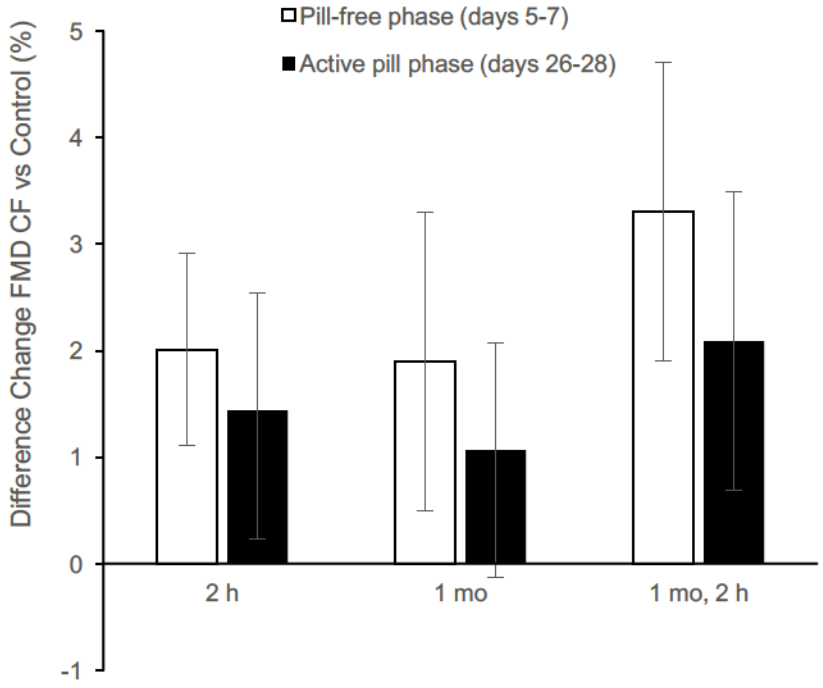

Figure 9: Effect of cocoa flavanol (CF) on flow-mediated dilation (FMD) in healthy women on oral contraception (OC). Bars show differences between FMD value changes in CF as compared to control from baseline before (fasted) to 2 h after acute ingestion of single CF dose (410 mg), fasted after 1 mo bi-daily CF, and to 1 mo, 2 h after additional ingestion of a single CF dose during the active pill and pill-free phase of the oral contraceptive cycle (see Table 3 for compositions of intervention drinks and table 5 for absolute values).

P values refer to repeated measurements ANOVA with 2 within-subject factors (time, pill-phase) and 1 between subject factor (intervention); p (main effect of intervention) < 0.001; p interaction (time × intervention) < 0.001; p interaction (pill-phase × intervention) = 0.019. Values are mean ± confidence interval. FMD = flow-mediated dilation.

Table 5: Overview of vascular end points in oral contraception - chronic study. Mean values and standard errors of flow-mediated dilation (FMD), systolic blood pressure (SBP), diastolic blood pressure (DBP), and blood lipids at baseline and 2 h after acute consumption of control or cocoa flavanol (CF) drink before and after 1 month bi-daily ingestion of respective intervention during active pill and pill-free cycle phase. Estimated marginal means of differences CF and Control related changes from baseline in active pill and pill-free phase. P values refer to repeated measurements ANOVA with 2 within-subject factors (time, pill phase) and 1 between subject factor (intervention).

	Control								CF								Difference (CF vs Control)						P		
	Pill-free phase (days 5-7)				Active pill phase (days 26-28)				Pill-free phase (days 5-7)				Active pill phase (days 26-28)				Pill-free phase (days 5-7)			Active pill phase (days 26-28)					
	BL (0 h)	2 h	1 M	1 M, 2 h	0 h	2 h	1 M	1 M, 2 h	BL (0 h)	2 h	1 M	1 M, 2 h	0 h	2 h	1 M	1 M, 2 h	Delta 2 h	Delta 1 M	Delta 1 M, 2 h	Delta 2 h	Delta 1 M	Delta 1 M, 2 h	Intervention	time x interv.	pill x interv.
FMD (%)	7.8 (0.3)	7.8 (0.3)	7.6 (0.4)	7.5 (0.4)	7.5 (0.4)	7.6 (0.3)	7.8 (0.4)	7.8 (0.4)	8.0 (0.3)	10.0 (0.3)	9.7 (0.4)	11.0 (0.4)	7.8 (0.4)	9.3 (0.3)	9.0 (0.4)	10.1 (0.4)	2.0 (1.1, 2.9)	1.9 (0.5, 3.3)	3.3 (1.9, 4.7)	1.4 (0.3, 2.6)	1.1 (-0.1, 2.3)	2.1 (0.7, 3.5)	<0.001	<0.001	0.019
SBP (mmHg)	114 (4)	114 (4)	111 (3)	111 (3)	114 (3)	115 (3)	108 (3)	111 (3)	111 (4)	107 (4)	111 (3)	111 (3)	113 (3)	112 (3)	110 (3)	112 (3)	-4 (-16, 9)	2 (-4, 9)	3 (-8, 13)	-2 (-6, 2)	2 (-4, 9)	4.0 (-8, 15)	0.824	0.178	0.567
DBP (mmHg)	72 (2)	72 (3)	70 (2)	68 (2)	72 (2)	69 (2)	63 (2)	67 (2)	71 (2)	72 (3)	67 (2)	69 (3)	72 (2)	69 (2)	64 (2)	65 (2)	2 (-5, 10)	-2 (-8, 3)	2 (-4, 9)	0 (-5, 6)	1 (-4, 7)	1 (-4, 6)	0.691	0.710	0.977
Total cholesterol (mg/dl)	209 (9)		208 (11)		196 (11)		193 (7)		196 (9)		195 (10)		186 (10)		182 (6)			1 (-30, 32)			-2 (-25, 25)		0.980		0.883
LDL cholesterol (mg/dl)	113 (12)		123 (15)		112 (10)		119 (9)		123 (12)		117 (15)		123 (10)		102 (9)			-16 (-81, 50)			-27 (-51, -3)		0.764		0.735
HDL (mg/dl)	107 (10)		116 (13)		104 (7)		98 (8)		83 (10)		81 (13)		75 (7)		77 (8)			-11 (-47, 25)			8 (-10, 27)		0.918		0.158
Triglycerides (mg/dl)	88 (13)		94 (11)		105 (10)		95 (12)		85 (12)		92 (11)		99 (10)		116 (11)			0 (-25, 25)			27 (-5, 58)		0.261		0.206

3.2.2 Flavanol intake has no effect on blood pressure and blood lipids

No significant changes in blood pressure, heart rate and selected blood parameters were observed between flavanol and control groups at 2 hours, after one month and after acute on chronic consumption of flavanols (Table 5).

All paramaters were within normal ranges for young healthy women and all given baseline paramaters were not significantly different between FLAVANOL and CONTROL (Table 4).

Fig. 10, 11 and 12 reflects the effect of cocoa flavanol (CF) on systolic blood pressure (SBP), diastolic blood pressure (DBP) and on blood lipids, respectively. Bars show differences between SBP value changes in CF as compared to control from baseline before (fasted) to 2 h after acute ingestion of single CF dose (410 mg), fasted after 1 mo bi-daily CF, and to 1 mo, 2 h after additional ingestion of a single CF dose during the active pill and pill-free phase of the oral contraceptive cycle (see Table 3 for compositions of intervention drinks and table 5 for absolute values).

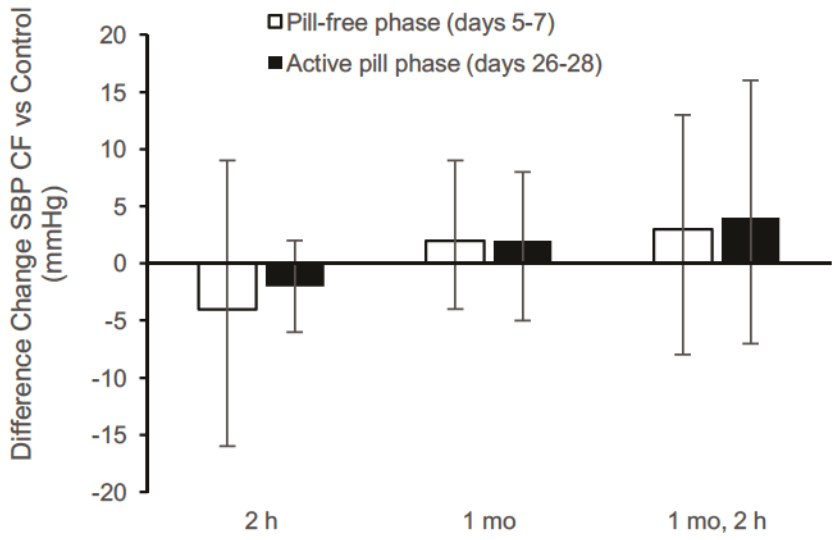

Figure 10: Effect of cocoa flavanol (CF) on systolic blood pressure (SBP) in healthy women on oral contraception (OC). Values are mean ± confidence interval.

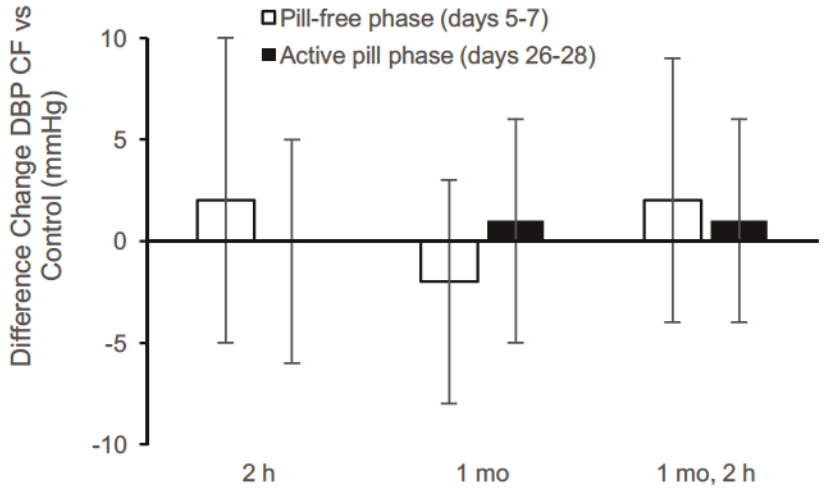

Figure 11: Effect of cocoa flavanol (CF) on systolic blood preussre (SBP) in healthy women on oral contraception (OC). Values are mean ± confidence interval.

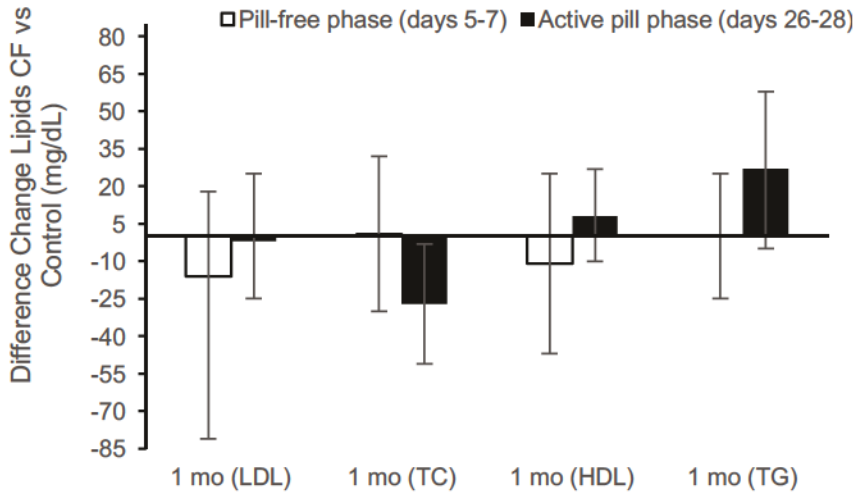

Figure 12: Effect of cocoa flavanol (CF) on low density lipoprotein (LDL), high density lipoprotein (HDL) and total cholesterol (TC) in healthy women on oral contraception (OC). Values are mean ± confidence interval.

3.2.3 Plasma levels of structurally related flavanol metabolites (SREM)

In order to determine levels of plasma flavanols, blood was sampled on each intervention day at baseline and at 2 hours after cocoa flavanol consumption, acute and chronic. Flavanol metabolites were quantified by high performance liquid chromatography (HPLC) using authentic standards. At baseline on visit 1, no flavanols were detected in plasma in all 24 participants, showing good compliance with the 24 h low polyphenol diet the volunteers were asked to follow prior to the study first. After one month of bi-daily flavanol consumption, followed by an overnight fasting,

flavanolmetabolites were below the limit of detection in baseline plasma in all subjects, during the pill- and pill-free phase.

The major metabolites detected in plasma of volunteers at two hours after consumption were (-) - epicatechin-3'-D-glucuronides (E3'G), (-) epicatechin 3 'sulfates (E3'S), 3′-*O*-methyl-(−)-epicatechin-5-sulfate (3'ME5S), and 3' -O-methyl - (-) epicatechin 7 sulfates (3'ME7S), (Fig. 13 A). These four metabolites accounted for approximately 94% of the sum of total cocoa flavanol metabolites quantified.

No significant differences in plasma flavanol levels were found between pill and pill-free phase visits and before and after one month twice-daily consumption of a 410mg CF-dose. (Fig. 13 B). Control group values were below limit of detection at all phases (data not shown).

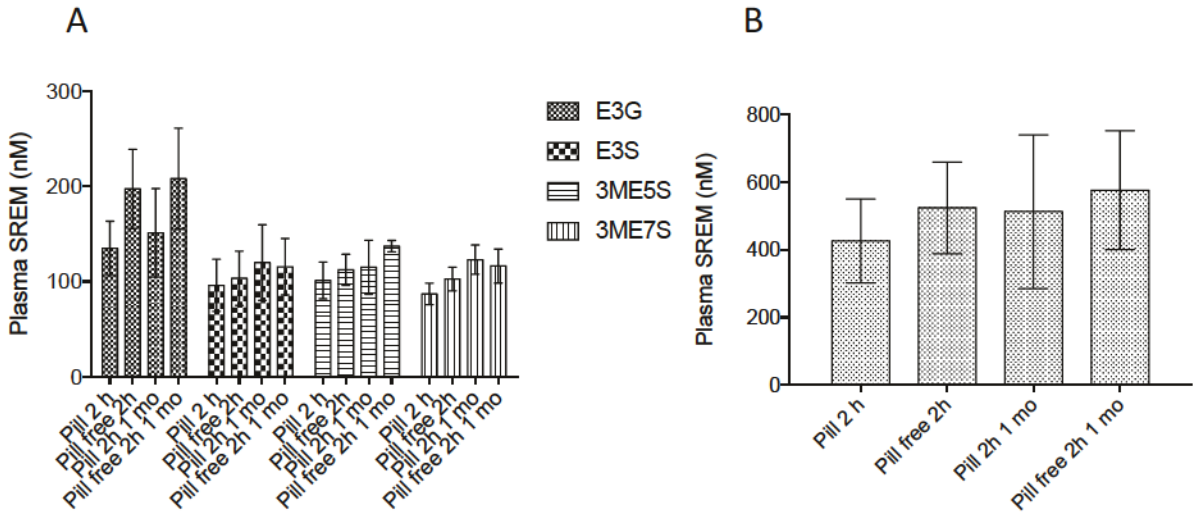

Figure 13: *A* Individual levels of SREM (mean values and confidence interval) after consumption of cocoa flavanol (CF) drink before and after 1 month bi-daily ingestion during active pill and pill-free cycle phase. ((-) epicatechin 3'-D-glucuronides (E3G), (-) epicatechin 3 'sulfates (E3S), 3′-*O*-methyl-(−)-epicatechin-5-sulfate (3ME5S), and 3' -O-methyl - (-) epicatechin 7 sulfates (3ME7S)). *B* Total Plasma levels (mean values and confidence interval) of sum of SREM after consumption of cocoa flavanol (CF) drink before and after 1 month bi-daily ingestion during active pill and pill-free cycle phase.

3.3 Effects of progestin type on flavanol-mediated improvements in endothelial function

3.3.1 Baseline characteristics of the acute study population

A double blind randomized parallel acute dietary intervention trial with 62 healthy, young premenopausal women using combined oral contraception was carried out. Recrutation took place with special regard to specific types of COC. Thus, we concentrated on different progestin types. We enrolled 15 subjects with drospirenone,

16 with dienogest, 16 with levonorgestrel and 15 with chlormadinone acetate. Altogether, two intervention groups were formed out of 62 participants.

Fig. 14 shows the acute study population flow diagram through all phases of the parallel randomized intervention trial. Participants age was 23±2 and 23±2 with a BMI of 21±3 and 21±2 in the flavanol- or control-group, respectively. There was no statistically significant difference of mean BMIs of the two groups, as evidenced by $p > 0.05$. Data not shown. Demographic and anthropometric parameters were all in normal limits and baseline blood values were in the range for healthy female individuals (Table 6B). Reference values according to our central laboratory is depicted in the Appendix. At the beginning of the study, clinical and laboratory parameters listed in Table 6A were collected to estimate cardiovascular risk. Baseline parameters indicated no group differences ($p > 0.05$) but statistically significant differences were detectable among users of COCs between the pill-free compared to the active pill phase. (Table 6A)

As compared to the active pill phase, FMD was significantly higher ($p = 0.001$), while systolic blood pressure (SBP) showed significant lower values ($p = 0.43$) during the pill-free period.

Blood lipids such as high density lipoprotein ($p = 0.002$), low density lipoprotein ($p = 0.002$) and total cholesterol ($p < 0.001$) were significantly at higher levels during the pill-free phase, while trigylcerides showed lower values during the pill free interval as compared to the active pill period, ($p < 0.001$).

Leukocytes were also at higher detectable levels during the active pill interval, $p = 0.035$. We also tested whether the proportions of people who were within the normal reference ranges for adults differed between the pill phases or intervention groups (Tabel 6B).

Neither in comparison of both intervention groups nor within each intervention group significant differences occurred. No adverse events were reported and all test drinks provided were well tolerated. No clinical signs or symptoms of CVD and none of the subjects were receiving medical treatment. Smoking was prohibited during the entire study phase. Pregnancy was not reported.

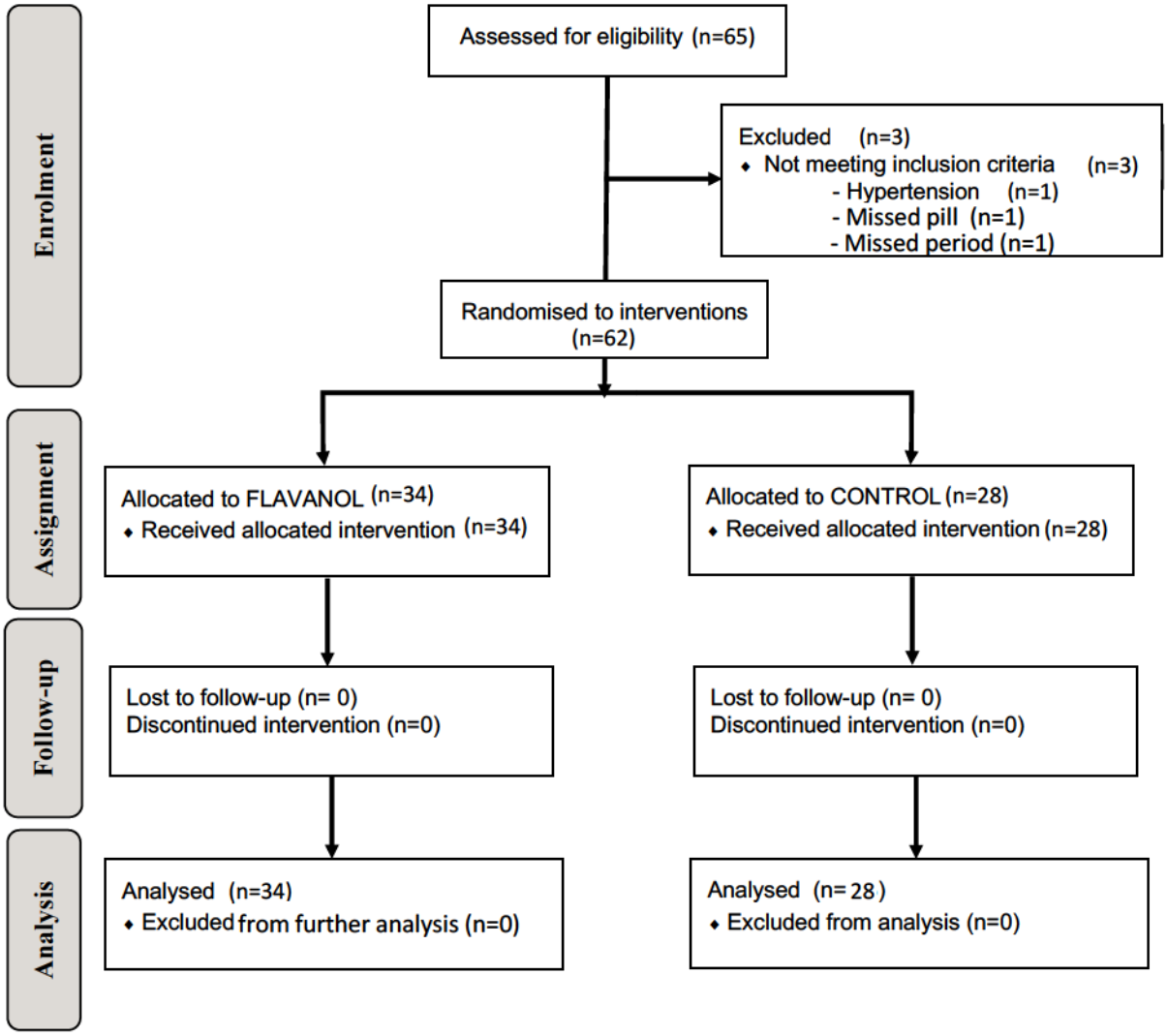

Figure 14: Flow diagram for the ACUTE Study.

Table 6: Baseline characteristics of the acute study population

	Control			Cocoa flavanol			p		
A	N	Pill-free phase (days 5–7)	Active pill phase (days 26–28)	N	Pill-free phase (days 5–7)	Active pill phase (days 26–28)	Intervention	Pill	Pill × Intervention
FMD (%)	28	7.57 (1.06)	7.16 (1.26)	33	8.16 (1.30)	7.61 (1.32)	0.079	0.001	0.585
SBP (mmHg)	28	116.02 (9.26)	118.43 (10.05)	34	113.90 (12.95)	115.67 (9.72)	0.339	0.043	0.752
DBP (mmHg)	28	76.18 (8.43)	74.80 (7.55)	34	72.76 (9.71)	72.77 (9.31)	0.196	0.448	0.441
Heart Rate	28	72.13 (11.57)	73.30 (10.58)	34	71.21 (10.16)	73.98 (10.00)	0.961	0.087	0.480
Total cholesterol (mg/dl)	28	197.75 (34.64)	180.39 (33.16)	34	198.68 (26.48)	187.82 (24.60)	0.552	<0.001	0.265
LDL cholesterol (mg/dl)	19	92.42 (23.13)	87.68 (20.47)	27	83.81 (22.81)	78.67 (18.61)	0.160	0.002	0.894
HDL cholesterol (mg/dl)	19	112.79 (26.94)	101.47 (23.54)	26	115.88 (26.33)	107.92 (24.41)	0.502	0.002	0.578
Triglycerides (mg/dl)	28	81.39 (28.19)	95.89 (29.32)	34	83.03 (42.68)	103.91 (44.34)	0.590	<0.001	0.376
Glucose (mg/dl)	28	81.89 (5.74)	83.04 (6.97)	33	79.97 (6.03)	80.91 (6.12)	0.170	0.116	0.877
Hb (g/dl)	28	13.35 (1.04)	13.31 (0.92)	34	13.13 (1.01)	13.21 (0.90)	0.488	0.753	0.459
CRP [mg/dl]	28	0.31 (0.21)	0.44 (0.36)	34	0.38 (0.49)	0.33 (0.24)	0.744	0.519	0.146
Leukocytes (x1000/µl)	28	5.92 (2.13)	6.37 (1.78)	34	5.89 (1.48)	6.38 (1.31)	0.976	0.035	0.920
Thrombocytes (x1000/µl)	28	267.64 (51.22)	261.25 (41.93)	34	270.59 (51.54)	278.50 (53.50)	0.413	0.835	0.054

B	Control			Cocoa flavanol (CF)			p Control vs. CF	
	Pill-free phase (days 5–7)	Active pill phase (days 26–28)	p	Pill-free phase (days 5–7)	Active pill phase (days 26–28)	p	Pill-free phase (days 5–7)	Active pill phase (days 26–28)
Total Cholesterol ≤ 200 mg/dl	61% (17/28)	71% (20/28)	0.453	65% (22/34)	74% (25/34)	0.508	0.796	>0.999
HDL ≥ 35 mg/dl	100% (19/19)	100% (19/19)	>0.999	100% (27/27)	100% (27/27)	>0.999	>0.999	>0.999
LDL ≤ 160 mg/dl	95% (18/19)	100% (19/19)	>0.999	92% (24/26)	100% (26/26)	0.500	>0.999	>0.999
Triglycerides ≤ 150 mg/dl	100% (28/28)	96% (27/28)	>0.999	88% (30/34)	91% (31/34)	>0.999	0.120	0.620
Glucose ≤ 100 mg/dl	100% (28/28)	100% (28/28)	>0.999	100% (33/33)	100% (33/33)	>0.999	>0.999	>0.999
Hb 11.9–14.6 g/d	82% (23/28)	79% (22/28)	>0.999	85% (29/34)	91% (31/34)	0.687	0.744	0.277
CRP ≤ 0.5 mg/dl	93% (26/28)	71% (20/28)	0.070	88% (30/34)	91% (31/34)	>0.999	0.681	0.053
Leukocytes 4.5–12.7 x1000/µl	75% (21/28)	86% (24/28)	0.453	82% (28/34)	97% (33/34)	0.063	0.541	0.166
Thrombocytes 173–390 x1000/µl	100% (28/28)	100% (28/28)	>0.999	97% (33/34)	97% (33/34)	>0.999	>0.999	>0.999

Mean values and standard deviations of selected clinical parameters before the start of respective intervention during active pill and pill-free cycle phase. SBP (systolic blood pressure), DBP (diastolic blood pressure), FMD (flow mediated dilation), HDL (high density lipoprotein), LDL (low density lipoprotein), Hb (hemoglobin), CRP (C-reactive protein). P values refer to 2-way repeated measures ANOVA with 1 within-subject factors (pill phase) and 1 between subject factor (intervention).

A – relevant clinical- and blood parameters at the beginning of the study. Comparison of baseline measurements in the different intervention groups at different pill phases.

B – reflects the the proportions of subjects within the normal laboratory reference ranges, expressed as % and absolute values.

3.3.2 Endothelial function fluctuates during the oral contraception cycle but can be acutely improved by cocoa flavanols in premenopausal women

Measurements of endothelial function, performed as FMD of the brachial artery, were collected in 62 subjects on two days, during the pill phase (with oral contraception, days between 19-21) and pill-free phase (without oral contraception, days 26-28). On each examination day, a cocoa-flavanol rich (410mg) - or a placebo-drink was provided. Basal FMD values were obtained before the administration of the test drink. The determined baseline values of 62 premenopausal women are shown in Table 6. To demonstrate the effect of different progestins contained in the most common oral contraceptives used in Germany, 62 volunteers using several different types of OC were recruited.

As already confirmed in the chronic study, a main effect of the intervention (CF) (p = 0.038) and an interaction between the intervention and pill (p < 0.001) was observed in all pill types after 2h. There was no significant difference among the different pill types (p = 0.720). (Table 7) and (Fig. 16).

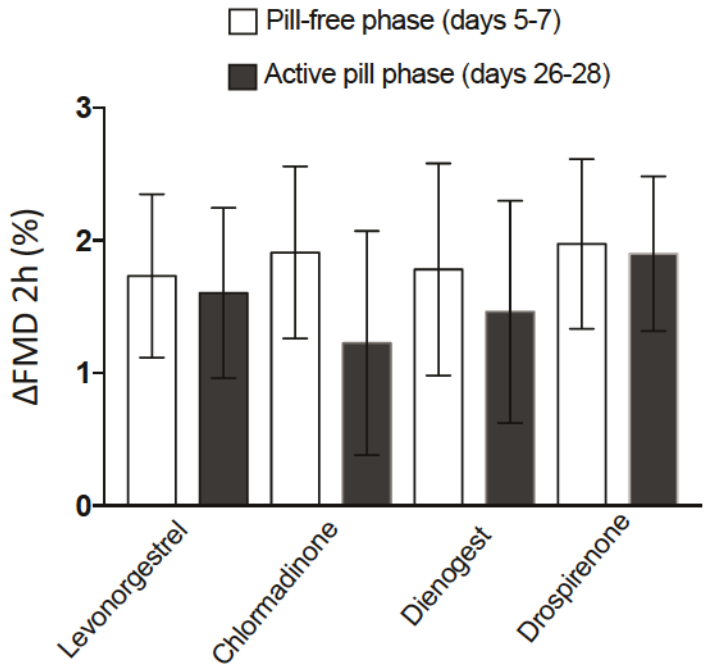

Figure 14: Flow mediated dilation (FMD) Changes 2 hours after cocoa flavanol (CF) consumption from baseline in all pill-types (shown as progestin-type). All data are expressed as means with confidence interval represented by vertical bars.

Table 7: Overview of vascular end points in oral contraception - acute study. Mean values and standard errors of flow-mediated dilation (FMD), systolic blood pressure (SBP), diastolic blood pressure (DBP), and blood lipids at baseline and 2 h after acute consumption of control or cocoa flavanol (CF) drink before and after 1 month bi-daily ingestion of respective intervention during active pill and pill-free cycle phase. Estimated marginal means of differences CF and Control related changes from baseline in active pill and pill-free phase. P values refer to repeated measurements ANOVA with 2 within-subject factors (pill type, pill phase) and 1 between subject factor (intervention).

	Control				CF				Difference (CF vs Control)				
	Pill-free phase (days 5-7)		Active pill phase (days 26-28)		Pill-free phase (days 5-7)		Active pill phase (days 26-28)		Pill-free phase (days 5-7)	Active pill phase (days 26-28)	P		
	BL (0 h)	2 h	0 h	2 h	BL (0 h)	2 h	0 h	2 h	Delta 2 h	Delta 2 h	Intervention	pill type	pill x interv.
FMD (%)	7.6 (0.2)	7.6 (0.2)	7.2 (0.2)	7.3 (0.3)	8.0 (0.2)	9.9 (0.2)	7.5 (0.2)	9.1 (0.2)	2.0 (1.6, 2.3)	1.5 (1.8, 1.5)	<0.001	0.720	0.038

3.3.3 Plasma levels of structurally related flavanol metabolites (SREM)

On each intervention day, selected flavanols and flavanol metabolites (SREM) were determined in plasma. At baseline, fasting levels of total plasma flavanol metabolites were below the limit of detection. After the acute cocoa intake, a significant increase in flavanol plasma levels were detected and quantified by HLPC. The maximum plasma concentration was 953 nM during the pill phase and 1049 nM during the pill free phase. No significant differences between pill and pill free phase were found in the amount of SREM in plasma, or in the individual metabolites (Fig. 16 A and B). The highest level was observed in E3G metabolites with 297 nM and 363 nM in the pill and pill free phase, respectively. Control group values were below limit of detection at all phases (data not shown).

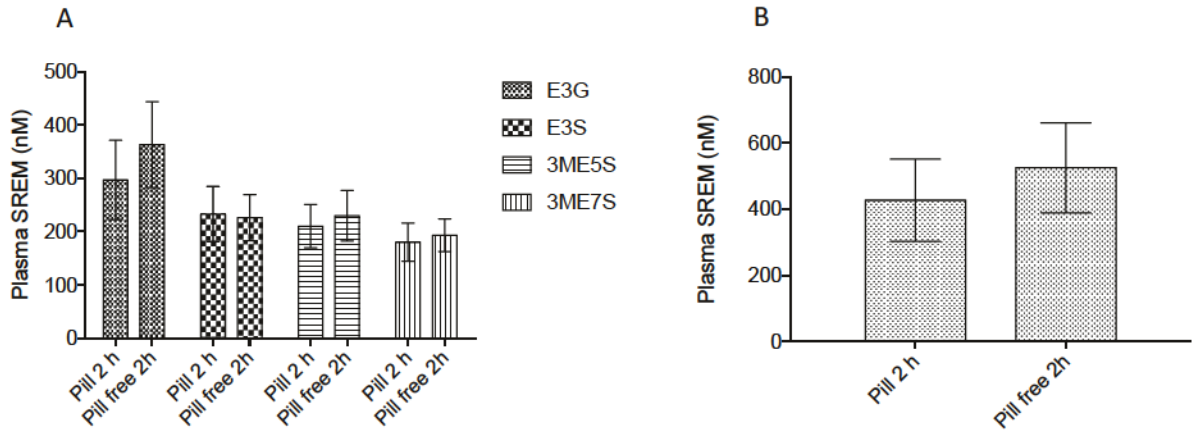

Figure 15: Plasma levels of structurally related epicatechin metabolites (SREM) 2h after consumption of cocoa flavanols (CF) during the pill and pill free phase. All data are expressed as means with confidence interval represented by vertical bars. **A)** Individual levels of epicatechin-3-glucuronide (E3G), (-) epicatechin-3-sulfate (E3S), 3-*O*-methyl-(−)-epicatechin-5-sulfate(3ME5S), and 3-O-methyl - (-) epicatechin 7 sulfate (3ME7S); **B)** Sum of total plasma SREM

Fig. 17 shows relevant plasma levels of mentioned SREMs with regard to the different progestogens. No significant differences were observed between different pill types at the pill and pill free phases.

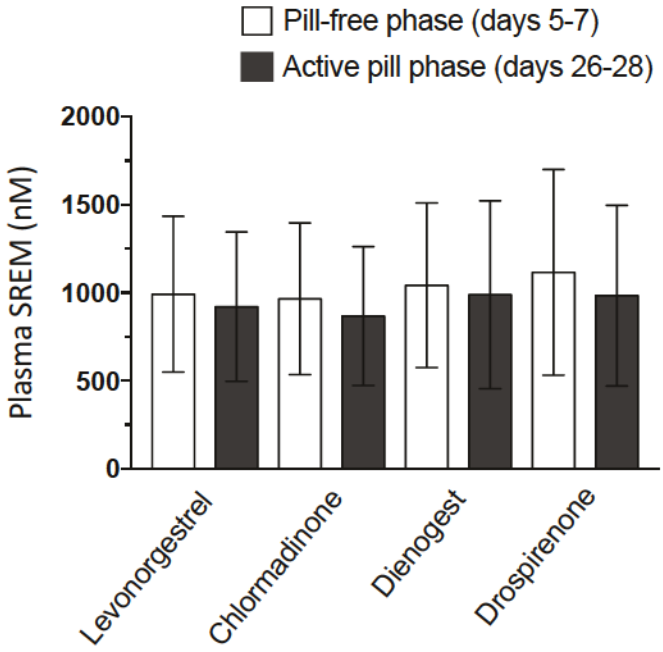

Figure 16: **Total plasma levels of structurally-related epicatechin metabolites (SREM) at 2 hours post-consumption of 410 mg cocoa flavanols on women taking different types of oral contraception.** All data are expressed as means with confidence interval represented by vertical bars.

No significants differences were seen in plasma levels between different pills or between pill and pill free phase ($p>0.05$). P values refer to repeated measurements ANOVA with 2 within-subject factors (time, pill phase) and 1 between subject factor (intervention).

3.3.4 Cocoa flavanols have no acute effect on office blood pressure and heart rate in healthy young women

No significant changes were found in blood pressure or heart rate after acute consumption of CF- or control drink for any of the progestin groups, as confirmed in the chronic study (data not shown).

4 Discussion

According to the United Nations worldwide more than 100 million women are current users of combined hormonal contraceptives, but only limited data about their effect on endothelial function is available. To our knowledge, this is the first study that investigates the effects of cocoa flavanols on endothelial function in healthy, premenopausal women with a regular combined oral contraceptive cycle.

The main findings of this work are:

I. Endothelial response is influenced by the OC cycle – significantly impaired FMD occurred during the active pill phase as compared to the pill free phase. At baseline ($p = 0.001$) and after CF intervention ($p = 0.019$)

II. There is a main effect of the intervention ($p < 0.001$) - endothelial function improved during pill-free- and active pill phase (2h, 1mo, 1mo, 2h) after consumption of 410mg CF as compared to the control

III. Responses on endothelial function did not differ between the 5 different pill-types ($p = 0.720$)

IV. COCs result in a mild but significant elevation of systolic blood pressure, while CF did not affect blood pressure at both pill phases

V. Significant blood lipid profile alteration in the composition of the plasma lipids caused by COCs is not affected by CF

VI. No significant difference between CF metabolites at different timepoints in the OC cycle occurred

The effect of oral contraceptives containing synthetic forms of estrogen (ethinylestradiol) and progesterone (drospirenon, levonorgestrel, chlormadinone acetate, dienogest) on endothelial function in premenopausal women is not established yet.

The purpose of our research was to highlight the effect of CF on endothelial function during a regular COC cycle and to study in depth the effect may be linked with the impaired endothelial function.

Our findings that endothelial function can be improved by CF, in the acute- and short-term (one month), are consistent with previous studies which are linked with a healthy study population [72, 130]. A meta-analysis of forty-two randomized controlled dietary intervention trials demonstrated significant acute and chronic (less than 18 weeks) improvements in endothelial function after CF ingestion [122]. Moreover, no additive effect was seen in both pill phases when flavanols were administered acutely after one month of bi-daily CF consumption. Sansone et al.[130] suggests a saturation of CF-related 'chronic' effects. We could confirm those results in terms of acute and chronic FMD responses linked with CF supplementation.

However, endothelial function, assessed with brachial flow-mediated dilation (FMD), was attenuated during the pill phase of the OC cycle as compared to the pill free interval. The control group showed no effect in both phases at different time points.

4.1 Oral contraception and cardiovascular risk

The use of hormonal contraception is still contentious when it comes to cardiovascular safety. Available data from observational studies, epidemiological studies and meta-analyses investigating the impact of estrogen combined with progestins on endothelial function in women on oral contraception have controversial results. Compared to non-users, higher risk of experiencing arterial thrombotic events (myocardial infarction and stroke) [171, 172] and the association with a higher risk of venous thromb-embolism (VTE) [173-176] are mainly in focus.

While some conclude different types of progestins exert different effects in the endothelium [175-177] others point out that risk only increases with higher estrogen doses [172, 174, 178]. Overall, the steady reduction in estrogen content has significantly increased the safety of COCs and the arterial complications have become rare in the current preparations. Altogether, arterial health is dependent of endothelial function, which plays an important role in the development of cardiovascular events.

So far, less is known about the impact of COCs on the endothelium. FMD as a marker of endothelial dysfunction has been shown to be predictive for adverse arterial events [179].

In controlled clinical studies the use of COC was negatively correlated with brachial flow-mediated dilation. Users experienced a mean FMD reduction compared to non-users. [180-182]

This is in agreement with our findings when it comes to baseline diameter of FMD. Measurements in 62 women on oral contraception resulted in significantly lower endothelial response during the active pill phase as compared to the pill free phase (7.4% vs. 7.9%, $p = 0.001$).

In 2009, a cross-sectional study on endothelial function in 100 premenopausal women between the ages of 18 and 30 years was conducted by Lizeralli et al. [181]. Twenty-five women were current users of a COC, another twenty-five were users of depot medroxyprogesterone acetate and fifty subjects were non-users. The authors showed that endothelium-dependent dilation was significantly lower in COC user compared to the control group (6.4% vs. 8.7%, $p < 0.01$). A similar effect was demonstrated by Heidarzadeh et al. [182] in 2014. They detected a significantly reduced FMD% in 30 premenopausal women on COC, in comparison with the control group of 30 subjects. Both authors concluded that COC use may lead to endothelial dysfunction.

With our work we support these findings and even point to further results on disturbed effects on endothelial function in women taking COC.

In our first study, 24 healthy premenopausal female subjects taking COC were randomly allocated to either a CF drink (410 mg) or flavanol-free control drink. FMD was measured during the active pill phase (days 26-28) and pill-free phase (days 5-7) of the OC cycle at different time points (2h, 1mo and 1mo, 2h). Although endothelial function significantly improved as compared to control (p main effect intervention < 0.001) in the pill-free (2 h: 2.0±0.4%; 1 mo: 1.9±0.7%; 1 mo, 2 h: 3.3±0.7%) and active pill phase (2 h: 1.4±0.5%; 1 mo: 1.1±0.6%; 1 mo, 2 h: 2.1±0.7%) of the OC cycle, responses were significantly lower during the active pill phase as compared to the pill-free phase (p interaction [pill x intervention] = 0.019).

Again, the impaired endothelial responses were confirmed in our second study with 62 healthy female subjects, in the active pill phase (1.5±0.2%) as compared to pill-free phase (2.0±0.2%; p interaction [pill x intervention] = 0.038).

Despite the relevance of the above mentioned publications, it should be noted that both studies were carried out with a low dose formulation of 0.03 mg ethinylestradiol (EE) and the progestin levonorgestrel (LNG). In our study we included different pill types with different EE dosage (0.02-0.03 mg). However, our results also indicate an impaired vessel function with the use of COCs, even when combined with CFs.

Furthermore, a growing body of evidence suggest that OCs with different types of progestin and different EE doses may have different effects on endothelial function [183-185].

Meendering et al. [186] and Torgrimson et al. [183] assessed endothelial function in young and healthy women with different COC formulations. They put focus on low dose (0.03 mg) and very low-dose (0.02 mg) concentrations of EE. Results revealed that 0.03 mg formulations altered the arterial endothelium positively while 0.02 mg showed no effect as compared to the control group. These findings are in agreement with a publication from Giribela et al. [187], where no significant differences during active pill and placebo phase were observed when a formulation of 3 mg drospirenone and 0.02 mg EE was used by 71 women.

The authors pointed out that progestogens may antagonize the beneficial properties of EE.

In our second study, we investigated if differences existed between different COC types in terms of attenuation of acute CF-related FMD improvements.

We could demonstrate that COCs with different progestins do not exert different effects in the vasculature, (p interaction [pill-type x intervention] = 0.720). One limitation may be, that we did not take EE dosage into account. EE-dosage varied from 0.02 to 0.03 mg. With a total of 62 women and 5 different pill types, the analyzed groups may have been too small to find any differences.

Altogether, available data is still inconsistent regarding the effect of COC duration on endothelial function. Some studies found non-significant relationships between short- and long-term (6 months) use [187, 188], while others demonstrate the influence of the duration of COC use on endothelial function [182, 185].

In our study population, the average duration of COC intake was 6.1 $\pm$ 2.6 years. The duration of intake may therefore possibly have an influence on the outcome of our study.

4.2 Potential mechanisms of interaction between combined oral contraceptives and cocoa flavanols

4.2.1 Progestin versus estrogen

The mechanism why estro-progestinic treatments can blunt the increase in FMD after flavanol consumption is currently unknown. Numerous recent studies highlighted the beneficial effects of EE on the endothelium [183, 189, 190]. However, paired with certain progestins an antagonizing effect has been described [183, 184]. Existing evidence supports a strong role for estrogen in generating positive FMD response by increasing eNOS activity in endothelial cells through genomic and non-genomic pathways [191-193].

In vivo studies also demonstrated that estrogen supplementation has been shown to improve endothelial function via a NO-dependent mechanism in premenopausal women [194, 195].

Furthermore, available data indicates that estrogen produces vasodilation of blood vessels through the Phosphoinositid-3-kinase-Akt (PI3K/Akt) cascade in endothelial cells and increases NO production in human umbilical vein endothelial cells (HUVEC) mediated by Estrogen Receptor α (ERα) [196-198].

In a recent study by Polidor et al. [199], the endothelium-dependent vasorelaxation induced by red wine polyphenols was suppressed in aortas of ERα deficient mice, suggesting that ERα is also required for the endothelium-dependent relaxation caused by polyphenols. Furtherly, the increase in NO production in endothelial cells through the increased phosphorylation of tyrosine-protein kinase (Src), extracelluar signal-regulated kinases 1 and 2 (ERK1/2), eNOS and caveolin-1 was suppressed in presence of an ERα inhibitor.

Moreover, recent work also pointed out that beneficial effects of estrogen on the endothelium are related to the stimulation of G-protein-coupled estrogen receptor (GPER) [200]. The activation of GPER leads to signaling pathways in endothelial cells via PI3K/Akt/eNOS [201].

Interestingly, Moreno Ulloa et al. [202] provided evidence that endothelial cell NO production derived by flavanols may be also linked with the membrane-bound GPER. The authors assume this receptor as being a potential mediator of EC, the major active metabolite of cocoa flavanols [78].

Thus, NO is not only a major target of estrogen but also flavanols have been reported to stimulate NO production at the endothelial level [78, 203].

Ramirez-Sanchez et al. [82] demonstrated EC-induced eNOS activation in human endothelial cells by Akt and Protein Kinase A (PKA), both known members of the PI3K pathway, while the addition of EC to endothelial cells together with an ERα antagonist blunted the increase in eNOS activation and NO production [204]. However, the validity of these findings are limited by the fact that those experiments were performed with pure EC, which is practically not present in plasma after flavanol consumption as opposed to its glucuronidated, methylated and sulphated metabolites [97]. Therefore, synergistic effects of estrogen and flavanols can be assumed.

Our data support the hypothesis that in women taking COC, the synthetic progesterone possibily attenuates flavanol-induced nitric oxide production in vivo via an interaction with the nitric oxide pathway.

The observed effect could be explained by the heterogeneous family of steroids which can also bind strongly to other steroid receptors, such as those for androgens, mineralocorticoids, and glucocorticoids, to exhibit differential genomic effects and thus biological responses, including nitric oxide synthesis in endothelial cells. [205]

The interaction may be thru steroid receptor modulation (glucocorticoid receptor, progesterone receptor) and the PI3K/Akt, thus inhibiting endothelium dependent vasorelaxation.

Although existing evidence has been suggested that natural progesterone may mediate vasodilatory effects by PI3K-Akt-eNOS pathway [206-208], *in vitro* experiments have shown that certain progestins can inhibit estrogen-induced NO production and eNOS activity in HUVEC via the PI3-kinase/Akt cascade [209, 210].

In summary, it can be hypothesized that the reason for these discrepancies might be due to different peculiar cellular effects exerted by certain progestins. Progesterone effects are mediated by its nuclear receptor, of which two isoforms exist, PR-A and PR-B [211]. Progesterone receptors (PR) have been localized in non-reproductive tissues, such as the myocardium [212], in peripheral vascular tissues [213] and in vascular smooth muscle cells [214] whereas progesterone effects range between potent vasodilation, no effect and inhibition of VSMC-relaxation.

The ability of PR to activate signaling pathways, such as PI3K-Akt cascade, only in the presence of progesterone but not all progestins, may depend on the different PR conformations. [210, 215]

In addition, the binding preference of certain progestins to the glucocorticoid receptor might also play a crucial role [209, 216, 217]. Hence, downregulation of eNOS expression via activation of glucocorticoid receptors or PRs could be a possible mechanism of interaction with cocoa flavanols, which leads to distinct alterations of NO synthesis. Progestins with glucocorticoid activity do not only decrease eNOS gene transcription [218] but also NO bioavailability through increasing generation of reactive oxygen species (ROS) [219]. Consequentially, endothelial dysfunction is the key player in the development of vascular events.

4.2.2 Oxidative stress

Another possible explanation might be the increased oxidative stress associated with oral contraception. Recently, Chen et al. [219] assumed that OCs may increase oxidative stress levels, independent of traditional cardiovascular risk factors, in premenopausal women. The reactive oxygen species then carry e.g. to a faster breakdown of NO in the vascular system.

It should be noted that unlike in our study, the OC used was a triphasic preparation that consisted of EE in combination with norethisterone and the study cohort was Japanese.

Andozia et al. [220] investigated in an experimental study where EE and natural occurring estradiol were compared in order to find different effects on NO production and protection against oxidative stress in human endothelial cell cultures. Concentrations were similar to those used in our study (0.03 mg EE). Finally, their results showed that EE was unable to protect endothelial cells against oxidative stress and it did not increase the production of NO, in contrast to estradiol.

Interestingly, we coud demonstrate an improvement in endothelial function after CF supplementation during the active pill phase of the OC cycle.

Lastly, experimental studies on canine coronary arteries [221] and ovariectomized mice [222] suggested that progesterone may modify vascular effects of estrogen on nitric oxide production and VSMC relaxation as well as on antioxidant enzyme

expression. Indeed, progesterone enhances NADPH oxidase activity and therefore the production of reactive oxygen species. The excess production of oxidants lead to decreased nitric oxide (NO) bioavailability in the vasculature.

Nevertheless, *in vivo* studies have shown that progesterone does not noticeably attenuate the estrogen-induced endothelium-dependent vasodilation in postmenopausal women [223]. In contrast, our study was conducted in premenopausal women.

Several studies have described that flavanols have the ability to scavenge free radicals and react with O_2. This prevents a direct reaction of O2 with NO and thus reduces a decrease in NO bioavailability [224, 225].

Alvarez-Cilleros et al. [226] recently elucidated that the mechanism underlying is mediated via adenosinemonophosphate-activated protein kinase (AMPK) and PI3K/Akt pathways and the subsequent eNOS phosphorylation/activation.

The authors investigated whether cocoa flavanol metabolites derived from colonic microbiota, could influence endothelial function and prevent oxidative stress in human endothelial cells. Results revealed that a mixture of the derived metabolites significantly increased phosphorylation of eNOS and NO production. They concluded that flavanols can protect against oxidative stress-induced endothelial dysfunction by preventing increased ROS generation and activation of signaling pathways related to oxidative stress.

In theory we can conclude from our study, that increased NO bioavailability occurred with the administration of CF, via the above mentioned mechanisms. However presumtively, the effectiveness of CF may be blunted due to the increased prooxidative activity of COCs, as a possible result of heightened oxidative stress.

4.3 Influence on blood pressure

Hypertension increases five to six times the risk for endothelial dysfunction in young women using COC [227]. In the present study, we observed significant changes in blood pressure related to COCs. As reported by other studies in normotensive women, where moderate but significant increases in blood pressure was associated with COC use. [228-230] This is in agreement with our findings. We also found moderate but significant lower systolic blood pressure in 62 normotensive women during the pill free

phase as compared to the active pill phase, (114,86mmHg vs. 116,91mmHg, p = 0.043). However, studies are scarcely available.

Literature with studies examining the effects of flavanol-rich cocoa products has previously shown significant reductions in BP, relative to controls, in the short-term. Indeed, in a meta-analysis of the blood pressure lowering-effects of flavanols, Ried et al. [121] pointed out that the blood pressure status (high - or normal blood pressure) seems to be a likely factor in the effect size of cocoa on blood pressure. In fact, studies with flavanols have not only failed to show a significant lowering in blood pressure in healthy young subjects, results were also strongly associated with hypertensive or pre-hypertensive populations. [121] In this trial baseline blood pressure levels were at normale range (SBP: 114 – 118 mmHg and DBP: 73 – 76 mmHg) and all volunteers were healthy and active. Moreover, as compared to the control group, no statistically significant effects on blood pressure were seen in our intervention group after short-term (2h) or long-term CF administration (one month) in 62 normotensive (< 120/80 mmHg) women on estro-progestinic treatment.

While the underlying putative mechanism is not fully understood yet, we can only postulate that CFs do not override control of normal BP in a young and healthy female population during COC treatment.

4.4 Blood lipid profile and the influence of cocoa flavanols

Research has shown that in healthy COC user, lipoprotein levels vary depending on the relative concentrations of EE and the progestin used in given formulations. Generally, progestins lead to a slight increase in LDL-cholesterol levels and a reduction in HDL-levels while EE exerts opposite effects. [231-233] This is in agreement with results from Lizarelli et al. [181], Endrikat et al. [234] and Berenson et al. [235] who showed that COC user had the most risky lipid-profile when a combination of EE/LNG or EE/Desogestrel was used, in contrast to non-hormonal user. The authors found an increase in LDL-C levels and decrease in HDL-C levels.

To support the theory that different progestins may have different effects on lipid metabolism, we refer here to the study by Taneepanichskul et al. [236], as a contrast to the previous mentioned studies. The authors collected and analyzed lipid profile levels in 92 women with EE/DRSP combination. They found significant decreased LDL-levels by 9.9% at cycle six compared with baseline.

In our study, alterations in the composition of the plasma lipids likely caused by COCs were characterized by a significant decrease in total cholesterol (184,47mg/dl vs. 198,26mg/dl, $p < 0.001$), in the HDL (82,39mg/dl vs. 87,37mg/dl, $p = 0.002$) and LDL (105,20mg/dl vs. 114,58mg/dl, $p = 0.002$), and an increase in triglyceride levels (100,29mg/dl vs. 82,29mg/dl, $p < 0.001$) without leaving the reverence range (Table 6B). Our findings are in agreement with previous observational studies and randomized trials for increased circulating triglycerides, lower LDL, HDL and total cholesterol to be associated with COCP [237-239].

At least, our results indicate that COCs can cause disorder in blood lipid profile in the serum of premenopausal women taking COCs.

It should be noted, that several factors, such as age, smoking, BMI, diabetes mellitus, hypertension, predispose COC users to dyslipidemias and these factors contribute to a higher risk [240]. Nevertheless, in our work, lipid-parameters were at normal ranges and none of these pre-existing conditions were enrolled.

In the end, caution should be taken with this interpretation due to scarce and inconsistent studies and the differences in the number of studies carried out with different formulation of combined OC.

So, our findings can not be generalized to other types of birth control pills with different doses of estrogen or other progestins.

After consuming the flavanol-rich cocoa drink, total cholesterol, LDL- and HDL-cholesterol as well as triglyceride levels did not show any significant differences during the active pill and pill free phase as compared to control.

Based on these findings, we envisioned that cocoa flavanols may do not exert effects on blood lipids in young and healthy females.

Although results of previous studies are not consistent, there is evidence that catechins lead to LDL reduction. Evidence from meta-analysis, human- and animal intervention studies suggested that cocoa flavanols are effective at reducing low-density and increasing high-density lipoprotein-cholesterol concentrations in serum. [241-244] The underlying mechanism is not fully understood yet. We did not detect any significant differences with respect to HDL-cholesterol and LDL-cholesterol levels after CF administration. One limitation is that most of the existing studies included health

disparities and hyperlipoproteina and was conducted in elderly. Therefore, no general statement can be made about a positive CF effect on lipid improvement.

4.5 Circulating cocoa flavanol metabolites

We provided evidence that EC is transformed into various conjugated metabolites after gastro-intestinal absorption during a COC cycle, with peak plasma concentrations detectable after two hours. This is in agreement with Rodriguez-Mateos et al. [245], Ottaviani et al. [97] and Actis-Goretta et al. [91] who elucidated EC metabolites after cocoa ingestion and found (−)-epicatechin-3′-O-β-D-glucuronide (E3′G), (−)-epicatechin-3′-sulfate (E3′S), 3′-O-methyl-(−)-epicatechin-5-sulfate (3′ME5S) and 3′-O-methyl-(−)-epicatechin-7-sulfate (3′ME7S) accounted for the most relevant EC metabolites in plasma.

The levels of plasma SREMs are consistent with those recently reported. [91, 97] E3′G concentrations were highest, whereas methyl-sulfated metabolites were at low concentration.

However, during combined OC regimen, concentrations of SREM did not differ significantly as compared to the pill-free interval. This leads to the proposition that estro-progestinic treatments, as used in this work, do not interfere with cocoa flavanol metabolites and that CF exert relevant effects on endothelial function when administered during a COC cycle.

5 Conclusion

Our data largely support the existing data on the moderate but nevertheless increased cardiovascular risk profile in women under combined oral contraception.

Furthermore, our results support consistent benefits of cocoa flavanols to exert protective effects on endothelial function in healthy premenopausal women taking combined oral contraceptives. However, the positive response exerted by cocoa flavanols on endothelial function is impaired by COC. This study provides important information for future dietary recommendations on flavanol intake, points to new mechanisms by which COC may affect cardiovascular health and also adds to the debate over the safety of oral contraceptives. Larger trials are required to confirm the potential cardiovascular benefits of cocoa flavanols when supplied with combined oral contraceptives. Additionally, more research is needed in order to investigate the exact mechanism COC can attenuate the CF improved endothelial function.

6 Future aspects / Perspectives

While combined oral contraceptives are generally not recommended for cardiovascular safety, due to the risk of vascular diseases over long-term use, it is important to emphasize the positive effects cocoa flavanols have on blood vessels. Improved vasodilation can decrease endothelial dysfunction and inhibit dozens of other cardiovascular diseases. Thus, realizing that a combination with oral contraceptives may be vasoprotective for premenopausal females. With such a large population of females currently taking oral contraceptives, these findings impact millions of premenopausal women worldwide.

A complete understanding of the molecular mechanism of action of (−)-epicatechin will be a crucial step in elucidating the impaired FMD response when combined with COCs. There is a noticeable dearth of research surrounding the young healthy female population, with regard to the endothelial actions of combined oral contraceptives.

7 Limitations

The main limitations of this study include the non-performance of an evaluation of the endothelium-independent vasodilation through pharmacological stimulation, the population's small age range, between 18 and 35 years old, and the relatively small number of volunteers when comparing different pill types. An increased number of subjects could have increased the power of the study.

In addition, it had not been possible to analyze this group of women prior to COC use and the average number of cigarettes smoked per week was about 4.6. This might be a possible confounder although smoking was forbidden 12 hours before measurements were performed.

It should be noted, that in our study the EE-dosage varied (0.02 – 0.03 mg) and the median duration of COC used in our study population was 6.1 years. There are references which indicate that the duration of COC use might has negative impact on brachial flow-mediated dilation [180, 182, 246]. Furtherly, no automatic analysing technique was used. Investigator-related measurement inaccuracies in the evaluation of endothelial function can not be excluded.

Lastly, intra subject variability, due to the parallel study design, has to be taken into consideration.

Our results can only be interpreted as indications and must be validated by future studies with a larger cohort.

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9 Appendix

The blood collection protocol was identical for both, the chronic- (one month) as well as the acute (2h) intervention study. Each study visit was followed by two blood samples at baseline (0h) and two hours (2h) after the intake of the test drink. Disposable cannulas (Venofix Safety G21 / 0.8 mm, Braun Melsungen AG) were used for venous puncture while vacuum tubes (Becton Dickinson, Vacutainer Systems, Plymouth, UK) were used to determine clinical blood parameters (small blood count, creatinine, uric acid, total cholesterol, HDL- and LDL- cholesterol, triglycerides, glucose, CRP, GOT, GPT, γ-GT, HbA1c, differential leukocyte count, progesterone, oestradiol, testosterone). Another EDTA vacuum tube was collected to determine plasma flavanol levels. Plasma was obtained by whole-blood centrifugation (800 rcf, 10 min, 4 C), portioned into several 1.2 mL aliquots and spiked with 6µl ascorbic acid. Subsequently, samples were frozen at -80 C. Clinical blood parameters were immediately processed under standard conditions by the Central Institute for Clinical Chemistry and Laboratory Diagnostics at the Heinrich Heine University in Duesseldorf.

Normal values according to our central laboratory:

Name	Unit	Reference value
Total cholesterol	mg/dL	<= 200
HDL	mg/dL	>= 35
LDL	mg/dL	<= 160
Triglycerides	mg/dL	<= 150
Glucose	mg/dL	<= 100
Hemoglobin	g/dL	11.9 – 14.6
Leucocytes	x1000/µL	4.5 – 12.7
Thrombocytes	x1000/µL	173 - 390
CRP	mg/dL	<= 0.5

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