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Effects of flavanol monomers and procyanidins on vascular function

Dissertation

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„Assessing the respective contributions of dietary flavanol monomers and procyanidins in mediating cardiovascular effects in humans: randomized, controlled, double-masked intervention trial.“

Ana Rodriguez-Mateos, Timon Weber, Simon S Skene, Javier I Ottaviani, Alan Crozier, Malte Kelm, Hagen Schroeter and Christian Heiss

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Die Auswirkungen von Flavanol-Monomeren und Procyaniden auf die Gefäßfunktion - Zusammenfassung

Flavanole sind sekundäre Pflanzenstoffe und Bestandteile unserer Nahrungszufuhr, vornehmlich im Rahmen der Ingestion von Kakaobohnen, aber auch von roten Trauben, Rotwein, grünem Tee, Äpfeln und Beeren. Vorangegangene Studien zeigen einen Zusammenhang zwischen der Einnahme von Kakaoflavanolen (CF) und der Verbesserung der Gefäßfunktion, selbst bei gesunden, jungen Probanden. Abhängig vom Grad der Polymerisation (DP) besteht die Zusammensetzung der CF größtenteils aus dem Monomer (–)-Epicatechin (~20%, DP1) und den oligomerischen Procyaniden (~80%, DP2–10).

Ziel dieser Studie ist es, den relativen Beitrag der Flavanol-Monomere und Procyanide zur Verbesserung der Gefäßfunktion im Zusammenhang mit der Aufnahme von Kakaoflavanolen bei jungen und gesunden Probanden zu bestimmen.

Demzufolge wurde eine randomisierte, doppelblinde, kontrollierte, dreiarmige Interventionsstudie im Parallelgruppendesign durchgeführt. Es wurden 45 junge, gesunde männliche Probanden rekrutiert. Der primäre Endpunkt der Studie war das Ergebnis der Fluss-vermittelten Vasodilatation (FMD). Als sekundäre Endpunkte wurden die Konzentrationen der strukturverwandten Epicatechin-Metaboliten (SREMs) in Blut und Urin festgelegt. Tertiäre Endpunkte waren die weiteren Gradmesser der Gefäßfunktion, wie Pulswellengeschwindigkeit (PWV), Blutdruck (BP), Cholesterinwerte und Blutzuckerwerte.

Nach Messung der Ausgangswerte erfolgte die Einnahme von Kapseln, die entweder einen DP1-10 Kakaoextrakt, einen DP2-10 Kakaoextrakt oder keinerlei CF beinhalteten. Die Kapseln ohne CF (Kontrolle) bestanden im Übrigen aus den gleichen Makro- und Mikronährstoffen wie die übrigen Testsubstanzen. Nach 2 Stunden wurden die Messungen wiederholt. Nach täglicher Einnahme der jeweiligen Kapseln über 30 Tage erfolgte eine Wiederholung der Messreihen zur Bestimmung von chronischen und akut auf chronischen Effekten.

Die Einnahme von DP1-10 führte zu einem signifikanten Anstieg der FMD-Werte, erhöhten Konzentrationen von SREMs im Blut sowie Abnahme der PWV und der BP-Werte. Diese Veränderungen konnten sowohl nach der Einnahme von DP2-10 als auch der Kontrolle nicht verzeichnet werden. Eine signifikante Reduzierung der Cholesterinwerte war nach der Einnahme von DP1-10 und DP 2-10 ersichtlich, verglichen mit der Kontrollgruppe.

Die Verbesserung der Gefäßfunktion nach der Einnahme von CF ist vornehmlich mit der Einnahme des Flavanolmonomers in Verbindung zu bringen und dem daraus resultierenden Anstieg der zirkulierenden Metaboliten im Blutplasma junger und gesunder Männer. Der vorrangige Anteil der CF, die Procyanide, als auch derer vom Mikrobiom abgeleiteten Kataboliten zeigen keinen signifikanten Effekt hinsichtlich der Gefäßfunktion. Die Reduzierung der Cholesterinwerte kann mit der Einnahme von Procyaniden in Verbindung gebracht werden, ist allerdings nicht sicher einem Effekt der Monomere zuzuordnen. Hinsichtlich dessen, als auch der Wirkung auf andere Kohorten, ist weitere Forschung notwendig.

Effects of flavanol monomers and procyanidins on vascular function – summary

Flavanols are plant secondary metabolites particularly abundant in cocoa, red grapes, red wine, green tea, apples, and berries. Earlier studies strongly suggest that consumption of cocoa flavanols (CF) can improve endothelial function both acutely and after short-term daily consumption. Such improvements have not only been observed in subjects with endothelial dysfunction but also in healthy and young subjects. CFs comprise different compounds, which are classified according to their degree of polymerisation (DP) in monomers, of which the most abundant are (–)-epicatechin (~20%, DP1) and the oligomeric procyanidins (~80%, DP2–10). Despite efforts to elucidate mechanisms of action, currently, it is unknown whether the monomers, the oligomers, or both are the bioactive compounds in cocoa exerting beneficial effects on vascular function.

This study aims to determine the relative contribution of flavanol monomers and procyanidins to the improvement of vascular function associated with CF intake in young and healthy subjects.

Accordingly, a randomised, double-blind, controlled, three-arm intervention study was conducted in a parallel-group design. Forty-five young, healthy male subjects were recruited. The primary endpoint of the study was the outcome of flow-mediated vasodilation (FMD). Secondary endpoints were the concentrations of structurally related epicatechin metabolites (SREMs) in blood and urine. Tertiary endpoints were the other grade measures of vascular function, such as pulse wave velocity (PWV), blood pressure (BP), cholesterol levels and blood glucose levels.

After baseline measurements were taken, capsules containing either DP1-10 cocoa extract, DP2-10 cocoa extract, or no CF were taken. The capsules without CF (control) consisted of the same macro- and micronutrients as the other test substances. The measurements were repeated after 2 hours. After daily intake of the respective capsules for 30 days, the series of measurements was repeated to determine chronic and acute on chronic effects.

Consumption of DP1-10 led to a significant increase in FMD values (at 2 h and 1 month post-consumption), raised concentration of SREMs in blood plasma, and decreases in PWV, BP, and total cholesterol levels. Meanwhile, the consumption of DP2-10 had no significant effects in any parameter, except for a significant decrease in total cholesterol levels compared to the control.

The current findings indicate that the improvements in vascular function after CF consumption are linked to the flavanol monomers and consecutively the increase of SREMs in the blood plasma of young and healthy humans. The cocoa procyanidins did not seem to affect vascular function. However, the reduction of total cholesterol can be linked to their consumption. Further work is, therefore, necessary to understand the mechanisms of action and generalisability of our findings to the general population.

List of Abbreviation

ANCOVA..... analysis of covariances
BA ..... brachial artery
BMI..... mean body mass index
BP ..... blood pressure
bpm..... beats per minute
CAD.....coronary artery disease
CF..... cocoa flavanols
CHD..... coronary heart disease
CI..... confidence intervall
CONSORT .....
Consolidated Standards of Reportings of Trials
COSMOS .....
COcoa Supplement and Multivitamin Outcomes Study
CVD.....cardiovascular disease
d days
DBP..... diastolic blood pressure
DP .....degree of polymerisation
eBASIS .....
Bioactive Substances in Food Information System
ECG.....electrocardiography
EDTA.....
..... Ethylenediaminetetraacetic acid
EuroFIR .....
European Food Information Resource
FDA..... Food and Drug Administration
FMD.....flow mediated dilation
g grams
GDP.....gross domestic product
GGT.....gamma-glutamyltransferase
GOT.....
glutamate oxaloacetate transaminase
GPT.....glutamyl pyruvate transferase
h hours
HbA1c..... Glycated haemoglobin
HDL..... high-density lipoprotein
HF..... heart failure
HPLC.....
High - performance - liquid chromatography
IR..... insulin resistance
L litre
LDL..... low-density lipoprotein
mmHg .....millimetre of mercury
mo ..... month
n Number
NADPH.....
nicotinamide adenine dinucleotide phosphate
nmol..... nanomoles
NO ..... nitric oxide
OR ..... odds ratio
PREDIMED.....
..Prevención con Dieta Meditérranea
PTA .....peripheral arterial tonometry
PWA ..... pulse wave amplitude
PWV .....pulse wave velocity
RCT ..... randomised controlled trials
RH-PAT.....
reactive hyperemia - peripheral arterial tonometry
RR .....risk reduction
SARS-CoV.....
severe acute respiratory syndrome coronavirus
SBP ..... systolic blood pressure
SD..... standard derivation
SEM..... standard error of mean
SOP..... Standard Operating Procedure
SREM .....
structurally related (-)-epicatechin metabolite
WHO.....World Health Organization
wk.....week
γVL..... γ-valerolactone

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1 Introduction

1.1 Cardiovascular Diseases

Cardiovascular disease (CVD) is defined as a causal sequence of modifiable and non-modifiable risk factors that develop into organ damage on behalf of increasing pathological vascular changes due to ongoing processes on a cellular and molecular scale. These vascular alterations are caused by various events, including inflammatory responses, increasing vasoactive mediators and vascular remodelling, which can lead to end-organ failure and ultimately death. Since the development is portrayed as a continuum, it is hypothesised that intervention to improve cardiovascular health can have an effect anywhere along the chain of pathological events (Dahlöf, 2010; Dzau et al., 2006). The most significant pathological change on a vascular basis is the development of fibrous fatty lesions in the wall of the arteries, known as atherosclerosis. As a result, this can lead to the formation of atherosclerotic plaques, which can rupture and lead to the adhesion of thrombotic material and consequent blockage of the vessels (De Caterina et al., 2006; Libby et al., 2019).

CVDs are the number one killer worldwide, with around 27% (15.2 million) of all deaths caused by only two diseases – ischaemic heart disease and stroke (WHO, 2018). They have been the leading cause of death for the last 15 years, and overall, CVDs are accountable for about 32% of all global deaths (WHO, 2021). In high-income countries, the situation is even more severe. Half of the population will die from a CVD in well-developed environments (WHO, 2018).

Notwithstanding the progress in modern medicine with pharmaceutical improvements, new technological developments, and the resulting reduction of mortality regarding CVDs, the burden caused by these diseases remains enormous. This results from increased life expectancy and poor adherence to prevention guidelines (Van Camp, 2014). This displays the importance of implementing prevention strategies to reduce immense health care spending and extend life expectancy.

The financial burden related to CVDs is also a big concern, especially in low- and mid-income countries, and makes up a considerable share of their gross domestic product (GDP). Noteworthy, in this case, are the sub-Saharan African countries (7%) and the Russian Federation (2,8 %), which spent around 45% of its total health care spending (6,2 % of the GDP) solely on the costs that emerged due to CVDs (Cook et al., 2014; Gheorghe et al., 2018). A literature review supports this data with a display of the worldwide economic burden due to heart failure (HF), just one disease within the group of CVDs, which causes an estimated cost of $108 billion per year (Cook et al., 2014).

A significant aim of modern medicine is the prevention of chronic diseases like coronary heart disease (CHD), which start developing in childhood even though symptoms onset is in middle age (Jokinen, 2015). Many CVDs can be avoided by reducing individual behavioural risk factors, primarily tobacco use, unhealthy diet, physical inactivity and obesity (WHO, 2021). Studies suggest that a suboptimal diet is the main CVD risk factor (Benjamin et al., 2017). Consequently, dietary strategies to prevent CVDs are a major public health goal to reduce the burden of those diseases.

1.2 Endothelial Dysfunction

The vascular endothelium is an active autocrine, paracrine and endocrine organ vital for maintaining vascular homeostasis and adjusting vascular tone. The imbalance of the complex endothelial physiology is titled endothelial dysfunction, characterised by impaired endothelium-dependent vasodilatation (Hadi et al., 2005). It is a well-observed response to cardiovascular risk factors and a progressor of atherosclerosis and, therefore, a liable predictor regarding the development of a CVD (Endemann, 2004). Most known risk factors for CVDs are linked to endothelial dysfunction, including modifiable factors, such as smoking, diet, arterial hypertension, dyslipidaemia, hyperglycaemia, hyperhomocysteinemia, chronic inflammation and obesity, as well as non-modifiable factors of risk, like age and genetic factors (**Figure 1**) (Su, 2015) (Heiss et al., 2015a). The primary pathophysiological characterisation is the reduction in the bioavailability of nitric oxide (NO) (Sitia et al., 2010; Weiss et al.,

2002). Nitric oxide is formed from L-arginine and has an essential impact on regulating BP, mainly due to vasodilatation (Vallance et al., 1992). Studies have discovered that NO, besides being a vasodilator, has essential functions in the cardiovascular system. NO is involved in the reduction of leukocyte adhesion and uptake from the bloodstream into the walls of the vessels, prevention of cell death, reduction of tissue injury during ischemia-reperfusion, as well as the prevention of developing pro-atherogenic phenotype in vascular smooth muscle cells and endothelial cells (Tritto and Ambrosio, 2004).

Different approaches exist for the measurement of endothelial function. The most commonly used method is flow-mediated dilation (FMD), a non-invasive but sensitive technique to measure vascular function, which is strictly NO-dependent. Therefore, FMD is an indirect assessment of NO-bioavailability in vivo, demonstrating endothelial cells' capability to release NO to an adequate physiological trigger. Consequently, FMD is a liable predictor of endothelial dysfunction (Joannides et al., 1995). An alternative scientific concept of evaluating endothelial dysfunction is done by assessing peripheral artery tonometry.

Contrary to FMD, which obtains data from conduit arteries, this approach focuses on the resistance vessels of the fingertip (microvasculature). The technique called reactive hyperaemia - peripheral arterial tonometry (RH-PAT) has shown comparable prognostic magnitude regarding the prediction of cardiovascular events compared to FMD in a meta-analysis (Matsuzawa et al., 2015). In comparison to FMD, RH-PAT correlates less with NO-bioavailability and depends more on endothelium-derived hyperpolarising factors, the approaches are proven to have a negative correlation in a population diseased with multi-vessel coronary artery disease (CAD). However, within a group of individuals with normal FMD, no correlation was detectable (Tajima et al., 2020). An advantage of RH-PAT is the reduced technical complexity compared to FMD. However, it lacks to represent age-associated reductions in endothelial function of large vessels (Babcock et al., 2021).

The endothelial function is modulated by modifiable risk factors such as smoking, medical therapy and diet. Diet, in particular, has been shown to modulate endothelial function in both a positive and a negative way. For example, high-fat diets are proven to lead to an impairment of endothelial function, while healthy dietary patterns rich in fish, fruits and vegetables are associated with a lower endothelial dysfunction (Davis et al., 2007; van Bussel et al., 2015). Furthermore, it has also been described that an intake of certain foods such as red wine, cocoa and black tea can reverse endothelial dysfunction (Duffy et al., 2001; Agewall, 2000; Heiss et al., 2007).

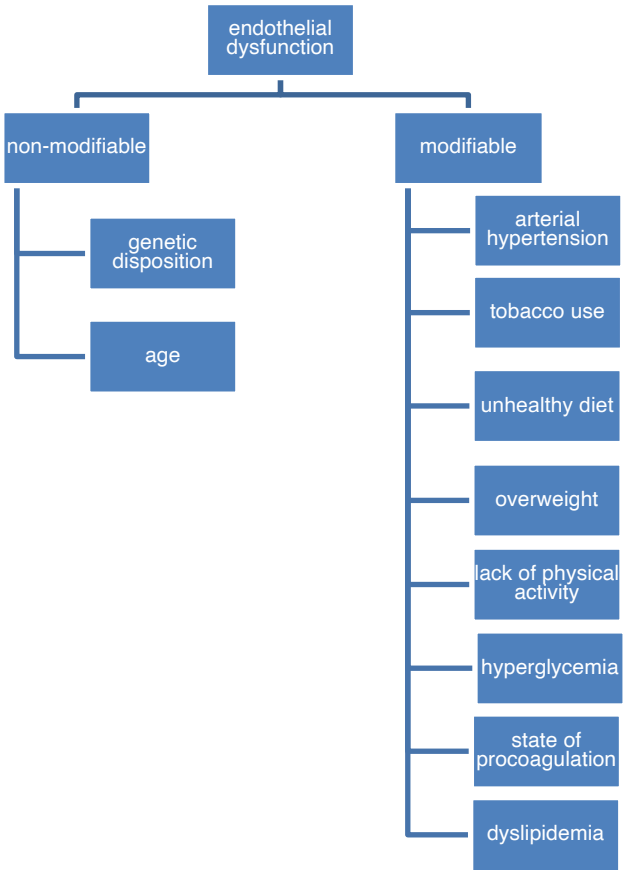

Figure 1 Modifiable and non-modifiable endothelial dysfunction risk factors

1.3 Dietary Strategies for CVD Prevention

The Global Burden of Disease Study 2017 emphasised that no other risk factor is responsible for more deaths worldwide than unhealthy eating habits. In particular, diets containing low amounts of fruits, nuts, seeds, vegetables, whole grains and high amounts of sodium were the top factors responsible for early deaths (GBD 2017 Diet Collaborators, 2019). These findings are supported by numerous observational studies showing that higher consumption of vegetables and fruit is linked to a lower risk of cardiovascular disease, cancer and premature mortality.(Aune et al., 2017).

This means that the global burden of CVDs has a significant variance between different parts of the world, which to a certain degree is connected to regional diet differences and the variations in fruit and vegetable intake (Hartley et al., 2013). Underlining this, in a prospective cohort study with middle-aged women, a nutritional pattern consisting of an increased intake of fruits, vegetables, legumes, poultry, whole grain and fish had a significantly lower risk of cancer, cardiovascular and total mortality compared to a nutritional pattern, which relies on a high consumption level of refined grains, refined sugar and red and processed meat (Western dietary pattern) (Heidemann et al., 2008).

Supporting the effects of dietary patterns on CVD mortality have been observations of the Mediterranean diet. This diet typically includes high consumption of fruits, nuts, vegetables and olive oil, while consumption of red meats, dairy products and sweets is low (Willett et al., 1995). A noticeable study observing the effects of the Mediterranean diet is the PREDIMED Study, which surveyed the incidence of major cardiovascular events in elderly people at risk of CVD. This study concluded that the Mediterranean dietary pattern accompanied with either nuts or olive oil led to a significant risk reduction for serious cardiovascular events among people with a high-risk profile for CVD (Estruch et al., 2018).

Recent studies in older Chinese adults have shown that moderate consumption of a “traditional Chinese food pattern” is linked with decreased cholesterol levels

and BP. This diet is characterised by a relevant proportion of vegetables, fruit, fish, pork and rice. The significant changes were monitored by comparing this dietary style to another common Chinese diet, the “fast and processed food pattern”, which comprises processed or fast food, confectionery and sugar (Sun et al., 2013).

The observed diets in the studies conducted by Heidemann et al. (Diet of a middle-aged woman), Estruch et al. (Mediterranean Diet), and Sun et al. (Diet of the Chinese elderly) have in common a high consumption of fruits and vegetables, which were associated to a CVD risk reduction, a lower probability of occurrence to encounter a major cardiovascular event and a lower risk of CVD-mortality.

A large number of meta-analyses have linked increased consumption of vegetables and fruits with a decreasing risk of developing CVD in observational studies (Aune, 2019). However, still, there is a current lack of observations exclusively on the effects of increased consumption of fruits and vegetables in randomised controlled trials (RCT) demonstrating cause and effect relationships (Hartley et al., 2013). Recent observations suggest an optimum intake of 800 g/d for fruits, or ten a day; however, little is known about which types of plant food may be more beneficial (Aune, 2019).

Novel advances focused on investigating the multiple bioactive compounds in plant food (e. g. cocoa), which may be responsible for health benefits. Hence attention was gathered by a group of natural substances called flavonoids. These plant derivatives have been linked to a decrease in cardiovascular mortality rate and the prevention of CHD (Panche et al., 2016).

1.4 Flavonoids – Overview

Flavonoids are part of a group of phytochemicals (chemical compounds present in plants) called polyphenols, which are widely found in our diet, especially in fruits and vegetables together with certain drinks like coffee, tea and wine. Polyphenols can be separated into four major subgroups: lignans, stilbenes, phenolic acids and flavonoids (**Figure 2**) (Scalbert and Williamson, 2000;

Manach et al., 2004). They all share the polyphenol base structure characterised by several hydroxyl groups on aromatic rings. The most common subgroup of polyphenols is flavonoids. These phytochemicals have the typical structure of two aromatic rings (ring A and B) joined by three carbon atoms forming an oxygenated heterocycle (ring C) (Manach et al., 2004). The group of flavonoids represents a variety of plant metabolites with over 10.000 compounds, of which a tiny portion has been studied until this date (Kozłowska and Szostak-Wegierek, 2014). Six subtypes of flavonoids are known namely: flavanones, flavonols, isoflavones, flavones, anthocyanidins and flavanols. (**Figure 3**)

Flavonoids are formed in plants for various reasons like their regulation and signalling properties in gene expression and enzyme regulation, ultraviolet ray protection and their antibacterial, antiviral and antioxidant characteristics (Pollastri and Tattini, 2011). Even for human pathogen viral diseases, there is increasing evidence that flavonoids may offer health benefits. In 2019, beforehand the pandemic of SARS-CoV-19, researchers conducted a study that showed significant inhibitory compounds of flavonoids regarding the viral protease of SARS-CoV 3CL (Jo et al., 2020).

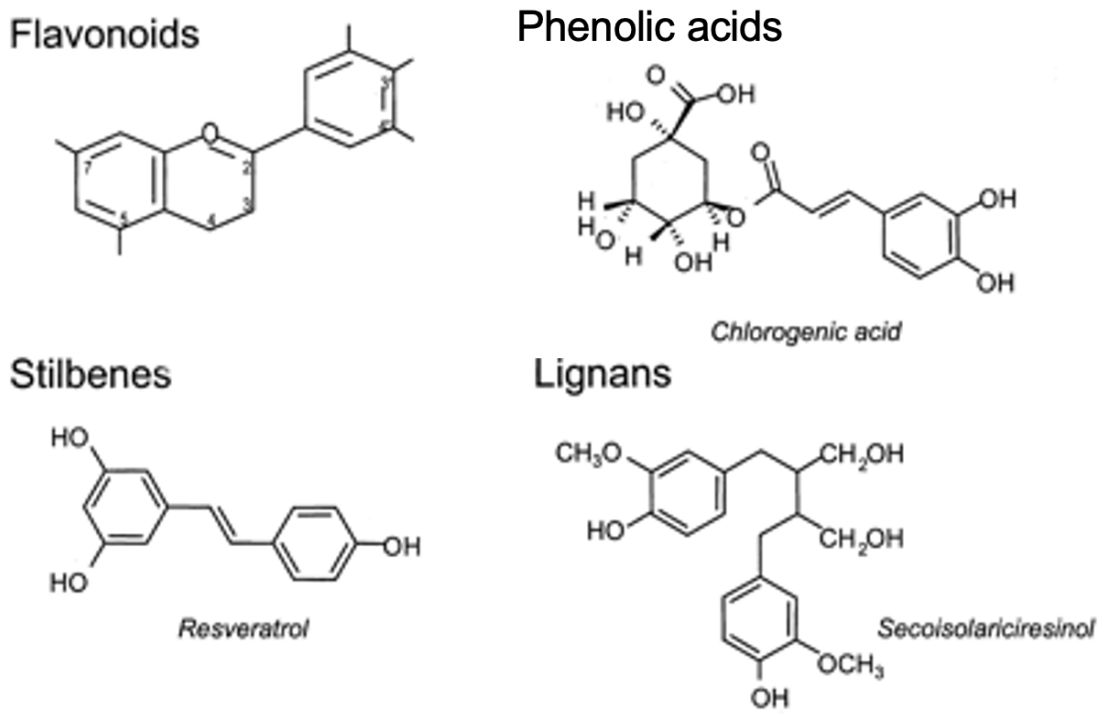

Figure 2 Main classes of polyphenols characterised by their chemical Structures. (adapted from Manach et al., 2004)

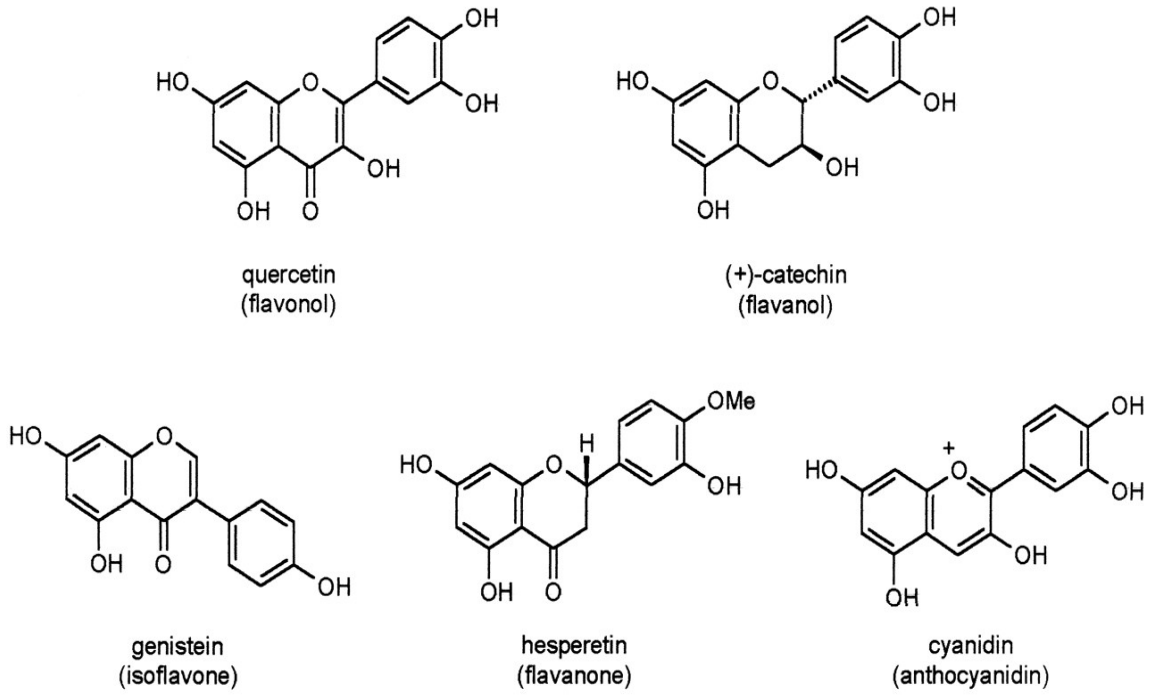

Figure 3 Assortment of the main subgroups of flavonoids characterised by their chemical structures. (adapted from Scalbert and Williamson, 2000)

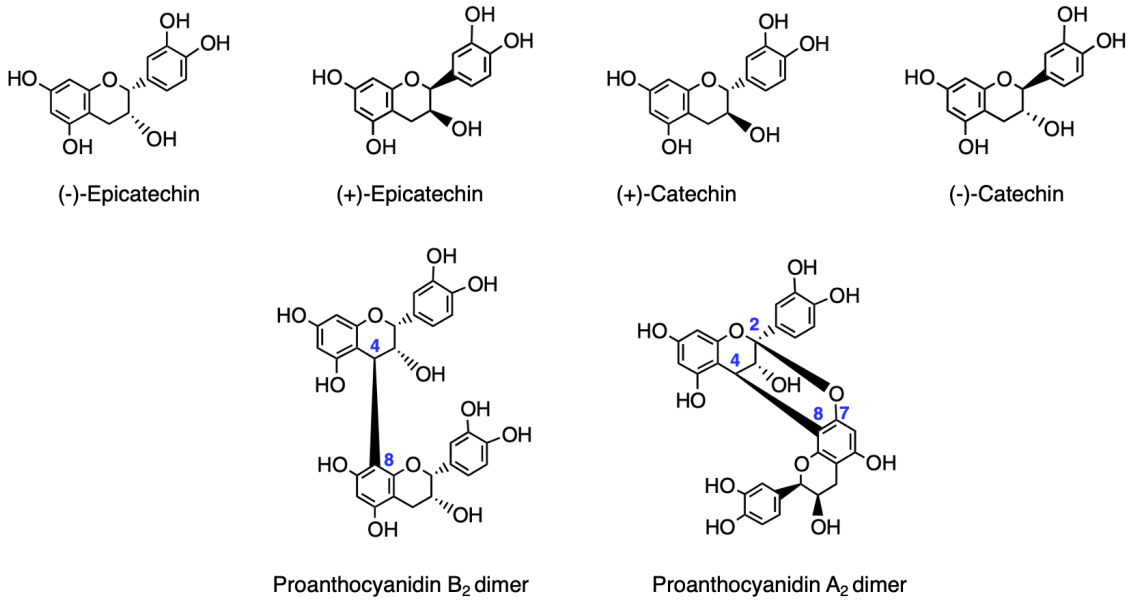

Figure 4 Chemical structures of flavanol monomers (epicatechin) and oligomers (proanthocyanidins). (adapted from Rodriguez-Mateos et. al 2014)

The characteristic structural features of flavanols are the absence of an oxygen group at the 4-position of the heterocyclic C-ring, which they have in common only with anthocyanidins, as well as the absence of a double bond at the 2-3-position and the presence of a 3-hydroxyl group, creating two centres of asymmetry in succession. (**Figure 3**) (Hollman and Arts, 2000). The flavanols can be found in monomeric forms, such as (-)-epicatechin and (+)-catechin, typically found in smaller concentrations, along with the polymeric form named procyanidins found in higher amounts in foods. With analytical techniques such as normal-phase high-performance liquid chromatography (HPLC), scientists identified oligomeric procyanidins up to a degree of polymerisation (DP) of 22 from silver birch bark (Rue et al., 2018) In the Range of DP1-10, a relatively evenly percentual contribution by weight, besides a clear drop for decamers, has been shown (Robbins et al., 2012)(Adamson et al., 1999). In relative number (-)-epicatechin makes up between 15% and 20% (by weight), while procyanidins have a weight contribution of up to 80-85% within cocoa flavanols (CFs)

(Ottaviani et al., 2018b). ***Table 1*** shows the commonly consumed foods containing procyanidins.

Flavanols are widely found in plant foods and particularly abundant in *Camellia sinensis* (tea), *Vitis vinifera* (grape wine) and *Theobroma cacao* (cocoa), and a wide variety of fruits (Schroeter et al., 2006). In particular, the flavonoids derived from *Theobroma cacao* (cocoa) have been studied for several years and have shown significant improvements in endothelial function associated with an increase in metabolites of (-)-epicatechin in plasma (Engler et al., 2004; Buijsse et al., 2006). The final content of flavanols in certain plant foods depends to a degree on planting, growing, the chosen harvesting technique, the different types of processing, and finally, the form of preparation, if necessary. Cooking plant foods in water, predominantly tea, leads to a significant loss of flavanols. This can be explained by the solubility of flavanols, since the water is a polar solvent, along with the possible ease of exiting the plant cells (Beecher, 2003). Also, enzymatic transformations may play a significant role in foods' final content of flavonoids. A 25 to 33% loss of quercetin has been observed within 12 days (d) of storing onions (Beecher, 2003).

Table1 Commonly consumed foods containing procyanidins (adapted from Rue et al., 2018).

	Fruits	
apple	grape	quinces
apricot	kiwi fruit	raspberry
avocado	lingonberry	red currant
banana	lychee	rhubarb
bilberry	mango	rose hip
black currant	marionberry	rowanberry
blackberry	nectarine	Saskatoon berry
blueberry	orange	sead buckthorn
cherry	peach	strawberry
chokeberry	pear	sweet rowanberry
cloudberry	persimmons	tangerine
chokeberry	pear	sweet rowanberry
cloudberry	persimmons	tangerine
cranberry	pineapple	tomatoes
crowberry	plum	watermelon
dates, deglet noor	pomegranate	whortleberry
gooseberry		

	Vegetables	
carrots	indian squash	pepper
eggplant	lettuce	potato
figs	onion	zucchini
Nuts / Grains	**Legumes**	**Misc.**
almonds	chickpeas	cinnamon
cashews	faba beans	dark/milk chocolate
hazelnut	french beans	hops
peanuts	kidney beans	tea beverage
pecan	lentils	red wine
pistachio	pinto beans	white wine
walnuts	white beans	rose wine
barley flour	black beans	sherry wine
buckwheat grits/flour	black eye peas	
rice	cacao beans	
wheat flours		
sorghum		

1.4.1 Bioavailability of CFs

In general, the bioavailability CFs is modulated by different factors. These factors can be related to the food and its preparation, such as the processing, the level of intake and the food matrix. For instance, different food matrices lead to faster or slower uptake of flavanol metabolites, especially specific carbohydrate matrices that may increase flavanol absorption (Rodriguez-Mateos et al., 2012). Furthermore, the presence of specific lipids and acids tends to have a positive effect on the bioavailability of flavanols (Scholz and Williamson, 2007). Other modulating factors are related to the individual, such as age, gender, dietary background, genetic polymorphisms and drug-flavanol interactions (Di Lorenzo et al., 2021; Rodriguez-Mateos et al., 2012; Scholz and Williamson, 2007), although little is known on the effects of some of those factors.

Studies have shown that CF can be found in circulation shortly after consumption. In humans, derivatives of flavanol monomers in plasma samples were found just 1 hour (h) after consuming a cocoa drink, while peak levels were found 2 h post-consumption (Baba et al., 2000). In recent years, the knowledge of the pharmacokinetics of CF in humans has increased, particularly after a study using radiolabelled (-)-epicatechin, which showed that 82 ± 5% of the consumed flavanol monomer was absorbed. Radioactivity was detectable in the circulation just 15 min after ingestion. Two peak levels were observable in blood plasma and full blood at 1h and 6h post-intake. The formed metabolites were almost exclusively found in the blood plasma (Ottaviani et al., 2016; Borges et al., 2018).

Within the flavonoid classes, the flavanols inherit distinguished features which characterise their way of uptake into human circulation. Flavanols are primarily found esterified with gallic acid, as oligomers or in their aglycone form. Contrary to most other flavonoids, flavanols never appear to be glycosylated nor methylated (Kühnau, 1976). Despite initial assumptions, CFs are not degraded by gastric acid and remain stable under these conditions (Rios et al., 2002; Javier I Ottaviani et al., 2012). Absorption of flavanols has been described by epithelial cells of the jejunal lumen. This is done primarily by passive diffusion for the aglycone forms, partly mediated through the lactase phlorizin hydrolase in the

brush border of mammalian epithelial cells (Day et al., 2000). Following absorption, flavanols undergo the common phase II enzymatic metabolism pathway determined for exogenous organic substances. Therefore sulfation, methylation and conjugation with glucuronic acid may take place. These reactions are catalysed by sulfotransferases, catechol-O-methyltransferases and uridine-5´-diphosphate glucuronosyltransferases (Del Rio et al., 2013; Richelle et al., 1999). The predominantly form of CF is conjugated while entering the mesenteric circulation (Spencer, 2003). Further phase II enzymatic conversions may take action in the liver after passing through the portal vein. Scientists are still debating a possible recycling transfer back into the small intestine via biliary enterohepatic recirculation (Crozier, 2013).

The majority of flavanol uptake in the small intestine is in the form of the monomer (-)-epicatechin, which is transformed into structurally related (-)-epicatechin metabolites (SREM), including (–)-epicatechin-3′-sulfate, (–)-epicatechin-3′-O-glucuronide and 3′-O-methyl-(–)-epicatechin-5- and -7-sulfate (Ottaviani et al., 2018a). SREM can be found in the human systemic circulation as soon as 30min post-consumption. Most of the conversion before absorption may take place in the enterocytes with further hepatic metabolisation post-absorption. These derivates maintain characteristic flavanol ring and ring-fission metabolites (Javier I. Ottaviani et al., 2012; Borges et al., 2018) (**Figure 5**).

Further investigations regarding the metabolism of oligomeric procyanidins (DP2-10) have shown that they do not contribute to the pool of circulating SREMs. No absorption of procyanidins occurs in their intact form within the proximal gastrointestinal tract, as well as no breakdown to the monomeric form. (Javier I Ottaviani et al., 2012) (**Figure 5**).

On the other hand, recent studies indicate that the human gut microbiome, predominantly in the colon, plays an essential role in the bioavailability of CFs. Some advances even state that the gut microbiome may be the key driver of CF metabolism, demonstrating that 90-95 % of the ingested polyphenols accumulate in the colon (Cardona et al., 2013).

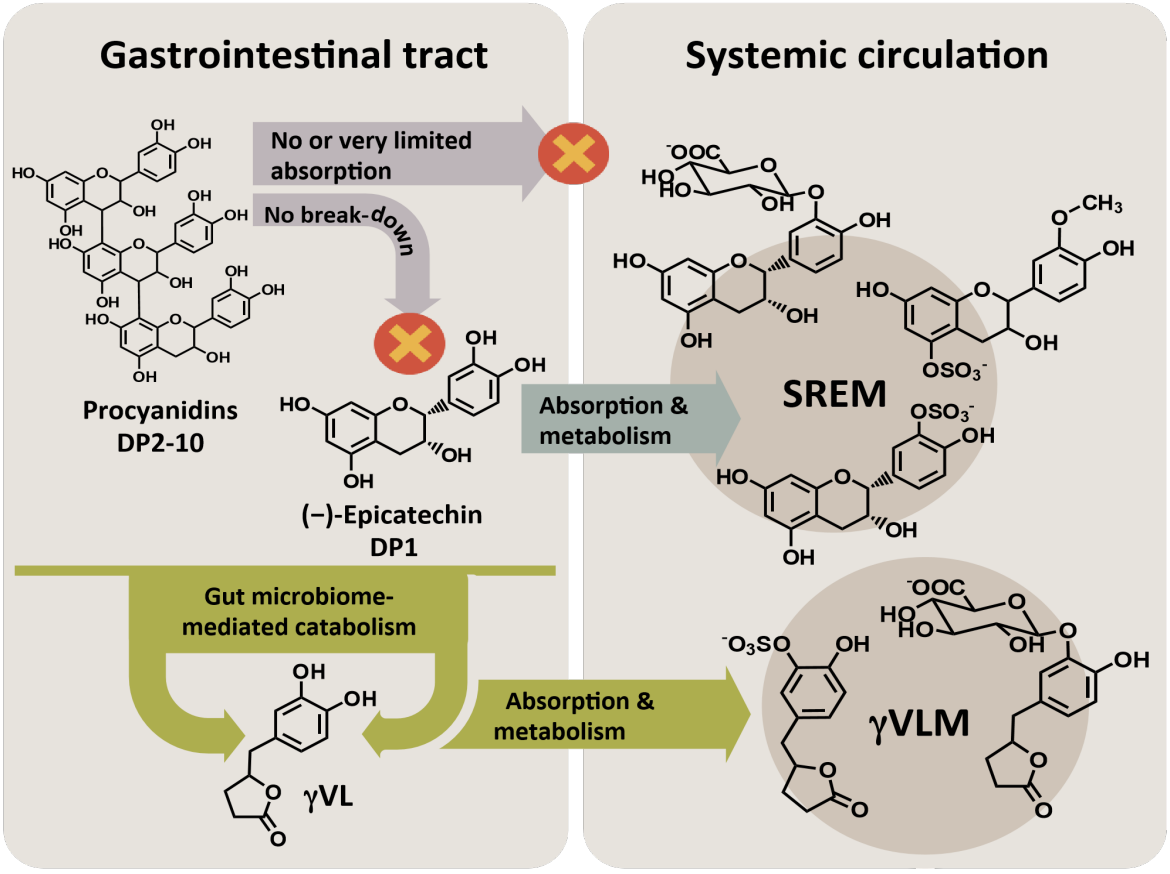

Figure 5 Uptake of monomeric and oligomeric CF into the systemic circulation. (used with permission from Rodriguez-Mateos et al., 2018)

Further studies laid out that over two-thirds of the ingested (-)-epicatechin is absorbed after microbiome-catabolism in the large intestine. (Ottaviani et al., 2016). The most abundant gut microbial metabolites are sulfated and glucuronidated metabolites of 5 - (3′,4′-dihydroxyphenyl)-γ-hydroxyvaleric acid and 5 - (3′,4′-dihydroxyphenyl)-γ-valerolactone (γVL). This contribution corresponds to almost half of the total (-)-epicatechin consumption (42% ± 5%) (Ottaviani et al., 2016) (**Figure 5**).

The procyanidins also undergo gut microbial catabolism in the colon, with a significant detectable increment of γVL concentrations in blood plasma. Novel advances detected that the consumption of equimolar amounts of (-)-epicatechin and procyanidin B-2 leads to comparable values of γVL metabolites in blood plasma samples (Ottaviani et al., 2018a) (**Figure 5**).

Novel acquired knowledge regarding the bioavailability of flavanols showed that within the upper gastrointestinal tract, there is an exclusive uptake of the monomeric flavanol (-)-epicatechin, which then is metabolised to SREMs. In the large intestine, there is proven catabolism of flavanol monomers and oligomeric procyanidins by gut microbes in the colon to several ring fission products like γVL, which then are absorbed and metabolised to γVL metabolites present in the human circulation (Rodriguez-Mateos et al., 2018).

1.4.2 CFs and Cardiovascular Health - The Evidence

In recent years flavonoids have gained more attention due to their health effects. A recent meta-analysis of 15 prospective cohort studies has shown a strong correlation between a high flavonoid intake and reduction in CVD mortality and total mortality (RR = 0.86, 95% CI: 0.73, 1.00). These effects can be considered in all flavonoid subclasses, excluding flavonols and isoflavones (Kim and Je, 2017). Accordingly, meta-analyses have associated foods with a high flavonoid content with a reduced risk of CVD. For instance, consumption of chocolate (< 100 g/week) displayed within 14 publications, with 405,304 participants, was associated with beneficial effects on the occurrence of CVDs (Ren et al., 2019). Similar results are obtained in a meta-analysis (9 studies/total of 259,267 volunteers) regarding the effects of green tea on cardiovascular and ischemic-related diseases, which identified significant higher risks of CVD for people who do not consume green tea compared to people who drink more than one cup of green tea per day (OR=1.19, 95% CI: 1.09-1.29) (Pang et al., 2016). Further reviews regarding wine (Chiva-Blanch et al., 2013) and apples (Gayer et al., 2019) confirm the positive effects of flavonoid-rich foods on CVDs.

Recent systematic reviews and meta-analyses focussed specifically on flavanols. A meta-analysis of 15 prospective cohorts (23 publications) showed that flavanol intake was linked to a decrease of CVD mortality [RR= 0.87 (0.77, 0.98)] and lower risk of chronic heart disease [RR= 0,81 (0,66, 0,99)]. Flavanol monomers were associated with a positive effect on CVD mortality with comparable significance to total flavanols [RR= 0.86 (0.76, 0.97)]. At the same time, this analysis displays the lack of studies on procyanidins as only two studies were

included with no significant results regarding CVD mortality (Raman et al., 2019). These findings are supported by the most extensive study, which examined 21442 volunteers for an average of 3.6 years. The COcoa Supplement and Multivitamin Outcomes Study (COSMOS) (ClinicalTrials.gov: NCT02422745) presented a 27 % reduction in deaths of CVD (Sesso et al., 2022).

The association of flavanol consumption with a reduction in CVD mortality seen in observational studies can be linked to a decrease in cardiometabolic biomarkers that have been identified by several systemic reviews and meta-analyses of randomised controlled trials, which observed reductions in BP, triglycerides and mean arterial pressure, as well as improvements in FMD (Hooper et al., 2012; Vlachojannis et al., 2016). The exact mechanism of these beneficial effects on vascular function has not been decoded yet. One of the many proposed mechanisms is an increase in NO bioavailability due to the downregulation of neutrophil NADPH oxidase activity (Rodriguez-Mateos et al., 2013). Novel advances provide evidence that the intake of CFs can adjust genes in whole blood cells, which are seen to interact with the endothelium due to modulation of DNA methylation profile of gene implicates in actin cytoskeleton organisation, cell signalling or cell adhesion. Also, CF-metabolites may have the potential to bind transcription factors and cell signalling proteins, as seen in silico docking analyses. These capabilities may lead to beneficial cardiovascular health effects, as seen in a clinical trial by preserving the immunological barrier functions of the endothelium (Milenkovic et al., 2022).

1.4.3 CFs and BP- The Evidence

BP is one of the most relevant prognostic surrogate markers of CVDs, with a positive correlation between higher BP values and the occurrence of CVDs (Wilson et al., 1998; D'Agostino et al., 2013). Due to this importance, a moderate number of studies are referring to possible lowering effects on BP by CF.

The Flaviola Health Study in 2015 displayed significant decreases in systolic blood pressure (SBP) and diastolic blood pressure (DBP) by 4.4 mmHg (95 % CI 7.9, 0.9 mmHg) and 3.9 mmHg (95 % CI 6.7, 0.9 mmHg) after consuming 450

mg of CF twice a day over 1 month compared to a control group (Sansone et al., 2015). Similar changes have been shown in people at risk, for instance, within a group of volunteers with hypertension (Grassi et al., 2008). A dose-dependency study within a group of individuals with mild untreated hypertension laid out positive changes in 24-h mean arterial BP only observable in the highest tested dose of 1052 mg CF over 6 weeks (Davison et al., 2010). There is heterogeneity regarding the amounts of flavanols applied since different studies showed relevant BP changes in far lower doses of CF. In 2007 it was described that doses of just 30 mg CF per day over 18 weeks have relevant positive effects on BP (Taubert et al., 2007). The heterogeneity between these studies may be caused due to the different periods of supplement.

Further investigations displayed the modulating effects of possible supplements within the tested CFs. Added sugar seems to diminish the positive changes in BP compared to non-sugared CF test substances within a group of overweight adults (mean body mass index (BMI): 30 kg/m^2) (Faridi et al., 2008). Given this information, future studies can find the best way of administering CF.

Conflicting results exist with several studies unable to detect significant changes in BP following the intake of CF. This may be due to a too short period of supplementation, inadequate doses, using purified compounds and because significant changes are primarily seen in populations at risk (Rees et al., 2018).

Overall, a meta-analysis updated in 2017 provides the best evidence. This review included 35 studies that provided moderate-quality results that flavanol-containing foods such as cocoa and chocolate have a blood pressure-lowering effect of 2 mmHg. However, the review highlights the limitations of this finding due to heterogeneity between studies and emphasises the need for further research, particularly concerning long-term studies (Ried et al., 2017).

The data concentrated on procyanidins is rare and controversial. In 2018 it was suggested that flavanols separated into monomers and oligomers did not affect BP on its own (Hollands et al., 2018).In contrast, a meta-analysis in 2021, which included six studies with a total of 367 subjects, concluded that a supplement of

procyanidins is capable of a significant reduction of systolic BP (-4.598 mmHg; 95 % CI: -8.037, -1.159) and diastolic BP (-2.750 mmHg; 95 % CI: -5.087, -0.412) (Ren et al., 2019). Animal studies suggest that the vasodilatation effect may be due to an obstruction of potential-dependent calcium channels and inhibition of calcium release next to the impact caused by a higher NO bioavailability (Zhang et al., 2008). The procyanidins derived from CFs have not been studied in this context until this study.

1.4.4 CFs and Endothelial Function - The Evidence

Endothelial dysfunction is a reliable predictor of the occurrence of CVDs. Therefore the impact of flavonoids on endothelial function has been widely studied. Significant evidence can be found regarding the positive changes in endothelial function due to the intake of CF. These effects are primarily measured by FMD, which reliably mirrors NO bioavailability in vivo.

In 2012 a meta-analysis and systemic review of 42 RCTs (total of 1297 volunteers) regarding the effects of CF on cardiovascular function detected significant improvements in FMD following chronic (1.34%; 95% CI: 1.00, 1.68%) and acute (3.19 %; 95% CI: 2.04%, 4,33%) consumption (Hooper et al., 2012). A more recent analysis of 13 systematic reviews from 2010 to November 2015 on the clinical effects of chocolate consumption confirmed the positive changes in FMD in a dose-dependent manner (Vlachojannis et al., 2016). ***Table 2*** displays various studies regarding the effects of CF on FMD in different cohorts.

In general, higher doses of CF are associated with a greater improvement in FMD. In 2007 it was shown that a consumption of 306 mg of CF three times a day (918 mg daily dose) leads to an FMD improvement from 3.7 +/- 0.4% to 6.6 +/- 0.5% (each $p < 0.05$) in young healthy men. This study also stated that the dose to reach a half-maximal FMD response was 616 mg. It must be outlined that this was only seen in a minimal number of volunteers (n=6) (Heiss et al., 2007). Coherently, the following study of the same research group with about half the dose (450 mg) of CF led to smaller changes (6.1 ± 0.7 vs 7.6 ± 0.7 %, $p < 0.001$) of FMD after an intake of over 14 d in young, healthy men (Heiss et al., 2015b).

Correlating with the early detection of SREMs in blood plasma, maximal effects of FMD are found 2 h after the intake of CF (Heiss et al., 2005).

The role of (-)-epicatechin and the beneficial effects on FMD is still being discussed. It has been shown that oral administration of pure (-)-epicatechin can show similar acute vascular effects compared to changes due to the intake of flavanol-rich cocoa (Schroeter et al., 2006). But other studies focussing on pure epicatechin showed no significant effects on FMD (1.1% absolute; 95% CI: -0.1%, 2.3%; P= 0.07) after supplementing 100mg of (-)-epicatechin per day (Dower et al., 2015). A recent review and meta-analysis concluded that peak effects on FMD can be seen after the intake of (-)-epicatechin ranging from 50 to 150 mg. Modelling the data from 15 articles, it was concluded that strongest effect might be seen at 710 mg total flavanols with 95 mg of (-)-epicatechin and 25 mg (+)-catechin. Also, this analysis stated that it could not differentiate the impacts of (-)-epicatechin and (+)-catechin on improving FMD due to correlating levels in the substances (r= 0.78) (Sun et al., 2019). To date, there is limited data regarding the impact of cocoa procyanidins on vascular function.

Table 2 RCTs observing effects of CF on FMD, statistical data obtained from Sun et al. 2019

YEAR	FIRST AUTHOR	SUBJECTS	DURATION	TREATMENT	CHG FMD BY TREATMENT SMD (95%CI)
2005	Grassi (1)	30 normotensives	15 d	550 mg CF/ chocolate	1.56 (0.73, 2.38)
2005	Grassi (2)	30 hypertensives	15 d	550 mg CF/ chocolate	1.71 (0.86, 2.55)
2006	Wang-Polagruto	17 women w/ hypercholesterolemia	42 d	403 mg CF/ cocoa drink	0.56 (-0.41, 1.54)
2008	Balzer	41 elderly w/ medicated diabetes	30 d	888 mg CF/ cocoa drink	1.19 (0.78, 1.48)
2008	Davison (1)	26 unhealthy	84 d	866mg CF/ cocoa drink & exercise	0.76 (-0.04, 1.55)
2008	Davison (2)	23 unhealthy	84 d	866mg CF/ cocoa drink	0.86 (0.01, 1.72)
2010	Heiss (1)	32 elderly w/ CAD	30 d	750 mg CF/ cocoa drink	0.93 (0.19, 1.66)
2011	Njike	78 overweight	42 d	796 mg CF/ cocoa drink (w vs w/o sugar)	0.91 (0.44, 1.38)
2012	Flammer	20 w/ congestive heart failure	28 d	1248 mg CF / chocolate	1.72 (0.68, 2.76)
2013	Mogollon	42 healthy pregnant	84 d	340 mg CF/ Chocolate	-0.13 (-0.73, 0,48)
2014	Esser	82 overweight men	28 d	819 mg CF/ Chocolate	0.08 (-0.35, 0.52)
2015	Heiss (2)	22 young men	14 d	900mg CF/ cocoa drink	1.16 (0.25, 2.07)
2015	Heiss (3)	20 elderly men	14 d	900mg CF/ cocoa drink	1.18 (0.22, 2.14)
2015	Sansone	100 healthy	28 d	900mg CF/ cocoa drink	2.42 (1.19, 2.94)
2016	Rassaf	49 w/ haemodialysis	20 d	900mg CF/ cocoa drink	1.12 (0.51, 1.72)

1.5 Goal of this Investigation

As discussed in the previous sections, strong epidemiological and clinical evidence suggests flavanol consumption can improve human vascular function. These findings are of high interest since impairments in FMD, a parameter for vascular function, are strongly correlated with the risk of cardiovascular events. Meta-analyses have shown that an increase of 1 % in brachial FMD is linked to a reduction of around 13% in CVD risk (95% CI, 0.83-0.91) (Inaba et al., 2010).

Up to now, there is still substantial uncertainty regarding the exact mechanisms of how flavanols can influence the cardiovascular system and about the relative impact of the different compounds belonging to the flavanol group. Particularly a lack of knowledge can be found about oligomeric flavanols with a DP up to 10 – the procyanidins. It has been shown that monomeric and oligomeric flavanols are taken up differently in human circulation and lead to different levels of metabolites in blood plasma. However, it is unknown whether they all are bioactive or act synergistically, exerting health effects.

Consequently, this study has been conducted to assess the individual specific contributions of flavanol monomers and the oligomeric procyanidins regarding the observed cardiovascular effects and the correlation of effects with the main circulating SREMs and γVL metabolites. The obtained data may help uncover the best way to administer CF in dietary supplements and provide a more profound understanding of the mechanisms of action of CF in the vascular system.

The main hypothesis is that procyanidins mediate the cardiovascular effects of cocoa intake in healthy individuals due to their microbiome-derived metabolites γVL.

Specific objectives are:

- to examine changes in endothelial function after the intake of CF when (-)-epicatechin is present or not

- to measure plasma and urinary flavanol metabolites after the intake of CF when (-)-epicatechin is present or not
- to examine control parameters reflecting endothelial function, flavanol absorption, and exploratory mechanistic parameters (nutrigenomics).

2 Methods and Materials

2.1 Declaration of Personal Contributions

Hereby I, Timon Weber, declare that I performed the following tasks to obtain the data needed to conduct this study: recruiting and scheduling all volunteers, holding consent discussions with each subject, execution of vascular measurements, collection of blood and urine samples, evaluating vascular parameters, preparation and storage of blood plasma and urine samples and performing various statistical analyses. Dr Javier Ottaviani analysed flavanol metabolites in urine and plasma samples at the University of California Davis. To determine the clinical chemistry parameters, the blood draws were sent and analysed by the central laboratory of the Düsseldorf University Hospital. The study was designed by Dr Hagen Schroeter, Prof. Christian Heiss and Dr Ana Rodriguez-Mateos.

2.2 Volunteers

A total of 60 volunteers were assessed for eligibility while planning this study. Recruiting was conducted via social media (e. g. Facebook), distributing flyers and word-of-mouth communication. Due to the known relationship between age, female hormonal cycle and endothelial function, and to minimise inter-individual variability in vascular response, only young and healthy males were considered as participants. Inclusion criteria were age from 18 to 35 years, Caucasian, BMI ($kg/m^{2)}$ of 23-27, good general health evaluated by a brief physical examination and a health and lifestyle questionnaire, no present acute infection, non-pathological blood values including a regular hemogram and standard values for liver enzymes, blood fats, HbA1c and blood coagulation. Agreeing to the terms and conditions of the consent form was also mandatory for inclusion. Exclusion criteria were a history of malignant diseases, assumed or manifested CVDs and other diseases affecting the vascular function such as peripheral, cerebrovascular and renal occlusive diseases, heart arrhythmia, chronic heart failure, acute or chronic renal failure, diabetes mellitus and arterial hypertension.

Furthermore, participants were excluded when following extreme diets, taking dietary supplements, extensive consumption of alcohol (> 210 g alcohol/wk) and due to the chronic consumption of antibiotics and antihypertensive drugs. Also, known allergies to milk products and sensitivities to theobromine, caffeine and methylxanthines led to exclusion. Every participant was informed about the planned intervention via comprehensible verbal and written explanations, which outlined the purpose of the trial and collateral risks as well as possible side effects.

The final study population consisted of 45 young and healthy males. Five volunteers were excluded due to the consumption of medicaments which matched the exclusion criteria. Ten individuals declined to participate after being provided with additional information. For the CONSORT Flow Diagram see **Figure 6**. All of the 45 enrolled volunteers acknowledged the terms and conditions, and a declaration of consent was signed by each before the start of this intervention.

The Ethics committee of Heinrich Heine University examined and judged the study protocol together with the consent form. It was concluded that there were no ethical or legal concerns resulting in a positive ethics committee vote.

(No. of study: 4392R Registration ID: 2013081291)

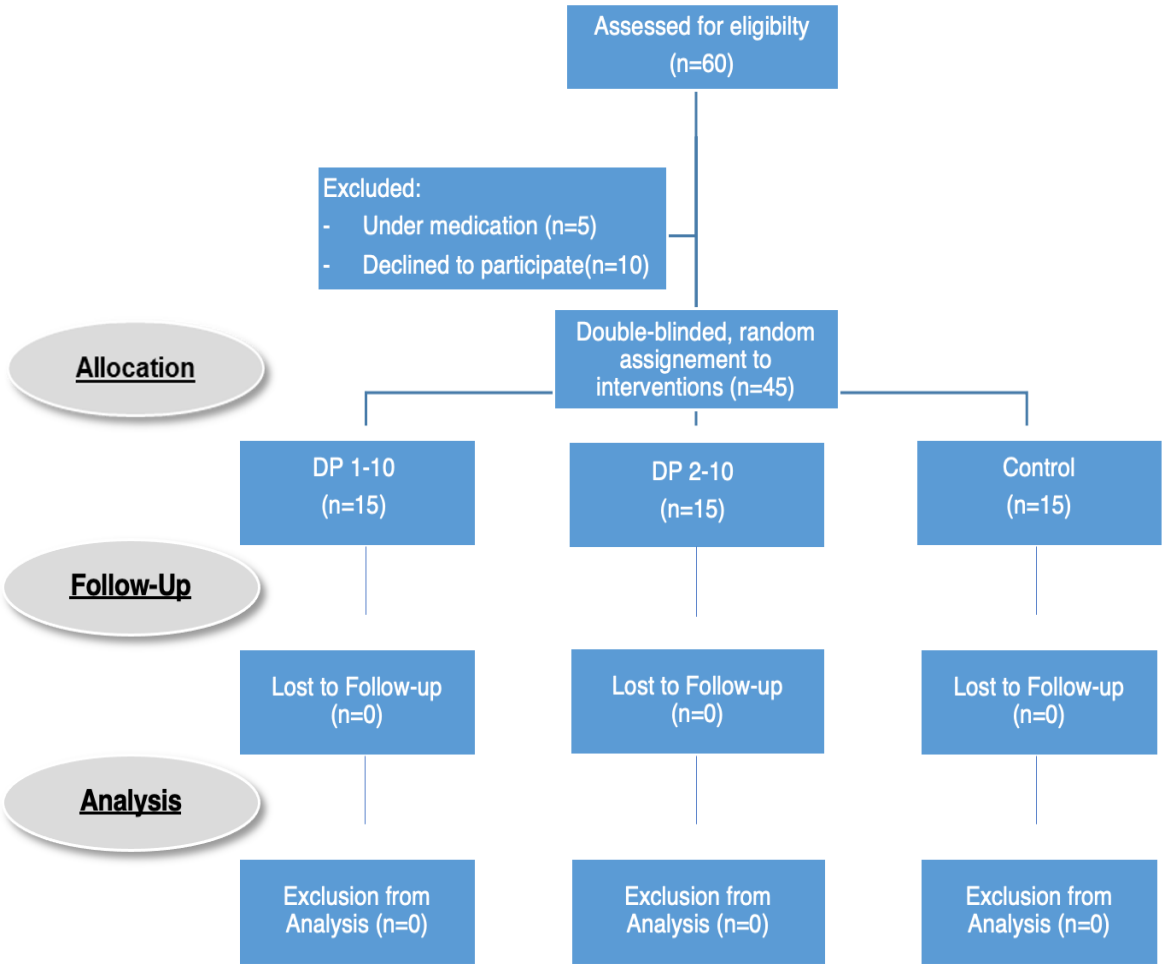

Figure 6: Study flow according to the Consolidated Standards of Reporting Trials (CONSORT), DP, degree of polymerisation.

2.4 Study Design

The conducted parallel-group dietary intervention trial was randomised, placebo-controlled and double-blinded. It contained three interventions, which were performed at the Department of Cardiology, Pulmonology and Angiology at the University Hospital Duesseldorf under the leadership of Prof. Dr Malte Kelm. The study population consisting of 45 volunteers was divided into three parallel arms. Fifteen volunteers were assigned to each arm, the control arm, the DP 1-10 arm and the DP 2-10 arm. Every involved researcher conducting the study or

analysing the outcomes was blinded regarding the identification of each test product.

Randomisation was done with the help of a freeware named GraphPad (GraphPad Software, Inc.), available on www.graphpad.com/quickcalcs/randomize2. This software produces „pseudo-random“ numbers that are random enough for analytical test methods, simulating data and randomised assignments. The time of the day is used by this program to generate the first random number. That way, every application run will result in a different set of random numbers (GraphPad Software, Inc., 2021).

Before starting the study, every volunteer was instructed to maintain a low-flavonoid diet for 24 h. Volunteers were asked to avoid the consumption of foods containing relevant shares of flavanols such as vegetables and fruits as well as coffee, tea, alcohol and cocoa during this timeframe. The restriction was prolonged to 24 h after every intervention since the urine, gathered over 24 h, was collected until the following morning. To assess compliance with the diet, volunteers were asked to fill a 48-h dietary recall. During the time of the study, the volunteers were asked not to change their usual fluid intake and diet, plus to avoid vigorous physical exercise (> 3 x 20 mins/week).

Each group was asked to consume two capsules with their daily breakfast over one month (see **Figure7**). Volunteers were randomly assigned to the three treatments tested consisting of:

1) DP1-10: capsules containing flavanol monomers and procyanidins
2) DP2-10: consisting of flavanol procyanidins only
3) Control: capsules matched in micro- and macronutrient composition to the flavanol capsules but without flavanols

To evaluate the compliance of the volunteers, the empty bottles with the spare capsules were collected at the second visit.

The data was obtained in a temperature-controlled room to design a reproducible and suitable study environment. Each participant had an appointment in the

morning that started between 7.30 am, and 8.30 am and ended between 10.00 am and 11.00 am. The urine was collected exactly 24 h after the consumption of the test substances on the first day and the last day of the study. The volunteers were instructed to collect the urine for 24 h in prepared containers. To do so, they had a cooler bag and ice packs to maintain the urine refrigerated.

Before any measurements, the participants were asked to lay down for 10 minutes in a supine position and remain in this position until all examinations were completed. The resting time was followed by BP, FMD and pulse wave velocity (PWV) measurements. A blood draw followed each data collection. BP values and the collected blood were obtained from the left arm. FMD measurements were performed on the right brachial artery.

FMD was the primary endpoint of this intervention. Secondary endpoints are flavanol metabolites and catabolites in plasma and urine. Further evaluations of vascular function, such as PWV and BP, blood lipids including total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides, and fasting glucose, were set to be the tertiary endpoints.

Every researcher engaged in this trial was blinded concerning the identity of the tested substances. The guidelines described by the Declaration of Helsinki were followed by this study. This trial was catalogued with the National Institutes of Health registry for randomised trials at clinicaltrials.gov as NCT02728466.

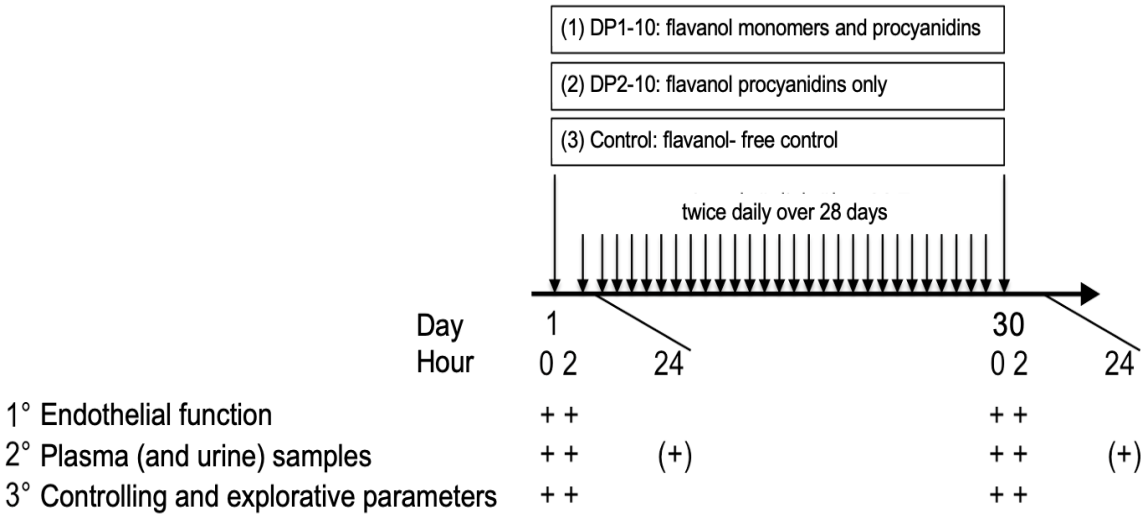

Figure 7 Flow chart of the study design, DP, degree of polymerisation;

2.5 Intervention Products

All the inspected test materials were provided by Mars Incorporated and are commercially available dietary supplements such as COCOAVIA™ based on CF to “support healthy blood flow” (Mars, Inc., 2021).

The test materials were delivered in capsules, which could not be differentiated from each other in size, colour, weight or haptic. The capsules were stored in identical, plain white bottles. The bottles were labelled with three alpha-numeric codes (1Q, 3T, 7J) and contained 70 capsules each. Every volunteer ingested two capsules per day with their breakfast. At the end of the intervention, the bottles were collected, and the remaining capsules were counted to evaluate each participant’s compliance.

Each capsule in the DP1-10 arm contained a cocoa extract with a total CF value of 345 mg, comprised of 65 mg (-)-epicatechin and 280 mg procyanidins (dimers-decamers). A total of 280 mg CF were included in every capsule handed out in the DP2-10 arm, of which just 10 mg were (-)-epicatechin. The main share was

the procyanidins (dimers-decamers) with a value of 270 mg. The placebo capsules of the control arm were comprised of identical amounts of theobromine (40 mg) and caffeine (10 mg) compared to capsules in the DP1-10 and DP2-10 arm while lacking any content of CF. (See **Table 3** for the total values of the daily intake).

Table 3 Daily intake of the test interventions (composition of 2 capsules) (Rodriguez-Mateos et al., 2018)

	DP1-10	DP2-10	Control
Total Flavanols (mg)	680	560	0
(-)-Epicatechin (mg)	130	20	0
Dimers-decamers (mg)	560	540	0
Theobromine (mg)	80	80	80
Caffeine (mg)	20	20	20

2.6 Primary Endpoint – FMD Assessment

To create comparable and reliable results of conducted FMD measurements, our workgroup created a standard operating procedure, which was followed by this study.

The assessments were scheduled around the same time of the day for each volunteer and in-between the acute and chronic time points to avoid circadian variances. This has been done precautionary though observations have shown no significant differences in FMD during the daytime in healthy volunteers (ter AVEST et al., 2005). Before any measurements, each participant rested for 15 minutes in a supine position in a temperature-controlled room at 21 °C. The same operator conducted each FMD. The designated measurement area was the right brachial artery (BA) within the Sulcus bicipitalis medialis. The vessel was displayed with the help of an ultrasound scanner (10-MHz transducer; Vivid I, GE)

connected to a linear probe designed for vascular imaging (12L-RS, GE). The artery was observed on the sagittal axis at the point of the maximum width. Only areas with a defined intima and media on the ultrasound image were used for measurement. The quality of the images had to be high enough to assess the intima-media-thickness. Furthermore, zones with specific landmarks were preferred to ensure the same region of interest for analysis in-between the different time points (**Figure 8**). Besides that, a 3-Channel-ECG was parallelly performed and recorded by the ultrasound machine to guarantee the analysis could be executed at the same time of the cardiac cycle for every measurement. The arm of the volunteer was comfortably rested with the cubital joint fully extended to avoid artefacts caused by movement. Distal to the triangle of the elbow (chelidon) of the participant, a manual inflatable blood pressure cuff (Boso clinicus, Jungingen, Germany) was placed to induce reactive hyperaemia.

FMD was obtained following this protocol:

- Baseline: At least three complete cardiac cycles. B-Mode.
- Inflation of the blood pressure cuff to 250 mmHg for 5 minutes
- Recording of BA-dilatation at 0s, 20s, 40s, 60s and 80s.

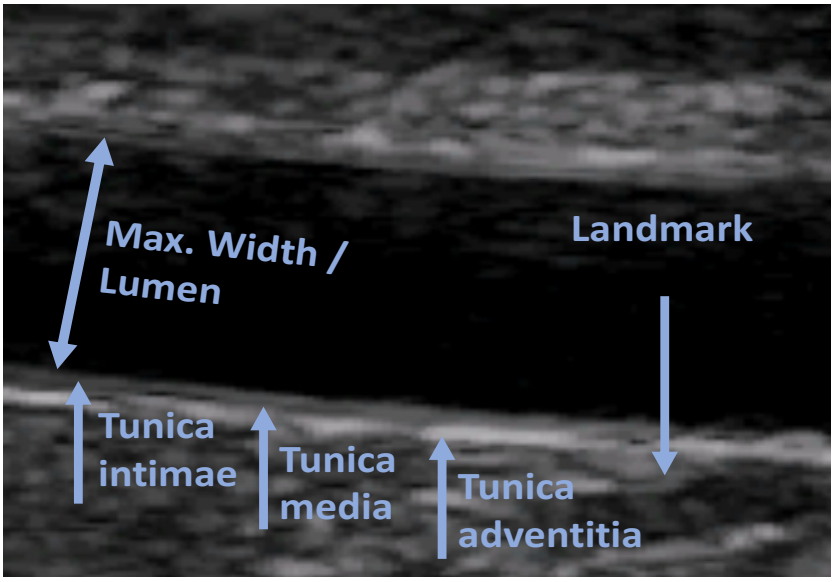

Figure 8 Obtained sagittal image of the brachial artery used for FMD

To analyse the obtained sonographic images, a semiautomated system was used (Brachial Analyzer, Medical Imaging Applications, USA). The software is FDA approved for the assessment of vascular function and has been designed to measure FMD. Due to complex algorithms, it ensures accuracy and consistency in detecting the width of a vessel with independently validated diameter errors of 0.034 ± 0.066 mm (Medical Imaging Applications, 2021). The validity of this edge detection software has been reported (Mancini et al., 2002). The relative percentage increase is expressed as FMD, according to the following formula [($max_{diameter}$ –$baseline_{diameter}$)/$baseline_{diameter}$ * 100)] (Heiss et al., 2010).

2.7 Secondary Endpoints - Biochemical Analysis

To assess the values of SREMs in the blood plasma of each volunteer, blood draws were performed before the ingestion of the test substances and 2h post-consumption. This occurred on the first visit and after 30 d of daily flavanol consumption. The blood draws were performed, primarily on the left arm, using a butterfly cannula (Vacutainer Safety-Lok, BD, USA). To obtain plasma, full blood collected in EDTA-coated tubes was centrifuged at 4°C for 15 min at 1800 x g. The plasma samples were collected in Eppendorf® tubes (1.2 ml), which were prepared with ascorbic acid resulting in a concentration of 1 mg/ml. The aliquots were stored at -80°C until analysis.

Urine samples were also collected for flavanol metabolite analysis. To do so, each volunteer was asked to collect urine for 24h after the consumption of the test substances on day 1 and day 30. The containers were specifically designed to contain body fluids, such as urine, and were stored in a cooling bag or a fridge. Following the collection, the total volumes were measured, and the urine was transferred to 15 ml tubes and acidified with 56 µl thymol and 0.56 ml sodium acetate. Until analysis, the prepared urine samples were preserved at -80°C. The quantification of γVL metabolites and SREMs was done via HPLC with fluorescence detection using a validated method. The full procedure is described in Ottaviani et. al. (Ottaviani et al., 2012a; Ottaviani et al., 2012b).

Further biochemical measurements such as evaluating fasting plasma glucose, total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides were performed at the Institute of Clinical Chemistry, University of Düsseldorf, using validated and standard procedures.

2.8 Tertiary Endpoints – PWV

To this date, PWV is the gold standard for assessing vascular stiffness. This study executed the measurements following the protocol laid out by an expert consensus document published by Van Bortel et al. in 2012. The document recommended a user protocol, which was adapted from various studies. Therefore, all measurements were conducted after the volunteer rested in a supine position for at least 10 min in a quiet and temperature-controlled room. The observations were scheduled around the same time of the day to diminish circadian effects. Since studies have shown the most accuracy using the carotid-femoral distance, the assessment was preferably done on the right carotid artery and ipsilateral femoral artery. The distance was measured in a straight line between the areas of interest and multiplied by 0.8 to receive a well-estimated vessel length. The calculation of the PVW is achieved by the following formula: PWV = d/t (expressed as m/s) (Van Bortel et al., 2012) (Heiss et al., 2015b). The pulse waves were recorded via applanation tonometry with the SphygmoCor® system (AtCor Medical Pty Ltd, Australia).

2.9 Tertiary Endpoints – BP

Coherent with the evaluation of other parameters of vascular function, each BP assessment was preceded by a resting time in a supine position for at least 15 min. It was taken precise care that the environmental conditions were stable and comparable throughout all time points and for each volunteer. BP was measured by a fully automated clinical digital sphygmomanometer (Boso medicus PC2, Jungingen, Germany) on the upper left arm, which was placed at the height of the heart. At each time point, 3 BP measurements were performed on every participant. The first result was not used for the analysis. The numerical mean was calculated from the data obtained by the second and third measurements.

2.10 Statistical Analysis

To evaluate the effects in the RCT, the primary test conducted was a univariate analysis of covariances (ANCOVA), which was succeeded by a post hoc pairwise comparison to compare the changes after the intake of DP1-10, DP2-10 and the control (fixed factors) after 1 month (dependent) of consumption to baseline values, which were designed as covariates to account for differences to the baseline results (Rodriguez-Mateos et al., 2018). The detected and calculated effects of the treatment were expressed in their respective parameters (e.g., FMD): the baseline values obtained on day one were subtracted from the results gathered after one month. In addition, we also completed a statistical analysis for changes found 2h after CF intake on day 1 and day 30 using repeated measurements ANCOVA with baseline values as covariates. SPSS 24 (IBM Corp.) was used to conduct the analyses.

The standard error of mean (SEM) is enclosed to the mean values of parameters (means ± SEMs). 95% confidence intervals (CI) adjusted to Bonferroni are attached to the calculated means of the relative changes. Means with standard derivations (SD) in parentheses are used to display the characteristics of the study population (See **Table 4**).

For the primary endpoint, a power analysis was conducted. After the intake of CF, we expected a change of 1.34% in FMD due to observations made in prior studies analysed by a metanalysis in 2012 (Hooper et al., 2012). In 2015, a study within our workgroup exposed inter- and intraindividual variabilities in FMD measurements by an SD of 0,9% within a group of 20 healthy individuals with repeating measurements (Heiss et al., 2015b). Another work conducted in our laboratory described similar variations in FMD of 1% SD within a group of 12 young and healthy subjects (Horn et al., 2014). Taking to account the excepted change in FMD (1,34%) and the observed SD of FMD-change in our laboratory (1%), at least 15 people had to be assigned to each arm to detect an absolute FMD-change of 1,2 % with sufficient power (power = 0.8, 2 sided α of 0.0167%) between the different arms using a Bonferroni correction in pairwise comparisons. These power calculations have been done with the help of the *PS*

power and sample size program, a freely available software developed by scientists at Vanderbilt University (Dupont and Plummer, 1990).

3 Results

3.1 The Study Population – Baseline Characteristics

A total of 45 volunteers formed the final study population after the exclusion (n=5) and the refusal to participate (n=10) of individuals, as in the CONSORT study flow chart (see ***Figure 6***). The baseline characteristics of the study population are described in ***Table 4***. No significant differences between the assigned groups were found. All obtained values were within the acceptable range for healthy males of young age. Coherently, an increase in risk for cardiovascular events in the future, which can be assessed with the Framingham risk score, was not detectable (Vandvik et al., 2012). The test substances were well tolerated, and no adverse effects were reported. All the enrolled participants completed the study (n=45). Given the quantity and quality of the collected data, it was possible to analyse the primary endpoint for each volunteer at every timepoint.

Table 4. Baseline characteristics of the study population.[1]

[1]BMI, body mass index; GPT, glutamyl pyruvate transferase; GOT, glutamate oxaloacetate transaminase; GGT, gamma-glutamyltransferase;
[2]values are mean and standard deviation in parentheses (Rodriguez-Mateos et al., 2018)

	DP1-10	**DP2-10**	**Control**
n	15	15	15
Age (years)	23 (2)[2]	25 (2)	23 (2)
Height (m)	1.81 (0.05)	1.81 (0.07)	1.85 (0.07)
Weight (kg)	78 (8)	79 (10)	79 (10)
BMI (kg/m^2)	23.6 (0.5)	24.1 (2.2)	23.1 (2.4)
Heart rate (bpm)	65 (6)	69 (8)	70 (6)
Exercise (h per wk)	3.2 (1.4)	2.1 (1.2)	2.7 (1.3)
Smoker (n)	0	3	5
Alcohol (g per wk)	89 (43)	88 (49)	97 (41)
Vegetarian (n)	0	0	1
Total cholesterol (mg/dL)	177 (12)	170 (7)	173 (7)
Creatinin (mg/dL)	0.9 (0.1)	0.9 (0.1)	0.9 (0.1)
Bilirubin (mg/dL)	0.9 (0.6)	0.7 (0.3)	1.1 (1)
Urate (mg/dL)	5.3 (1.2)	5.9 (1.0)	5.8 (0.9)
GGT (mg/dL)	30 (26)	28 (31)	33 (14)
GPT (mg/dL)	32 (34)	30 (23)	32 (15)
GOT (mg/dL)	28 (12)	37 (30)	45 (37)

3.2 Changes in Endothelial Function

The primary endpoint of this double-masked, parallel-group RCT was to detect significant changes in FMD after consuming CF and to investigate the relative contributions of (-)-epicatechin and procyanidins in improving endothelial function following acute, chronic and acute chronic intake. Participants consumed for 30 d either a regular composition of CF (DP 1-10), including flavanol monomers (≈ 19%) and oligomers (procyanidins) or a monomer-reduced CF-composition (DP 2-10), which had a significantly reduced share of (-)-epicatechin of approximately 3.6%. This trial was placebo-controlled by a third group consuming flavanol-free, micro- and macronutrient matched control capsules.

After chronic consumption during 1 month, an increase in FMD was detected within the group consuming DP1-10 capsules compared to DP2-10 and control – the baseline values of the FMD measurements were included as covariates. (***Table 5).*** The improvement in FMD following the intake of DP1-10 over the control at one month was significant – 1.4 % (95% CI: 0.7%, 2.2%). Enhancement in FMD after intake of DP1-10 over DP2-10 at 1 month was 1.7% (95% CI: 0.9%, 2.5%). As displayed in **Figure 9,** comparable changes were seen on day 1, 2h after the consumption of the first DP1-10 capsules. Further enhancements in FMD at 1 month and 2h (acute on chronic), following the intake of the last DP1-10 capsules, were not observable. The consumption of capsules belonging to DP2-10 or the control did not result in any significant changes in FMD at any time throughout the intervention, as seen in **Figure 9.** To determine the possible effects of DP1-10 between the time points, the time x intervention interaction was assessed, which showed no significant variances during the trial (P = 0.101) (Rodriguez-Mateos et al., 2018).

Table 5. Baseline characteristics of the study population

	DP1-10 (n=15)		DP2-10 (n=15)		Control (n=15)		Change (DP1-10 vs Control) at 1 month[4]	Change (DP2-10 vs Control) at 1 month[4]	Change (DP1-10 vs DP2-10) at 1 month[4]	*P*[2]
	Baseline[3]	1 month[3]	Baseline[3]	1 month[3]	Baseline[3]	1 month[3]				
Primary endpoint										
FMD (%)	6.4 (0.4)	8.2 (0.3)	7.3 (0.4)	7.2 (0.5)	6.5 (0.4)	6.9 (0.3)	**1.4 (0.7, 2.2)**	-0.2 (-1.0, 0.6)	**1.7 (0.9, 2.5)**	***<0.001***
Secondary endpoints										
Plasma γVL (nmol/L)	55 (14)	150 (34)	26 (5)	76 (15)	46 (10)	55 (8)	**94 (15, 174)**	22 (-58, 103)	72 (-10, 155)	***0.014***
Plasma SREM (nmol/L)	2 (2)	86 (52)	2 (2)	4 (3)	0 (0)	7 (7)	48 (-26, 123)	-26 (-101, 48)	**75 (1, 148)**	***0.048***
Tertiary endpoint										
PWV (m/s)	5.7 (0.2)	5.3 (0.2)	6.0 (0.2)	5.6 (0.2)	5.4 (0.1)	5.6 (0.3)	**-1.0 (-1.6, -0.4)**	-0.1 (-0.7, 0.5)	**-0.8 (-1.4, -0.2)**	***0.001***
Office SBP (mmHg)	134 (3)	125 (2)	126 (2)	126 (2)	126 (2)	126 (2)	**-6.7 (-12.6, -0.9)**	-0.3 (-5.8, 5.3)	**-6.5 (-12.4, -0.6)**	***0.010***
Office DBP (mmHg)	78 (2)	70 (2)	73 (2)	75 (2)	73 (2)	74 (2)	-5.5 (-11.6, 0.6)	3.2 (-4.8, 11.1)	**-6.9 (-13.0, -0.8)**	***0.020***
Glucose (mg/dL)	83 (2)	77 (2)	81 (1)	81 (1)	80 (3)	80 (2)	-1 (7, 4)	2 (-3, 7)	-3 (-9, 2)	*0.247*
Triglycerides (mg/dL)	121 (25)	108 (23)	71 (7)	82 (8)	109 (18)	113 (13)	-24 (-64, 16)	-11 (-50, 27)	-13 (-54, -29)	*0.330*
Total cholesterol (mg/dL)	177 (12)	163 (12)	170 (7)	160 (6)	173 (7)	182 (7)	**-22 (-40, -4)**	**-19 (-37, -1)**	-3 (-20, 15)	***0.007***
HDL cholesterol (mg/dL)	65 (4)	59 (4)	60 (2)	56 (2)	61 (2)	62 (3)	-6 (-13, 1)	-5 (-12, 2)	-1 (-9, 6)	*0.114*
LDL cholesterol (mg/dL)	100 (14)	100 (11)	107 (9)	100 (5)	113 (8)	109 (8)	-15 (-34, 4)	-15 (-35, 4)	0 (-17, 18)	*0.102*

[1] DP, degree of polymerisation; FMD, flow-mediated dilation; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PWV, pulse wave velocity; SBP, systolic blood pressure; DBP, diastolic blood pressure; SREM, structurally related epicatechin metabolites; γVL, valerolactone metabolites;

[2] one-factor ANCOVA with baseline value as a covariate; significant values are in bold (Rodriguez-Mateos et al., 2018)

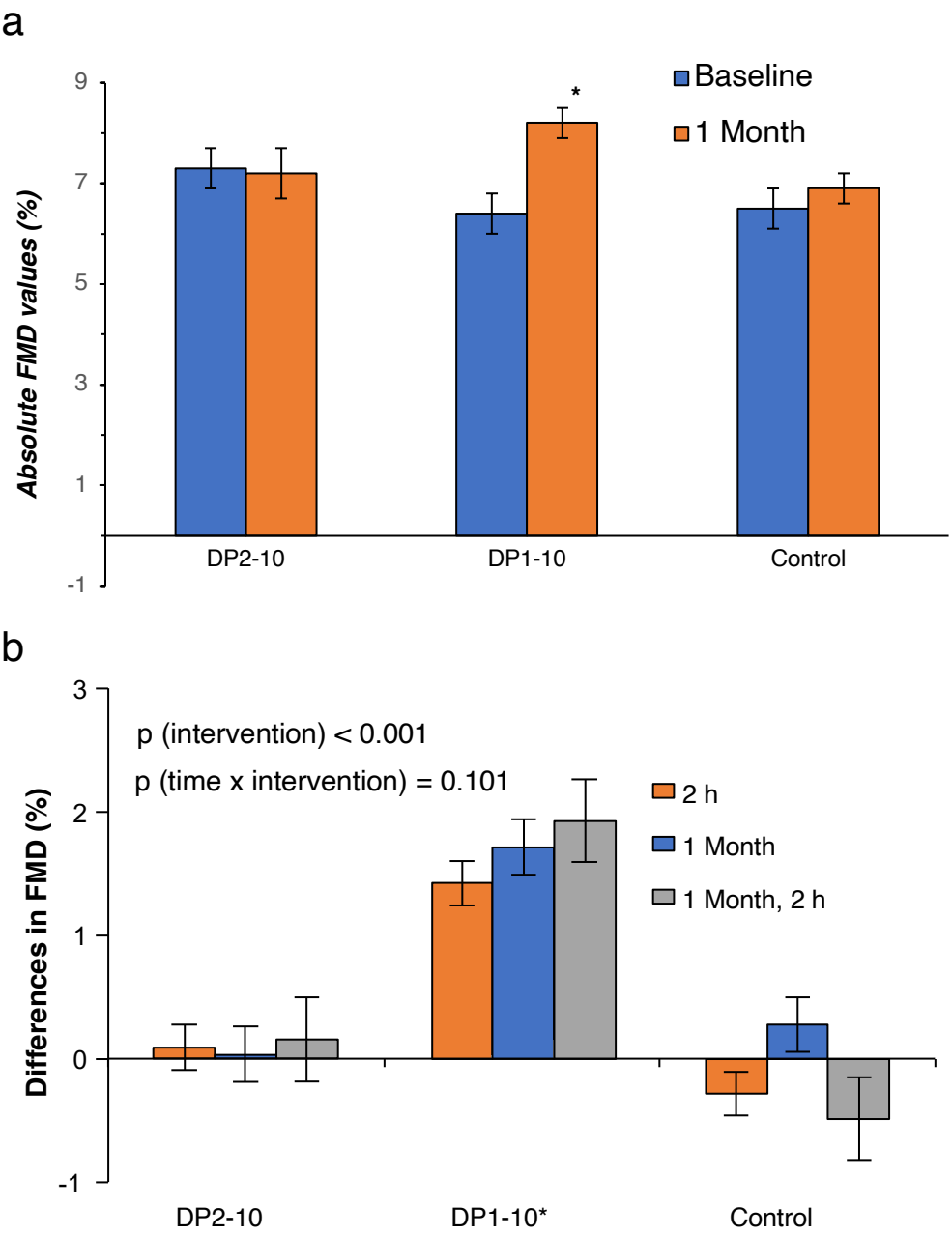

Figure 9 (a) Absolute FMD values at baseline and the chronic time point. (b) Relative differences of FMD at baseline compared to the acute (2h), chronic (1 mo) and acute on chronic (1 mo and 2h) timepoint. Collection of data following the consumption of: 1) DP2-10, specially designed extract of cocoa containing mainly procyanidins with a range of the DP between 2 and 10; 2) DP1-10 extract of cocoa with standardised shares of monomeric flavanols, predominantly (-)-epicatechin (DP1) and procyanidins; 3) Control containing no CF but with the identical amounts of micro- and macronutrients compared to DP1-10 and DP 2-10. Statistical analysis was performed by repeated measurements of ANCOVA using baseline values as covariates, followed by a Bonferroni post hoc test. Main effect of intervention, $P < 0.001$, time x intervention, $P = 0.101$. Values are presented as means with SEMs. *$P < 0.05$ compared to Control or DP2-10. FMD, flow-mediated vasodilation; DP, polymerisation; SEM, standard error of mean (Rodriguez-Mateos et al., 2018).

3.3 Changes in SREMs and γVL Metabolites

The secondary endpoint of this study was to detect changes in SREMs and γVL metabolites following the intake of the different test substances. Quantification was done via fluorescence detection by HPLC. Baseline values of SREMS were below the level of detection in all but two volunteers, as seen in Table 4. These findings are explained by the low-flavonoid diet, which was accomplished by the participants 24h before the first and second visits.

A significant increase of plasma SREMs by 611 nmol/L (95% CI: 483, 739 nmol/L) was identified following the intake of DP1-10, two h after consumption on the first visit. The ingestion of DP2-10 or the control did not result in significant changes in SREMs in blood plasma (**Figure 10a**). Comparing the three arms after one month of regular consumption of the test materials, no significant differences between them were detectable. These findings can be explained by the 24 h fasting period of the volunteers before the second visit combined with the short half-life of SREMs (Actis-Goretta et al., 2012). In addition, this displays the good compliance of the study subjects. The acute on chronic consumption was followed by a significant increase of plasma SREM to 847 nmol/L (95% CI: 679, 1016 nmol/L), compared to the effect on the first visit. It was impossible to identify any significant deviations.

Coherently, the acute on chronic intake of DP2-10 and the control was not followed by a change in concentration of SREMs. Supporting the obtained data from blood plasma, the urine analysis presented a significant increase in excretion of SREMs in 24-h urine after the ingestion of DP1-10 compared to the control. The intake of DP2-10 showed no significant difference from the placebo. The results after chronic consumption led to similar values and were not significantly different to the data obtained on day 1. (**Figure 12a**).

Plasma concentrations of γVL metabolites derived from gut microbiome-mediated catabolism (Ottaviani et al., 2018a) were significantly elevated by 94 nmol/L (95% CI: 15, 174 nmol/L) following the consumption of DP1-10 after one month when compared to the control. The detected increase in concentration

after the intake of DP2-10 was insignificant (**Figure 11**). Nevertheless, the renal elimination of γVL metabolites in urine was significantly increased in DP2-10 and DP1-10 after one month (**Figure 12b**). Comparing the effects of the different arms to each other, the consumption of DP1-10 leads to a significant larger excretion of γVL metabolites (Rodriguez-Mateos et al., 2018).

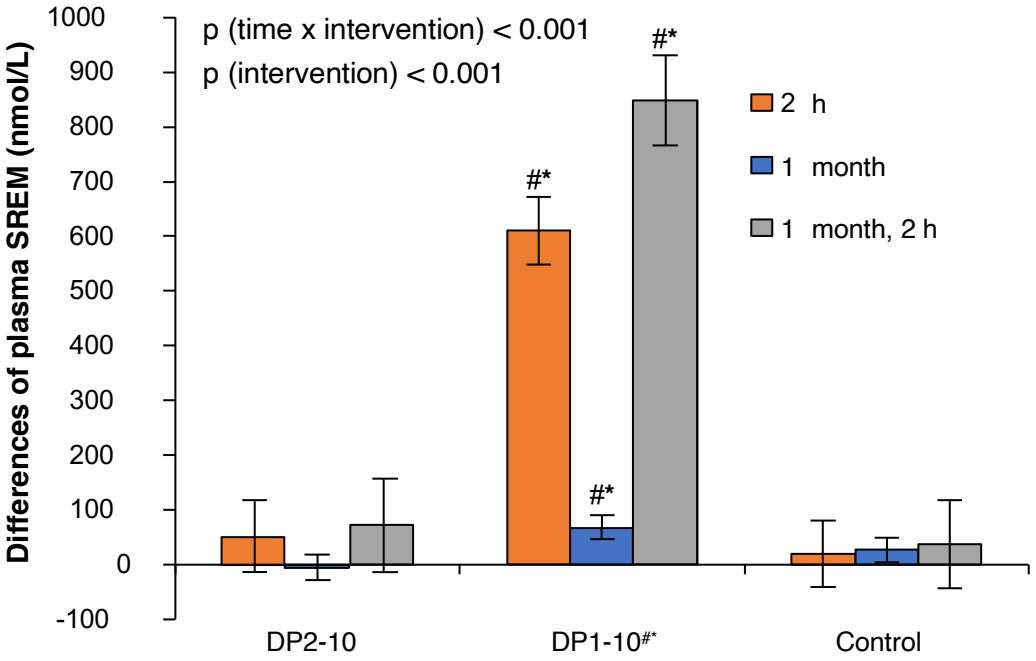

Figure 10 Differences in plasma SREMs concentration from baseline compared to the acute (2h), chronic (1 mo) and acute on chronic (1 mo and 2h) timepoint following the intake of 1) DP2-10, specially designed extract of cocoa containing mainly procyanidins with a range of the DP between 2 and 10; 2) DP1-10 extract of cocoa with standardised shares of monomeric flavanols, predominantly (-)-epicatechin (DP1) and procyanidins; 3) Control containing no CF but with the identical amounts of micro- and macronutrients compared to DP1-10 and DP 2-10. Statistical analysis was performed by repeated measurements ANCOVA using baseline values as covariates, followed by a Bonferroni post hoc test. Main effect of intervention, $P < 0.001$, time x intervention, $P < 0.001$. Values are presented as means with SEMs. *$P < 0.05$ compared to DP2-10. # $P < 0.05$ compared to Control. DP, degree of polymerisation, SEM, standard error of mean (Rodriguez-Mateos et al., 2018).

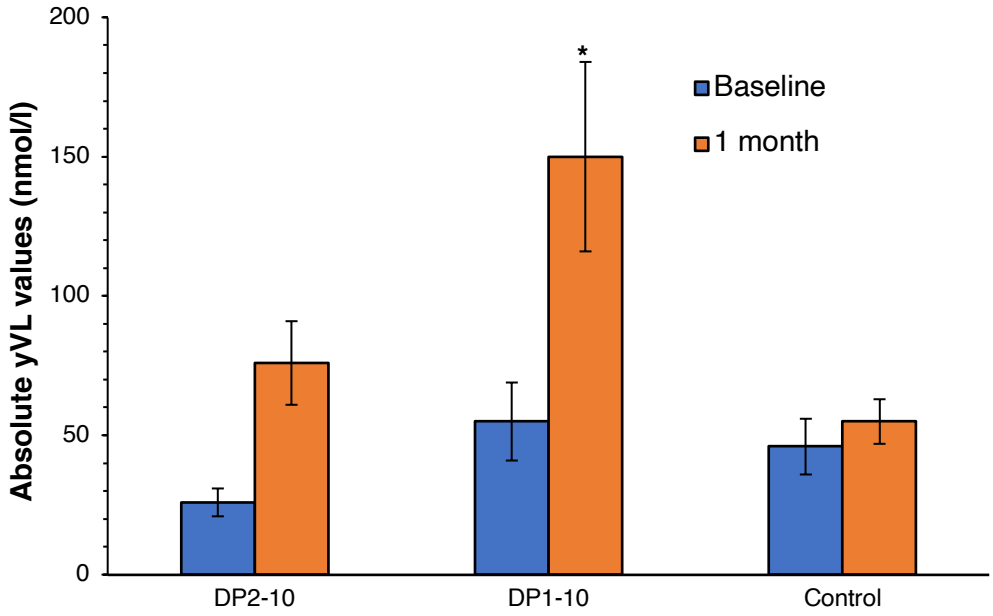

Figure 11 Absolute values of plasma γVL metabolites in blood plasma samples collected at baseline and the chronic time point (1mo) following the daily intake of 1) DP2-10, specially designed extract of cocoa containing mainly procyanidins with a range of the DP between 2 and 10; 2) DP1-10 extract of cocoa with standardised shares of monomeric flavanols, predominantly (-)-epicatechin (DP1) and procyanidins; 3) Control containing no CF but with the identical amounts of micro- and macronutrients compared to DP1-10 and DP 2-10. Statistical analysis was performed using a one-factor ANCOVA with baseline values as covariates. Values are presented as means with SEMs. *P= 0.014. DP, degree of polymerisation, SEM, standard error of mean (Rodriguez-Mateos et al., 2018).

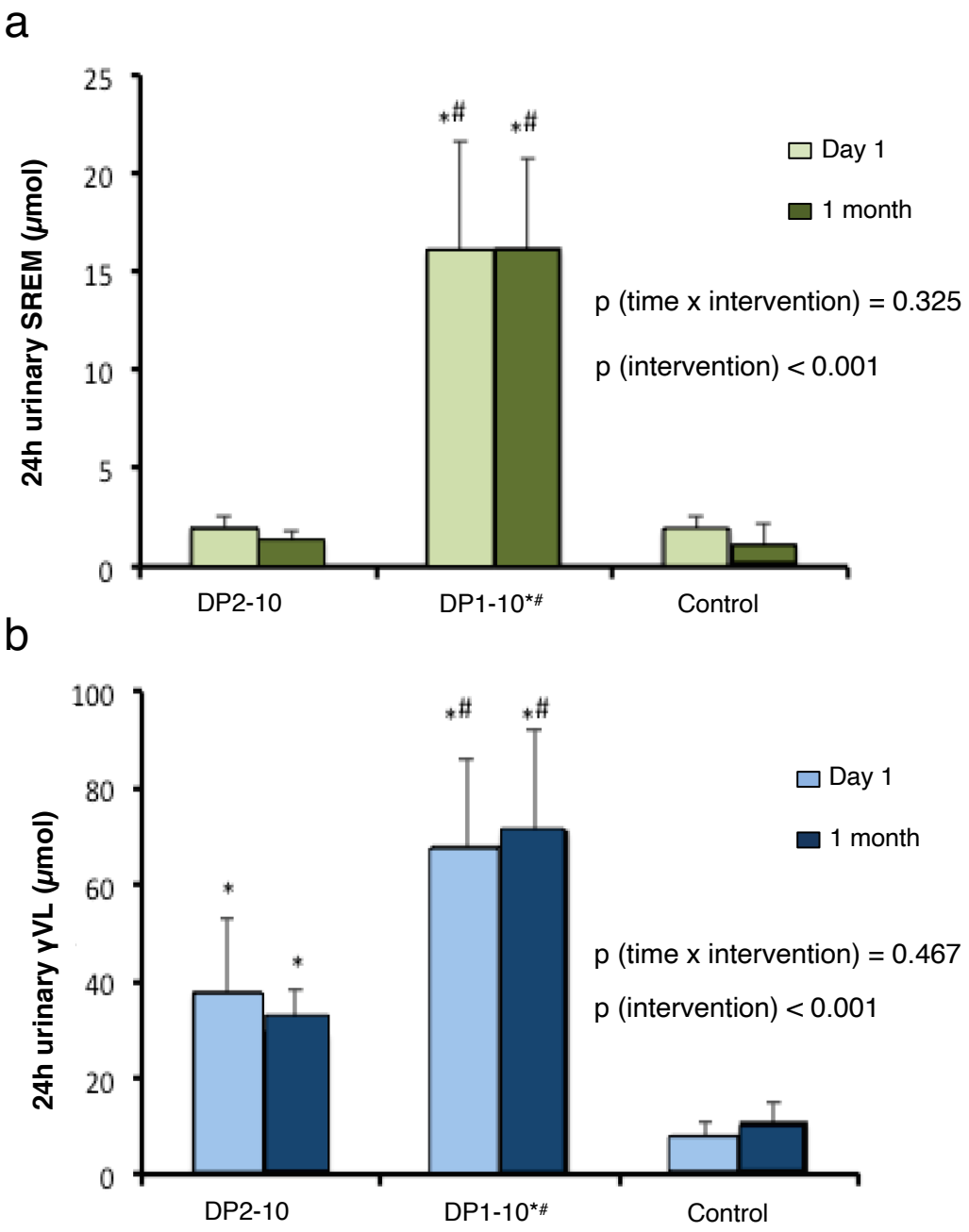

Figure 12 Absolute values of excreted of SREMs (a) and γVL (b) at baseline and after 1 mo 24 h after consumption of the intake of 1) DP2-10, specially designed extract of cocoa containing mainly procyanidins with a range of the DP between 2 and 10; 2) DP1-10 extract of cocoa with standardised shares of monomeric flavanols, predominantly (-)-epicatechin (DP1) and procyanidins; 3) Control containing no CF but with the identical amounts of micro- and macronutrients compared to DP1-10 and DP 2-10. Statistical analysis was performed by repeated measurements ANCOVA using baseline values as covariates, followed by a Bonferroni post hoc test. Main effect of intervention: (a) $P < 0.001$; (b) $P < 0.001$, time x intervention: (a) $P < 0.325$; (b) $P < 0.467$. Values are presented as means with SEMs. *$P < 0.05$ compared to control. $^{\#}$ $P < 0.05$ compared to DP2-10. DP, degree of polymerisation, SEM, standard error of mean (Rodriguez-Mateos et al., 2018).

3.4 Changes in BP and PWV

A decrease in SBP values was seen after 30 days of daily consumption of DP1-10. Significant changes were detected compared to the control by a reduction of 6.7 mm Hg (95% CI: -12.6, -0.9 mm Hg), and compared to the DP2-10 arm by a decrease of 6.5 mmHg (95% CI: -12.4, -0.6 mm Hg). Compared with the control, chronic consumption of DP2-10 had no significant effect on SBP (-0.3 mm Hg [95% CI: –5.8, 5.3 mm Hg]). It was impossible to detect any significant differences in the effect caused by DP1-10 at the different time points [p (time x intervention) = 0.291]) (**Figure 13a**).

DBP was significantly decreased by 6.9 mm Hg (95% CI -13.0, -0.8 mm Hg) following the chronic consumption of DP1-10 when compared with DP2-10. During the same period, a nonsignificant decrease of 5.5 mm Hg was observed compared with the control group (95% CI: -11.6, -0.6 mm Hg). The analysis of the PWV data revealed a significant reduction by 1.0 m/s (95% CI: -1.6, -0.4 m/s) following the chronic intake of DP1-10 compared with the control and by 0.8m/s in contrast to DP2-10 (95%CI: -1.4, -0.2 m/s). The analysis showed that the effect of DP1-10 did not change between the individual time points. No significant impact was seen in the time x intervention interaction (*P*= 0.176). The consumption of DP2-10 over 1 month was not followed by any significant changes when compared to the control (-0.1 m/s; 95% CI: -0.7, 0.5 m/s) (**Figure 13 b**) (**Table 4)** (Rodriguez-Mateos et al., 2018).

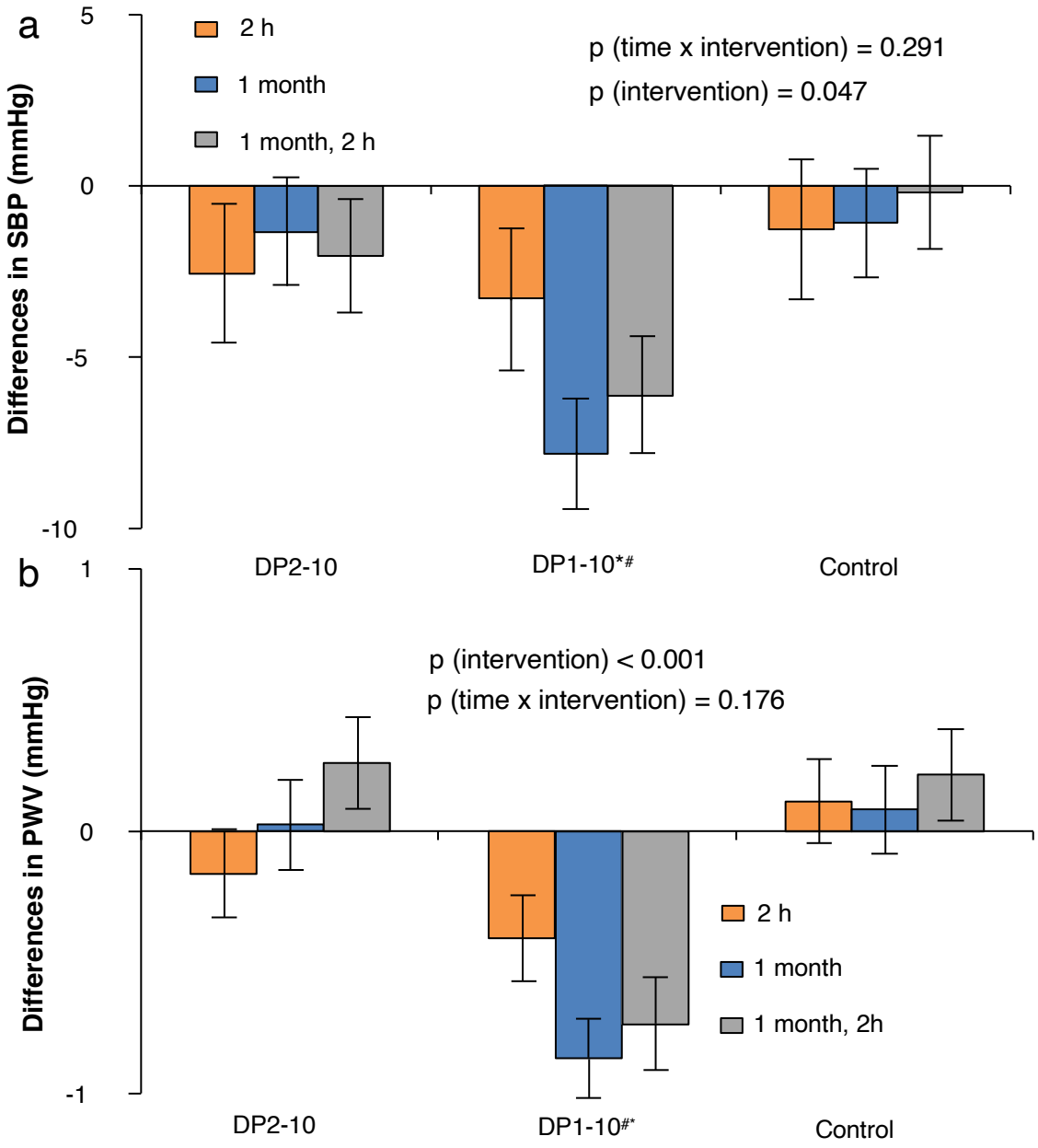

Figure 13 Differences in SBP (a) and PVW (b) from baseline compared to the acute (2h), chronic (1 mo) and acute on chronic (1 mo and 2h) timepoint following the intake of 1) DP2-10, specially designed extract of cocoa containing mainly procyanidins with a range of the DP between 2 and 10; 2) DP1-10 extract of cocoa with standardised shares of monomeric flavanols, predominantly (-)-epicatechin (DP1) and procyanidins; 3) Control containing no CF but with the identical amounts of micro- and macronutrients compared to DP1-10 and DP 2-10. Statistical analysis was performed by repeated measurements of ANCOVA using baseline values as covariates, followed by a Bonferroni post hoc test. (A) Main effect of intervention, $P = 0.047$, time x intervention, $P = 0.291$. (B) Main effect of intervention, $P < 0.001$, time x intervention, $P = 0.001$. Values are presented as means with SEMs. *$P < 0.05$ compared to DP2-10. $^{\#}$ $P < 0.05$ compared to Control. DP, degree of polymerisation, SEM, standard error of mean (Rodriguez-Mateos et al., 2018).

3.5 Changes in Glucose and Blood Lipids

Significant changes in total cholesterol were seen following the chronic consumption of DP2-10 and DP1-10. The intake of DP1-10 for 30 d helped to significantly reduce 22 mg/dl (95% CI: -40, -4 mg/dl) in total cholesterol. The intake of DP2-10 over one month resulted in a decrease of 19 mg/dl (95% CI -37, -1 mg/dl) in total cholesterol (**Figure 14**). A significant difference in the effects caused by the D2-10 and DP1-10 was not seen. No significant impact on plasma glucose, triglycerides, LDL, or HDL cholesterol was found after consuming any test material (Rodriguez-Mateos et al., 2018).

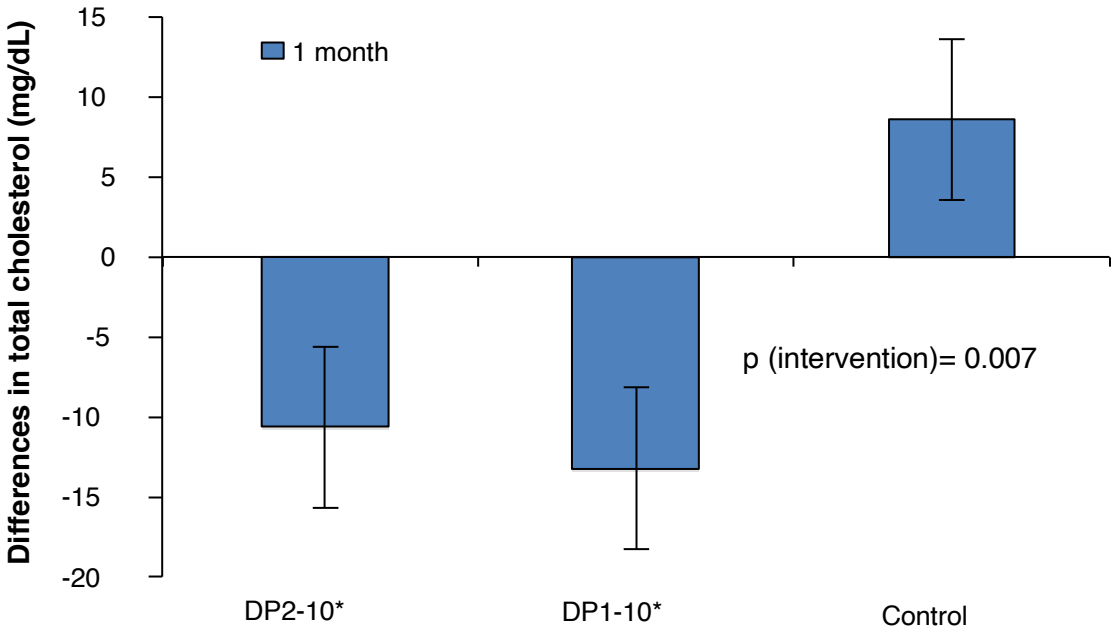

Figure 14 Differences in total cholesterol after the daily consumption of the following consumption of 1) DP2-10, specially designed extract of cocoa containing mainly procyanidins with a range of the DP between 2 and 10; 2) DP1-10 extract of cocoa with standardised shares of monomeric flavanols, predominantly (-)-epicatechin (DP1) and procyanidins; 3) Control containing no CF but with the identical amounts of micro- and macronutrients compared to DP1-10 and DP 2-10. Statistical analysis was performed using a one-factor ANCOVA with baseline values as covariates. Values are presented as means with SEMs. *P= 0.007. DP, degree of polymerisation, SEM, standard error of mean (Rodriguez-Mateos et al., 2018).

3.6 Changes in Framingham Risk Scores

To display the findings of this trial and the resulting impact on the risk for the occurrence of future CVDs, calculations evaluating the 10-year risk via the Framingham risk score were performed. As described in the characteristics of the study population, at baseline, there was no elevated risk in the study population for CVD (overall average 0.25±0.34%) and CHD (0.28±0.39%). Coherently, the incidence of encountering a stroke (0.08±0.04%), myocardial infarction (0.05±0.14%) or death from CVD (0.19±0.23%) or CHD (<0.01±<0.01%) was very low among all arms of this study. The healthy and young study population can explain this. Comparing the groups revealed no significant differences regarding the risk assessment. Following the chronic consumption of DP1-10, we detected significant decreases in the 10-year risk for CHD (relative change -48%), stroke (-22%), CVD (-46%), and CVD death (-45%), which are graphically displayed in **Figure 15**. These changes can be evaluated regarding the control group and the DP2-10 arm. The risk reduction is due to the observed changes in SBP and total cholesterol (as described in 3.4 and 3.5). Notwithstanding the detected significant decrease in total cholesterol after the chronic intake of DP2-10, the consumption of this test substance was not followed by any significant changes in the Framingham risk scores.

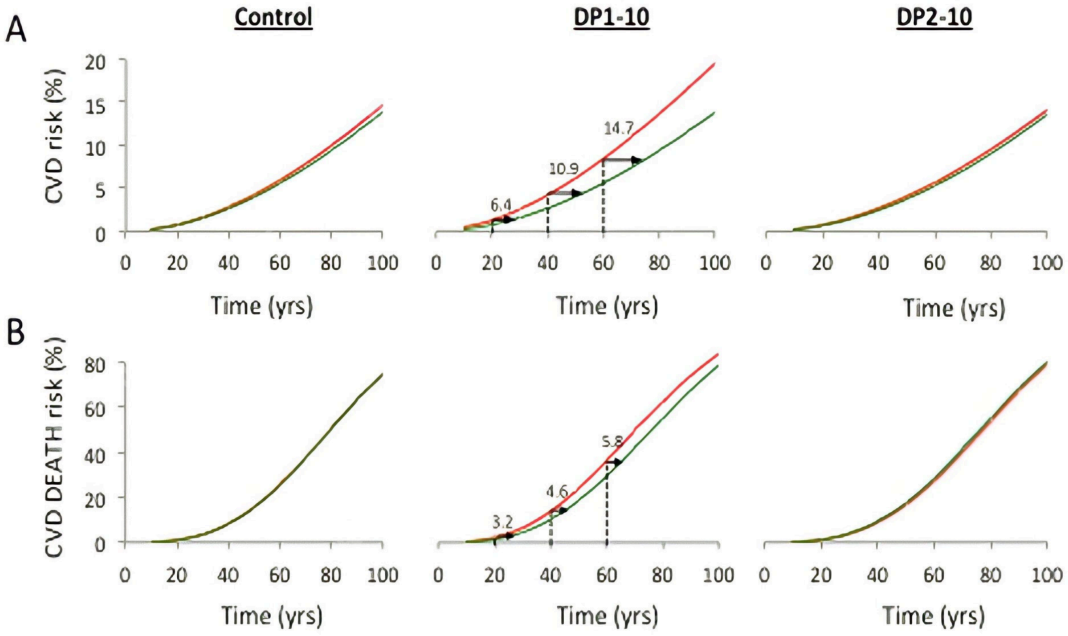

Figure 15 Framingham risk scores calculated to demonstrate the risk of suffering from cardiovascular disease (CVD) (A) or death from CVD (B). Changes outlined by displaying values before (red line) and after (green lines) the chronic intake of 1) DP2-10, specially designed extract of cocoa containing mainly procyanidins with a range of the DP between 2 and 10; 2) DP1-10 extract of cocoa with standardised shares of monomeric flavanols, predominantly (-)-epicatechin (DP1) and procyanidins; 3) Control containing no CF but with the identical amounts of micro- and macronutrients compared to DP1-10 and DP 2-10. The projected potential and leverage effect of DP1-10 to extend CVD-free life (A) and prolong survival (B) are illustrated by black arrows.

4 Discussion

This work aimed to investigate the relative contribution of flavanol monomers and oligomeric procyanidins on the observed overall effects of CF consumption on vascular function.

With the collected and presented data, the main discoveries of this trial are:

1. The acute and chronic intake of CF with a daily dose consisting of 130 mg (-)-epicatechin and 560 mg procyanidins (DP1-10) improves endothelial function ($p < 0.001$) with an increase in FMD after 2h by 1.4% (95% CI: 0.7%, 2.2%) and after 1 month by 1.7% (95% CI: 0.9%, 2.5%).
2. The acute and chronic intake of CF with a daily dose consisting of a reduced share of (-)-epicatechin (20 mg) and 540 mg procyanidins (DP2-10) had no significant on endothelial function evaluated via FMD.
3. Positive effects on systolic BP ($p = 0.047$) and PWV ($p < 0.001$) were only observable following the intake of DP1-10, but not DP2-10.
4. Consumption of DP1-10, but not DP 2-10, is followed by a significant elevation of SREM concentrations in blood and urine ($p < 0.001$). γVL concentrations were increased after intake of DP1-10 and DP2-10 but significantly greater due to the consumption of DP1-10.
5. Total cholesterol was reduced following the intake of DP1-10 and DP2-10 ($p=0.007$).

4.1 Contributions of Procyanidins and Epicatechin

One of our hypotheses was that an intake of procyanidins might have beneficial effects on vascular function. This assumption was based on findings of a resulting increase in γVL concentration, a circulating metabolite formed and absorbed in the large intestine after microbiome-derived catabolism post-consumption of procyanidins (Ottaviani et al., 2018a). Procyanidins represent a large share of CFs, which have shown positive health effects in several studies (see **Table 2**), and therefore, these metabolites were studied to evaluate possible beneficial health effects.

Besides that, earlier *in vitro* experiments provided data suggesting that consumption of procyanidins would contribute to the pool of SREMs via breakdown in the small and large intestines to the monomer (-)-epicatechin (Richelle et al., 1999). This theory was not confirmed by more recent *in vivo* experiments (Javier I Ottaviani et al., 2012). A trial observing the uptake of procyanidins in rats could not detect derivates by CF-monomers. The concentration of auxiliary microbial metabolites was decreased by 21 times when comparing the intake of procyanidins to monomeric CFs (Gonthier et al., 2003).
Studies observing the bioavailability of procyanidins in humans stated that oligomeric CF do not yield to the pool of circulation SREMs in blood plasma (Ottaviani et al., 2012). After a stable gastric transit, procyanidins form various metabolites following the fermentation by gut microbiota in the large intestines. (Rios et al., 2002; Tao et al., 2019). A relevant share of these formed and absorbed represent the γVL metabolites. A significant increase in SREMs was not detectable (Ottaviani et al., 2012).
The conducted trial confirmed these findings, and a relevant increase in SREMs was only detectable due to the consumption of the test material containing a relevant proportion of (-)-epicatechin (DP1-10). γVL metabolites were present in urine and blood plasma after consuming substances containing (-)-epicatechin and procyanidins (DP1-10). The intake of CFs containing mainly procyanidins (DP 2-10) only resulted in elevated γVL metabolites in urine.

In combination with the observed beneficial effects following the intake of DP1-10, this leads to the conclusion that the beneficial influence on vascular function due to CFs is linked to the ingestion of (-)-epicatechin but not to the intake of procyanidins. Coherently, an increase in FMD and a decrease in BP and PWV seem influenced by SREMs but not γVL – the metabolites of procyanidins. Since the study spanned for 30 d and in conjunction with the wide variety of dependent variables regarding vascular function, it can be confidently assumed that responses to an increase in γVL post-consumption of procyanidins would have been measurable if any bioactivity in context to alteration of vascular function originated from procyanidins and respectively γVLs.

Despite seemingly inheriting no effects on vascular function, the intake of procyanidins containing CF led to significantly decreased total cholesterol levels, even when containing a significantly reduced proportion of (-)-epicatechin. These findings have been described by prior studies evaluating the effects of plasma cholesterol due to the consumption of cacao procyanidins in rats (Yasuda et al., 2008). This indicates that a long-term intake of procyanidins may have positive effects because of a decrease in blood lipids. These effects may be due to inhibiting properties of CFs on cholesterol biosynthesis (e.g., reduction of HMG-CoA synthase) or a higher expression of cholesterol receptors in the liver. A different theory assumes that CFs inhibit cholesterol uptake in the digestive tract on behalf of a decreased micellar cholesterol solubility (Yasuda et al., 2008). Consecutively, it needs to be highlighted that this trial did not assess possible health benefits caused by procyanidins and γVL beyond potential improvements in vascular function. Furthermore, recent investigations concluded that γVL-metabolites could be used as biomarkers to measure and evaluate the consumption of CF – monomers and oligomers (Ottaviani et al., 2018a). Combined with the growing interest in the observed positive health effects, this will be of great interest for further studies.

Even though, in this trial, it was impossible to identify positive changes in endothelial function due to the intake of CF mainly consisting of procyanidins, it cannot be concluded that these procyanidins have no positive effect on vascular function. The positive effects seen in CFs containing (-)-epicatechin and procyanidins may depend on the presence of both subgroups. For example, procyanidins may indirectly affect the bioavailability of (-)-epicatechin. This may be because procyanidins inhibit the degradation of CFs during storage, food processing or at the time of digestion. For Instance, this could be accomplished by acting as an antioxidant, as a strong antioxidant effect has been attributed to the procyanidins originating from *Litchi Chinesis (Sui et al., 2021).* Therefore, it is impossible to unmistakably determine the potential effect strength of procyanidins since they may indirectly affect the bioavailability of (-)-epicatechin.

Recent observations support this conclusive assumption. In 2018, a trial observed the possible effects of pure epicatechin in a group of 48 overweight to

obese non-smoking individuals. After a daily supplementation of 25 mg of pure epicatechin, over 14 d, no changes were found in BP or blood lipids (Kirch et al., 2018). Similar results were obtained by a study evaluating the effects of 100 mg pure epicatechin per day for 4 weeks in 37 middle-aged to elderly people. The chronic intake of the test material was not followed by significant changes in FMD, BP or insulin resistance (IR) (Dower et al., 2015).

At the same time, it must be highlighted that the results obtained by Dower regarding FMD were close to significant (an increase of 1.1 %; $p = 0.07$), and the study population had a high heterogeneity, with more than one-fifth of the population being hypertensive, which may have confounded the results. Also, the assessment of FMD in this trial was not ideal since it was obtained using a probe with a lower frequency (7.5 MHz) than recommended (10-14 MHz) (Harris et al., 2010) (Schroeter et al., 2015). In addition, a recent observation displayed significant changes in vascular function evaluated regarding FMD post-consumption of pure epicatechin. This study supplied a population of healthy young to middle-aged men with pure epicatechin in different doses depending on their body weight. This trial demonstrated that the acute intake of 0.5 and 1 mg/kg of pure epicatechin is followed by a significant increase in FMD (< 1.2 %; $p <$ 0.01-0.001). Therefore, it was concluded that pure epicatechin inherits capabilities of improving vascular function on its own by emitting significant alterations in acute FMD (Castle, 2017). An earlier trial supports the results obtained by Castle (Schroeter et al., 2006).

A comprehensive valuation of this heterogeneity in recent literature points out that various aspects should be considered when attempting to create a deeper understanding of CFs, especially in terms of the relative contributions of the individual components and their circulating metabolites. First, it has been described that observable beneficial changes post-consumption of CFs are dose-dependent (Heiss et al., 2007) (Sun et al., 2019). Additionally, novel advances link more dependable factors than the dose of CFs to the observable positive health effects. These factors should be considered when designing studies which try to assess the effects of CFs respectively, their monomeric and oligomeric components. According to a recent study, one factor seems to be the way of

administration regarding the used food matrix (Scholz and Williamson, 2007). The composition of the CFs appears to significantly impact their abilities to enhance vascular function. It is worth mentioning in this context are the methylxanthines, in the case of cocoa, the naturally most abundant compounds are caffeine and theobromine (Franco et al., 2013). Sansone et al. have shown these methylxanthines inherit capabilities to mediate the effect size of the described beneficial changes of vascular function caused by the intake of CFs, probably on the level of absorption due to increased uptake of (-)-epicatechin and consecutively higher concentrations of SREMs in blood plasma (Sansone et al., 2017).

Therefore, this study took precise care in the design and composition of the administered test materials. The test substances contained identical amounts of micro- and macronutrients, especially in terms of methylxanthines, such as theobromine and caffeine (see Table 3), to diminish the potential effects caused by these purine-based derivates of xanthine. The importance of this task is highlighted by studies, which suggest that methylxanthines inherit effects on vascular function on their own (Karatzis et al., 2005) (Papaioannou et al., 2005), which may interact with the impact caused by CFs. The outlined observations emphasise the complexity in this field and underline the difficulties in assessing the relative contributions of single compounds in CF regarding the beneficial health effects. This may explain the heterogeneity in this field to a certain degree. Hence administering pure compounds (e.g., epicatechin) may have a more negligible effect absent mediating factors such as methylxanthines or procyanidins.

4.2 Interaction of the Gut Microbiome and CFs

To date, the research about the impact of the gut microbiome on our health is limited, particularly regarding nutrition-based benefits and consecutively the prevention of diseases because of these advantages. In context to polyphenols, it has been observed that flavanols obtain the ability to inflect the function and architecture of the gut microbiome while acting as a prebiotic (Martín and Ramos,

2021). Consecutively, it has been demonstrated that CFs inhibit the growth of pathological gut bacteria, like *Clostridium perfringens*, whereas upregulating the presence of advantageous bacteria, like *Bifidobacterium* and *Lactobacillus*. This is of high interest since these beneficial bacterial groups are associated with the ability to prevent the growth of pathogens and stimulate the production of advantageous organic acids such as acetate and lactate. Especially an accelerated growth of bifidobacteria has been linked to positive changes due to the upregulation of the synthesis of various vitamins and the reduction of plasma cholesterol concentrations (LeBlanc et al., 2013; Bordoni et al., 2013).

On top of that, it is proposed that the flavanol-induced change of the microbiome may be partly responsible for reductions of CRP concentrations in plasma (Tzounis et al., 2011; Sorrenti et al., 2020*)*. Also, the impact of flavanols on the gut microbiome seems to have a low-grade anti-inflammatory effect on chronic inflammation in the course of upregulation of immune responses and metabolic pathways (Martín and Ramos, 2021). This modulation may decrease the risk for cardiovascular events due to the reduction of plasma cholesterol and plasma CRP. These findings may explain to a certain degree the positive cardiovascular effects observable after consuming CFs.

Furthermore, the change in microbiome architecture may lead to increased uptake of flavanol-derived metabolites accompanying possible effects on the human metabolism. Flavanols show a dual relationship with the human microbiome by inflecting with its architecture and function on the one hand. On the other hand, a relevant share of the flavanol metabolites in the circulation are derived from the microbiome (De Bruyne et al., 2019)

The impact of the gut microbiome on cardiovascular health has been brought to attention through different studies. The microbiome may influence the endothelial function due to effects on vascular homeostasis in key areas like NO production, thrombosis and inflammation (Leslie and Annex, 2018). Furthermore, gut microbiome-derived metabolites, such as Trimethylamine N-oxide, seem to increase SBP and induce aortic stiffening, which is highly associated with developing an CVD (Brunt et al., 2021). In 2016 a group of researchers

discovered that an important precursing condition of CVDs and type 2 diabetes is largely impacted by the gut microbiome - IR. The trial concluded that the gut microbiome might have the ability to reduce IR and therefore help to avoid CVDs and type 2 diabetes (Pedersen et al., 2016). Recent studies underline the impact of the gut microbiome on vascular health by influencing risk factors for the complex pathological called metabolic syndrome, a known forerunner of CVDs, certain types of cancer, chronic kidney disease and type 2 diabetes (Croci et al., 2021; Carrizales-Sánchez et al., 2021).

It must be stated that the profound examination of the gut microbiome and an in-depth evaluation of the composition of its different components was not part of this work. But the performed analysis of the microbiome-derived metabolite γVL was able to detect the metabolites in the volunteers. Even in the population consuming the placebo containing no flavanols, it was possible to identify γVL-metabolites. These findings suggest that the occurrence of γVLs is a reaction mediated by the gut microbiome within a wide range of the human population and across various dietary patterns and global lifestyles. This assumption is backed by an earlier investigation, which traced the metabolome of flavanols in humans and discovered the possibility of accurately evaluating the bioavailability of flavanols helped by circulating γVL-metabolites (Ottaviani et al., 2016). Confirming the data in this work, a recent study presented that procyanidins are less efficiently metabolised to γVL by the gut microbiome compared to (-)-epicatechin (Hollands et al., 2020).

The fact that valecterones are metabolised by all volunteers must be highlighted since there is a wide variety in the human microbiome, which has recently been getting more attention from various research groups. In fact, it is proposed that the gut microbiome may play a key role in preventing CVDs due to nutritional strategies. Since the gut microbiome significantly differs between individuals and therefore microbiome-derived metabolites are not consistent for everyone, a strategy called *metabotyping* has been introduced. Recognising the possible influence of the gut microbiome, the goal of this approach is to personalise the nutrition to improve the beneficial effects of various nutritional interventions. It has

been hypothesised that people at risk for CVDs inherit a certain *metabotype* and therefore respond differently to dietary interventions (Riedl et al., 2017; Palmnäs et al., 2020). On behalf of that, it is noteworthy that the metabolisation of γVL is happening across many metabotype and consecutively may inherit positive effects on a broad range of individuals.

Besides the possible usefulness as a flavanol biomarker, the direct biological impact of γVL metabolites is still under debate and to be further evaluated. Executed experiments in vitro have presented promising results. A recent trial demonstrated that γVL-metabolites protect brown adipocytes from oxidative stress (Mele et al., 2017). Further in vitro studies concluded that γVL-metabolites inherit more than half of the antioxidant activity in the flavanol monomer (Sánchez-Patán et al., 2011). Besides that, γVL-metabolites have also been linked to the beneficial effects of green and black tea consumption, which strongly correlates with an increase in urine and blood plasma concentrations of γVL-metabolites (Henning et al., 2013). A recent investigation detected a protective influence of γVL-metabolites on neurodegenerative diseases by affecting intracellular proteolysis in neuronal cells (Cecarini et al., 2021). In the future, these promising results must be validated by in vivo experiments to evaluate if, or to what degree, these bioactivities can be translated into interactions on human health.

4.4 Cardiovascular Risk Assessment

We applied the Framingham framework of cardiovascular risk assessment to the collected data to generate a more visual display and estimate the impact of the detected changes of vascular biomarkers, which originated from CF, on cardiovascular health. Prior investigations have made similar approaches to outline the relevance of the observed changes (Sansone et al., 2015). It must be mentioned that certain limitations apply to these calculations. Primarily this assessment assumes that the effect-strength of CF is stable over a chronic intake, which is impossible to prove with this 30-day trial. Additionally, the focus would rely solely on cardiovascular outcomes. Also, the Framingham risk score is not validated for a population under the age of 30. Still, it could be expected

that changes measurable in a group of young and healthy individuals may have a leverage effect over a greater timespan and when extrapolated to a large population.

With the obtained data, we portrayed that due to the consumption of CF, but not procyanidins, it may be possible to reduce the 10-year-risk of cardiovascular mortality and morbidity by 45-46% and 22-48%. Worthy of mention in this context is these changes were observable in this young and healthy study population, which may be an indicator of the strength of the detected effects. The risk assessment according to the Framingham framework displays that even small changes in BP and cholesterol, which were observable after the intake of CF, may have the power to significantly extend the timespan without the encounter of an CVD ('healthspan') and consecutively extend survival to a relevant degree. Numerical, due to the observed changes, it is proposed that diagnosis of CVD will be delayed by 6.4 years at 20 years, by 10.9 years at 40 years and by 14.7 years at 60 years.

Despite the limitations outlined above to implementing the Framingham risk score concerning our study population, the outcomes of this calculations provide a helpful foundation for the potential effects of CF on the maintenance of health. In addition to that, these results can be used as food for thought for discussion and serve as a foundation for potential future studies. Furthermore, these findings set a base for speculations about the potential impact when extrapolated to a population level.

4.5 Strengths and Limitations

With the implementation of an RCT, the major limitations of scientific studies were bypassed. The randomisation was done by a standardised computer program (GraphPad Software, Inc.). For this trial, an appropriate control determined the effects caused by CFs adequately. The placebo was matched to be composed of the same micro- and macronutrients while containing no CFs. Every person involved in the conduction of this study, as well as the volunteers, was blinded regarding the identity of the test material, resulting in a double-blinded situation.

The materials were encapsulated in identical-looking capsules. Compliance was tested by collecting the reaming test substances at the end of the intervention, which exposed overall good compliance.

Although this study was conducted with adequate scientific means, some limitations must be considered. A significant limitation is the interindividual variability regarding the bioavailability of polyphenols. As outlined beforehand, this has to be highlighted in terms of microbiome-derived metabolites since studies have shown that individuals respond differently to the same dietary input and, therefore, may benefit from diets tailored to their metabolic phenotype (Palmnäs et al., 2020). Consequently, the *metabotype* present in each volunteer may mediate the effects seen in this study. The narrowly defined study population, consisting of young, healthy men, may intensify this limitation. In general, the bioavailability of polyphenols depends on several variables, as described by earlier investigations, like sex, age, weight, diet, genotype and enzymatic patterns (Di Lorenzo et al., 2021; Manach et al., 2017; Rodriguez-Mateos et al., 2015). Therefore, the data in this study must be interpreted cautiously when extrapolated to a larger, more diverse population.

These findings lead to another minor limitation. Since the bioavailability of CFs may differ in the study population, the time point of blood collection post-consumption of the test materials may not fit each volunteer to the same degree. Resulting in earlier or later and possibly missed peaks of CF-metabolites in blood plasma. The time point 2 h after the intake of the test substances was chosen due to the data obtained by prior investigations (Rodriguez-Mateos et al., 2012). In an ideal environment, multiple blood draws, each followed by quantification of metabolites via HPLC with fluorescence detection, could be performed for more accurate analyses.

A limitation of this trial regards the diet of the participants. We asked the volunteers to follow a low-flavanol diet 24 h before and after the day of the intervention. Each volunteer was asked to complete a detailed dietary questionnaire, and additional random telephone calls were performed over one month to assess the study population's diet. It must be mentioned that there are

still various inaccuracies when determining the polyphenol intake helped by questionnaires, for example, due to inconsistencies in food tables and due to regional differences. This has to be emphasised since an in-depth survey was designed in England at the University of Reading.

Nonetheless, the participants were not asked to change their diet or follow a low-flavanol diet between the acute and chronic periods. This strategy was chosen to evaluate the effects in a realistic environment and obtain representative data. The selected approach helps to significantly limit this study. It may be possible that the persisting chronic polyphenol intake may mediate the effects on vascular function seen in this intervention. This impact has been mentioned by an earlier investigation, which hypothesised that the regular consumption of different natural antioxidants supplied by vegetables and fruits, such as apples, oranges and onions, may affect the changes seen post-consumption of CFs (Kusano Bucalen Ferrari et al., 2015). In the case of this study, these effects may be accelerated due to the recruitment of healthy volunteers interested in participating in dietary interventions. Therefore, it may be assumed that these individuals follow a healthy diet with various polyphenol sources. This assumption is supported by the relatively high baseline values of γVL (see **Table 4**), which have been validated to be a biomarker of flavanol intake (Ottaviani et al., 2018a).

The relatively small number of participants per arm must be considered when interpreting the data collected in this trial. The primary endpoint was defined to be FMD. According to earlier investigations, a change of 1.34 % in FMD was anticipated (Hooper et al., 2012). Combined with the inter- and intraindividual variabilities observed by FMD measurements in our workgroup, we calculated power, which concluded that at least 15 participants had to be assigned to each arm to detect the changes in FMD with sufficient power (Horn et al., 2014) (Heiss et al., 2015). Due to the wide variety of dependable variables in this trial, including endothelial function, BP, PWV, blood lipids and metabolites of CFs, we concentrated on a relatively small number of volunteers. Naturally, the obtained data must be taken cautiously when extrapolated to a greater population, although we matched the self-imposed requirements.

Beforehand, it has been taken special care in the design of the test materials. But it must be outlined that the DP2-10 arm contained fewer flavanols overall when compared to the DP1-10 arm (560 mg vs 690 mg). On top of that, the number of procyanidins was slightly different when comparing the arms to each other (540 mg vs 560 mg). It cannot be precluded that these differences may cause a variance in effect strength since earlier studies have shown a dose-dependency regarding the intake of CFs and improvement in FMD (Heiss et al., 2007). According to a recent metanalysis, these slight discrepancies in doses cannot explain the result. Hence the applied doses of both flavanol-containing test substances should result in measurable effects on FMD if inheriting any bioactive compounds (Sun et al., 2019).

As a methodological limitation, assessing the endothelial function via FMD must be considered. Although FMD is the most commonly evaluated vascular function, and it has been proven to be a valid assessment when compared to invasive methods, it remains an indirect substitute measurement for predicting the NO availability *in vivo* (Takase et al., 1998; Anderson et al., 1995; Kasprzak et al., 2006). Although the FMD measurements were conducted in 2013, the followed SOP in our laboratory was mostly coherent with recent guidelines regarding preparation and execution (Thijssen et al., 2019). Consequently, the recommendations regarding the study preparation were obeyed and only fastened participants, at the same time of the day, in a climate-controlled room and after an adequate time of rest were assessed for FMD. All measurements were executed by the same operator with sufficient experience in this method. The implementation of the FMD measurement was standardised. The manual inflatable blood pressure cuff was placed distal to the imaged vessel because this has been the most accurate regarding NO-bioavailability (Doshi et al., 2001). It was taken precise care to the localisation and size of the area of ischemia because studies have shown a direct impact regarding this, on the extent and duration of vascular dilatation (Naylor et al., 2005; Doshi et al., 2001). In addition, the time of occlusion of 5 minutes in the protocol used is still recommended by today's guidelines. Since the occlusion time can affect the strength of dilatation, standardisation is vitally important (Leeson et al., 1997). The consensus to work

with a time of ischemia of 5 min remains a compromise between maximum dilatation and pain tolerance of the participant.

Besides the fact that many requirements of recent guidelines regarding the conduction of FMD were accomplished, it must be mentioned that some recommendations were not met, and the assessment of vascular function could be improved. First, a mounted holding device for the ultrasound probe could be implemented into our SOP for FMD. According to a metanalysis, which assessed the variability of this measurement, reproducibility is significantly improved when performed with a stereostatic holding device compared to the handheld method (Greyling et al., 2016). But evidence states that there is no significant difference when comparing both techniques regarding reproducibility, as long as the measurement is conducted in a professional environment with strict guidelines (van Mil et al., 2016).

Second, the capture of the peak dilatation post occlusion was not ideal in the study we conducted. We saved images at multiple timepoints (0s, 20s, 40s, 60s, 80s post occlusion) to reduce the possibility of missed points of maximum vessel diameter. This is an extensive increase compared to earlier measurements, which only focused on a solitary timepoint between 45 and 60s (Celermajer et al., 1992). More recent advances suggest obtaining continuous data post occlusion to confidently identify peak dilatation, which will be missed by 42% if only using a single time point and therefore underestimates FMD values (Black et al., 2008).

Third, we used a semi-automated edge-detection software to analyse the obtained images (Brachial Analyzer, Medical Imaging Applications, USA). Compared to the manual technique, this approach significantly reduces errors related to the operator and the intra-observer variety (Woodman et al., 2001). Additionally, this procedure is far more time-efficient (Preik et al., 2003). Going a step further, the recently published expert consensus recommends using an automatic analysis during the ultrasound scan in real-time. This concept will allow identification and adjustment to low-quality images concerning evaluation, which may lead to more accurate and reproducible results (Thijssen et al., 2019).

5 Conclusion

To gain a greater understanding of the mechanism of action of CFs, the individual contribution to each of their main components, (-)-epicatechin and the procyanidins, were investigated. Based on the data obtained in this work, we conclude that the beneficial effects of CF consumption on vascular function can primarily be attributed to the bioactivity of (-)-epicatechin, the flavanol monomer. Thus, our data demonstrate that procyanidins are not, or at least not directly, responsible for the positive changes in endothelial function post-consumption of CFs, as observed by us and many studies conducted priorly. This discovery is important regarding nutritional recommendations for using flavanols to maintain health and prevent CVDs. According to our results, the potential to extend a healthy lifespan can be attributed to the flavanol monomer.

Future investigations on larger scales have to confirm these findings, which will eliminate a large part of the relevant limitations described. Recently published studies like the COSMOS trial support the effect of CF on vascular health but still fail to assess the specific components' bioactivity.

Additionally, further research is needed regarding identifying bioactive food compounds in our diet. Also, the content of these components in various foods must be evaluated to generate validated nutritional recommendations regarding intake values and the consumption of certain foods. The importance of this task reflects the effort of different institutions. To be highlighted in this context would be the eBASIS (Bioactive Substances in Food Information System), founded by the European Food Information Resource (EuroFIR), a collection of data regarding food composition and the content of phytochemicals derived by plants. The data is mainly extracted from human intervention studies, evaluated by experts, and then compiled. The data collection aims to verify whether the claimed health benefits of bioactive substances are scientifically substantiated (on behalf of the EuroFIR Consortium et al., 2010). Another database worth mentioning is the Phenol Explorer, which is freely available and constantly updated. Next to the compilation of nutritional polyphenol content, it holds

information about the metabolites and the pharmacokinetics (Durazzo et al., 2019). Despite these databases' constant expansion and updates, our understanding of polyphenol intake is still highly restricted. It must be emphasised that using such data collections after the ingestion of various substances leads to estimated values and thus to significant limitations. Helped by validated biomarkers, these limitations could be reduced and therefore contribute to better comparability and reproducibility of nutritional studies (Oteiza et al., 2021; Kuhnle, 2012).

Even though the study provided us with a deeper insight into the mechanisms of action of flavanols, this experiment also underlines a tremendous scientific need for quantitatively determining flavanols to predict the possible beneficial health effects better and to implement dietary advice more validly.

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