Aus der Klinik für Kardiologie, Pneumologie und Angiologie der
Heinrich - Heine - Universität Düsseldorf
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Effects of cranberry (poly)phenols on vascular function

Dissertation

zur Erlangung des Grades eines Doktors der Medizin der
Medizinischen Fakultät der
Heinrich – Heine – Universität Düsseldorf

vorgelegt von
Albert Maximilian Böres
2023

Als Inauguraldissertation gedruckt mit Genehmigung der Medizinischen Fakult t der Heinrich-Heine-Universit t D sseldorf

gez.:
Dekan: Prof. Dr. med. Nikolaj Klöcker

Erstgutachter: Prof. Dr. med. Christian Heiß

Zweitgutacherin: Prof. Dr. rer. nat. Judith (Jojo) Haendeler

Meiner Familie und deren Geduld gewidmet

Parts of this work have been published:

„Cranberry (poly)phenol metabolites correlate with improvements in vascular function: A double-blind, randomized, controlled, dose-response, crossover study"

Ana Rodriguez-Mateos, Rodrigo P. Feliciano, Albert Boeres, Timon Weber, Claudia Nunes dos Santos, M. Rita Ventura and Christian Heiss

Published 16.05.2016

„Identification and quantification of novel cranberry-derived plasma and urinary (poly)phenols"

Rodrigo P. Feliciano, Albert Boeres, Luca Massacessi, Geoffrey Istas, M. Rita Ventura, Claudia Nunes dos Santos, Christian Heiss and Ana Rodriguez-Mateos

Published 04.02.2016

Effects of cranberry (poly)phenols on vascular function – summary

Background: (Poly)phenols are natural compounds present in various fruits and vegetables such as grapes and berries. According to epidemiological research, a higher intake of (poly)phenols is associated with a lower risk of developing cardiovascular diseases (CVD). This work hypothesizes that increased consumption of cranberry (poly)phenols (CP) improves vascular function and thus reduces cardiovascular risk.

Methods: A randomized, placebo-controlled, double-blind, six-arm cross-over, time and dose-response study was conducted in 10 healthy males. The studies primary endpoint was to evaluate vascular function using flow-mediated dilation (FMD). Secondary endpoints included measuring central and peripheral blood pressure (BP), pulse wave velocity (PWV), and aortic augmentation index (AIX). Tertiary endpoints included analyzing CP metabolites in urine and plasma. The measurements were taken at baseline before and at 1, 2-, 4-, 6-, and 8-hours post-consumption of a cranberry drink containing 409, 787, 1,238, 1,534, or 1,910 mg of total CP or a (poly)phenol-free control drink. Plasma and urine metabolites were analyzed by ultra-performance liquid chromatography quadrupole time of flight mass spectrometry using authentic standards.

Results: Consumption of the CP containing drinks, but not the control drink, led to an acute, dose-dependent increase of the FMD. Maximum effects were observed for juice containing 1,238 mg CP at 1, 2, 4, 6, and 8 h post-consumption. No significant results were observed for the secondary endpoints. Sixty (poly)phenol metabolites were identified in plasma. Correlation analysis showed significant correlations between 12 phenolic metabolites and changes in FMD.

Conclusion: CP increase vascular function acutely and dose-dependently in healthy young males and therefore reduce cardiovascular risk. However, further studies are needed to investigate whether this effect persists with chronic consumption and if this translates into a reduction of reduce cardiovascular risk. Furthermore, the influence of factors such as sex and age, as well as the basic (poly)phenol intake in the usual diet, must be noted and could be subject of further investigations

Auswirkungen von Kranbeer(poly)phenolen auf die Gefäßfunktion - Zusammenfassung

(Poly)phenole sind Nahrungsmittelbestandteile, die vor allem in Obst und Gemüse, wie zum Beispiel in Trauben und Beeren vorkommen. Epidemiologische Studien deuten darauf hin, dass ein erhöhter Konsum von (Poly)phenolen mit einem reduzierten Risiko für Herz-Kreislauf-Erkrankungen verbunden ist. Die Hypothese dieser Arbeit ist, dass der erhöhte Konsum von Kranbeer(poly)phenolen die Gefäßfunktion verbessert und damit das kardiovaskuläre Risiko reduziert.

Es wurde eine randomisierte, placebokontrollierte, doppelblinde, sechsarmige Crossover-Studie mit zehn gesunden Männern durchgeführt. Die Gefäßfunktion wurde durch flow-mediated dilation (FMD) ermittelt. Blutdruck und Pulswellengeschwindigkeit wurden gemessen, sowie Blut- und Urinproben zu Studienbeginn entnommen. Die Messungen wurden 1, 2, 4, 6 und 8 Stunden nach dem Verzehr eines Cranberry-Getränks mit 409, 787, 1238, 1534 oder 1910 mg Gesamt-CP oder eines (poly)phenolfreien Kontrollgetränks wiederholt. Plasma- und Urinmetabolite wurden mittels Ultra-performance liquid chromatography quadrupole time of flight mass spectrometry unter Verwendung authentischer Standards analysiert. Der primäre Endpunkt war die FMD. Die sekundären Endpunkte waren Blutdruck (peripher und zentral), Pulswellengeschwindigkeit sowie Augmentationsindex. Tertiäre Endpunkte umfassten die Quantifizierung von Kranbeeren-Metaboliten in Korrelation mit der flussvermittelten Dilatation.

Die Einnahme eines (poly)phenolhaltigen Saftes, nicht aber des Kontrollsaftes führte zu einem dosisabhängigen Anstieg der FMD. Maximale Effekte konnten für den Saft beobachtet werden der 1238 mg Kranbeeren(poly)phenole enthielt. Diese Effekte wurden jeweils 1, 2, 4, 6 und 8h nach dem Konsum beobachtet. Signifikante Effekte auf die sekundären Endpunkte konnten nicht erhoben werden. Insgesamt sechzig (Poly)phenolmetabolite konnten im Plasma nachgewiesen werden. Es zeigten sich signifikante Korrelationen zwischen 12 phenolischen Metaboliten und Veränderungen der flussvermittelten Dilatation.

Kranbeeren(poly)phenole können bei gesunden jungen Männern kurzfristig sowie dosisabhängig die vaskuläre Funktion verbessern und damit das kardiovaskuläre Risiko reduzieren. Weitere Studien sind erforderlich, um zu untersuchen, ob der Effekt bei regelmäßigem Konsum anhält, welche Dosis erforderlich ist, um das kardiovaskuläre Risiko am besten zu senken und inwieweit Geschlecht und Alter diesen Effekt beeinflussen.

List of abbreviations

γ - GT .....gamma - glutamyltransferase
μL..... microliter
°C.....degree celcius
ACN .....anthocyanidins
ADME .....
absorption, distribution, metabolism, and excretion
AHA .....American Heart Association
AIX..... augmentation index
ANOVA.....analysis of variance
BA.....brachial artery
BP.....blood pressure
CA.....carotid artery
CBG..... cytosolic β-glucosidase
CHD.....coronary heart disease
C_{max} ..... Maximal concentration
COMT ....catechol-O-methyltransferase
CP.....cranberry (poly)phenols
CRP ..... c-reactive protein
CVD .....cardiovascular diseases
CVRF.....cardiovascular risk factors
DP.....degree of polymerization
ECG.....electrocardiography
EDTA.....Ethylendiamintetraacetate
eNOS. endothelial nitric oxide synthase
EVOO .....extra virgin olive oil
ExPEC.....
extraintestinal pathogenic Escherichia coli
FA .....femoral artery
FMD.....flow-mediated dilation
g .....gram
GOT.....glutamic-oxaloacetic transaminase
GPT ... glutamic-pyruvate transaminase
h .....hour
HDL ..... high-density lipoprotein
HR .....heart rate
L .....liter
LDL..... low-density lipoprotein
LPH .....lactase-phlorizin hydrolase
MD.....Mediterranean diet
min.....minute
mm .....millimeter
mmHg.....millimeters of mercury
MPM..... microbial phenolic metabolite
NO .....nitric oxide
Nrf2 ..... NF-E2–related factor 2
NZBC .....
.....New Zealand blackcurrant extract
PACs .....proanthocyanidins
PAT .....peripheral arterial tonometry
PREDIMED .....
PREvención con DIetaMEDiterránea
psig.....pound-force per square inch
PWA .....pulse wave analysis
PWV .....pulse wave velocity
RA ..... radial artery
RCT..... randomized controlled trial
RHI .....reactive hyperemia index
s .....seconds
SLGT 1.....
sodium-dependent glucose transporter 1
SULT ..... sulfotransferase
TG .....triglyceride
UDPuridine 5'-diphospho-glucuronosyltransferase
UGT.....uridine-5´-diphosphate glucuronosyltransferase
UPLC-Q-TOF MS. ...Ultra-performance liquid chromatography quadrupole time of flight mass spectrometry
UTI..... urinary tract infection

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1 Introduction

1.1 Diet and cardiovascular disease

In 2017, worldwide 17.8 million people died of cardiovascular diseases (CVD), 82.9% of which were due to myocardial infarction and stroke[1]. CVDs are thus responsible for almost half of all deaths of noncommunicable diseases[2]. Along with smoking and lack of physical activity, an unhealthy diet is one of the biggest modifiable risk factors for CVDs[3,4]. Evidence to date suggests that CVDs are highly preventable by addressing known risk factors such as high blood pressure, diabetes mellitus, high cholesterol, adiposity, smoking, and lack of physical activity[5]. Dietary strategies, as part of a lifestyle change, therefore, are a promising strategy for the prevention of CVDs, which, however, needs to be further explored by randomized controlled trials (RCT) and prospective observational studies.

In a systematic review and meta-analysis of observational studies by Aune et al. in 2017, consumption of 200g/day fruits and vegetables was associated with reduced risk of cardiovascular disease, cancer, and all-cause mortality[6]. A reduction could be observed up to an amount of 800g/day, for all outcomes, except for cancer (600g/day). It was also calculated that 5.6 and 7.8 million premature deaths worldwide in 2013 are caused by reduced consumption of fruits and vegetables[6]. A diet that includes more whole grains, legumes, fish, poultry, vegetables, and fruits is associated with 28% lower cardiovascular mortality (95% CI; 0,60–0,87) and 17% lower all-cause mortality (95% CI; 0,76-0,90)[7]. Whereas in the same study, a Western diet based on increased consumption of red meat, processed meat, refined grains, french fries, and sweets/desserts was associated with a 22% increase in cardiovascular mortality (95% CI, 1.01-1.48)[7].

The Mediterranean diet (MD) is one of the dietary patterns that has increasingly been the subject of research in recent years due to its broad health-promoting effects[8]. The MD consists mainly of vegetables, fruits, nuts, legumes, whole grains, monounsaturated fats from olive oil, and the moderate consumption of fish and wine[9,10]. Although epidemiological data indicates that the increased consumption of these foods leads to a reduction in the risk of CVD, it is not yet clear exactly which food components are responsible for this positive effect[8]. The positive effect of the Mediterranean diet on the prevention of cardiovascular diseases was first demonstrated in a large scale randomized controlled trial: the PREvención con DIetaMEDiterránea (Prevention with Mediterranean diet; PREDIMED) study. In a multicenter trial in Spain, 7477 participants at high cardiovascular risk, but without CVD, were assigned to either a MD

supplemented with extra virgin olive oil (EVOO), a MD supplemented with mixed nuts, or a control diet consisting of reduced-fat content[8]. Primary endpoint was a major cardiovascular event (myocardial infarction, stroke, or death from cardiovascular causes). The incidence of the primary endpoint was 30 % lower among those assigned to a Mediterranean diet supplemented with EVOO or nuts than among those assigned to a reduced-fat diet[8].

The results are in line with a meta-analysis of observational and RCTs conducted by Grosso et al. in 2017. Twenty prospective studies and 4 RCTs were examined for an association between MD adherence and the incidence and mortality of CVD. In the group with the highest adherence to MD, a lower incidence (RR: 0.76, 95% CI: 0.68-0.83) and mortality (RR: 0.76, 95% CI: 0.68-0.83) from CVD was detected, compared to the group with the lowest adherence[11]. In addition, significant reductions of risk were also observed for coronary heart disease, myocardial infarction, and stroke (RR: 0.72, 95% CI: 0.60-0.86; RR: 0.67, 95% CI: 0.54-0.83; RR: 0.76, 95% CI: 0.60-0.96; respectively). Furthermore, the individual components of the MD were examined in a pooled analysis. It was found that the protective effects of the MD are mainly attributable to olive oil, fruits, vegetables, and legumes[11]. This knowledge is also reflected in various guidelines. For example, the American Heart Association (AHA) recommends a diet high in fruits, vegetables, and whole grains and highlights that most cardiovascular events are avoidable by primordial prevention[12]

In summary, the increased consumption of fruits and vegetables has a positive effect on the prevention of cardiovascular diseases. But which components of these foods are responsible for this effect? Fruits and vegetables are rich in vitamins, fibers, and minerals, but also phytochemicals such as (poly)phenols, especially flavonoids. The latter have increasingly become the focus of research in recent years, as they are thought to have positive health effects[13]. It should be noted that different fruits, vegetables, and nuts also have a different (poly)phenol composition. Therefore, it is necessary to investigate the individual foods for their (poly)phenol composition and their effects on human health. If specific health-promoting effects can be attributed to individual (poly)phenols, this would have important consequences for dietary recommendations and could thus be incorporated into primary prevention strategies.

1.2 Endothelial dysfunction

Endothelial dysfunction is one of the earliest detectable changes leading to atherosclerosis[14,15]. As a consequence of these changes, complex

pathophysiological processes are initiated, which ultimately lead to increased cardiovascular risk[16]. In 1976, Russell Ross formulated his *"Response to injury"* hypothesis, which states that the destruction of the barrier function of the intima marks the beginning of atherosclerosis[17]. Pathophysiological processes that promote the development of endothelial dysfunction have been the subject of intensive research. These include diabetes mellitus (via glycolyzed end products, oxygen radicals), metabolic syndrome, hypercholesterolemia (via oxidatively modified lipoproteins), imbalance of sexual hormones (via estrogen deficiency and menopause), age, oxidative stress, proinflammatory cytokines (interleukin-1, tumor necrosis factor), environmental factors (e.g., tobacco smoke, air pollution), and also hemodynamic changes (e.g. changes in blood flow caused by hypertension)[17–20]. All these pathophysiological processes ultimately lead to reduced availability or activity of endothelium-derived nitric oxide (NO). A basal level production of NO by endothelial cells contributes to regulating vasomotor tone. Endothelial dysfunction is promoted by a reduced availability of NO, which is either a result of reduced NO production or increased degradation by oxidative stress[21]. Endothelial dysfunction can be detected prior to the development of structural atherosclerotic changes and thus offers a possibility to intervene in the early phase of atherosclerosis[22,23]. The following section illustrates some of the methods used to determine endothelial dysfunction.

Flow-mediated dilation (FMD) is the most used method, considered the gold standard for non-invasive methods. It based on high resolution ultrasound of the brachial artery (BA). Celermejer et al. demonstrated first in 1992 that asymptomatic children, and young adults who had risk factors for atherosclerosis presented altered FMD values before anatomical or clinical evidence of atherosclerosis was detected[24]. The method is easily available and has been shown to be NO-dependent, making it suitable for exploring the L-arginine/NO pathway in clinical studies[25]. The FMD values correlate well with the endothelial function tests in cardiac catheterization, which require the intraarterial injection of vasoactive substances and are therefore significantly more invasive[22,26]. FMD is suitable for the evaluation of endothelial function and thus for cardiovascular risk prediction as well as for the prediction of cardiovascular events[27]. A meta-analysis published in 2013 showed that an increase in FMD of 1% leads to a reduction in cardiovascular risk of 10%[28]. Despite existing guidelines for the implementation of the FMD, the same protocol is not always used[29]. Different protocols can influence FMD[30]. Reference values have been established for Japanese subjects, analyzing the data of more than 6000 study subjects who underwent FMD measurements following the same protocol[31]. Furthermore, there are no reference values for FMD in Western countries[32]. However, based

on the analysis of 44 studies conducted in Western countries that assessed FMD values, Heiss et al. now propose standard values for Western countries. As in Japan, the measurements were all carried out according to the same protocol and yielded similar results[33].

As previously mentioned, endothelial function can also be measured directly on the coronary arteries by cardiac catheterization. NO release facilitating substances are injected intraarterially and allow the measurement of vasodilatation. The greatest disadvantage of this method is that it is highly invasive and therefore not suitable for the evaluation of endothelial function in asymptomatic volunteers[34].

Another method to evaluate endothelial function is peripheral arterial tonometry (PAT) and it has been shown that this method can identify patients with coronary endothelial dysfunction[35]. Furthermore, PAT has been shown to be an independent predictor of cardiovascular events and all-cause mortality[36]. However, further investigations are necessary to evaluate their prognostic utility in comparison to FMD. At this stage, however, it should be noted that FMD is currently the best non-invasive method and therefore the gold standard for measuring endothelial function[29,37].

In addition to vasoactive drugs and environmental toxins such as nicotine, food components also influence endothelial function and, thus, FMD values. Among these food components, (poly)phenols play a significant role[38]. In recent years, studies showed that the intake of (poly)phenol-rich foods leads to an increase in FMD and, thus, to a reduction in cardiovascular risk[28,38,39]. Cigarette smoking, unhealthy diet, and obesity lead to a decrease in FMD values and, conversely, to an increase in cardiovascular risk[24,40,41].

1.3 Dietary (poly)phenols

The consumption of fruits and vegetables as part of a healthy diet is a broad consensus [42,43]. (Poly)phenols are a type of bioactive substances mainly found in fruits and vegetables[44]. Among the foods with the highest concentration of (poly)phenols are green and black tea, red wine, coffee, cocoa or chocolate, and many fruits and vegetables such as berries[45].

1.3.1 Classification, sources, and structure

(Poly)phenols are aromatic compounds consisting of a phenyl group to which one or more hydroxyl group is attached. They can be classified into two groups based on their structure and complexity (*i.e.*, the number of phenolic rings and substituting groups), the flavonoids and non-flavonoids[44,46].

Flavonoids consist of two aromatic rings (A- and B-ring) connected by a tetrahydropyran ring. Depending on the arrangement of the hydroxyl, methoxy, and glycosidic side groups and the conjugation between the A and B rings, different subclasses are distinguished[47]. The main subclasses are flavonols, flavones, isoflavones, flavanones, anthocyanidins (ACN), and flavanols[48–50]. Other, less common and abundant subclasses are the chalcones, dihydrochalcones, dihydroflavanols, flavan-3,4-diols, coumarins, and aurones, but little is known regarding their bioactive and relevance for human health[49].

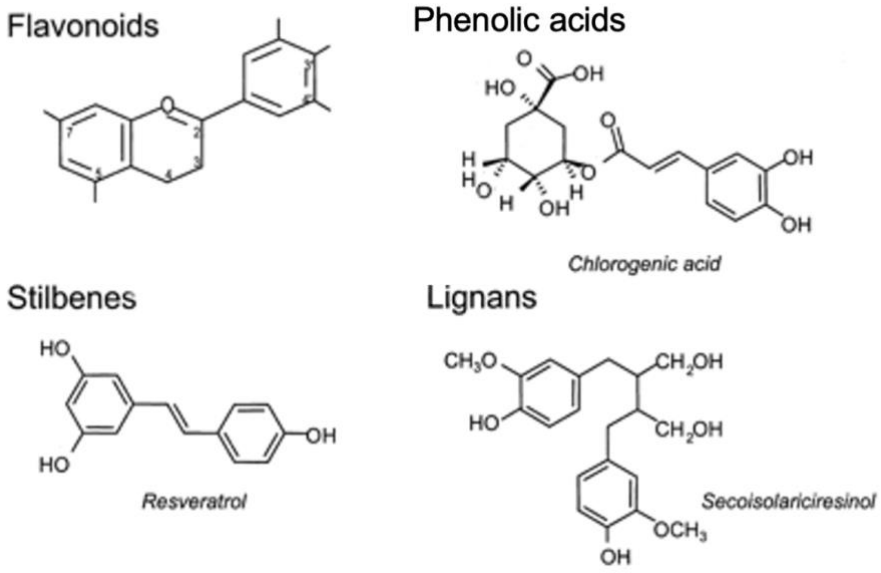

Figure 1: Chemical structure of (poly)phenols (adapted from Manach et al. 2004[45])

It has been reported that flavonols are widely spread in the plant kingdom[49,51,52]. The most common dietary flavonols are kaempferol, quercetin, isorhamnetin, and myricetin, which are found as glycosides in large quantities in yellow and red onions[44]. Representatives of the flavones are, for example, apigenin and luteolin. They are not very widespread, but relevant concentrations of flavones can be found in celery, parsley and some herbs[44,53,54]. Isoflavones are almost exclusively found in soy products such as soybeans and have a structural similarity to estrogen[55,56]. These structures, also called phytoestrogens, are associated with a reduction in mortality in women with breast cancer and are also associated with the relief of post-menopausal symptoms[57,58]. Flavanones, such as naringenin and hesperetin, are mainly found in vegetables, fruits, especially in the flavedo of citrus fruits, and spices. They are associated with a reduced risk of diabetes and potential health benefits on cardiovascular and metabolic diseases[49,59–63]. Anthocyanins are water-soluble plant components that are responsible for the purple blue and red color of the skin and flesh of fruits and vegetables. The most common anthocyanidin aglycones are cyanidin, delphinidin, pelargonidin, petunidin, and malvidin [49,64]. Epidemiological and human dietary intervention studies have shown that they may have positive effects on cardiometabolic diseases [65]. Flavanols are the structurally most complex subclass of flavonoids, consisting of monomers such as (+)-catechin or the isomer (-)-epicatechin, but also oligomeric and polymeric proanthocyanidins (PACs)[44]. They are present in plants and the oligomers are classified as A or B type according to their interflavan bonds[66]. A-type are less commonly found in nature and have an additional ether bond[66,67]. B-type are mainly found in cocoa and tea but also in fruits and vegetables such as apples, grapes and strawberries[66]. At this point it should be noted that 94.5% of PACs in cranberries contain one or more A-type bonds, whereas in apples 88.3% of PACs have exclusively B-type bonds[68].

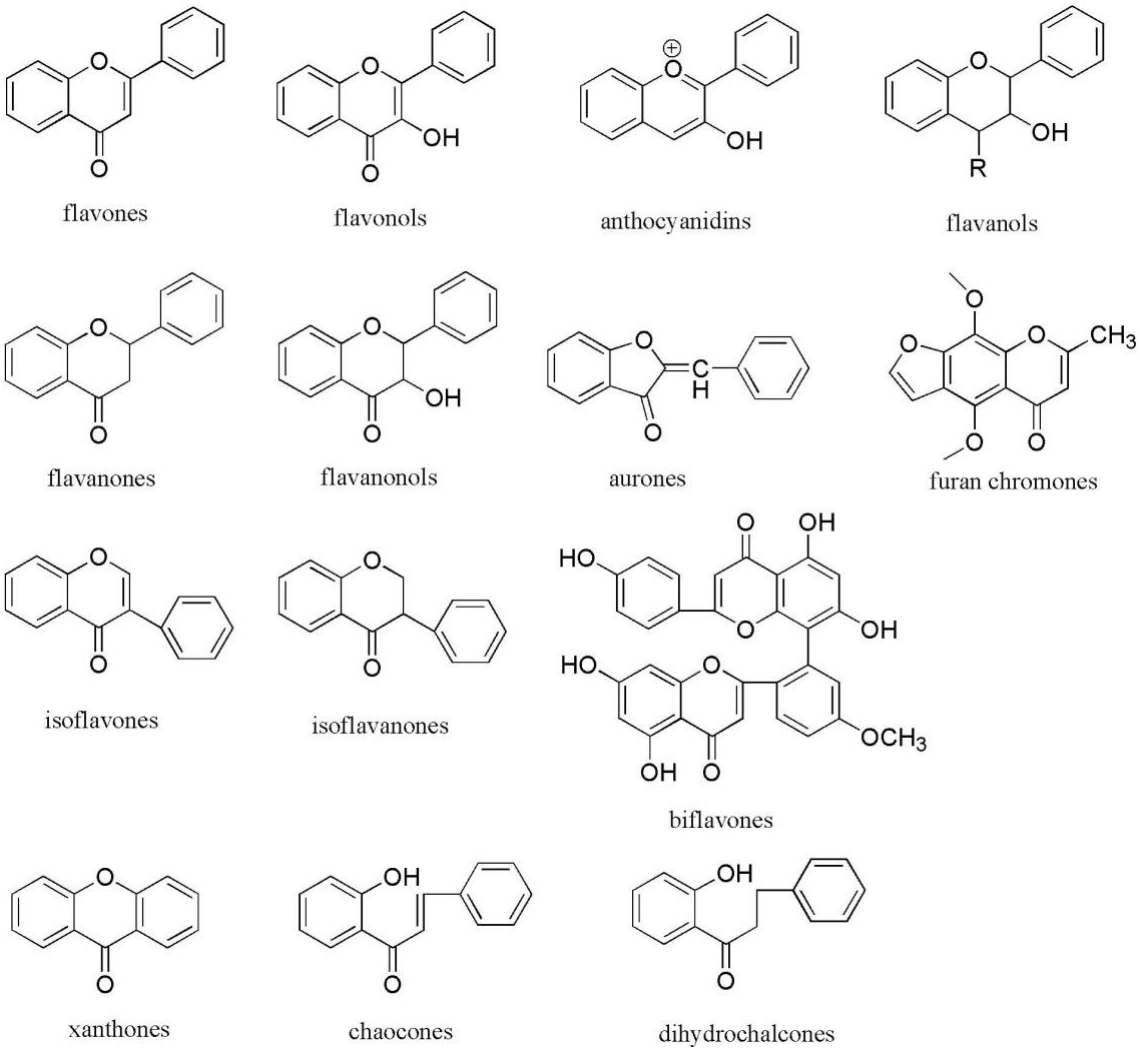

Figure 2: Chemical structures and classification of flavonoids (adapted from Wang et al. 2018[69])

The most important non-flavonoids with dietary significance are phenolic acids[44] Especially gallic acid, as biochemical precursor of the water-soluble tannins and most common phenolic acid, the hydroxycinnamates and their derivatives. Gallic acid is found in small amounts in foods as complex sugar esters in gallotannins, whereas non-sugar galloyl esters are the main source of gallic acid in the human diet[44]. The most important representatives of hydroxycinnamates are p-coumaric acid, caffeic acid, ferulic acid and sinapic acid. These are found mainly in their conjugated form and are then called chlorogenic acids, which can be detected in larger concentrations in coffee beans [44]. Other types of non-flavonoids include lignans, ellagitannins and stilbenes. Stilbenes are phytoallexins that are synthesized by plants in response to disease, injury, or stress. Their most important representative, relevant in the human diet, is resveratrol. It can be found in red wine as well as in berries and spinach, although in very small amounts. There are two different isomers, cis-resveratrol and trans-resveratrol, the latter being of great interest as it has been shown to slow down or even inhibit the development of CVDs and cancer[44].

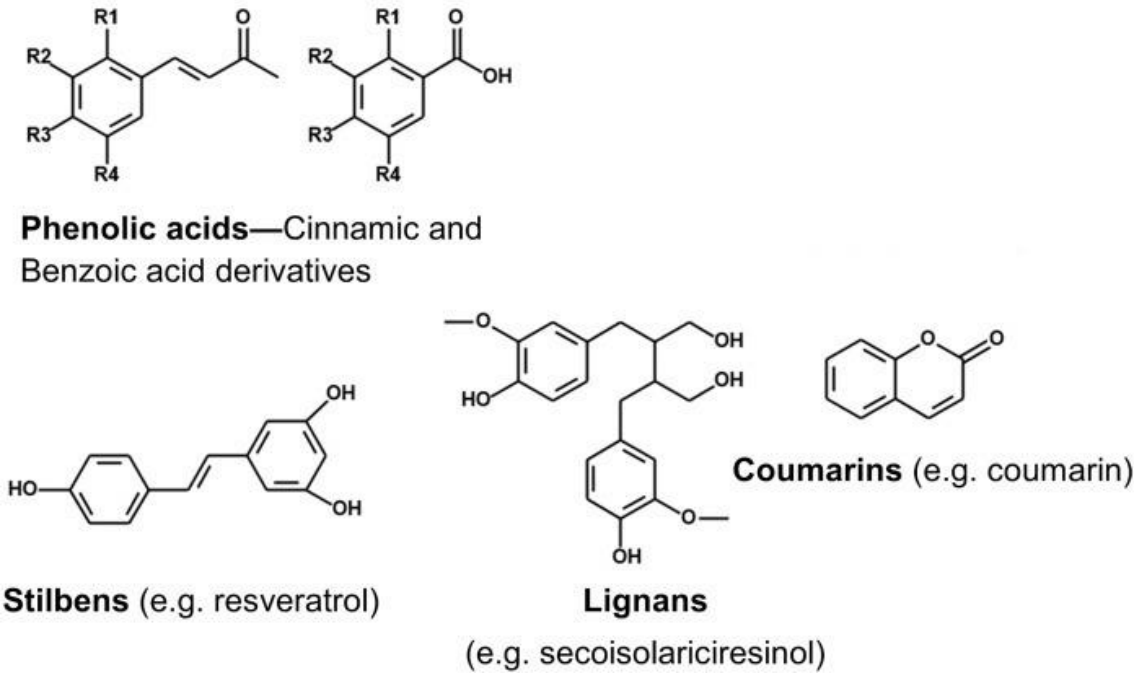

Figure 3: Chemical structure of main non-flavanoids (adapted from Puangpraphant et al., 2022[70])

1.3.2 Absorption and metabolism of (poly)phenols

After oral intake, the glucose-bound (poly)phenols enter the stomach where, despite the low pH, they are only partially hydrolyzed[71]. They then enter the small intestine, where they are deglycosylated by lactase-phlorizin hydrolase (LPH) in the brush border of the small intestine and then enter the small intestine cells via passive diffusion, based on the increased lipophilicity[72]. Alternatively, the (poly)phenols which are not hydrolyzed by LPH may be transported via the sodium-dependent glucose transporter 1 (SLGT 1) into the small intestine cells and hydrolyzed by cytosolic β-glucosidase (CBG). Before they enter the portal venous circulation, they are partly conjugated by phase II enzymes, forming glucuronide, sulfate an/or methylated metabolites. This conversion is mediated by sulfotransferases (SULT), uridine-5´-diphosphate glucuronosyltransferases (UGT), and catechol-O-methyltransferases (COMT)[44,49]. After being transported to the liver, they can be subjected to further phase II metabolism[73]. After metabolization in the liver they either enter the into the blood circulation or return to the digestive tract via the enterohepatic circulation[74]. But just 5–10% of the total (poly)phenol intake, mainly those with monomeric and dimeric structures, is directly absorbed in the small intestine[73]. Most (poly)phenols, including those bound to a sugar other than glucose, are not hydrolyzed by LPH or CBG and reach the colon unchanged where they are catabolized by the gut microbia[75]. Around 90-95 % of the polyphenols reach the colon unchanged, where they are enzymatically cleaved by different bacteria and converted into a large number of low molecular weight metabolites. Here, the heterocyclic polyphenol backbone and the glycosidic linkages are broken down[46].

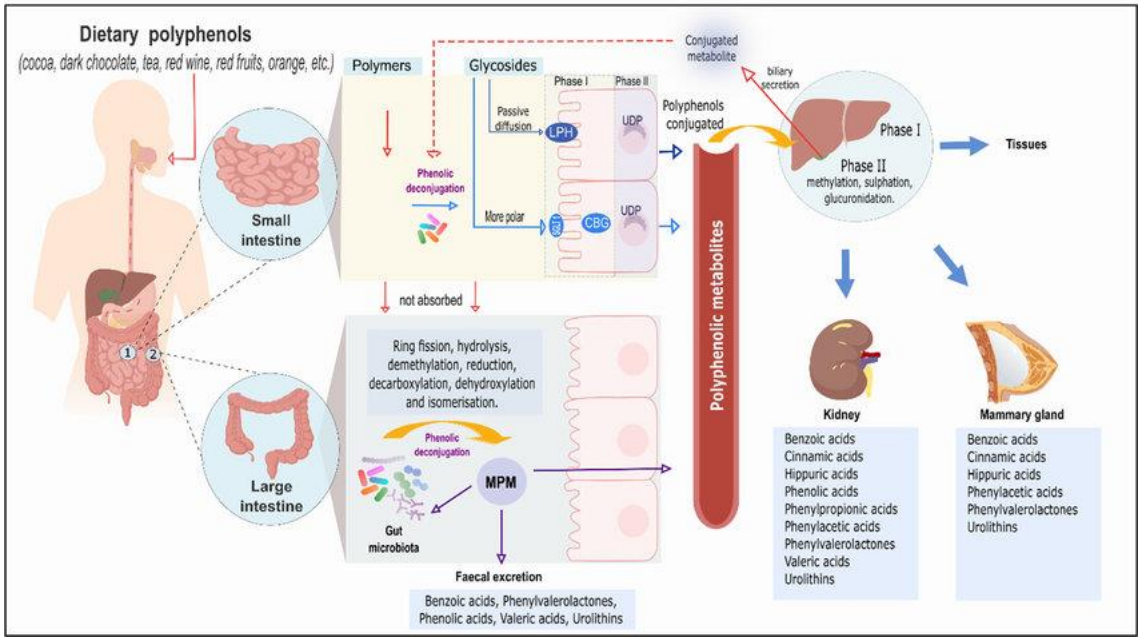

Figure 4: Metabolic pathways of polyphenols produced by the human gut microbiota. LPH: lactase-phlorizin hydrolase; CBG: cytosolic β-glucosidase; UDP: uridine 5'-diphospho-glucuronosyltransferase; MPM: microbial phenolic metabolites (adapted from Marhuenda-Muñoz et al[76]

By now only a limited number of trials have investigated in detail the absorption, distribution, metabolism, and excretion (ADME) of CP in the human body[77–81]. They focused on the analysis of some of the metabolites, especially the analysis of structurally-related anthocyanin metabolites. They are present in plasma in very low amounts compared to their phenolic acid metabolites.

1.4 (Poly)phenols and cardiovascular health

Recent data suggest that the consumption of (poly)phenol-rich foods has a positive effect on cardiovascular health[13]. Numerous epidemiological studies have investigated the relationship between the consumption of (poly)phenol-rich foods (e.g., fruits and vegetables, tea, cocoa, olive oil, and red wine) and cardiovascular health.

1.4.1 Epidemiological evidence

To assess the strength of the association between (poly)phenols and CVD risk, Wang et al. in 2014, conducted a systematic review with meta-analysis. The review suggests that the intake of six different classes of (poly)phenols significantly decreases the risk of CVD. They included 14 cohort studies that investigated the intake of flavonoids, namely anthocyanidins, proanthocyanidins, flavones, flavanones and flavanols on fatal or non-fatal CVD events, but not on CVD risk factors, as the primary outcome and assessed and reported relative risk (RR) and the corresponding 95 % CI for CVD. The meta-analysis found that anthocyanidins (RR 0-89, 95 % CI 0-83, 0-96), proanthocyanidins (RR 0-90, 95 % CI 0-82, 0-98), flavones (RR 0-88, 95 % CI 0-82, 0-96), flavanones (RR 0-88, 95 % CI 0-82, 0-96) and flavanols (RR 0-87, 95 % CI 0-80, 0-95) were inversely associated with the risk of CVD. Furthermore, an inverse association between flavonol consumption and CVD risk was demonstrated[82].

A substudy of the PREDIMED study, conducted by Medina-Remón et al., investigated the relationship between (poly)phenol intake and circulating inflammatory biomarkers in the blood of 1139 participants as well as cardiovascular risk factors[83]. They observed a correlation between increased (poly)phenol intake, measured as (poly)phenol concentration in urine, and decreased inflammatory biomarkers, as well as decreasing blood pressure values, and increasing plasma high-density lipoprotein (HDL)[83]. Also an observational study within the PREDIMED study showed a 46% reduction of CVRF comparing the group with the highest (poly)phenol-intake versus the group with the lowest intake[84].

Similar results were obtained by Grosso in 2021[85]. They conducted a dose-response meta-analysis to determine the association between dietary intake of flavanoids and risk of CVD. They included 39 studies with more than 1.5 million indivduals. There were 33 637 cases of CVD, 23 664 of coronary heart disease (CHD), and 11 860 of stroke. They found a linear association between increased

flavonoid intake and a lower risk of CVD. The intake of anthocyanins and flavan-3-ols was inversely associated with the risk of CVD, whilst an increased intake of flavonols and flavones was inversely associated with the risk of coronary heart disease.

There are also studies which did not observe any positive correlation between the consumption of (poly)phenols and a reduction of cardiovascular risk [86,87]. Ikeda et al. conducted a prospective, nested case-control study including 29,876 men and women between 40 and 69 years without CVD or stroke history from 1990-94 until follow-up 2008. There was no correlation between plasma levels of tea catechins and a risk reduction of stroke or CHD[87].

In summary, although mixed results exist, most of the meta-analysis of observational studies have found an inverse association between (poly)phenol consumption and CVD risk.

1.4.2 Evidence from randomized controlled trials

Epidemiological data, as described above, suggest that there is a link between the consumption of (poly)phenol-rich foods and cardiovascular health. However, randomized controlled trials (RCT) are much more suitable for demonstrating direct, dose-dependent effects, although it should be noted that they are also subject to some limitations. A direct correlation between the consumption of (poly)phenol-rich foods and hard clinical endpoints for CVDs, such as myocardial infarction or stroke, is difficult to prove due to time and financial limitations[88]. For this reason, surrogate markers of cardiovascular risk such as endothelial function, arterial stiffness, blood pressure and blood lipids are used to demonstrate cause-and-effect relationships[27]. In the following section, the most relevant RCTs that have investigated the effect of berry (poly)phenols on one or more markers of vascular function in humans, and on blood pressure, are discussed.

A total of 22 RCTs have investigated the effect of berry (poly)phenols on biomarkers of vascular function like FMD, reactive hyperemia index (RHI) or on arterial stiffness, measured by pulse wave velocity (PWV), augmentation index (AIX) or peripheral resistance. Eight studies investigated the effects of (poly)phenols in blueberries (2 used freeze dried blueberry powder, 2 used blueberry drinks, 1 used thawed blueberries, 1 used fresh blueberries, 1 used homogenized blueberries and 1 used backed products with blueberry in a blueberry bun)[39,89–95], 5 studies used blackcurrants (3 used blackcurrant drinks or juice, 1 used New Zealand blackcurrant extract (NZBC) and 1 used NZBC powder in water)[96–100], 4 of them used cranberry juice[101–104], 3 of them used

freeze-dried strawberry powder[105–107], 1 used an açai smoothie[108] and 1 used frozen raspberries in water[109]. The study populations were heterogeneous, 11 studies investigated healthy non-smokers, 1 study included healthy smokers, 1 study smokers and non-smokers with peripheral arterial dysfunction, and the remaining 9 studies included subjects with at least one cardiovascular risk factors (CVRF) or manifest CVD. Thirteen studies investigated short-term effects, whereas 9 studies examined long-term effects on vascular function.

1.4.3 Effects on vascular function

Of the studies investigating short-term effects, 5 studies measured FMD values, 5 studies measured RHI values, and 3 studies measured other values such as PWV. Four studies showed an increase in FMD after consumption of (poly)phenols[94,104,108,109]. Rodriguez-Mateos et al. found that the consumption of 3 pieces of baked blueberry-containing products and/or a blueberry drink (both containing the equivalent of 240 g of fresh blueberries) increased FMD at 1, 2, and 6 hours post-consumption on healthy individuals[94]. Alqurashi et al. found that the consumption of an acai smoothie containing 150g of açai pulp significantly increased FMD 2h (+ 1.4%; $p = 0.001$) and at 6h (+ 0.8%; $p < 0.001$) post-consumption on healthy individuals[108].
Istas et al. also found that the intake of either 200g or 400g of red raspberries improved FMD at 2h (+1,6% and +1,2%, respectively) and 24h (+1,0% and +0,7%, respectively) post-consumption on healthy males, whereby there was no difference between the two doses and thus no dose-dependent relationship could be established[109]. Three studies showed a significant increase in RHI values[90,91], whereas 2 studies did not report significant effects on RHI following short-term interventions with berries[89,97].

Looking at the study investigating long-term effects, one can see a clear difference to the acute studies. Only 2 studies could detect a significant effect on FMD or RHI after chronic consumption[95,99]. Khan et al. detected a significant increase in FMD (5.8 ± 3.1 to 6.9 ± 3.1%, $p = 0.022$) after consumption of blackcurrant juice (1 L/d, containing 815mg of total (poly)phenols) for 6 weeks compared with the placebo group (6.0 ± 2.2 to 5.1 ± 2.4%) on healthy individuals[99]. Khan et al.'s results are consistent with those of Stull et al., who reported significant improvements in RHI on participants with metabolic syndrome, after six weeks of consumption of two blueberry smoothies containing 800mg total (poly)phenol equivalent compared with the placebo group (0.32 ± 0.13 vs. – 0.33 ± 0.14; respectively; $p = 0.0023$)[95]. Four studies could not find a significant effect on FMD or RHI as explained in the following section[93,101,102].

Riso et al. couldn't find any significant improvement in vascular function measured by RHI in healthy males with at least one risk factor for CVD (1.83 ± 0.43 before vs. 1.86 ± 0.55 after six weeks of consumption wild blueberry drink; p = 0.452). Comparing with placebo there was also no significant increase[93]. Flammer et al. conducted a study in participants over the age of 18 years with endothelial dysfunction and cardiovascular risk factors or known CVD. Measurements were performed at baseline, 45 minutes after intake and after four months of daily intake of two cranberry drinks. No change in RHI values could be detected after four months of daily intake (1.7 ± 0.4 before vs. 1.7 ± 0.3 after four months of consumption of the cranberry drink; p = 0,37). No effect was detected comparing with placebo[101]. Dohadwala et al. conducted a crossover study investigating the effect of cranberry juice in subjects with CAD. No effect was detected after four weeks of daily intake of a cranberry drink (6.3 ± 4.4 before vs. 6.7 ± 4.4 after four weeks)[102].

1.4.4 Effects on arterial stiffness

Regarding arterial stiffness none of the 5 acute studies measuring pulse wave velocity (PWV) or augmentation index (AIX) after berry consumption showed a significant effect[39,90,91,106]. In contrast, 6 out of 7 chronic studies reported positive modulation of PWV and AIX[92,93,98,100,102,103,107].

Johnson et al. found a significant decrease in brachial artery (BA) PWV (from 1498 ± 179 cm/s to 1401 ± 122 cm/s at about 6.5%; p < 0.05) after 8 weeks of blueberry drink intake (containing 22g of freeze-dried blueberry powder) on participants with prehypertension[92]. Ferresin et al. describe significantly decreased BA PWV and femoral artery PWV in postmenopausal women with prehypertension or stage 1 hypertension (- 0.73 m/s, p = 0.03, and - 0.55 m/s, p = 0.02, respectively) after 8 weeks of consumption of a strawberry drink (containing 25g of freeze-dried strawberry powder)[107]. Dohadwala et al. found that the carotid - femoral PWV decreased after cranberry juice (450ml) consumption (8.3 ± 2.3 to 7.8 ± 2.2 m/s; p = 0.003) in contrast with an increase after placebo (8.0 ± 2.0 to 8.4 ± 2.8 m/s; p = 0.003) in patients with coronary artery disease[102]. Ruel et al. could determine a significant decrease in AIX in participants with CVRF (- 10.8% ± 6.4%; p < 0.0001) after 4 weeks of cranberry drink consumption (500mL/d), whereas Riso et al. could not determine a significant decrease after 4 weeks of consumption of a wild blueberry drink (250mL/d, containing 250mg anthocyanins)[93,103]. Significant decrease in peripheral resistance could be found in two studies[98,100].

1.4.5 Effects on blood pressure

Rodriguez-Mateos et al. reviewed in 2014 the effect of different berry (poly)phenols on blood pressure[88]. It was possible to identify 19 RCTs and include them in the review, although these differed significantly in terms of the berries tested, the study population, and the duration of the study[39,89,93,97,102,103,110–122]. However, blood pressure decreases could only be detected in two double-blind RCTs[115,122]. In 44 myocardial infarction survivors on statin therapy, who took chokeberry extract (containing 64 mg of anthocyanins, 128 mg of procyanidins, and 23 mg of phenolic acids) for six weeks, decreases in both SBP and DBP by 11 and 7.2 mmHg could be observed[115]. In another study, a decrease in SBP of 6.7 mmHg compared to the baseline in hypercholesteremic individuals after 12 weeks of daily consumption of 320 mg of berry anthocyanins was shown[122]. However, it should be noted that blood pressure was not the primary endpoint of the studies mentioned here. The 24 h ambulatory blood pressure, gold standard for blood pressure measurements, was only used by Hasselund et al.[120]. No changes in blood pressure were observed after four weeks of supplementation with purified berry anthocyanins (2x320mg). In conclusion, there is not yet enough evidence to make a clear statement about a potential antihypertensive effect of berry (poly)phenols.

1.5 Cranberry (poly)phenols

Cranberries, also called *Vaccinium macrocarpon Ait.*, are an important source for phenolic compounds[123]. They mainly contain flavonoids such as flavanols, anthocyanins, flavonols and non-flavonoids as hydroxycinnamic and other phenolic acids[67,124–126]. In ascending order, cranberries contain the following classes of (poly)phenols: phenolic acids (5.0-12.1%), anthocyanins (8.0-24.4%), flavonols (18.6-30.5%), and flavan-3-ols (41.5-52.2%). However, the concentration depends strongly on the ripeness of the fruit. The lowest (poly)phenol concentration was measured in immature and semi-mature ripening stages and the highest concentrations in the overripe state.[127].

Flavan-3-ols contained in cranberries are mostly oligomers or polymers, which are then referred to as proanthocyanidins (PACs). With 85% they make up the largest part of the (poly)phenols in cranberries and differ in their structure by constitutive units, types of linkage, and degree of polymerization (DP)[123,126,128].

The monomer (-)-epicatechin is the main constitutive unit in cranberry PACs, while (+)-catechin and (epi)gallocatechin are present in small amounts[123]. Depending on the types of linkage, A-type PACs and B-type PACs are distinguished, whereby PACs with an A-type inter-flavan bond account for 51-

91% of all PACs in cranberries[129,130]. Other studies assume that up to 94.5% of cranberry PACs have A-type bonds[68]. This differentiation is crucial because the antimicrobial properties of cranberry PACs have been associated with their ratio of A- to B-type linkages.

Feliciano et al. have compared A-type PACs from cranberries and B-type PACs from apples to investigate whether these affect extraintestinal pathogenic Escherichia coli (ExPEC) agglutination and invasion of enterocytes. Both increased agglutination and reduced epithelial cell invasion, the higher the proportion of A-type PACs, the stronger the effect[68]. Furthermore, the degree of polymerization (DP) plays a significant role. Thus, it was found that cranberry PACs with a greater DP inhibited the growth of Candida spp. more effective, than those with a smaller DP[131]. Although ExPEC does not cause acute intestinal disease, they are one of the main intestinal germs and play a major role in extraintestinal pathologies such as urinary tract infections (UTI), possibly subsequent (uro-)sepsis and postoperative wound infections[128,132,133]. Here, cranberry PACs, as described above, seem to offer a possibility to decrease intestinal colonization with ExPEC.

Cranberries contain glycosides of the 6 aglycones of the anthocyanidin family: cyanidin, peonidin, malvidin, pelargonidin, delphinidin, and petunidin[134]. The anthocyanins most commonly found in cranberries are 3-O-galactosides and 3-O-arabinosides of cyanidin and peonidin[125]. The amount of anthocyanin is reported to be about 3.6 to 171 mg/100 g fresh weight[125]. They are responsible for the red color, but also play a significant role in potential health benefits[123]. In larger quantities they are found in rare cranberries, but their proportion, for example in juice, decreases due to the processing techniques used [128,135].

In the public perception, cranberry products and their consumption are therefore known mainly because of their potential UTI preventive or therapeutical effect[136]. However, there are some studies that point to potential cardiovascular health benefits, but there is a lack of strong evidence due to the low number of human intervention trials and their heterogeneity[88,102,137].

1.5.1 Cranberry (poly)phenols and cardiovascular health

So far, not many studies have been performed that focus on the cardiovascular effects of CP. Most studies focus on CVRF such as serum lipid profiles, blood pressure (BP), endothelial function, glucoregulation, and a variety of biomarkers of inflammation and oxidative stress. Especially double-blind randomized controlled trials on the effects of CP on clinically relevant and accredited markers of vascular function are rare[101–103,112,116,137]. Flammer et al. reported that a daily

supplementation with cranberry juice for 4 weeks did not improve vascular function detected by peripheral arterial tonometry (PAT) in patients with manifest CAD, endothelial dysfunction or CVRF101. In contrast, Dohadwala et al. could demonstrate an acute increase in FMD 4 h after cranberry juice intake (Baseline 7.7 ± 2.9% vs. 4h 8.7 ± 3.1%; p = 0.003). In addition, improvements in PAT could be observed after 2 and 4 h (Baseline 0.10 ± 0.12, 2h 0.22 ± 0.14, 4h 0.23 ± 0.16; p = 0.006). The results were statistically significant but were obtained in the uncontrolled pilot study. In contrast in the RCT, they were just able to demonstrate a positive effect on PWV more than 12 h (defined as chronic effect) after cranberry juice consumption. The carotid - femoral PWV decreased after cranberry juice consumption (8.3 ± 2.3 to 7.8 ± 2.2 m/s; p = 0.003) in contrast with an increase after placebo (8.0 ± 2.0 to 8.4 ± 2.8 m/s; p = 0.003) in patients with CAD[102].

Effects on BP were recently detected by Novotny et al. in healthy volunteers. Low-calorie cranberry juice lowered diastolic BP by 4.7mmHg after 8 weeks of daily consumption, compared with the placebo beverage[137]. These results are consistent with those of Ruel et al., who reported a significant decrease in systolic BP by 3mmHg in obese men after consumption of a low-calorie cranberry drink, compared to the baseline[138].

In the above-mentioned study, Novotny et al. reported reduced triglyceride and c-reactive protein (CRP) levels in plasma in the cranberry group. Simão et al. reported that the daily consumption of a low-calorie cranberry drink for 8 weeks does not influence proinflammatory cytokines such as IL-1, IL-6 and TNF alpha[139]. However, they were able to demonstrate a reduction of the biomarkers of lipid peroxidation and advanced oxidation protein products. A study by Basu et al. on female patients with metabolic syndrome came to similar results[112]. The 36 participants consumed a low-calorie cranberry juice daily for 8 weeks. Afterwards an increase in biomarkers of antioxidant capacity and decreases in lipid peroxidation in plasma were observed.

Furthermore, most of these studies were conducted on subjects with CVRF or with manifest CVD, which results in the fact that the effect on healthy individuals cannot be estimated.

1.6 Aims of this thesis

Epidemiological and clinical data suggest that there is a link between the consumption of food rich in (poly)phenols and a reduced CVR. Berries have a high content of (poly)phenols. Cranberries are rich in procyanidins, anthocyanins, and phenolic acids. Whether CP consumption can improve vascular function and

health when given over relevant periods and in proper amounts is not known. Therefore, it is necessary to know the exact (poly)phenolic composition of cranberries and their absorption, distribution, metabolism, and excretion (ADME) to understand their effect on vascular biomarkers better.

This work hypothesizes that CP improve vascular function in healthy individuals in a time- and dose-dependent manner. Therefore, a double-blind, randomized controlled trial was performed, which assessed this effect on clinical determinants of vascular function (FMD; primary endpoint), as well as PWV, AIX, and BP (secondary endpoints). A further aim was to investigate the absorption and metabolism of cranberry polyphenols and investigate relationships with the measured vascular effects (tertiary endpoint).

2 Materials and methods

2.1 Materials

If not further described all chemicals and reagents as well as flavonoid and phenolic acid standards were obtained from Sigma-Aldrich Co. (Steinheim, Germany). Homovanillic acid sulfate sodium salt, caffeic acid 3-O-β-D-glucuronide, caffeic acid 4-O-β-D-glucuronide, dihydro caffeic acid 3-O-sulfate sodium salt, dihydro caffeic acid 3-O-β-D glucuronide diammonium salt, ferulic acid 4-O-β-D-glucuronide disodium salt, ferulic acid 4-O-sulfate disodium salt, dihydro ferulic acid 4-O- sulfate sodium salt, dihydro ferulic acid 4-O-β-D-glucuronide, iso- ferulic acid 3-O-sulfate disodium salt, isoferulic acid 3-O-β-D-glucuronide, dihydro isoferulic acid 3-O-sulfate disodium salt, dihydro isoferulic acid 3-O-β-D-glucuronide and (5R)-5-(3´,4´ - dihydroxyphenyl)- γ-valerolactone-4´-O-sulfate sodium salt were obtained from Toronto Research Chemicals (Toronto, Canada). The valerolactone was provided without information regarding the exact position of the sulfate and will be designated in this work as (5R)-5-(3´-hydroxyphenyl)- γ-valerolactone-4´ -O-sulfate, according to the NMR spectrum provided by the supplier. Kaempferol-3-O-β-D-glucuronide was obtained from Extrasynthese (Genay, France). 1-Methylpyrogallol-O-sulfate, 2-methylpyrogallol-O-sulfate, 4-methylcatechol-O-sulfate, 4-methylgallic-3-O-sulfate, catechol-O-sulfate, pyrogallol-O-1-sulfate, pyrogallol-O-2-sulfate and vanillic acid-4-O-sulfate were kindly provided by Dr. Cláudia Nunes dos Santos and Dr. Rita Ventura, and their synthesis has been described elsewhere [140]. All the (poly)phenol and phenolic acid aglycones were obtained from Sigma-Aldrich Co. (Steinheim, Germany) and 2-, 3- and 4-hydroxyhippuric acids were purchased from Enamine (Kiev, Ukraine). Acetic acid was from Carl Roth (Karlsruhe, Germany) and Oasis HLB μElution plates (2 mg sorbent per well, 30 μm) were from Waters (Eschborn, Germany). Milli-Q system (Merck KGaA, Darmstadt, Germany) ultra-pure water was used.

2.2 Study participants

In this study, healthy men were recruited from the Düsseldorf area. They were contacted via word of mouth, adverts in the Heinrich-Heine University, and advertisements in social media (Facebook). The volunteers were screened for pre-existing conditions with a Health and Lifestyle Questionnaire and subjected to a general physical examination by a qualified researcher. Inclusion criteria included being male, age between 18-35 years, no pre-existing conditions based on the Health and Lifestyle Questionnaire and absence of CVD, normal blood lipid values, liver enzymes, hemoglobin, leukocyte values, and coagulation, and a signed consent form. Exclusion criteria included women (due to complex hormonal variability which could influence FMD), smokers, manifest cardiovascular diseases including coronary artery disease, peripheral, renal, and cerebrovascular occlusive disease, arterial hypertension, diabetes mellitus, renal insufficiency (acute and chronic) and malignant diseases as well as coagulation disorders. All individuals with heart rhythms, except sinus rhythm, were also excluded. Chronic intake of antihypertensive drugs, antibiotics, and dietary supplements such as vitamins in the last two months were also exclusion criteria, as was the intolerability of cranberry products.

Written informed consent was obtained from all subjects before participation in the study. Declarations of consent were signed by all study participants prior to the start of the study.

2.3 Study design

A six-arm randomized, double-blind, crossover, controlled intervention trial was conducted at the Division of Cardiology, Pulmonology and Vascular Medicine of D sseldorf University. Every volunteer took one of six different cranberry drinks on each of the six study days to investigate the time- and dose-dependent effect of CP on vascular function as determined by FMD (primary endpoint) of the BA. Secondary endpoints were effects on blood pressure (BP) (peripheral and central), pulse wave velocity (PWV), pulse wave analysis (PWA), and augmentation index (AIX). The tertiary endpoint included the quantification of CP metabolites in correlation with FMD. Blood and urine samples were collected for that purpose. Researchers involved in interventions or assessing outcomes, and volunteers were blinded to the interventions.

Volunteers were instructed not to alter their usual dietary or fluid intake for the time of the study. Those selected for the study were asked to refrain from the following for 72 h prior to, and during, the study: consumption of (poly)phenol-rich foods including fruits, vegetables, cocoa, chocolate, coffee, tea and wine, any

fruit juices and soya products, intake of nitrate-rich foods: leafy green vegetables and beetroot, participating in vigorous exercise (>3×20 min/week) and consuming more than 168 g of alcohol (any form) per week. 24 hours before the study day, the volunteers were asked not to drink energy drinks and soft drinks nor coffee or alcohol. Volunteers were also asked not to eat anthocyanin-rich foods such as berries or red wine for one week before starting (run-in) and until the completion of the study. They also were asked to be sober on every examination day. Sobriety was defined as abstinence from solid food and any liquids for 8 hours. After taking the cranberry juice and 8h post-consumption, the volunteers were served a white bread sandwich with cheese. Furthermore, they received a low (poly)phenol dinner consisting of long grain rice with fish to take home. Compliance with the diet and lifestyle restrictions was determined via 24 h-dietary recalls and via an interview on every intervention day.

To create appropriate and reproductive investigation conditions, every study day started at 7.30 am with the baseline measurements and ended with the 8h post-consumption measurements. The volunteers were asked to lie down quietly for 10 minutes in an air-conditioned and temperature-controlled room before every analysis. FMD, peripheral BP measurements, and blood samples were taken before (0 h) and at 1, 2, 4, 6, and 8 h post-acute consumption of each drink on six different days. PWV, PWA, AIX, and central blood pressure were measured at 0, 1.5, 4, 6 and 8 h post-consumption. An additional blood sample was taken after 24h. Urine was collected at baseline (0 h), from 0-8h and 8-24h post-consumption. The order of the measurements was standardized and is as follows: 10 minutes rest phase, BP measurement, PWA and PWV, FMD, blood sampling. A one-week washout separated each intervention day.

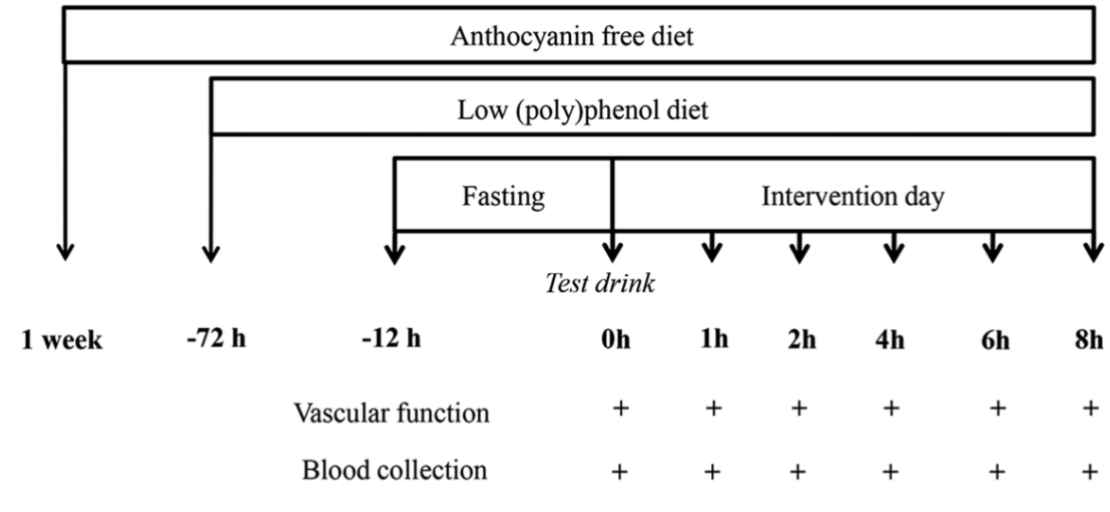

Figure 5: Study protocol

The software used for randomization is called GraphPad (GraphPad Software Inc.; https://www.graphpad.com/quickcalcs/randomize1.cfm). It is a free software that produces “pseudo-random” numbers that are random enough for statistical analytical tests.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the University of Düsseldorf Research Ethics Committee (ref: 14-012). This study was also registered with the National Institutes of Health (NIH)-randomized trial records held on the NIH Clinical Trials.gov website (NCT02517775).

2.4 Test materials

A total of 5 different cranberry drinks and one control drink were investigated. Ocean Spray supplied the drinks. The drinks had a total volume of 450ml and could not be distinguished in color or taste. The nutritional values of the drink with the highest (poly-)phenol concentration and the micro- and macro-nutrient isocaloric control drink are shown in Table 1. The (poly)phenol concentrations of the cranberry juices were 409, 787, 1238, 1534, and 1910 mg (equivalent to 25, 48, 76, 94, and 117% concentrated cranberry juice, respectively) and 3 mg for the control drink.

Table 1: Nutritional analysis of the control drink and the most concentrated cranberry juice (1910 mg CP) per serving (450 mL)

	Control Drink	**Drink 1910 mg CP**
Energy (kcal)	208	200
Energy from fat (kcal)	< 4.72	< 4.72
Total fat (g)	< 0.009	< 0.009
Saturated fat (g)	< 0.009	< 0.009
Trans fat (g)	< 0.009	< 0.009
Cholesterol (mg)	< 4.7	< 4.7
Total carbohydrates (g)	50.9	49.5
Total dietary fibre (g)	< 3.54	< 3.54
Total sugars (g)	42	35
Fructose (g)	35	20
Glucose (g)	7.1	15
Sucrose (g)	< 0.5	< 0.5
Lactose (g)	< 0.5	< 0.5
Maltose (g)	< 0.5	< 0.5
Galactose (g)	< 0.5	< 0.5
Protein (g)	0.90	0.75
Pro – Vitamin A (I.U.)	< 170	< 170
Vitamin C (mg)	< 4.7	< 4.7
Calcium (mg)	3.04	0.118
Copper (mg)	< 0.0590	0.118
Iron (mg)	< 0.235	0.689
Magnesium (mg)	< 2.35	19.2
Manganese (mg)	< 0.035	1.28
Phosphorus (mg)	< 2.35	19.2
Potassium (mg)	231	374
Sodium (mg)	88.2	11.3
Zinc (mg)	< 0.0472	0.26
ASH (g)	0.57	0.71
Moisture (g)	419	421

Table 2: (poly)phenol content of the cranberry drinks and the control drink, expressed in mg/450 mL (single dose)

	Control drink	**409 mg TP**	**787 mg TP**	**1238 mg TP**	**1534 mg TP**	**1910 mg TP**
Phenolic acids	**2.7**	**12.8**	**24.5**	**35.3**	**48.6**	**59.2**
Benzoic acid	2.3	4.5	7.8	11.1	15.5	17.1
Caffeic acid (3,4-dihydroxycinnamic acid)	0.0	0.6	1.2	1.9	2.5	3.1
Chlorogenic acid	0.0	2.6	5.2	7.5	10.3	12.8
Ferulic acid (3-methoxy-4-hydroxycinnamic acid	0.0	0.0	0.0	0.3	0.5	0.6
Gallic acid (3,4,5-trihydroxybenzoic acid)	0.0	0.0	0.1	0.1	0.2	0.2
Protocatechuic acid (3,4-dihydroxybenzoic acid)	0.0	0.4	1.1	1.8	2.4	3.1
Salicylic acid (2-hydroxybenzoic acid)	0.0	0.0	0.1	0.1	0.2	0.3
Vanillic acid (4-hydroxy-3-methoxybenzoic acid)	0.0	0.4	1.0	1.1	1.7	3.2
p-Coumaric acid (4-hydroxycinnamic acid)	0.0	3.5	6.9	9.6	13.3	16.2
t-Cinnamic acid	0.3	0.6	1.0	1.8	2.1	2.6
Flavanols	**0.0**	**2.5**	**5.0**	**6.8**	**10.1**	**12.3**
Catechin	0.0	0.2	0.5	0.8	1.2	1.5
Epicatechin	0.0	2.2	4.5	6.0	8.9	10.8
Flavonols	**0.2**	**14.5**	**31.3**	**48.9**	**62.8**	**76.9**
Quercetin	0.0	4.0	8.5	15.5	18.2	20.5
Quercitrin (Quercetin-3-O-rhamnoside)	0.0	2.2	4.6	6.5	8.1	10.7
Hyperoside (Quercetin-3-O-galactoside)	0.0	2.0	3.9	5.5	7.2	9.4
Myricetin	0.2	4.0	8.2	11.2	14.7	18.4
Myricetrin (Myricetin-3-O-rhamnoside)	0.0	1.6	3.2	4.8	6.4	7.4
Myricetin-3-O-galactoside	0.0	0.7	2.9	5.4	8.2	10.4
Anthocyanins	**0.0**	**6.8**	**16.2**	**23.2**	**26.3**	**32.3**
Cyanidin-3-arabinoside	0.0	3.7	6.8	9.5	12.1	14.7
Cyanidin-3-galactoside	0.0	0.0	1.7	2.4	2.9	3.6
Cyanidin-3-glucoside	0.0	0.0	0.0	0.0	0.0	0.0
Peonidin-3-arabinoside	0.0	2.0	2.1	3.6	7.0	8.4
Peonidin-3-galactoside	0.0	1.1	2.0	2.9	3.3	4.5
Peonidin-3-glucoside	0.0	0.0	0.0	4.8	1.0	1.1
Proanthocyanidins	**0.0**	**6.8**	**16.2**	**23.2**	**26.3**	**32.3**
BL-DMAC	0.0	124.8	242.5	278.2	420.9	485.5
OSC-DMAC	0.0	372.6	710.5	1124.0	1386.3	1729.1
Total Phenolics (Folin method)	0.0	180.0	517.5	778.5	1318.5	1521.0
Total sum (poly)phenols	**2.9**	**409.0**	**787.5**	**1238.1**	**1534.1**	**1909.9**
% Cranberry juice	0.0	25.1	48.2	75.8	94.0	117.0

The exact (poly)phenol concentration is shown in Table 2 and was analyzed by Ocean Spray according to established procedures previously described[141].

2.5 Flow-mediated dilation measurements

FMD was performed following the standard operating procedure established by our workgroup[142]. In order to create reproductive investigation conditions, the measurements were carried out at the same time of the day, always in the same, air-conditioned and temperature-controlled room [29]. FMD was performed on each examination day and timepoint after the BP and PWV measurements. The examination was performed on all subjects on the right BA by the same investigator. The arm was stored in a relaxed, outstretched position. A blood pressure/sphygmomanometric cuff was placed distal to the antecubital fossa. In order to relate the images to the cardiac cycle, electrocardiography (ECG) was performed simultaneously[29]. The ultrasound device used for the examination was a 10-MHz transducer (Vivid I, GE healthcare) coupled with a (GE 12L-RS) for vascular imaging.

First, baseline measurements were carried out. The ultrasound probe was placed approximately 2 cm proximal to the elbow bend, and B-mode ultrasound scans of the BA in longitudinal section were obtained. Care was taken to ensure that the vessel wall was represented in such a way that intima and media could be correctly differentiated. After a correct image of the artery had been taken, the blood pressure cuff was inflated to 250mmHg and left for 5 minutes. The ultrasound probe remained in the same position on the upper arm for the entire 5 minutes. A further image of the vessel was taken (0s), the pressure was released, and reactive hyperemia was induced. Further images were taken 20s, 40s, 60s, and 80s after deflation of the cuff[38,142]. The images were stored and analyzed separately.

This examination was carried out on each study day before (0h) and 1, 2, 4, 6, and 8h after the consumption of the drinks.

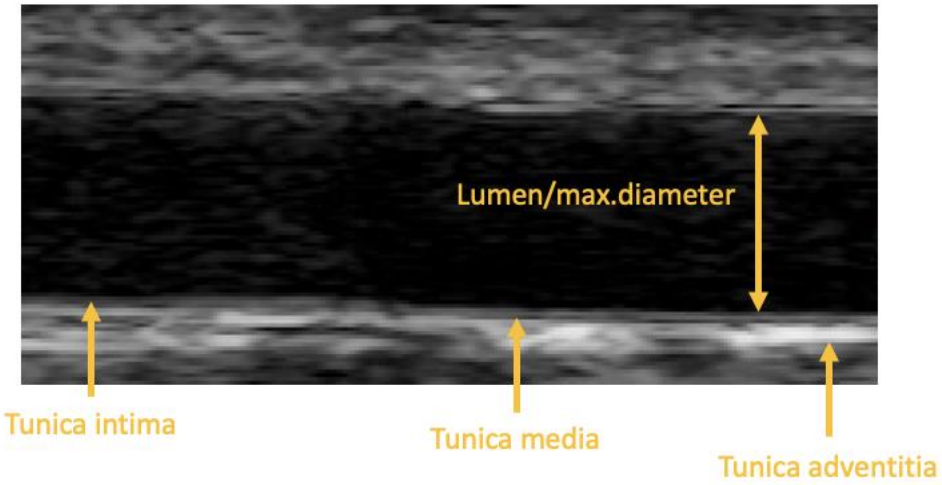

Figure 6: Ultrasound image obtained with 10-MHz transducer (Vivid I, GE healthcare) for FMD measurements

An automatic edge detection software (Brachial Analyzer, Medical Imaging Applications, Iowa City, IA, USA) was used to analyze the obtained images. The diameter was assessed, and FMD calculated as maximal relative diameter gain at 60s relative to baseline ((diameter$_{max}$ – diameter$_{baseline}$)/diameter$_{baseline}$ * 100). All images were compared at the beginning of the R-peak, defining the end of the diastole during the heart cycle[29,104].

2.6 Blood pressure

Blood pressure and heart rate (HR) measurements were the first examinations on each study day and were performed in lying position after a 10 minute resting period on the left upper arm before and 1, 2, 4, 6, 8 and 24h after consumption of the drinks using an electronic oscillometric device (Boso medicus PC2, Jungingen, Germany) with appropriately sized cuff[142]. Three measurements were performed with a pause of 60 seconds between each measurement. The mean value was calculated out of three measurements.

2.7 Pulse wave velocity

To determine arterial stiffness, PWV and PWA were measured with applanation tonometry, using the SphygmoCor® system (AtCor Medical, West Ryde, Australia).

After a resting phase of 10 minutes in a lying position, the examination was started with measurements of the BP as described above. Afterwards, PWA was performed on the right radial (RA) artery using a pressure sensor attached to the tip of a pencil-type probe. The ascending aortic pressure wave is calculated using a transfer function out of the pressure waveform of the RA. As these measurements depend on the heart rate, ECG was recorded simultaneously using self-adhesive electrode pads. The central blood pressure and the AIX can be derived non-invasively using the transfer function, as already described[143].

PWV can be assessed by measuring the pulse waveform of the carotid and femoral artery. As described above, the pencil-type probe was placed on the right side of the neck over the carotid artery (CA) and the right groin over the femoral artery (FA). To calculate the PWV, the distance from the suprasternal notch to the carotid artery and femoral artery was measured. The measurements were made with a tape measure[144,145]. These measurements were taken before (0h) 1.5, 4, 6 and 8h post-consumption of the investigated drinks.

2.8 Blood collection

All blood samples were taken using a butterfly cannula, which was placed in the elbow bend of the left arm by an experienced examiner. The blood sampling, as the most invasive of the examinations, was carried out after the FMD measurement as described above.

On the first visit, blood was taken for determination of parameters of clinical chemistry such as total cholesterol, low-density lipoprotein (LDL)- and high-density lipoprotein (HDL), triglycerides (TG), glucose, glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), gamma - glutamyltransferase (γ - GT), uric acid, creatinine, bilirubin, and CRP. The analyses were done by the central laboratory of the University of Düsseldorf.

Blood was also taken for the analysis of CP metabolites and plasma for analysis was obtained by whole-blood centrifugation (EDTA - containing vacutainers) at 1800 *g* for 15 min at 4°C. Afterwards 600µL of plasma was transferred to Eppendorf® tubes and spiked with 2% formic acid. Samples were then stored at -80°C until analysis [124].

2.9 Urine collection

Urine was collected in containers specially designed for this purpose. The containers were acidified using ascorbic acid (3.75g/2L container). After a baseline collection (0h), urine was collected for 24h (0-8h and 8-24h) post-

consumption of the drinks. The containers were always stored in an opaque cooling bag with cooling elements. 600μL of urine was then transferred to Eppendorf® tubes, acidified with formic acid until pH 2,5 was achieved and then stored at -80 °C until analysis.

2.10 Quantification of (poly)phenol metabolites

The following method for the quantification of (poly)phenol metabolites has been described in detail in various publications and is performed as described below[146]Using the Agilent 6550 iFunnel Accurate-Mass Quadrupole Time-of-Flight Mass Spectrometer (QTOF-MS), plasma and urine (poly)phenol metabolites were detected via an electrospray interface with jet stream technology after separation on a 1290 Infinity UPLC system (Agilent, Waldbronn, Germany), later referred to as UPLC-Q-TOF MS.

Plasma and urine (600 μL) were thawed in an ice bath and centrifuged at 15,000 g for 15 min at 4 °C. Supernatant (350 μL) was diluted (1:1) with phosphoric acid 4% to reduce phenolic-protein interactions and spiked with taxifolin as an internal standard (50 nM). Each sample was loaded (600 mL) on a 96 well microelution solid phase extraction plate, washed with 200 μL of water and 200 μL 0.2% acetic acid and finally eluted with methanol (30 μL) followed by 70% acidified acetone (30 μL). The 96 well collection plates were directly put in the UPLC autosampler for immediate analysis. Five ml of each sample were injected in a Zorbax Eclipse Plus RRHD column 2.1 x 50 mm, 1.8 mm with a compatible Eclipse Plus guard column 2.1 x 5 mm, 1.8 mm (Agilent, Waldbronn, Germany). The mobile phase consisted of 0.1% HCOOH (solvent A) and acetonitrile with 0.1% HCOOH (solvent B) in a 10 min gradient program. The elution profile (flow rate of 0.4 mL/min) started at 1% solvent B and increased to 10% after 5 min, to 25% at 8 min and to 99% at 9.1 min at which the percentage of solvent B was held constant for 0.9 min. The gradient was reverted to 1% solvent B for 2 min to equilibrate the column. Samples were analyzed in negative mode with gas temperature 150 °C, gas flow 20 l/min, nebulizer 25 psig, sheath gas temperature 350 °C, sheath gas flow 12 L/min and Vcap 3000 V. Mass accuracy was verified with a peptide reference solution and the instrument was calibrated daily with a standard mixture to provide mass resolution >20,000 and mass accuracy <1 ppm. Data was analyzed and processed using the Mass Hunter Workstation Quantitative Analysis software (version B.06.00, Agilent)[113].

2.11 Diet questionnaire/ Nutritional habits assessment

The nutritional habits of the study participants were asked using a standardized food-frequency questionnaire. 24-hours dietary recall questionnaires were completed at each visit in order to evaluate compliance with the (poly)phenol-free diet.

2.12 Power calculation and statistical analysis

Power calculations were performed for the primary endpoint, change in FMD response. Power was based on the intra-individual variability of the operator who performed the FMD analysis (5% CV, SD = 0.3). At 0.8 power, a 0.05 significance level and a mean FMD of 7.2%, the number of subjects required to detect a difference of 0.3% in the response of matched pairs in a crossover study is ten. This number is consistent with other studies carried out with similar endpoints and study design[39,147,148]. The characteristics of the study population are expressed as mean values and standard deviations. Results are presented as mean values and their standard error of means, and differences between responses are presented as mean values and 95% confidence intervals. Differences in the outcome variables were compared by one-way ANOVA using Tukey post-hoc test. Data not normally distributed were compared with Wilcoxon test. Statistical analysis was performed with GraphPad Prism (version 6.00, GraphPad software, CA, US), and JMP Pro (version 11.0.0; SAS institute Inc., NC, US). Correlations are presented as Pearson's *r*. "[104]. Pharmacokinetic parameters were calculated using the PKSolver add-in software for Microsoft Excel [149].

3 Results

3.1 Baseline characteristics of study population

A total of 15 male volunteers were examined for their suitability (Figure 2). Ten subjects were included. One study participant was excluded due to arterial hypertension (systolic BP > 160 mmHg), another participant was a vegetarian, and three volunteers were under medication. Ten volunteers did not show anamnestic evidence of cardiovascular disease or other pre-defined exclusion criteria. The Framingham risk score, which reflects the 10-year risk of suffering from CHD, was 1.0 ± 0.5%. The average age was 24 ± 2 years. The average body weight was 79 ± 8 kg and the average BMI was 24 ± 2 kg/m^2. The characteristics are shown in Table 3. The cardiovascular risk at baseline (10-year CAD risk) was low (1.0 ± 0.5%), according to the Framingham risk score.

Table 3: Baseline characteristics of study population (n = 10) and laboratory values. Males are mean and standard deviation

Characteristics	
Age (years)	24 ± 2
Body weight (kg)	79 ± 8
BMI (kg/m^2)	24 ± 2
Total cholesterol (mg/dL)	149 ± 33
LDL cholesterol (mg/dL)	90 ± 31
HDL cholesterol (mg/dL)	49 ± 7
Triglycerides (mg/dL)	66 ± 17
Glucose (mg/dL)	88 ± 5
GOT (U/L)	24 ± 5
GPT (U/L)	22 ± 6
γ - GT (U/L)	19 ±7
Uric acid (mg/dL)	6 ± 1
Creatinine (mg/dL)	1.0 ± 0.1
Bilirubin (mg/dL)	0.5 ± 0.3
Heart rate (bpm)	60 ± 8
Systolic blood pressure (mmHg)	119 ± 8
Diastolic blood pressure (mmHg)	66 ± 10
Flow-mediated dilation (%)	6.0 ± 1.4
Pulse wave velocity(m/s)	5.3 ± 0.8
Augmentation index (%)	-4.3 ± 14

The measured baseline values for systolic BP, FMD, PWV, and AIX of the participants did not differ significantly from each other and all values on Table 3 were all within the value range for healthy young subjects. All drinks were

tolerated by the volunteers and no side effects were observed[124]. All participants signed a consent form before starting the study.

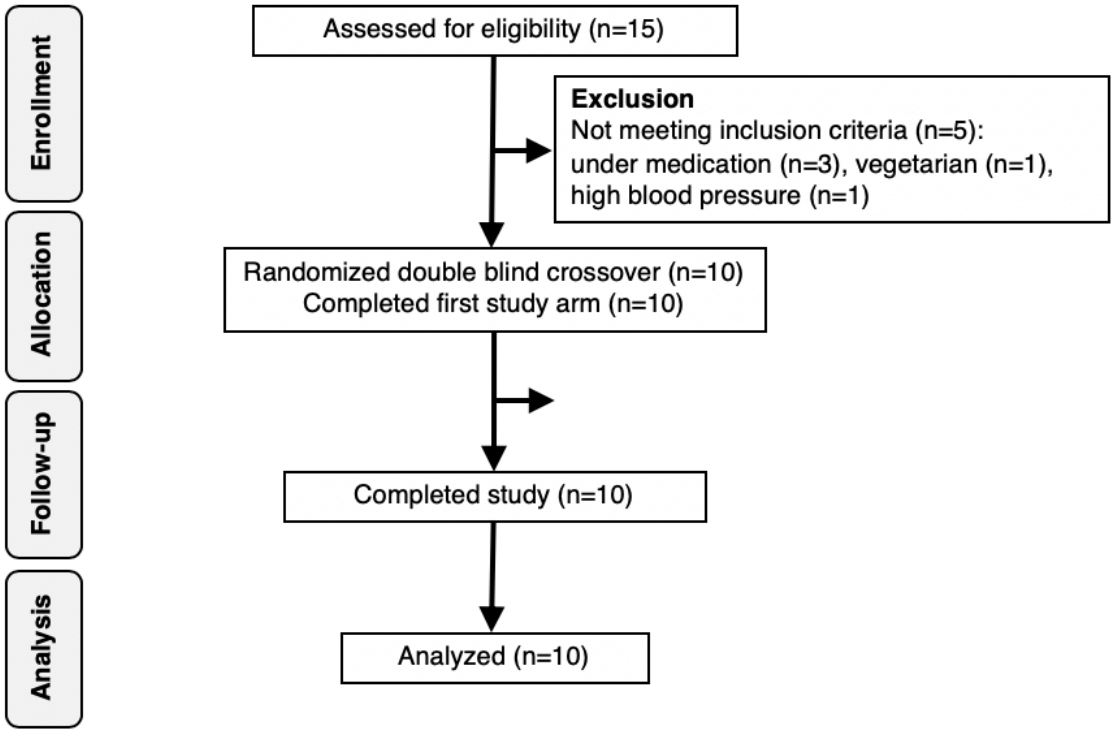

Figure 7: Study flow

3.2 Flow-mediated dilation

The participating volunteers received a cranberry drink or a control drink on six different days with one-week washout between the study days. FMD, as the primary endpoint, was measured 1, 2, 4, 6 and 8 h post-consumption of the cranberry(poly)phenol containing drink or the control drink. The participants did not differ significantly in their baseline values as seen in Table 3.

A significant increase in FMD was shown after single intake of a cranberry drink. This effect was observed for drinks containing 409 - 1910 mg total (poly)phenols (TP). The FMD increased continuously from 1h after consumption of cranberry(poly)phenol containing drinks with a maximum at 4 hours post-consumption. The increase in FMD was dose-dependent with the strongest effect after consumption of the drink containing 1238 mg TP. The cranberry (poly)phenol intake necessary to achieve half-maximal effects (ED_{50}) was calculated to be 436 mg (95% CI 226, 841 mg). When comparing the FMD change after consumption of the drinks containing 409 - 1910 mg TP with the FMD changes after consumption of the control drink, it is as follows:

- Significant increase of FMD at 2h after consumption of the drink containing 409 mg TP in comparison with the control drink (Figure 8 A, and B).
- Significant increases in FMD at 1, 2, 6, and 8h after consumption of the drink containing 787 mg TP in comparison with the control drink (Figure 8 A, and C)
- Significant increases in FMD at 1, 2, 4, and 6h after consumption of the drink containing 1238 mg TP in comparison with the control drink (Figure 8 A, and D)
- Significant increases in FMD at 1, 2, 4, 6, and 8h after consumption of the drinks containing 1534 and 1910 mg TP in comparison with the control drink (Figure 8 A, E, and F).

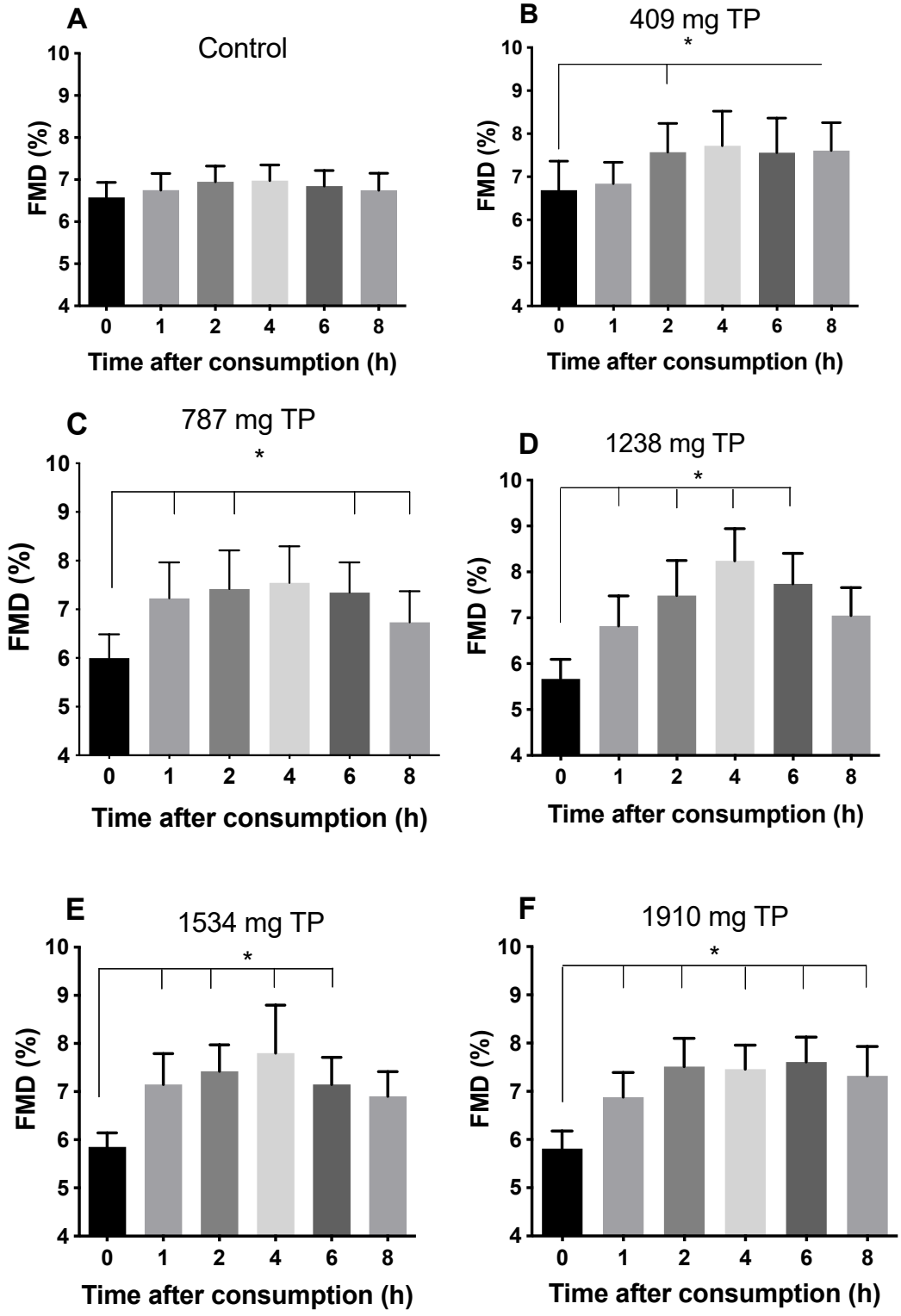

Figure 8:Flow-mediated dilation (FMD) before and after consumption of the cranberry juice drinks containing 1910, 1534, 1238, 787 and 409 mg of total (poly)phenols (TP), respectively, and the control drink (n=10). *p<0.05 significantly different from baseline

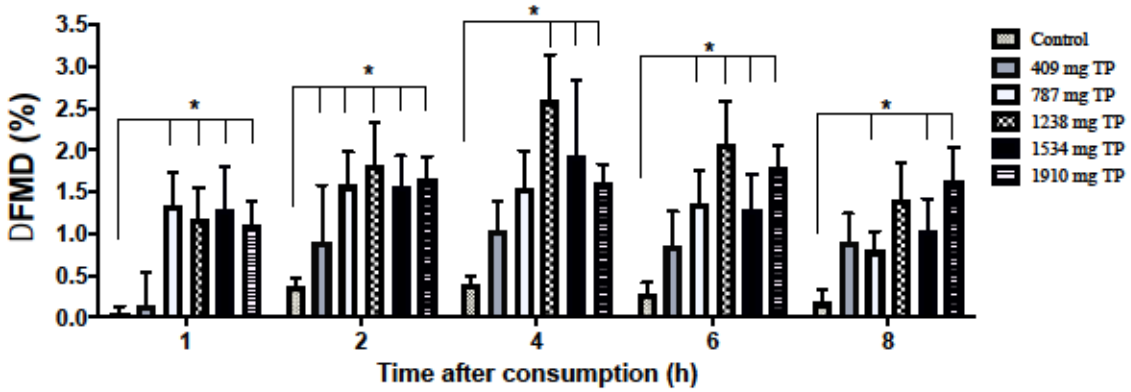

Figure 9: Changes in FMD respect to baseline after consumption of the cranberry juice drinks containing 409, 787, 1238, 1534, and 1910 mg of total (poly)phenols, respectively) and the control drink (n=10)

The area under the curve for FMD% versus time after consuming juice containing (poly)phenols showed a significant increase (p values of 0.025, 0.03, 0.004, and 0.002 for doses of 787, 1238, 1534, and 1910 mg TP respectively) compared to the control drink, except for the lowest dose of 409 mg TP.

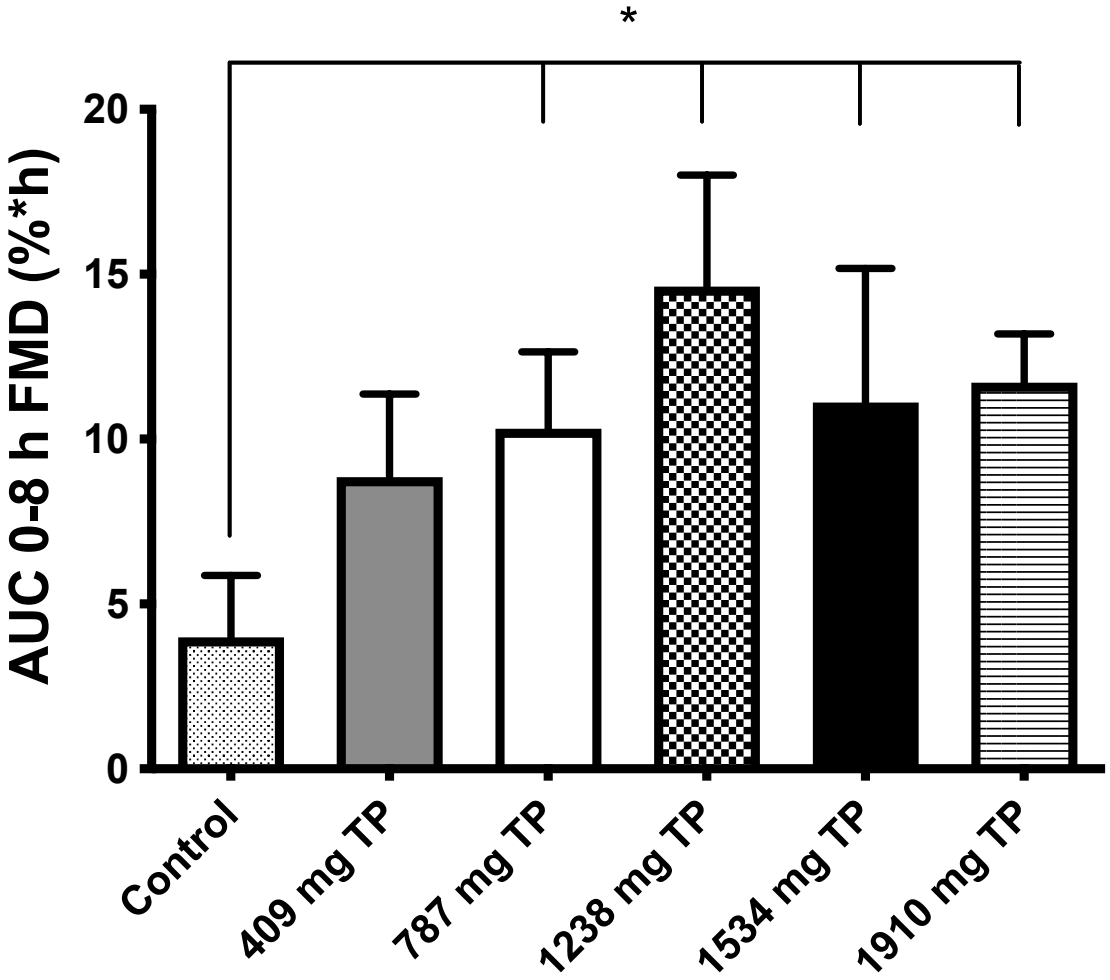

Figure 10: Changes in AUC of the FMD response over time after consumption of the cranberry juice drinks containing 409, 787, 1238, 1534, and 1910 mg of total (poly)phenols and the control drink (n = 10). *p<0.05 significantly different from baseline

3.3 Pulse wave velocity, PWA and AIX

PWV, PWA and AIX, as secondary endpoints, were assessed 1, 1.5, 4, 6, and 8 h post-consumption of the CP containing drinks or the control drink. The participants did not differ significantly in their baseline values as seen in Table 3: Baseline characteristics of study population (n = 10) and laboratory values. Males are mean and standard deviation.

A significant decrease in AIX with respect to baseline was also observed after 4 and 6 h post-consumption of drink containing 409 mg TP (–15 ± 3.4%, –16 ± 3.5% versus –6.1 ± 2.1%, p = 0.0341 and 0.0342, respectively)(Figure 11 A, and B), and after 1.5 and 6 h post-consumption of the cranberry drink containing 1534 mg TP (–14 ± 3.2%, –13 ± 2.9% versus –4.2 ± 2.5%, p = 0.0306 and 0.0483, respectively (Figure 11 A, and E).

No changes in carotid - femoral PWV were observed after consumption of any of the test drinks (data not shown).

Comparing changes in PWV after consumption of the cranberry drinks with changes in PWV after consumption of the control drink, no significant changes were observed (Figure 13)

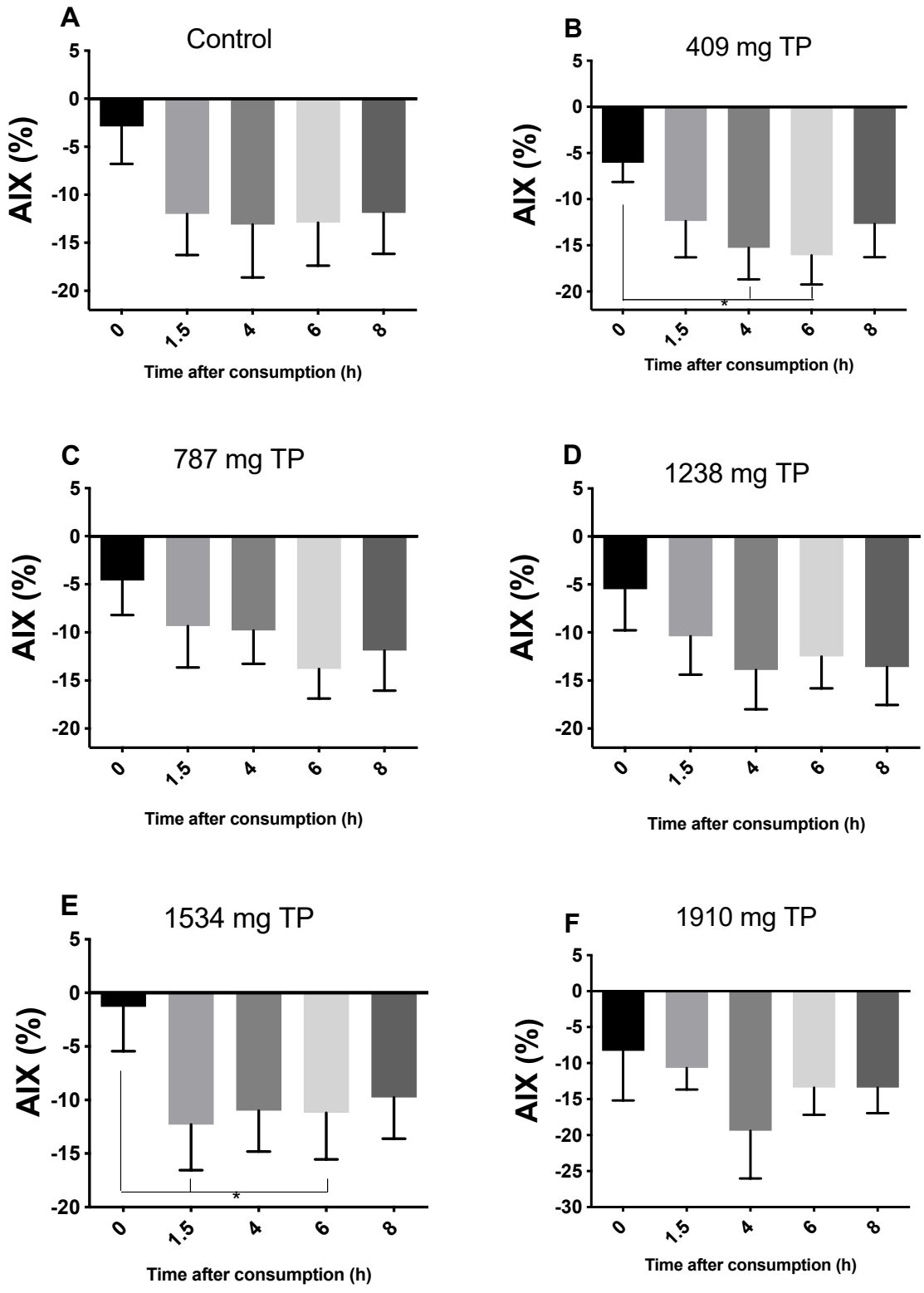

Figure 11: Augmentation Index (AIX) before and after consumption of the cranberry juice drinks containing 1910, 1534, 1238, 787 and 409 mg of total (poly)phenols, respectively) and the control drink (n=10). *p<0.05 significantly different from baseline

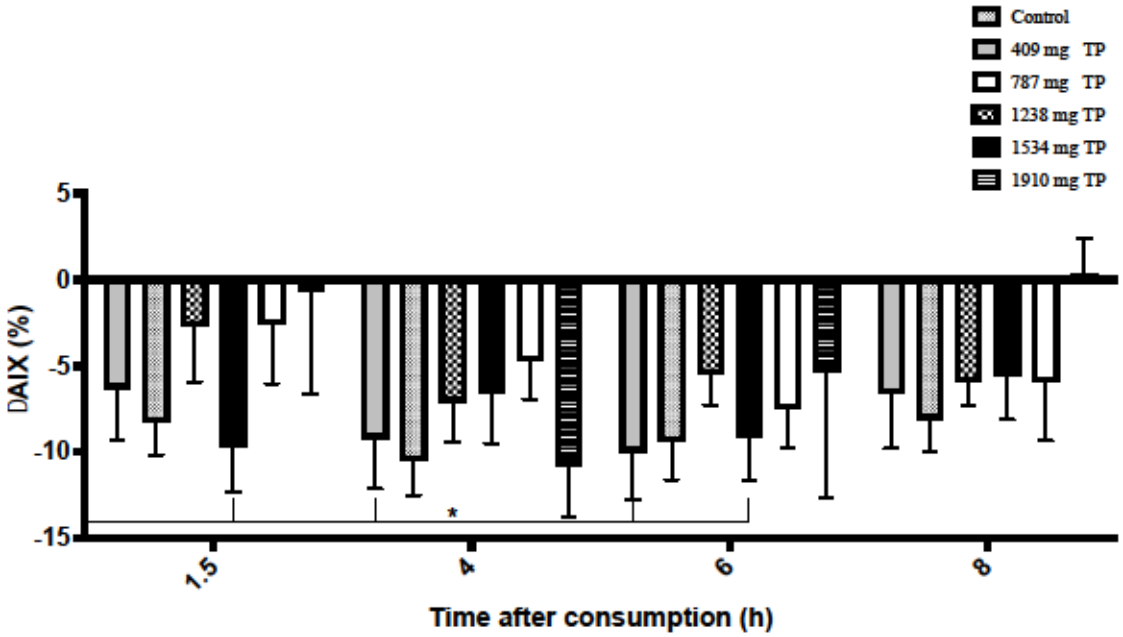

Figure 12: Changes in Augmentation Index (AIX) respect to baseline after consumption of the cranberry juice drinks containing 1910, 1534, 1238, 787 and 409 mg of total (poly)phenols, respectively) and the control drink (n=10)

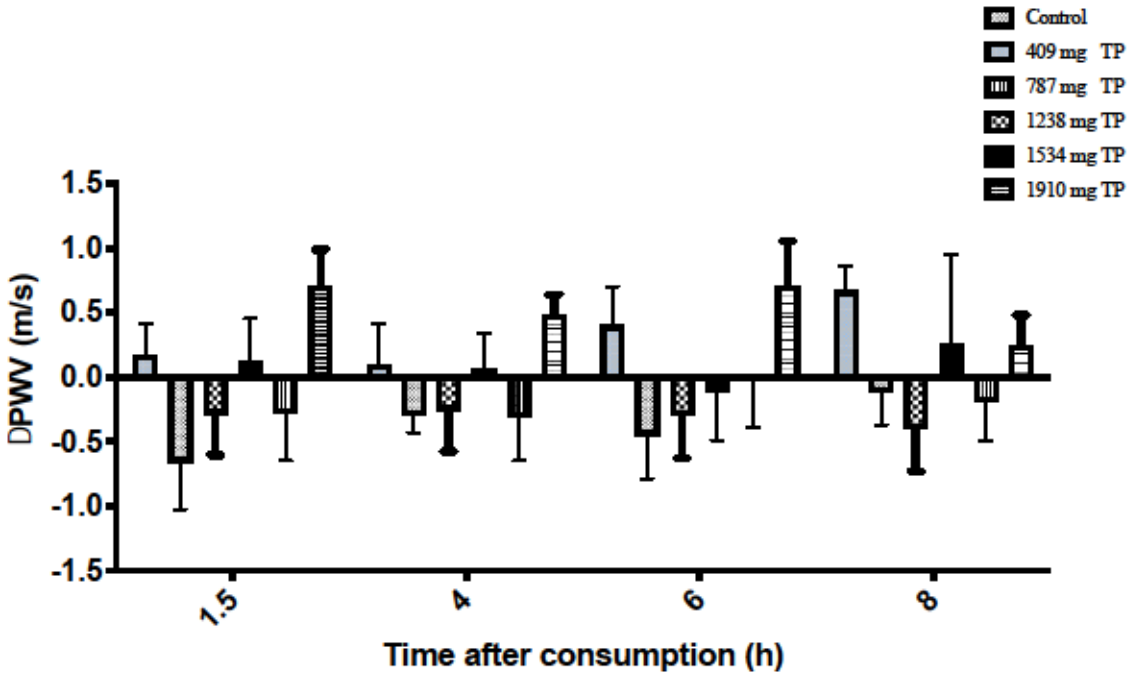

Figure 13: Changes in Pulse Wave Velocity (PWV) respect to baseline after consumption of the cranberry juice drinks containing 1910, 1534, 1238, 787 and 409 mg of total (poly)phenols, respectively) and the control drink (n=10)

3.4 Blood pressure

When comparing changes in blood pressure (BP) after consumption of the cranberry drinks containing 1910, 1534, 1238, 787 and 409 mg of total (poly)phenols with changes in BP after consumption of the control drink, no significant differences were found (data not shown).

A significant decrease in CSBP was only observed 6h after consumption of the drink containing the largest amount of TP (1910 mg TP) compared to the baseline (97 ± 1.6 mmHg versus 107 ± 2.6 mm Hg, p = 0.0351) (Figure 14)

No significant differences were found in diastolic blood pressure (CDBP), either comparing with the baseline or with the control drink (Figure 15)

No significant changes were observed in peripheral systolic and diastolic blood pressure at any time point after consumption of the test drinks, when compared with baseline or when compared with the control drink (data not shown).

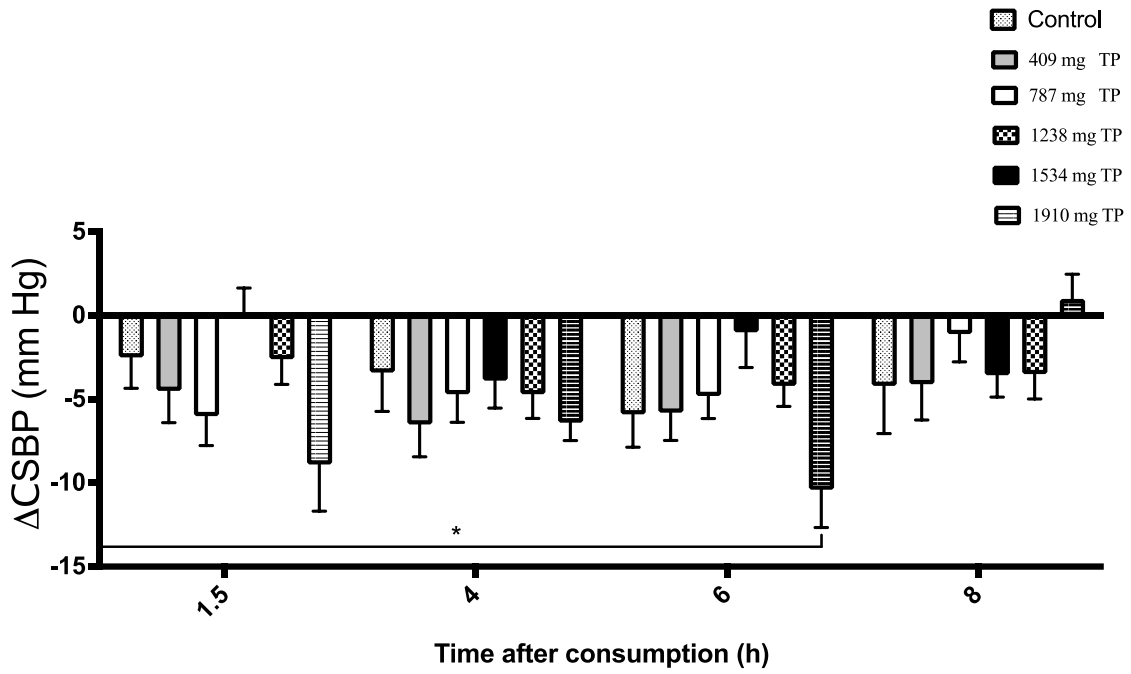

Figure 14: Changes in central systolic blood pressure (CSBP) respect to baseline after consumption of the cranberry juice drinks containing 1910, 1534, 1238, 787 and 409 mg of total (poly)phenols, respectively) and the control drink (n=10)

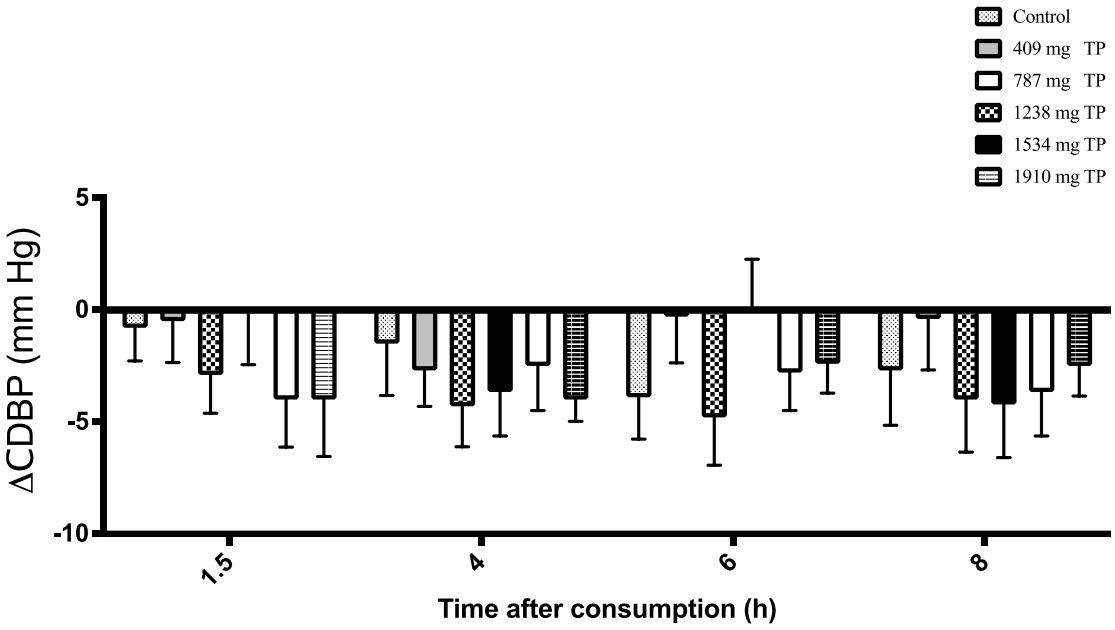

Figure 15: Changes in central diastolic blood pressure (CDBP) respect to baseline after consumption of the cranberry juice drinks 261, 558, 573, 519, 997 (containing 1910, 1534, 1238, 787 and 409 mg of total (poly)phenols, respectively) and the control drink 819

3.5 Plasma and urine analysis

The identification and quantification of cranberry-derived (poly)phenol metabolites in plasma and urine after consumption of cranberry juice was performed using UPLC-Q-TOF MS detection with authentic standards. 60 compounds were identified in plasma analyzing plasma after consumption the drinks containing 409, 787, 1238, 1534 and 1910 mg total (poly)phenols. To the best of our knowledge, 43 of these metabolites have never been previously reported in either urine or plasma after cranberry consumption.

3.5.1 Identification of novel cranberry (poly)phenol metabolites

Most of the detected metabolites were phenolic acid derivatives, both conjugated and non-conjugated, with only 3 being flavonoids, specifically flavonols (kaempferol, kaempferol-3-O-β-D-glucuronide, and quercetin-3O-β-D-glucuronide). Out of the 60 metabolites, 23 were cinnamic and hydroxycinnamic acid derivatives, including 11 ferulic acid, 6 caffeic acid, and 3 coumaric acid metabolites. Thirteen metabolites were benzoic and hydroxybenzoic acid derivatives, such as vanillic, syringic, protocatechuic, and gallic acid metabolites. Six compounds were derived from pyrogallol and catechol, while others were phenyl acetic acid (6 derivatives), hippuric acid (5 derivatives), flavonol (3 derivatives), and benzaldehyde related (2 derivatives). The γ-valerolactone metabolite (5R)-5(30-hydroxyphenyl-γ-valerolactone-40-O-sulfate) was also detected in both plasma and urine in significant concentrations. Fourteen sulfated and nine glucoronidated metabolites were identified. Among the sulfated, five were derivatives of small phenols (one catechol and four pyrogallol sulfates), four ferulic acid metabolites, two vanillic acid sulfates, one caffeic acid sulfate, one methylated gallic acid sulfate, and one (5R)-5'-(3'-hydroxyphenyl)- γ-valerolactone-4'-O-sulfates. The glucuronides included 7 from ferulic and caffeic acids and 2 flavonol glucuronides (kaempferol-3-O-β-D-glucuronide and quercetin-3O-β-D-glucuronide). In urine, a similar profile of metabolites was observed compared to plasma, with a few exceptions. Ferulic and cinnamic acids were not detected in urine, while quercetin and 2-(4-hydroxyphenoxy) propionic acid were found in urine but not in plasma.

3.5.2 Quantification of (poly)phenol metabolites in plasma

Maximal concentrations (C_{max}) for each metabolite varied between the low nanomolar and high micromolar range. The compounds that were detected in the highest concentration in plasma after cranberry consumption were hippuric acids, representing 45% of the total, followed by catechols (22%), benzoic acids (15%), phenylacetic acids (10%), and cinnamic acid derivatives (7%). For hippuric acid,

a C_{max} of 43.0 μM 22h post-consumption and for catechol-O-sulfate, a C_{max} of 24.6 μM 7h post-consumption could be measured. Other metabolites found in high concentrations were 2,3-dihydroxybenzoic acid (C_{max} = 12.0 μM), phenylacetic acid (C_{max} = 8.3 μM), isoferulic acid (C_{max} = 4.6 μM), 4-methylcatechol-O-sulfate (C_{max} = 3.5 μM), a-hydroxyhippuric acid (C_{max} = 3.0 μM), ferulic acid 4-O-sulfates (C_{max} = 2.3 μM), benzoic acid (C_{max} = 2.2 μM), 4-hydroxyphenylacetic acid (C_{max} = 1.8 μM), dihydro caffeic acid 3-O-sulfates (C_{max} = 1.7 μM), and vanillic acid-4-O-sulfates (C_{max} = 1.0 μM).

The metabolites reached their maximum in plasma at different times (T_{max}). Benzoic acids, such as protocatechuic acid, syringic acid, and vanillic acids, as well as cinnamic acid metabolites, such as caffeic, ferulic, isoferulic acids, and their glucuronidated forms reached T_{max} 1-2 h post-consumption. A similar T_{max} was observed for sinapic, chlorogenic, o- and p-coumaric acids, as well as for the glucuronides of quercetin and kaempferol. γ- valerolactone sulfate showed a T_{max} of 3h. All other phenylacetic acids, benzaldehydes, pyrogallols, catechols, and hippuric derivatives, as well as the sulfates and glucuronides of the remaining cinnamic acid metabolites, reached their T_{max} between 4-22h post-consumption of the cranberry drink[124].

The T_{max}, or the time required to reach C_{max}, differed depending on the metabolite and the concentration of (poly)phenols in the juice. The T_{max} of individual metabolites appeared to be influenced by the level of TP in cranberry juice. For instance, the T_{max} of 3-(4-hydroxyphenyl) propionic acid ranged from 7.2 hours (with 409 mg TP juice) to 13.8 hours (with 787 mg TP juice)[150].

Table 4: Kinetics of (poly)phenol metabolites identified in plasma after consumption of cranberry juice containing 787mg of (poly)phenols (n = 10). Compounds with an asterisk (*) represent novel metabolites reported here for the first time in plasma after cranberry juice consumption. Area under the curve (AUC) of the concentration in plasma over 24 h. Results are given as mean ± SEM (n = 10).

Pharmacokinetic parameters	**AUC (nM*h)**	**C_{max} (nM)**	**T_{max} (h)**
Benzoic acid derivatives			
Benzoic acid	42,207 ± 12,182	2,169 ± 608	8.6 ± 3.0
2-Hydroxybenzoic acid	4,857 ± 2261	308 ± 128	6.3 ± 3.3
3-Hydroxybenzoic acid*	1,114 ± 374	66 ± 23	6.1 ± 2.4
4-Hydroxybenzoic acid*	605 ± 101	42 ± 7	5.3 ± 2.5
2,3-Dihydroxybenzoic acid	185,530 ± 55,774	12,024 ± 4,055	7.3 ± 3.2
2,4-Dihydroxybenzoic acid	280 ± 54	22 ± 4	5.1 ± 2.4
2,5-Dihydroxybenzoic acid	5,888 ± 1,937	425 ± 137	4.4 ± 0.3
Protocatechuic acid	1,196 ± 442	109 ± 45	2.0 ± 0.6
Syringic acid*	21 ± 20	8±6	0.3 ± 0.2
Vanillic acid	3,700 ± 1,070	410 ± 115	1.8 ± 0.8
Vanillic acid-4-O-sulfate*	11,791 ± 3,372	1,054 ± 274	2.1 ± 0.8
Isovanillic acid*	3,087 ± 558	220 ± 44	9.9 ± 3.6
4-Methylgallic-3-O-sulfate*	2,188 ± 592	275 ± 82	2.1 ± 0.3
Phenylacetic acid derivatives			
Homovanillic acid	9,005 ± 2,913	511 ± 165	5.6 ± 0.9
Homovanillic acid sulfate*	9,005 ± 2,913	30 ± 10	10.2 ± 3.5
Phenylacetic acid*	123,805 ± 28,913	8,304 ± 1,886	9.4 ± 3.7
3,4-Dihydroxyphenyl acetic acid	6,348 ± 2,188	476 ± 138	7.3 ± 2.3
3-Hydroxyphenyl acetic acid*	9,293 ± 5,484	615 ± 360	10.9 ± 3.4
4-Hydroxyphenyl acetic acid	30,711 ± 12,082	1,849 ± 724	13.8 ± 4.0
Benzaldehyde derivatives			
4-Hydroxybenzaldehyde*	1,204 ± 359	77 ± 18	4.9 ± 2.5
3,4-Dihydroxybenzaldehyde*	618 ± 191	34 ± 10	4.1 ± 2.6
Pyrogallol derivatives			
Pyrogallol-O-1-sulfate*	3,393 ± 1,338	199 ± 79	8.7 ± 3.1
Pyrogallol-O-2-sulfate*	3,007 ± 1,095	339 ± 123	6.2 ± 2.3
1-Methylpyrogallol-O-sulfate*	5,613 ± 1,395	538 ± 172	9.1 ± 3.8
2-Methylpyrogallol-O-sulfate*	2,821 ± 596	185 ± 44	5.3 ± 2.6
Catechol derivatives			
Catechol-O-sulfate*	346,432 ± 61,239	24,555 ± 3,775	7.1 ± 0.4
4-Methylcatechol-O-sulfate*	54,214 ± 18,798	3,497 ± 1,192	7.7 ± 3.2
Hippuric acid derivatives			
Hippuric acid*	758,245 ± 223,886	42,926 ± 12,282	21.8 ± 2.2
2-Hydroxyhippuric acid*	89 ± 28	7 ± 2	6.4 ± 3.3
3-Hydroxyhippuric acid*	718 ± 128	45 ± 7	8.3 ± 3.9
4-Hydroxyhippuric acid*	6,111 ± 1439	592 ± 157	3.8 ± 2.6
α-Hydroxyhippuric acid*	34,824 ± 871	2,943 ± 587	3.9 ± 2.5
Cinnamic acid derivatives			

Cinnamic acid*	1,888 ± 399	123 ± 34	6.2 ± 2.4
Caffeic acid	1 ± 1	1±1	0.1 ± 0.1
Caffeic acid 3-O-β-D-glucuronide*	35 ± 10	16 ± 4	1.1 ± 0.2
Caffeic acid 4-O-β-D-glucuronide*	380 ± 69	59 ± 8	1.2 ± 0.1
Dihydro caffeic acid*	1,451 ± 535	93 ± 32	7.6 ± 3.3
Dihydro caffeic acid 3-O-sulfate*	15,079 ± 10,166	1,656 ± 1,116	8.1 ± 3.1
Dihydro caffeic acid 3-O-β-D-glucuronide*	1,291 ± 207	84 ± 11	22.2 ± 1.8
Ferulic acid	148 ± 51	47 ± 12	1.0 ± 0.0
Ferulic acid 4-O-glucuronide*	1,062 ± 181	165 ± 29	1.3 ± 0.2
Ferulic acid 4-O-sulfate*	11,484 ± 5522	2,268 ± 794	3.6 ± 2.6
Dihydro ferulic acid*	3,241 ± 1294	304 ± 122	6.1 ± 3.4
Dihydro ferulic acid 4-O-sulfate*	2,037 ± 950	197 ± 96	6.8 ± 3.3
Dihydro ferulic acid 4-O-β-D-glucuronide*	2,061 ± 512	201 ± 59	7.1 ± 3.3
Isoferulic acid*	79,971 ± 46,457	4,592 ± 2,251	3.8 ± 2.5
Isoferulic acid 3-O-sulfate*	529 ± 90	49 ± 6	4.1 ± 2.5
Isoferulic acid 3-O-β-D-glucuronide*	2,534 ± 538	387 ± 95	1.9 ± 0.6
Dihydro isoferulic acid 3-O-sulfate*	859 ± 449	97 ± 42	4.0 ± 2.5
Dihydro isoferulic acid 3-O-β-D-glucuronide*	287 ± 106	23 ± 10	5.1 ± 2.5
m-Coumaric acid*	322 ± 150	29 ± 14	3.2 ± 0.8
o-Coumaric acid*	99 ± 13	6±1	1.2 ± 0.3
p-Coumaric acid	320 ± 133	131 ± 51	1.0 ± 0.0
Sinapic acid	358 ± 84	46 ± 11	1.9 ± 0.8
Chlorogenic acid*	40 ± 35	5±2	0.7 ± 0.3
Flavonol derivatives			
Kaempferol	1,035 ± 305	59 ± 18	6.8 ± 2.3
Kaempferol-3-O-β-D-glucuronide*	118 ± 12	13 ± 1	2.1 ± 0.4
Quercetin-3-O-β-D-glucuronide*	910 ± 162	156 ± 35	1.8 ± 0.4
Valerolactone derivatives			
(5R)-5-(3´-hydroxyphenyl)-γ-valerolactone-4´-O-sulfate*	2,582 ± 447	306 ± 60	3.1 ± 0.6

After assessing the linear dose response for each plasma metabolite, 14 of them showed a linear dose-response curve in plasma.

Many of the compounds were absorbed in a dose-dependent manner. The plasma concentration increased depending on the TP content of the cranberry drinks.

3.5.3 Quantification of (poly)phenol metabolites in urine

Over 24h a total of 48.4 mg of (poly)phenol metabolites were excreted, with 28.2 mg in the first 8h and 20.2 mg between 8-24h post-consumption of the cranberry drink. Hippuric acids accounted for the majority (64%), 14% were phenylacetic acids, followed by benzoic acids (9%), catechols (5%), valerolactones (4%) and cinnamic acids (3%). The exact excreted amounts of (poly)phenol metabolites are listed in Table 5.

Table 5: Excretion of cranberry (poly)phenol metabolites in urine after 8 and 24h of cranberry juice consumption. Compounds with an asterisk (*) represent novel metabolites reported here for the first time in urine after cranberry juice consumption. Results are given as mean ± SEM (n = 10).

Urinary excretion	**nmoles**			**µg**		
	8 h	**24 h**	**Total**	**8 h**	**24 h**	**Total**
Benzoic acid derivatives						
Benzoic acid	3,279 ± 477	861 ± 200	4,141 ± 427	400 ± 58	105 ± 24	506 ± 52
2-Hydroxybenzoic acid	24 ± 17	4 ± 2	29 ± 17	3 ± 2	1 ± 0	4 ± 2
3-Hydroxybenzoic acid*	18 ± 4	23 ± 4	41 ± 7	2 ± 1	3 ± 1	6 ± 1
4-Hydroxybenzoic acid	1,101 ± 298	1,451 ± 317	2,552 ± 434	152 ± 41	200 ± 44	352 ± 60
2,3-Dihydroxybenzoic acid	3,373 ± 778	5,060 ± 1,756	8,432 ± 2,176	520 ± 120	780 ± 271	1,300 ± 335
2,4-Dihydroxybenzoic acid	71 ± 14	185 ± 96	257 ± 108	11 ± 2	29 ± 15	40 ± 17
2,5-Dihydroxybenzoic acid	2,184 ± 291	2,722 ± 682	4,906 ± 797	337 ± 45	420 ± 105	756 ± 123
Protocatechuic acid	626 ± 137	318 ± 57	944 ± 162	96 ± 21	49 ± 9	146 ± 25
Syringic acid*	113 ± 25	137 ± 23	249 ± 31	22 ± 5	27 ± 5	49 ± 6
Vanillic acid	330 ± 128	93 ± 53	423 ± 136	56 ± 22	16 ± 9	71 ± 23
Vanillic acid-4-*O*-sulfate*	128 ± 40	160 ± 57	288 ± 87	32 ± 10	40 ± 14	72 ± 22
Isovanillic acid*	481 ± 142	757 ± 163	1,238 ± 229	81 ± 24	127 ± 27	208 ± 39
4-Methylgallic-3-*O*-sulfate*	206 ± 64	91 ± 35	297 ± 74	54 ± 17	24 ± 9	79 ± 20
Phenylacetic acid derivatives						
Homovanillic acid	3,157 ± 943	3,483 ± 1,092	6,640 ± 1,472	575 ± 172	635 ± 199	1,210 ± 268
Homovanillic acid sulfate*	96 ± 27	110 ± 29	206 ± 41	25 ± 7	29 ± 8	54 ± 11
Phenylacetic acid*	6,436 ± 2,243	8,317 ± 4,083	14,753 ± 6,184	876 ± 305	1,132 ± 556	2,009 ± 842
3,4-Dihydroxyphenyl acetic acid	1,050 ± 219	547 ± 101	1,597 ± 297	176 ± 37	92 ± 17	269 ± 50
3-Hydroxyphenyl acetic acid*	1,900 ± 546	2,485 ± 679	4,384 ± 922	289 ± 83	378 ± 103	667 ± 140
4-Hydroxyphenyl acetic acid	4,330 ± 1,006	5,821 ± 2,360	10,152 ± 3,161	659 ± 153	886 ± 359	1,545 ± 481
Propionic acid derivatives						
2-(4-hydroxyphenoxy)propionic acid	247 ± 158	206 ± 57	453 ± 152	45 ± 29	37 ± 10	82 ± 28
Benzaldehyde derivatives						
4-Hydroxybenzaldehyde*	66 ± 28	78 ± 25	144 ± 41	8 ± 3	10 ± 3	18 ± 5
3,4-Dihydroxybenzaldehyde*	110 ± 27	112 ± 23	222 ± 36	15 ± 4	15 ± 3	31 ± 5

Urinary excretion	nmoles			µg		
	8 h	24 h	Total	8 h	24 h	Total
Pyrogallol derivatives						
Pyrogallol-*O*-1-sulfate*	34 ± 15	112 ± 64	146 ± 76	7 ± 3	23 ± 13	30 ± 16
Pyrogallol-*O*-2-sulfate*	127 ± 46	175 ± 70	302 ± 72	26 ± 9	36 ± 14	62 ± 15
1-Methylpyrogallol-*O*-sulfate*	165 ± 37	188 ± 70	353 ± 97	36 ± 8	41 ± 15	78 ± 21
2-Methylpyrogallol-*O*-sulfate*	53 ± 13	70 ± 20	123 ± 26	12 ± 3	16 ± 4	27 ± 6
Catechol derivatives						
Catechol-*O*-sulfate*	4,670 ± 1,439	5,826 ± 1,899	10,496 ± 2,257	888 ± 274	1,108 ± 361	1,996 ± 429
4-Methylcatechol-*O*-sulfate*	564 ± 219	1,061 ± 380	1,625 ± 518	115 ± 45	217 ± 78	332 ± 106
Hippuric acid derivatives						
Hippuric acid	38,236 ± 10,686	31,481 ± 6,044	69,717 ± 13,686	6,851 ± 1,915	5,640 ± 1,083	12,491 ± 2,452
2-Hydroxyhippuric acid	393 ± 89	418 ± 135	811 ± 172	77 ± 17	82 ± 26	158 ± 34
3-Hydroxyhippuric acid*	692 ± 109	791 ± 237	1,484 ± 270	135 ± 21	154 ± 46	290 ± 53
4-Hydroxyhippuric acid*	13,821 ± 4,842	5,674 ± 2,409	19,495 ± 6,618	2,697 ± 945	1,107 ± 470	3,805 ± 1,292
α-Hydroxyhippuric acid*	48,621 ± 18,907	25,917 ± 7,701	74,538 ± 20,636	9,489 ± 3,690	5,058 ± 1,503	14,548 ± 4,028
Cinnamic acid derivatives						
Caffeic acid	36 ± 8	39 ± 20	75 ± 22	7 ± 1	7 ± 4	13 ± 4
Caffeic acid 3-*O*-β-D-glucuronide*	35 ± 12	35 ± 9	70 ± 18	13 ± 4	13 ± 3	25 ± 6
Caffeic acid 4-*O*-β-D-glucuronide*	24 ± 5	25 ± 8	50 ± 12	9 ± 2	9 ± 3	18 ± 4
Dihydro caffeic acid*	80 ± 26	104 ± 26	184 ± 41	15 ± 5	19 ± 5	34 ± 7
Dihydro caffeic acid 3-*O*-sulfate*	255 ± 60	162 ± 54	417 ± 82	67 ± 16	42 ± 14	109 ± 22
Dihydro caffeic acid 3-O-β-D-glucuronide*	65 ± 15	64 ± 15	129 ± 20	23 ± 5	23 ± 5	46 ± 7
Ferulic acid 4-*O*-glucuronide*	50 ± 10	59 ± 15	109 ± 20	18 ± 4	22 ± 5	40 ± 7
Ferulic acid 4-*O*-sulfate*	731 ± 185	324 ± 149	1,055 ± 259	201 ± 51	89 ± 41	289 ± 71
Dihydro ferulic acid*	457 ± 373	67 ± 20	524 ± 368	90 ± 73	13 ± 4	103 ± 72
Dihydro ferulic acid 4-*O*-sulfate*	512 ± 111	489 ± 118	1,001 ± 176	141 ± 31	135 ± 33	276 ± 49
Dihydro ferulic acid 4-*O*-β-D-glucuronide*	211 ± 110	143 ± 40	353 ± 117	78 ± 41	53 ± 15	132 ± 44

Urinary excretion	nmoles			µg		
	8 h	24 h	Total	8 h	24 h	Total
Isoferulic acid*	889 ± 225	0 ± 0	889 ± 225	173 ± 44	0 ± 0	173 ± 44
Isoferulic acid 3-*O*-sulfate*	139 ± 29	140 ± 66	279 ± 88	38 ± 8	38 ± 18	76 ± 24
Isoferulic acid 3-*O*-β-D-glucuronide*	99 ± 20	115 ± 30	213 ± 41	37 ± 8	42 ± 11	79 ± 15
Dihydro isoferulic acid 3-*O*-sulfate*	126 ± 29	108 ± 23	234 ± 35	35 ± 8	30 ± 6	65 ± 10
Dihydro isoferulic acid 3-*O*-ß-D-glucuronide*	385 ± 161	374 ± 163	760 ± 318	143 ± 60	139 ± 61	283 ± 118
m-Coumaric acid*	121 ± 39	51 ± 19	172 ± 50	20 ± 6	8 ± 3	28 ± 8
o-Coumaric acid*	14 ± 4	8 ± 2	22 ± 4	2 ± 1	1 ± 0	4 ± 1
p-Coumaric acid	34 ± 9	2 ± 1	36 ± 9	6 ± 2	0 ± 0	6 ± 1
Sinapic acid*	79 ± 16	35 ± 8	114 ± 17	18 ± 4	8 ± 2	26 ± 4
Chlorogenic acid*	12 ± 4	40 ± 25	52 ± 25	4 ± 2	14 ± 9	19 ± 9
Flavonol derivatives						
Kaempferol	7 ± 2	4 ± 1	11 ± 3	2 ± 1	1 ± 0	3 ± 1
Kaempferol-3-*O*-ß-D-glucuronide*	145 ± 112	34 ± 14	180 ± 116	67 ± 52	16 ± 7	83 ± 54
Quercetin	13 ± 5	5 ± 4	18 ± 9	4 ± 1	1 ± 1	6 ± 3
Quercetin-3-O-ß-D-glucuronide*	79 ± 22	29 ± 12	108 ± 26	38 ± 11	14 ± 6	51 ± 13
Valerolactone derivatives						
(5R)-5-(3′-hydroxyphenyl)-γ-valerolactone-4′-*O*-sulfate*	7,577 ± 2,316	3,388 ± 1,227	10,965 ± 2,757	2,184 ± 668	977 ± 354	3,161 ± 795
Total amount excreted (mg)				28 ± 8	20 ± 4	48 ± 9
Recovery (%)				3.6 ± 1.0	2.6 ± 0.5	6.2 ± 1.1

3.6 Plasma (poly)phenol metabolites correlate with improvements in FMD

A correlation analysis was performed between the increases in % FMD after 1, 2, 4, 6, and 8h as the dependent variable and the concentrations of plasma metabolites as independent variables. In the analysis 12 phenolic metabolites were detected, which could predict the vascular effects (Table 6). Seven of them were cinnamic acid derivatives (caffeic acid, caffeic acid 4-O-ß-D-glucuronide, dihydro caffeic acid 3-O-sulfate, ferulic acid 4-O-sulfate, dihydroferulic acid 4-O-sulfate, dihydro isoferulic acid 3-O-sulfate, cinnamic acid), and three of them were benzoic acid derivatives (vanillic acid-4-O-sulfate, homovanillic acid sulfate, 4-methylgallic- 3-O-sulfate). The only conjugated flavanoid that correlated with changes in FMD was quercetin 3-O-β-D-glucuronide. The proanthocyanidin/flavan-3-ol-derived metabolite, (4R)-5-(3´-hydroxyphenyl)-γ-valerolactone-4´-O-sulfate, also correlated with changes in FMD. Six metabolites correlated with changes in FMD after 1 h, nine metabolites with values after 2 h, seven with 4 h values, eight with 6 h values, and six with 8 h values (Table 6). The AUC of the concentration of all these metabolites in plasma correlated with the AUC of the FMD responses between 0 and 8 h (r = 0.510, p= <0.0001).

Table 6: Significant correlations ($p<0.05$) between plasma cranberry-derived (poly)phenol metabolites and changes in FMD at different timepoints after consumption of cranberry juice with respect to baseline at 0 h (n = 10). Correlations correspond to Pearson's r. *$p<0.05$; #$p<0.01$; §$p<0.001$

Metabolites correlating with ΔFMD at respective timepoints	Pearson's *r*				
	1 h	2 h	4 h	6 h	8 h
(4*R*)-5-(3′-hydroxyphenyl)-γ-valerolactone-4′-*O*-sulfate			0.325*		0.345*
4-Methylgallic acid-3-*O*-sulfate	0.297*	0.460#	0.332*		
Caffeic acid			0.682§		0.337*
Caffeic acid 4-*O*-ß-D-glucuronide		0.320*			
Cinnamic acid		0.303*			
Dihydro caffeic acid 3-*O*-sulfate				0.395#	0.470§
Dihydro ferulic acid 4-*O*-sulfate				0.405#	0.477§
Dihydro isoferulic acid 3-*O*-sulfate	0.367#	0.397#	0.338*	0.371#	0.471§
Ferulic acid 4-*O*-sulfate	0.408#	0.437#	0.362#	0.369#	0.461§
Homovanillic acid sulfate	0.378#	0.428#	0.430#	0.455#	0.339*
Quercetin-3-*O*-ß-D-glucuronide	0.275*	0.423#	0.308*	0.371#	
Vanillic acid-4-O-sulfate	0.387#	0.494§	0.364#	0.347*	

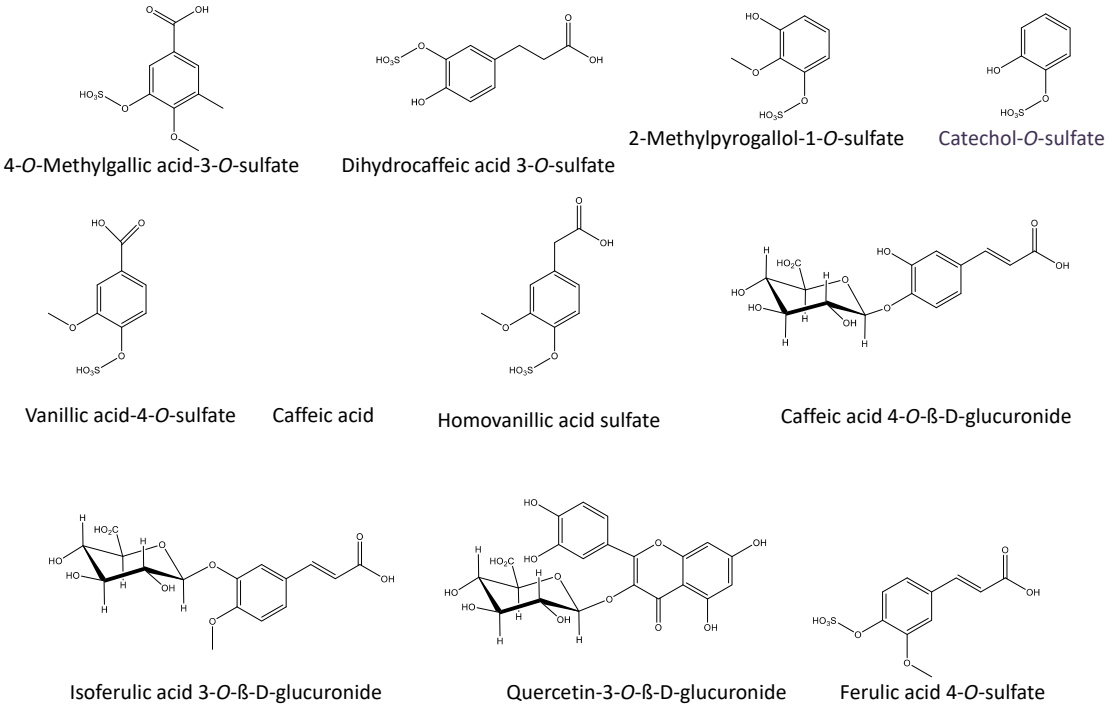

Figure 16: Structures of plasma (poly)phenol metabolites that correlated with changes in FMD after cranberry juice consumption

4 Discussion

The aim of the work was to investigate the effect of cranberry (poly)phenols (CP) on vascular function and give an insight of the ADME. The hypothesis of this work was that increased consumption of CP improves vascular function, which is considered a biomarker of cardiovascular disease risk. An RCT was performed in ten young, healthy men with vascular function measured by FMD as the primary endpoint. Other biomarkers of vascular function, such as PWV and AIX, as well as BP, were also assessed. Furthermore, plasma and urine samples were collected to investigate the absorption, metabolism, and excretion of CP.

The main results presented in this thesis are the following:

1. CP improve FMD up to 2.6% in a dose- and time-dependent manner
2. A total of 60 cranberry-derived (poly)phenol metabolites were identified in plasma and urine
3. Documentation of the absorption, metabolism, and excretion of 43 cranberry-derived (poly)phenol metabolites for the first time after consumption of cranberry juice
4. A total of 12 phenolic metabolites could predict the vascular effects

We could show that the intake of cranberry drinks containing (poly)phenols improve FMD up to 2,6% in a dose- and time dependent manner. To date, four studies have investigated the effect of CP on vascular function in RCTs[101–104]. This study is the first to demonstrate a short-term positive effect on vascular function in healthy volunteers. The other three studies mentioned here examined the effects in patients with CVRF and with different results. For example, Flammer et al. could not demonstrate a short-term or long-term positive effect on vascular function (measured by RHI) by the consumption of (poly)phenol-rich cranberry juice, compared to placebo[101]. Dohadwala et al. also could not demonstrate any long-term positive effects on vascular function (measured by FMD)[102].

Although there are few studies that have examined the effect of CP on vascular function via FMD, there are some that have examined the effect of other berries and their (poly)phenols, such as anthocyanin-rich foods on vascular function. However, since, as mentioned above, CP consist primarily of PACs, some conclusions can be drawn.

Rodriguez-Mateos et al. found a dose-dependent increase in FMD after consumption of pure anthocyanins (ACN). Changes could be observed 2h and 6h after consumption of 160mg of pure anthocyanins. The maximum changes in

FMD were of 1.3% (95% CI = 0.8% to 1.9%) 2h and 1.1% (95% CI = 0.6% to 1.5%) 6h after consumption[151]. These findings are not entirely consistent with our study. We observed the maximum increase in FMD 4h after consumption, whereas after the consumption of 160mg of pure anthocyanins there was no effect measured after 4 hours.

On the other hand, Rodriguez-Mateos et al. could observe a similar increase in FMD after consumption of a blueberry bun containing 637mg of total (poly)phenols (TP). The maximum improvement in FMD happened 2h post-consumption (2.6 ± 0.4%). After consumption of a drink containing freeze dried blueberries (692mg TP) they observed similar changes in FMD. However, the maximum effect on FMD was observed 1h after consumption. They explained this by the fact that solid foods take longer to be transported through the stomach and are therefore metabolized later. No significant effect could be observed after 4h, whereas after 6h there was again a significant increase in FMD observable[94].

Our findings are in agreement with a previous clinical trial where the dose-dependent effects of blueberry (poly)phenol consumption were investigated[39]. The study design, study population, and measurements of endothelial function are comparable to our study. They observed a time dependent increase in FMD, with significant increase at 1h (2.4 ± 0.5%), 2h (1.5 ± 0.4%) and after 6h (1.2 ± 0.6%) post-consumption of a blueberry drink containing 766mg of TP. No significant changes in FMD increase could be observed post-consumption of the drinks containing 1278- and 1791mg TP. The polyphenol intake needed to achieve half-maximal effects (ED_{50}) was found to be 482 mg, which is remarkably similar to this study (436 mg TP; (95% CI 226, 841 mg)). They also found a linear increase in FMD up to 766mg TP. The consumption of more TP did not lead to an improvement in FMD. In our study we observed a linear increase in FMD between 409 to 1238 mg TP, reaching a plateau at higher intake amounts.

Alqurashi et al. found that the consumption of an acai smoothie containing 694 mg of TP significantly increased FMD 2h (+ 1.4%; $p = 0.001$) and at 6h (+ 0.8%; $p < 0.001$) post-consumption on healthy individuals[108]. Istas et al. also found that the intake of red raspberrie drinks containing either 201 mg of TP or 403 mg of TP improved FMD at 2h (+1,6% and +1,2%, respectively) and 24h (+1,0% and +0,7%, respectively) post-consumption on healthy males, whereby there was no difference between the two doses and thus no dose-dependent relationship could be established[109]. Three studies showed a significant increase in RHI values[90,91,101]. In one study del Bo et al. investigated the effect of a single serving of blueberry on peripheral arterial function and on arterial stiffness in young smokers. They recruited 16 male smokers for a 3 – armed randomized-

controlled study, subjecting them either to smoking treatment (one cigarette), blueberry treatment (300g of blueberry) and smoking, or control treatment (300 mL of water with sugar) and smoking. There was one week washout period between each treatment. They measured peripheral arterial function (via RHI), arterial stiffness (digital AIX), blood pressure and heart rate before and 20min after the treatment. They found, that smoking impaired RHI, led to a temporary increase in systolic blood pressure and heart rate, but did not affect arterial stiffness. The consumption of 300g of blueberry reduced the impairment of the reactive hyperemia index induced by smoking (− 4.4 ± 0.8% blueberry treatment vs. − 22.0 ± 1.1% smoking treatment, $p < 0.01$) and Framingham reactive hyperemia (+ 28.3 ± 19.2% blueberry treatment vs. − 42.8 ± 20.0% smoking treatment, $p < 0.0001$), and the increase of systolic blood pressure (+8.4 ± 0.02% blueberry treatment vs. +13.1 ± 0.02% smoking treatment, mmHg, $p < 0.05$) after cigarette smoking[89]. In another study del Bo´et al. recruited 24 healthy males (12 smokers, 12 non-smokers) for two different randomized, controlled, crossover pilot acute studies. In the first study the non-smokers were randomly divided in two groups of six and were either to the control treatment (300ml of water with sugar) or the blueberry treatment (300g blueberry). In the second study the smokers were randomly assigned to 3 groups and underwent 3 different protocols: the smoking treatment, the control treatment (300 mL of water with sugar + smoking) or the blueberry treatment (300 g of blueberry + smoking). Every intervention day was separated by a one-week washout period. Measurements of blood pressure, heart rate, RHI and AIX were performed before and after (120min) each treatment. In the first study they could show that blueberry consumption significantly acute increases peripheral arterial function in non-smokers compared to the control treatment (54.8 ± 8.4% vs. 28.2 ± 8.3%; $p = 0.01$).

Several meta-analyses have shown that a 1% increase in FMD translates into a 10-13% decrease in CVD risk[28,152,153]. The increase in FMD of up to 2,6% showed in our study could be interpreted as a decrease in CVD risk of 20%. However, for a sustained positive effect on CVD risk, a sustained FMD improvement is necessary. To achieve this, a regular, perhaps even daily consumption of cranberry juice or other foods containing cranberry polyphenols would be necessary. Long-term studies are necessary to make a statement about a potentially long-lasting positive effect on CVD risk.

To assess the impact of cranberry (poly)phenols on vascular function, we conducted a study examining plasma levels of phenolic metabolites following cranberry consumption. A limited number of studies have investigated the

bioavailability of cranberry (poly)phenols, but none of them utilized authentic standards of phase II metabolites for quantification. Recently, we identified 60 cranberry-derived phenolic metabolites in human plasma after cranberry juice intake, with 43 of them being previously unknown. Using correlation analysis, we aimed to identify which of these metabolites could account for the observed vascular effects. Out of the 12 phenolic compounds found to be correlated with the vascular response, only one was a flavonoid conjugate from the flavonols present in cranberry (quercetin-3-O-β-D-glucuronide). It has been reported that valerolactone sulfate is likely derived from proanthocyanidins and flavan-3-ols, which are abundant in proanthocyanidin-rich foods such as cocoa flavanols and tea[154–156]. Other metabolites, such as derivatives of cinnamic, benzoic, and phenylacetic acids, were non-specific and could arise from any of the (poly)phenols found in cranberry juice, such as through the direct absorption of hydroxycinnamic acids, breakdown of anthocyanins, or gut microbial metabolism of proanthocyanidins. Furthermore, when the area under the curve (AUC) of all these metabolites were calculated together, a significant correlation with the AUC of the FMD response was also observed.

Our findings suggest that cranberry juice contains a variety of (poly)phenols that can lead to a complex profile of potential bioactive compounds in the bloodstream. These bioactive compounds may work together to improve endothelial function after cranberry juice consumption. However, further research is necessary to establish causality and identify the potential underlying mechanisms of action. Additionally, it's important to note that other nutrients or bioactives in cranberry juice may also contribute to the observed vascular effects. When designing future studies, it's important to consider the observed concentrations of compounds associated with vascular effects, which ranged from low nM to low μM. The mechanisms by which (poly)phenols improve vascular function are still not completely understood. However, it's likely that the improvements in endothelial function observed in our study are due to increased bioavailability of NO, which is known to partially mediate FMD[157,158]. In a recent study, we found that blueberry (poly)phenol metabolites correlated with FMD improvements and inhibited NADPH oxidase activity[39]. Thus, it's possible that cranberry phenolic metabolites may also improve NO bioavailability via NADPH oxidase inhibition. Many of the metabolites that correlated with FMD in our study have structural homologies to apocynin, a pharmacologic NADPH oxidase inhibitor, and have been proposed as potent inhibitors of NADPH oxidase in endothelial cells[159,160]. However, since most of these metabolites are not commercially available, further mechanistic studies are necessary.

Recent research has shown that quercetin and its major metabolites, including quercetin glucuronide, can activate the AMPK pathway and induce endothelial nitric oxide synthase (eNOS) activation, leading to acute endothelial protective effects[161]. It's possible that other potential mechanisms of action, such as regulation of heme oxygenase-1 and NF-E2–related factor 2 (Nrf2) signaling, may also contribute to the vascular effects of (poly)phenols[162].

4.1 Strengths and Limitations

The RCT designed to evaluate the effect of cranberry (poly)phenols on vascular function has some limitations. Nevertheless, we tried to avoid the most common ones as lack of unblinding or absence of adequate controls. The test drinks were indistinguishable from each other by sight or taste. The food that the volunteers got during the intervention was low in (poly)phenols or other known bioactive compounds. The vascular function measurements where always taken by the same investigator following a standard protocol. Nevertheless, there are some limitations. In this thesis, vascular function was investigated by FMD. This technique is commonly considered to be the non-invasive gold standard for measuring vascular function[29,37]. After attaching a blood pressure cuff on the forearm, the brachial artery (BA) segments are located, and the diameter is measured non-invasively using high-resolution ultrasound. The blood pressure cuff is then inflated to a pressure that exceeds the systolic blood pressure of the test person. This induces ischemia distal to the inflated cuff, which, after the pressure is released, causes consecutive vasodilation, and thus increases blood flow. It is assumed that the resulting increased shear stress opens calcium-activated potassium channels in the endothelial cell membrane[163–165]. As a result, the endothelial cells hyperpolarize, which causes calcium influx. The calcium influx activates the eNOS, which synthesizes NO[25,166]. This finally leads to vasodilation, which can be detected by high-resolution ultrasound, measured as the difference between the baseline diameter and the "dilation diameter"[29].Celermejer et al. demonstrated first in 1992 on asymptomatic children and young adults who had risk factors for atherosclerosis that they presented altered FMD values before anatomical or clinical evidence of atherosclerosis[24]. In a further study, Celermejer et al. investigated the effect of different risk factors on FMD values. The risk factors defined were smoking but also ex-smokers, elevated cholesterol levels (>240mg/dl) and/or positive family history of coronary artery disease (angina, myocardial infarction, or bypass surgery) at age ≤55 years, male sex and high blood pressure. Ultimately, it has been demonstrated that smoking, high cholesterol levels, family history, male gender, and arterial hypertension have a negative impact on FMD values even before the onset of actual CVD[167]. In recent years, different risk factors have been

investigated for their influence on vascular function. It has been demonstrated that, for example overweight[40], age[167,168], but also a single high-fat meal can have a negative impact on FMD values[169]. However, hormonal fluctuations in the female menstrual cycle also influence FMD values[170,171].

Furthermore, estrogen replacement therapy in postmenopausal women is associated with a reduced risk of cardiovascular disease, which is partly attributed to the augmented release on endothelium-derived nitric oxide by estrogens[172]. Liebermann et al. were able to demonstrate an increase in FMD values in women who had received estrogen therapy for nine weeks[173]. Even high-dose estrogen therapy in men (male to female transsexuals) had a positive effect on FMD values, although it is not entirely clear whether this is due to the increased estrogen levels or testosterone deficiency[174,175]. Furthermore, the circadian rhythm influences the FMD values, which are higher in the late afternoon than in the morning (FMD at 8:00 a.m. 4.0% vs. 9.7% at 5 p.m.)[176].

To keep these influencing variables as small as possible and to obtain comparable results, the FMD measurements were always carried out at the same time of day, 7.00 a.m. (baseline measurement) and 3 p.m. (8h measurement). After a sobriety period of 10 hours, the subjects came to each day of the study sobered, and all measurements were performed after a 10-minute resting period in a horizontal position. For the reasons mentioned above, all subjects recruited for the study were healthy, young, male non-smokers aged 24 $\pm$ 2 years

Apart from the physiological factors that influence FMD values, other sources of error must be considered. The most important source of variability in ultrasound imaging is the experience of the investigator. Considering that the average diameter of the BA is 4.36 mm, even minimal measurement errors are sufficient to obtain different values[177]. Sorensen et al. investigated the ability to detect the smallest deviations in diameter by using phantom arteries with differences in diameter of 0.1 - 0.2 mm and measuring the diameter with high-resolution ultrasound[178]. The investigators were able to determine the correct diameter in 162 of 264 cases (61%). In addition, the overall coefficient of variation in FMD was 1.8%. However, it is recommended to perform at least 100 scans per year to obtain an adequate image - and thus data quality[29]. In order to generate adequate ultrasound images, it is also necessary to use an ultrasound machine and probe suitable for vascular imaging. Most currently used ultrasound devices are suitable for this purpose. A linear transducer with a frequency of 7 - 12 MHz fulfills the requirements, adequate penetration depth, and resolution to measure endothelial function[29]. In this study, a linear transducer with a frequency of 10

MHz was used. The higher the frequency of a transducer, the better the resolution. However, this is accompanied by a reduction of the penetration depth. Since the BA is usually not very deep in young, healthy volunteers, the above-mentioned frequencies are, however, sufficient to achieve adequate penetration depth.

In this study the blood pressure cuff was positioned distal to the cubital fossa. The BA was visualized approximately 2cm proximal to the elbow and the blood pressure cuff was then inflated to 250mmHg and left for 5 minutes. The ultrasound probe was not moved during the 5 min of ischemia. Ultrasound images were then taken 20, 40, 60 and 80 seconds after the pressure was released. The position of the blood pressure cuff and thus the size of the ischemic area significantly influence the FMD.

Some working groups apply the cuff to the upper arm of the test person and thus generate a larger ischemic area, which is associated with higher FMD values[179]. However, this makes it more difficult to position the ultrasound probe and thus to visualize the BA, which in turn is at the expense of image quality[29]. One way to simplify the position of the ultrasound probe and thus increase image quality would be to use a stereostatic probe holder. In a recent systematic review that evaluated the reproducibility of FMD measurements it was shown that a stereostatic probe holder significantly improves the reproducibility of FMD measurements[180]. Nevertheless, another study showed that reproducibility was not affected by a probe holder, provided that the investigating team adhered strictly to the implemented guidelines[181].

The time of ischemia also plays an important role in FMD measurements. Already after one minute of ischemia flow increases can be measured, but statistically significant vasodilation can only be detected after 5 minutes[182]. Another variable to be considered is the change in vessel diameter during the cardiac cycle. To obtain the ultrasound images always at the same time during the cardiac cycle, more precisely at the end diastole (onset of QRS complex), an ECG is taken at the same time[29].

Not only the image collection but also the analysis of the images contains various sources of error regarding the diameter. For example, two different examiners can evaluate the images produced differently and thus measure different diameters. For this reason, it is recommended to use an automated, computer-based analysis program (Brachial Analyzer, Medical Imaging Applications, Iowa City, IA, USA), which reduces the coefficient of variability by more than 40% compared to manual readings[183]. The program detects the diameter of the vessel

at several points and calculates the average diameter. For this reason, the ultrasound images in this study were always obtained by the same examiner.

All these points suggest that the implementation of standard operating procedures (SOP) plays a prominent role. Until now, there are no international guidelines defining the exact criteria of FMD measurements, but there is an expert consensus which tries to replace the missing guidelines and to give recommendations for FMD measurement[184]. The procedure implemented in our SOP is similar to this expert consensus in many points, which also reflects the quality of the data collected.

The participants in this study were all male Central Europeans aged 24 ± 2 years. However, to measure effects that could be inferred for the entire population, female subjects, other age groups and different ethnicities would be necessary. Due to the heterogeneity of the studies mentioned here and the different study designs, it is therefore not possible to draw definitive conclusions. For this, uniform study designs on larger and less homogeneous groups of subjects would be necessary.

In addition, there are other factors that could influence these effects. Despite control of the diet of the participants, it cannot be excluded that some have nevertheless consumed fruits and vegetables despite the indication of a low (poly)phenol diet. An increased regular intake of (poly)phenol-rich foods certainly falsifies the parameters obtained here and allows false conclusions to be drawn. This is reflected, among other things, in the fact that despite following a low (poly)phenol diet for 72 hours before the study, the baseline levels of phenolic compounds were highly variable and significant. The plasma levels of some compounds, such as hippuric acid, were higher than those reported in previous studies on other berry fruits[39,94,185]. There are numerous factors, including body weight, sex, age, diet, genetic factors, gut microbiota, and others, that contribute to interindividual variability in (poly)phenol bioavailability[186].

5 Conclusion

In conclusion, cranberry polyphenols improve endothelial function acutely, in a time- and dose-dependent manner in young, healthy men. These results are consistent with those in many other studies.

Exploring the potential long-term impact of (poly)phenols on vascular function in individuals with an imbalanced diet would be a fascinating area of research. Cardiovascular disease is the leading cause of death globally, and many people who consume an unbalanced diet have lower baseline levels of (poly)phenols. Such individuals may benefit from a feasible intake of (poly)phenols, as demonstrated in recent studies, which could significantly reduce their cardiovascular risk. These studies indicate that (poly)phenols not only benefit those with existing conditions but also healthy individuals, highlighting their importance as an essential component of a healthy diet for primary prevention of cardiovascular disease. The consumption of (poly)phenols may improve the general health and well-being of many individuals.

The persistence of positive effects with chronic consumption and the potential health benefits in primary prevention, as well as their applicability to a wider segment of the population (generalizability), will be determined by future long-term studies.

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