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Effects of anthocyanins on vascular function

Dissertation

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Effects of dietary anthocyanins on vascular function - summary

Background and hypothesis: Anthocyanins are dietary polyphenols present in fruits and vegetables such as blueberries and grapes. Epidemiological studies indicate that there is an inverse association between increased intake of anthocyanin-rich foods and cardiovascular risk. The overall hypothesis of the present work was that anthocyanin consumption can improve vascular function in postmenopausal women.

Methods: Two clinical studies were performed.The first study was a randomized, double-blind six-arm clinical intervention trial to test the dose response of pure anthocyanins on endothelial function in healthy young men (n=10). Endothelial function was determined by flow-mediated vasodilation (FMD) along with measurements of blood pressure and anthocyanin metabolites in blood before (0h) and at 2 and 6 h hours after the ingestion of capsules containing 0 (control), 80, 160, 240, 320, and 480 mg pure anthocyanins. The metabolites of the anthocyanins were measured in blood using a validated UPLC-Q-TOF MS method. The second study was a randomized, double-blind controlled trial to test the acute and chronic effects of pure anthocyanins in healthy postmenopausal women (n=22). The volunteers received either 160 mg of pure anthocyanins or placebo capsules without anthocyanins bi-daily over one month. The measurements were performed at before (0 h) and at 2 h after intake of the capsules in the morning oft he first study day and at 1 month. The primary endpoint was FMD. Secondary endpoints were blood pressure and pulse wave velocity.

Results: Anthocyanin intake led to a dose-dependend increase in FMD at 2 and 6 h in healthy young males with significant increases (Δ: 1.1-1.3%) after intake of ≥160 mg pure anthocyanins. Fifty anthocyanin metabolites were identified in plasma. A correlation analysis showed significant correlations between FMD changes and ferulic acid 4-O-sulfate and homovanillic acid at 2 h post-ingestion and dihydro ferulic acid 4-O-sulfate at 6 h post-ingestion. However, anthocyanins failed to exert significant effects on FMD or any of the vascular measurements in postmenopausal women. The baseline polyphenol intake was low in the young males, it was significantly higher in the postmenopausal women.

Conclusion: While anthocyanins can acutely and dose-dependently increase vascular function healthy young men with low baseline polyphenol intake, these compounds did not affect vascular function in healthy postmenopausal women with a higher baseline polyphenol consumption. Further work is necessary to delineate the contribution of age, sex, and background polyphenol intake on the biological efficacy of anthocyanidins.

Effekte von Nahrungsanthocyanen auf die Gefäßfunktion – Zusammenfassung

Hintergrund und Hypothese. Anthocyane sind eine Gruppe von Polyphenolen, die in Nahrungsmitteln z.B. in Früchten wie Blaubeeren oder Weintrauben vorkommen. Epidemiologische Studien deuten darauf hin, dass es eine umgekehrt proportionale Relation zwischen hohem Anthocyan-Gehalt und kardiovaskulärem Risiko gibt. Die übergreifende Hypothese war, dass Anthocyan-Konsum die Gefäßfunktion gesunder postmenopausaler Frauen verbessern kann.

Methoden. Zwei klinische Studien wurden durchgeführt. Die erste Studie war eine randomisierte, doppelblinde, sechs-armige Studie, um die Dosis-Wirkungs-Beziehung von aufgereinigten Anthocyanen auf die Gefäßfunktion zu testen (n=10). Die endotheliale Fuktion wurde mittels Fluss-vermittelter Vasodilation (FMD) bestimmt, des Weiteren wurden Blutdruckmessungen und Messungen der Anthocyan-Metaboliten im Blut vor Einnahme (0h), zwei und sechs Stunden nach der Einnahme der Kapseln mit 0(Kontrolle), 80, 160, 240, 320, und 480 mg puren Anthocyanen durchgeführt. Die Metabolite wurden im Blutplasma mittels einer validierten UPLC-Q-TOF MS gemessen. Die zweite Studie war eine doppelblinde randomisierte kontrollierte Untersuchung, um die akuten und chronischen Effekte von Anthocyanen auf gesunde postmenopausale Frauen zu untersuchen (n=22). Die Probandinnen nahmen entweder 160 mg Anthocyane oder ein Placebo zweimal täglich für einen Monat ein. Die Messungen wurden vor (0h) und 2h nach Kapseleinnahme am ersten Studientag und nach einem Monat durchgeführt. Der primäre Endpunkt war FMD. Sekundäre Endpunkte waren Pulswellengeschwindigkeit und Pulswellenanalyse.

Ergebnisse. Anthocyan-Einnahme führte zu einem dosisabhängigen Anstieg in der FMD nach 2 und 6h in gesunden jungen Männern mit einem signifikanten Anstieg (Δ: 1.1-1.3%) nach der Einnahme ≥160 mg aufgereinigter Anthocyane. Eine Korrelationsanalyse zeigte eine signifikante Korrelation für Ferulasäure 4-O-Sulfat und Homovanillinsäure nach zwei Stunden und Dihydro-Ferulasäure 4-O-Sulfat nach sechs Stunden nach der Einnahme. Allerdings verfehlten Anthocyane signifikante Effekte auf jegliche Gefäßmesspukte bei postmenopausalen Frauen. Die Basis-Polyphenol-Einnahme bei jungen Männern war niedrig, bei den postmenopausalen Frauen war diese signifikant höher.

Schlußfolgerung. Während Anthocyane akut und dosisabhängig die Gefäßfunktion gesunder junger Männer mit einer geringen Basis-Polyphenol-Einnahme verbessern können, haben diese Verbindungen nicht die Gefäßfunktion gesunder postmenopausaler Frauen mit einer höheren Basis-Polyphenol-Einnahme verbessert. Weitere Forschung ist notwendig, um die Auswirkung von Alter, Geschlecht, und Hintergrund-Einnahme von Polyphenolen auf die biologische Wirkungskraft von Anthocyanen eingrenzen zu können.

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List of Abbreviations

(pre)hypertens ..... *(pre)hypertension*
Δ *difference*
ABPM ..... *ambulatory blood pressure monitoring*
ACE ..... *Angiotensin-converting enzyme*
ACN ..... *anthocyanin*
AIX ..... *Augmentation index*
ANOVA ..... *Analysis of variance*
AUC ..... *area under the curve*
BL ..... *blood levels*
BMI ..... *Body mass index*
BP ..... *blood pressure*
bpm ..... *beats per minute*
CAD ..... *coronary artery disease*
CI ..... *confidence interval*
CONSORT ..... *Consolidated Standards of Reporting of Trials*
CPK ..... *creatine phosphokinase*
CRP ..... *c-reactive protein*
cSBP ..... *central systolic blood pressure*
CVD ..... *cardiovascular disease*
Cy-3a *cyanidin-3-O-beta-arabinosides*
Cy-3g *cyanidin-3-O-beta-glucosides*
Cy-3gal *cyanidin-3-O-beta-galactosides*
Cy-3r *cyanidine-3-O-rutinoside*
d *days*
DBP ..... *diastolic blood pressure*
Dp-3a ... *delphinidin-3-O-beta-arabinosides*
Dp-3g *delphinidin-3-O-beta-glucosides*
Dp-3gal *delphinidin-3-O-beta-galctoside*
Dp-3r *delphinidin-3-O-rutinoside*
DSM-IV . *Diagnostic and Statistical Manual of Mental Disorders*
ECG ..... *electrocardiography*
EDTA ..... *Ethylenediaminetetraacetic acid*
FD ..... *freeze-dried*
Fig. ..... *Figure*
FMD ..... *Flow-mediated vasodilation*
FSH ..... *Follicle-stimulating hormone*
GBD ..... *Global Burden of Disease*
GFR ..... *glomerular filtration rate*
GRISS *Golombok Rust Inventory of Sexual Satisfaction*
h *hour*
HbA1c ..... *Glycated hemoglobin*
HDL ..... *High-density lipoprotein*
HLB ..... *Hydrophilic-Lipophilic-Balanced sorbent*
HPLC ..... *High-performance liquid chromatography*
HR ..... *heart rate*
HRQoL ..... *health-related quality of life*
HRT ..... *hormone replacement therapy*
hypercholesterolemics ..... *hypercholesterolemics*
LDL ..... *low-density lipoprotein*
LH ..... *Luteinizing hormone*
log ..... *logarithm*
M *month*
MDRD *modification of diet in renal disease*
MENQoL *menopause-related quality of life questionnaire*
MI ..... *myocardial infarction*
mIU ..... *milliunits*
mmHg ..... *millimeter of mercury*
MRS ..... *menopause rating scale*
MS ..... *metabolic syndrome*
Mv-3a *malvidin-3-O-beta-arabinoside*
Mv-3g *malvidin-3-O-beta-glucoside*
Mv-3gal *malvidin-3-O-beta-galactoside*
n *Number*
nd ..... *not determined*
NO ..... nitric oxide
OBPM ..... *office blood pressure*
PAT ..... *peripheral arterial tonometry*
Pe-3a *petunidin-3-O-beta-arabinoside*
Pe-3g *petunidin-3-O-beta-glucoside*
Pe-3gal *petunidin-3-O-beta-galactoside*
pH ..... *potential of hydrogen*
Pn-3a *peonidin-3-O-beta-arabinoside*
Pn-3g *peonidin-3-O-beta-glucoside*
Pn-3gal *peonidin-3-O-beta-galactoside*
postmen ..... *postmenopausal*
PREDIMED ..... "Prevención con Dieta Meditérranea"=Prevention with Mediterrenean Diet
PWA ..... *pulse wave analysis*
PWV ..... *pulse wave velocity*
RCT ..... *randomized controlled trial*
s *seconds*
SBP ..... *systolic blood pressure*
SEM ..... *Standard error of the mean*
StDev ..... *Standard deviation*
T2D ..... *type 2 diabetes mellitus*
TC ..... *total cholesterol*
TG ..... *triglycerids*
TLC ..... *therapeutic lifestyle changes*
TP ..... *total polyphenols*
TSH ..... *Thyroid-stimulating hormone*
UPLC-Q-TOF MS. *ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry*
VAS ..... *visual analogue scale*
vs ..... *versus*

1 Introduction

1.1 Diet and cardiovascular health

Cardiovascular disease (CVD) is the number one killer in the world. It is responsible for a third of all deaths worldwide (GBD 2013 Mortality and Causes of Death Collaborators, 2015). Ischemic heart disease and stroke in particular are the reason for most cardiovascular and circulatory deaths in almost all countries (Eyre et al., 2004; GBD 2013 Mortality and Causes of Death Collaborators, 2015; Mozaffarian et al., 2015).

An unhealthy diet, insufficient exercise and smoking are the major modifiable risk factors for CVD but also for cancer and diabetes (Eyre et al., 2004). Of these modifiable factors, diet is one of the most important (Mozaffarian et al., 2015), with suboptimal diet quality being the number one risk factor for death and disability In the United States of America (Mozaffarian et al., 2015). Dietary strategies are therefore an important working point in the primary and secondary prevention of these frequent diseases. A nutritionally balanced diet is crucial for a healthy lifestyle and helps to maintain an ideal weight, might decrease blood pressure, and improve plasma lipids and blood glucose levels (Eyre et al., 2004;Heiss et al., 2010). However, the existing evidence on the role of diet on CVD prevention is, at the moment, inconclusive, and more randomized controlled trials and prospective observational studies are needed in order to give adequate recommendations to the general public.

Evidence from epidemiological studies suggest that certain diets can have cardiovascular health benefits. For example, a dietary pattern characterized by higher intakes of vegetables, fruits, legumes, fish, poultry, and wholegrain was associated with a 28% lower cardiovascular mortality (95% CI, 0.60 to 0.87) and a 17% lower risk of all-cause mortality (Heidemann et al., 2008). In the same study a 22% increase in cardiovascular mortality was seen in the cohort following a dietary pattern characterized by higher intakes of processed meat, red meat, refined grains, French fries, and sweets/desserts (95% CI, 1.01–1.48) (Heidemann et al., 2008; Mozaffarian et al., 2015). Strong epidemiological evidence exists for diets rich in fruits and vegetables. A recent meta-analysis of nearly 50 prospective cohort studies including 1.5 million people found a dose response relationship between relative risk of CVD and fruit and vegetable intake (Zhan et al., 2017). People eating 800 grams of fruits and vegetables per day had a reduced risk of CVD of around 20%.

However, the evidence from randomized controlled trials is not so strong, with a recent meta-analysis finding the evidence available inconclusive (Hartley et al., 2013). Nevertheless, dietary guidelines of many countries advise to consume at least five servings of fruits and vegetables per day (Oyebode et al., 2014). The European Guidelines on CVD Prevention in Clinical Practice from the European Society of Cardiology recommend consumption of 400 grams of fruits and vegetables per day (Perk et al., 2012), including 30-45g of fibre per day from wholegrain products and fruits and vegetables, 200 g of fruit per day (2-3 servings) and 200 g of vegetables per day (2-3 servings)(Perk et al. 2012). In most Western countries people do not meet these recommendations. In Germany for example, only 12% of adult women and 6% of men consume at least five servings of fruits and vegetables every day (Rabenberg, M., Mensink, G., 2011; Mensink et al., 2013; Mozaffarian et al., 2015).

Another diet that has been shown to have cardiovascular benefits is the Mediterranean diet. Systematic reviews of epidemiological studies including more than half a million subjects have found associations between greater adherence to Mediterranean diet and significant improvement in health status. Most prominently, a 9% reduction of overall mortality and mortality from CVD was found (Sofi et al., 2008; Sofi et al., 2010). Greater adherence to a

Mediterranean dietary pattern was associated with lower cardiovascular mortality and morbidity and also reduction of major CVD risk factors, in particular hypertension, diabetes mellitus and metabolic syndrome (Nunez-Cordoba et al., 2009; Psaltopoulou et al., 2004; Rumawas et al., 2009; Sanchez-Tainta et al., 2008; Rees et al., 2013).

Few well-conducted and adequately powered randomized controlled trials (RCTs) have investigated the benefits of the Mediterranean Diet in the primary prevention of CVD (Rees et al., 2013; Serra-Majem et al., 2006). In a recent Cochrane review which included 11 RCTs (52,044 participants in total), subgroup analyses revealed statistically significant greater reductions in total cholesterol in those trials using a classical Mediterranean diet (-0.23 mmol/L, 95% CI -0.27 to -0.2) compared with control (-0.06 mmol/L, 95% CI - 0.13 to 0.01). Reductions in blood pressure were seen in three of five trials reporting this outcome. None of the trials reported adverse events (Rees et al., 2013). A recent large primary prevention trial in Spain called PREDIMED (Prevención con Dieta Mediterránea), which was not included in the meta-analysis, has reported a decrease of 30% in major cardiovascular events such as cardiovascular death, myocardial infarction or stroke in volunteers randomized to Mediterranean-style diets (Estruch et al., 2013). The Mediterranean diet mainly consists of a high intake of fruits and vegetables and certain foods such as olive oil, nuts and red wine (Arós and Estruch, 2013). Thus although evidence is accumulating on the cardiovascular health benefits of a Mediterranean-like dietary pattern, promotion of the Mediterranean diet has not been followed by changes in lifestyle in other parts of the Western world, most likely because of difficulties in fundamentally changing dietary patterns, limited accessibility to ingredients, and cultural differences in taste (Olsen et al., 2011).

Another example of diet that is believed to improve cardiovascular health is the Nordic diet. The Nordic diet includes a variation of traditional food items used in the Nordic countries due to the Nordic climate that restricts crop diversity, and the long coastline which provides rich sources of fish (Olsen et al., 2011). Wholegrain cereals such as rye, barley and oats and berries, especially blueberries and fruits such as apples, play an important role in the Nordic diet. Other important components are oily fish, e.g. salmon and herring, vegetables, root vegetables and legumes. Prospective cohort studies have shown a reduction of around 4% of total mortality in people following a Nordic diet (Olsen et al., 2011; Risérus, 2015). There is also some evidence from controlled trials conducted in various Nordic populations. For example, it has been shown that a healthy Nordic diet can improve blood lipid profile (-16% plasma cholesterol, -21% LDL cholesterol) and insulin sensitivity (-9% insulin), and lower blood pressure (-5% systolic blood pressure) at clinically relevant levels in hypercholesterolemics (Adamsson et al., 2011). This evidence suggests that the Nordic diet reduces cardiovascular risk factors to a similar extent as the Mediterranean diet (Adamsson et al., 2011; Risérus, 2015), but more RCTs are needed to confirm these findings.

Something both Mediterranean and Nordic diets have in common is that both are rich in fruits and vegetables. But which fruits and vegetables are the healthiest and should be favoured? Fruits and vegetables are rich in vitamins, minerals and fibres, but they also have a wide range of phytochemicals, in particular a group of phenolic compounds called flavonoids, which are receiving increasing attention due to their putative health benefits (Del Rio et al., 2013). Different fruits and vegetables contain different type and amounts of flavonoids. So, what if the positive effects of a diversified diet rich in vegetables, nuts, and fruits really depend on a limited number of flavonoids? That would have consequences for future dietary recommendations, dietary supplements and probably even on the therapeutic use of some foods and food components (Heiss et al., 2010).

1.2 Endothelial dysfunction

Endothelial dysfunction is an early marker of atherosclerosis and an independent predictor of cardiovascular events (Lavi et al., 2008; Sitia et al., 2010; Su, 2015). Widespread and important cardiovascular risk factors such as hypertension, hypercholesterolemia, smoking, ageing, type II diabetes mellitus, chronic inflammation, hyperglycaemia, oxidative stress are pathophysiologically linked to the development of endothelial dysfunction (Fig. 1) (Bonetti et al., 2003). The cause is an imbalance in the endothelial status due to atherogenic and atheroprotective factors interacting in an individual, including known as well as yet-unknown variables and genetic predisposition (Bonetti et al., 2003; Su et al., 2015). The pathophysiological background of endothelial dysfunction is traced back to reduced availability or activity of endothelium-derived nitric oxide (NO), an important endothelium-dependent vasodilator, in combination with an increase in vasoconstrictors. The decrease in NO dependent vasodilation may be caused by reduced NO production or alternatively increase in the degradation of NO by e.g. oxidative stress (Lavi et al., 2008). Measuring endothelial dysfunction can be seen as a way of identifying a vascular phenotype prone to atherogenesis. Endothelial dysfunction might not only help to identify people at risk for atherosclerosis and CVD but it might also be a marker of the inherent atherosclerotic risk of an individual. It could be used to identify people that should take preventive measures against CVD (Bambrick et al., 2016; Bonetti et al., 2003).

There are several methods to assess endothelial function. Flow-mediated vasodilation (FMD) is the most commonly used method for measuring endothelial function. It is sensitive and non-invasive. The physiological background was established in the late 1980s by Anderson and Mark and it was used for the first time by Celermajer et al. (Celermajer et al., 1992). The mechanism of FMD is NO-dependent, so it is also considered a measurement of NO bioavailability (Heiss, Rodriguez-Mateos et al., 2015). A very good correlation has been shown between coronary artery endothelium-dependent vasomotor responses to acetylcholine, the gold standard invasive method for measuring endothelial function, and FMD in patients referred to the catheterization laboratory for the evaluation of coronary artery disease (Anderson et al., 1995). FMD can be used to measure vascular function and to assess the predisposition to vascular dysfunction and atherosclerosis (Anderson et al. 1995). Recent meta-analysis have correlated FMD with CVD, and an increase in FMD of 1% is related to a 10% decrease in CVD risk (Ras et al., 2013).

There are guidelines for the performance of FMD (Corretti et al., 2002; Thijssen et al., 2011), but a full standardization has not been achieved yet (Arrebola-Moreno et al., 2012), resulting in significant differences between mean FMD in different studies with similar populations, using different protocols.

Peripheral arterial tonometry (PAT) is another method of measuring endothelial function, and studies have shown that correlate with coronary endothelial function (Schnabel et al., 2011), however no correlation with invasive endothelial function tests has been demonstrated (Schnabel et al., 2011). Clinical correlations with cardiovascular disease events such as death from CVD and myocardial infarction were found for both FMD and PAT in the Framingham Heart Study (Benjamin et al., 2004; Hamburg et al., 2008) and the Cardiovascular Health study (Yeboah et al., 2008). FMD and PAT correlate with each other but it is still questionable if their results are comparable with each other. FMD of the brachial artery is at the moment the best non-invasive method to assess large artery reactivity and is consequently the gold standard of non-invasive measurements of endothelial function.

Endothelial function is known to be modulated by various dietary components. In this context, polyphenol-rich foods such as fruits, cocoa, tea or red wine have generated a lot of attention

because of their capability to induce improvements in endothelial function after acute and chronic consumption (Rodriguez-Mateos, Heiss et al., 2014). On the other hand, acute or chronic consumption of high-fat meals induce a negative effect on endothelial function (with the exception of n3- fatty acids) (West et al., 2005), but if given simultaneously with polyphenol-rich foods, such as red wine, fruits and vegetables this negative effect on endothelial function is reversed (Bonaccio et al., 2016; Cerletti et al., 2016).

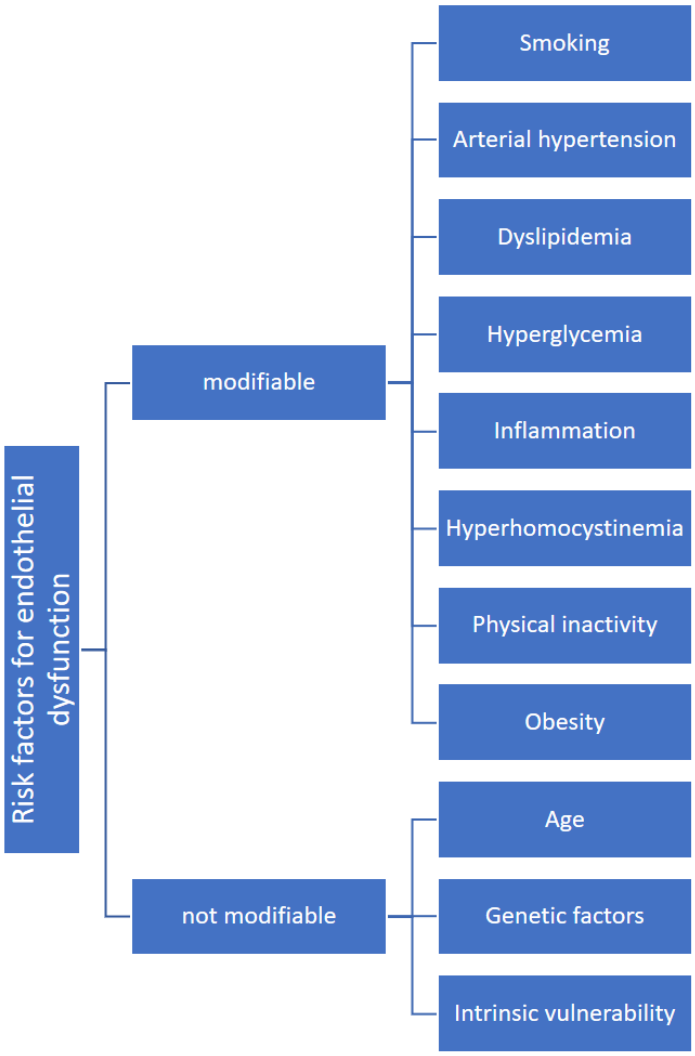


Fig. 1: Modifiable and non-modifiable risk factors of endothelial dysfunction

1.3 Dietary anthocyanins

1.3.1 Classification, food sources, and dietary intake

Polyphenols are a type of phytochemicals present in many fruits and vegetables of our diet (Manach et al., 2004). A polyphenolic structure consists of several hydroxyl groups on aromatic rings. Thousands of molecules with a polyphenol structure have been found in plants and several hundreds of these are present in edible plants that are an important part of our diet. There are four main groups of polyphenols: phenolic acids, flavonoids, stilbenes, and lignans (Fig. 2). Of all polyphenols, flavonoids are the most abundant group. The main subclasses of flavonoids are the flavones, flavonols, flavanols, isoflavones, flavanones, and anthocyanidins (Del Rio et al., 2013). Anthocyanidins are water-soluble colourful plant pigments, which are responsible for the orange, red, blue and purple colours of the skin and flesh of many fruits and vegetables (Del Rio et al., 2013). They generally occur in the plant as aglycones, glycosides and acylglycosides of anthocyanidins. They have a basic flavylium structure (2-phenylbenzopyrilium) that is altered by different hydroxyl or methoxyl substitutions (Wu and Prior, 2005). The most common anthocyanidin aglycones that are considered relevant for human diet are pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin (Fig. 3). The aglycones are usually attached to different sugars and organic acids forming a plurality of anthocyanins.

The average anthocyanin consumption in our diet is very variable within different countries, for example the estimated intake in the United States is 12.5 mg/day (Wu et al., 2006), whereas in Europe the average anthocyanin consumption differs significantly from 19.8 (Netherlands) to 64.9 mg/day (Italy) for men, and for women, from 18.4 (Spain) to 44.1 mg/day (Italy) (Zamora-Ros et al., 2011). Fruits, especially apples, pears, berries, stone fruit and grapes, and wine are the dominating sources of anthocyanins in European diet (Zamora-Ros et al., 2011). Berry fruits are rich sources of dietary anthocyanins (Wu and Prior, 2005) and can have up to 500 mg of anthocyanins in a single serving (McGhie and Walton, 2007; Rodriguez-Mateos, 2012). As anthocyanins are present in many commonly consumed fruits an increase in anthocyanin intake could be accomplished easily. Consuming 1-2 portions of either strawberries, raspberries or blueberries would significantly increase intakes of anthocyanins to levels that have been reported to be associated with a reduction in risk of CVD in epidemiological studies (Bhagwat, 2013; Cassidy et al., 2011; Cassidy et al., 2013; Cassidy, 2017; Jennings et al., 2012; McCullough et al., 2012).

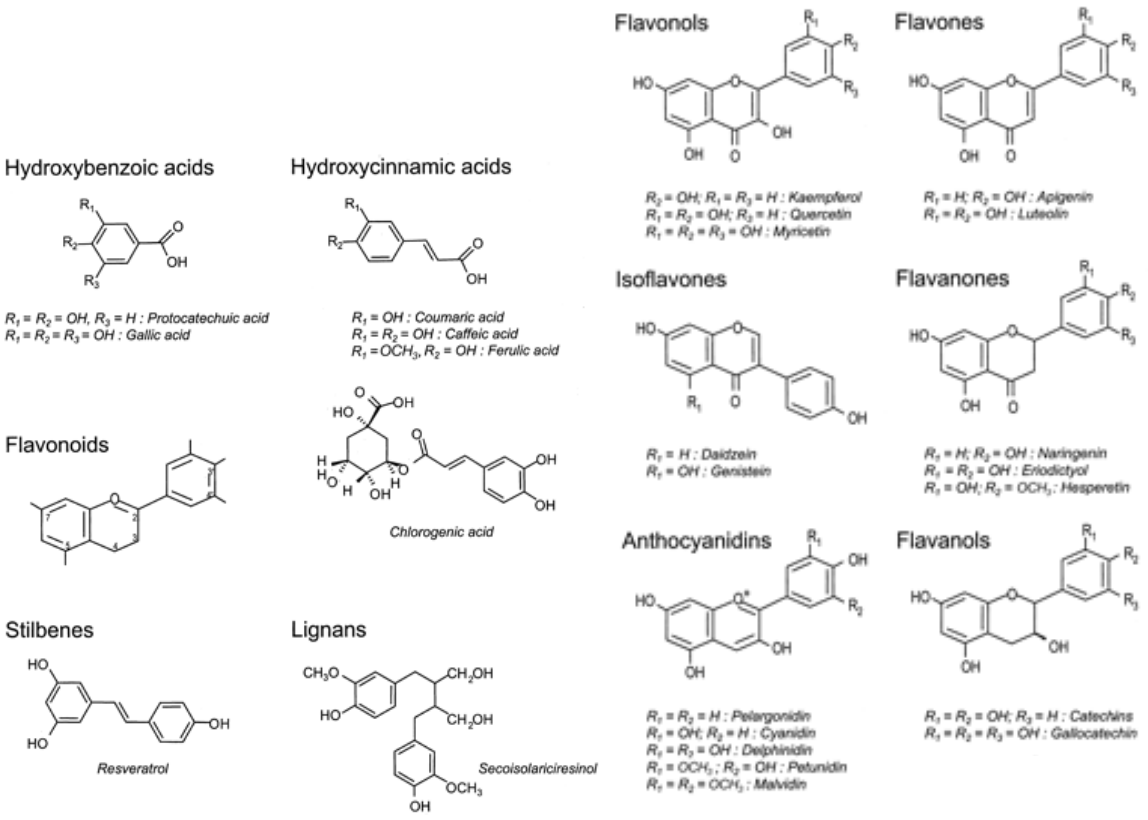
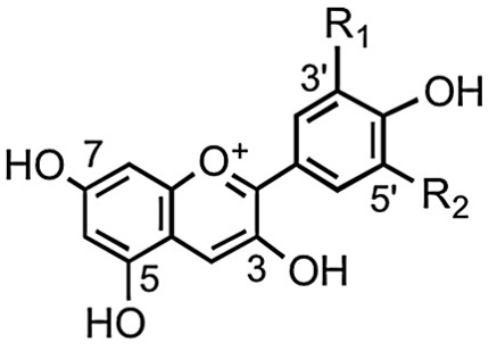


Fig. 2: Polyphenols are classified by their chemical structure (Manach et al., 2004).



Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	OH
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Fig. 3: Chemical structures of anthocyanins (modified after Del Rio et al. 2013)

1.3.2 Bioavailability of anthocyanins

The effects of polyphenols on cardiovascular health are very likely dependent on their bioavailability. After ingestion, polyphenols are absorbed in the small or large intestine where they are metabolized by phase II enzymes and the gut microflora leading to a wide array of metabolites that are circulating in blood and are excreted in urine (Kay et al., 2005; Manach et al., 2005; McGhie and Walton, 2007) (Fig. 4).

Due to their instability at neutral pH, anthocyanins were thought to have lower bioavailability than other polyphenols, such as flavanols, flavanones or flavonols, with recoveries in urine of less than 2% (Manach et al., 2005). Recent studies however suggest that the bioavailability of anthocyanins may have been underestimated (Czank et al., 2013; Nurmi et al., 2009; Rodriguez-Mateos et al., 2013; Vitaglione et al., 2007). Phenolic metabolites derived from the breakdown of anthocyanins in the small intestine and from the action of the gut microflora in the colon have been found in plasma and urine after consumption of anthocyanins-rich foods in much higher amounts than the parent compounds of anthocyanins (Rodriguez-Mateos, Vauzour et al., 2014).

While structurally-related anthocyanins and phase II conjugates have been found in blood in low nanomolar concentrations, phenolic acid metabolites have been found in micromolar concentrations (Czank et al., 2013; Feliciano et al., 2016; Ferrars et al., 2014; Rodriguez-Mateos et al., 2013; Rodriguez-Mateos et al., 2016; Rodriguez-Mateos, Vauzour et al., 2014). Czank et al. investigated the absorption, metabolism and excretion of isotope-labelled cyanidin-3-O-glucoside in healthy volunteers. $^{13}C_5$-labeled metabolite concentrations were measured in plasma, urine, breath and faeces for 48 hours post-consumption using isotope-ratio mass spectrometry and liquid chromatography–tandem mass spectrometry (Czank et al., 2013). The relative bioavailability was found to be 12 % (5% excreted in urine and around 7% in breath) (Czank et al., 2013). In faeces, 32 % of ingested ^{13}C cyanidin-3-O-glucoside was recovered. Approximately 50% of the radioactivity was recovered in plasma, urine, breath and faeces. However, the fate of the other 50% is unknown, suggesting that there are still many anthocyanin metabolites or breakdown products that have not been identified yet.

Rodriguez-Mateos et al. investigated the bioavailability of anthocyanins after consumption of a polyphenol-rich blueberry drink in a group of healthy individuals (Rodriguez-Mateos et al., 2013). Thirty-two compounds were found in plasma after blueberry consumption. The most abundant ones were hippuric and hydroxyhippuric acid with maximum concentrations ranging from 3 to 5 µmol/L. Some compounds appeared early in plasma (1-2 h post consumption), such as 2-hydroxybenzoic acid, benzoic acid, ferulic acid, isoferulic acid and vanillic acid, suggesting absorption in the small intestine, while others appear at a later time (6 h post consumption) suggesting absorption in the large intestine after metabolism by the gut microflora. Among the compounds appearing later after consumption, hippuric, hydroxyhippuric acid and homovanillic acid were the most abundant. Interestingly, some of these metabolites were found to correlate with the vascular response measured as FMD. Vanillic and benzoic acid correlated with the effects observed at 1-2 h post consumption, whereas metabolites derived from anthocyanin and chlorogenic acid, notably hippuric, hydroxyhippuric acid and homovanillic acid correlated with the effects at 6 hours post consumption. The authors speculate that a synergistic effect among several polyphenol metabolites could be responsible for the beneficial effects observed on endothelial function. However, although blueberries are rich in anthocyanins, they also have other polyphenol compounds such as flavanols, flavonols and chlorogenic acids in significant amounts. It is therefore unknown whether anthocyanins or other polyphenols from blueberry, or a combination of all, led to the improvements in endothelial function observed.

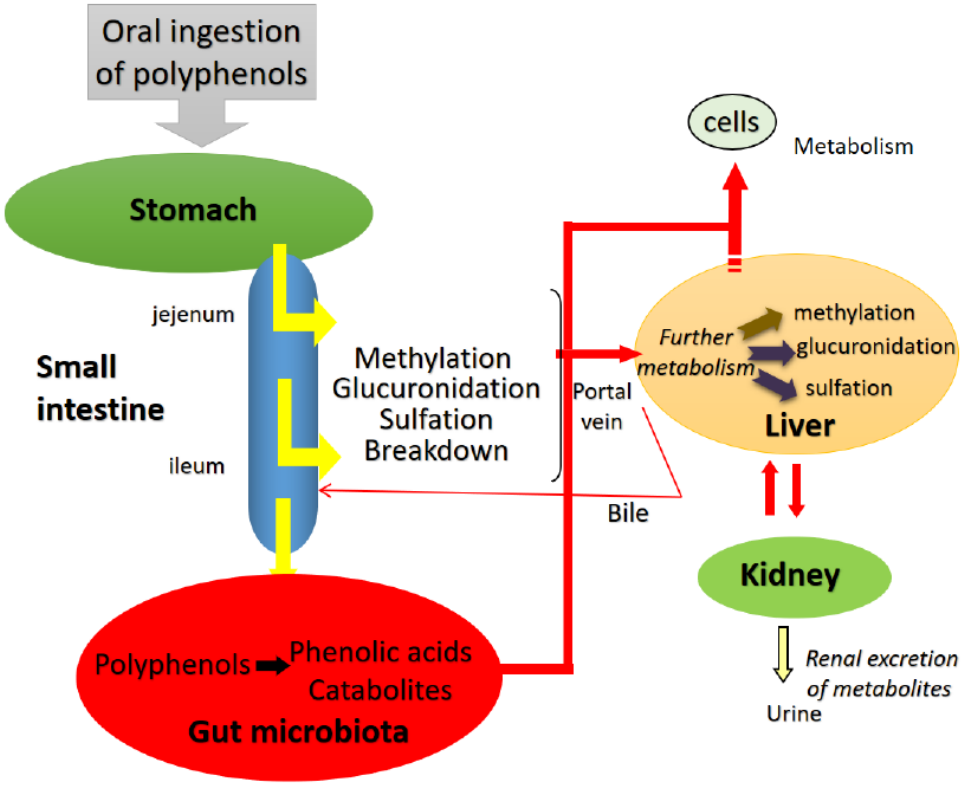


Fig. 4: Absorption, distribution, metabolism and elimination of anthocyanins in the body (modified after Rodriguez-Mateos et al., 2014; Spencer et al., 2004)

1.4 Anthocyanins and cardiovascular health

Increasing evidence from epidemiological and human intervention studies suggests that anthocyanin-rich foods such as berries may improve cardiovascular health (Rodriguez-Mateos, Heiss et al., 2014). In this section the most important studies conducted so far will be pointed out.

1.4.1 Epidemiological evidence

Several epidemiological studies have investigated associations between anthocyanin consumption and cardiovascular disease (CVD). One of the most recent studies examined the relation between habitual anthocyanin intake and coronary artery disease and stroke in nearly 44, 000 healthy men from the Health Professionals Follow-Up Study. Higher anthocyanin intake was not associated with total or fatal Myocardial infarction (MI) risk or stroke risk, but an inverse association was found with nonfatal MI especially in normotensive men (HR: 0.87; 95% CI: 0.75, 1.00; p = 0.04; p-trend = 0.098) (Cassidy et al., 2016). Mink and Scrafford investigated the relationship between flavonoid intake and cardiovascular disease mortality in postmenopausal women (Mink et al., 2007). A total of 34 000 healthy postmenopausal women were followed for 16 years in the Iowa Women's Health Study. A higher intake of anthocyanidins lowered the risk for coronary heart disease by 12%, cardiovascular disease by 9% and total mortality by 10% when compared to no intake. Among the individual foods that lowered the risk were bran, apples, peas, red wine, grapefruits, strawberries and chocolate (Mink et al., 2007, p. 895).

In one more recent study, 5 flavonoid classes—anthocyanidins, flavan-3-ols, flavones, flavonols, and proanthocyanidins—were individually associated with lower risk of fatal CVD in a large prospective trial including 38 000 elderly men and more than 60 000 elderly women conducted over 7 years in the US. The correlation was non-linear because the lowest risk was associated with intermediate flavonoid consumption, suggesting that even the intake of small amounts of flavonoid-rich foods support cardiovascular health (McCullough et al., 2012). Another recent study with more than 93,000 women observed over 18 years showed that women with the highest intake of anthocyanins in their diet had a reduction in the risk of having a myocardial infarction of 32% compared to the participants with the lowest intake (Cassidy et al., 2013). In this study, the intake of anthocyanins in the diet was very low, between 2 and 35 mg per day. Red wine and berries also correlated with a reduction of cardiovascular risk. A study by Jennings et al. examined the correlation between anthocyanin intake, arterial stiffness and central blood pressure in nearly 1, 900 women. A higher anthocyanin intake was associated with significantly lower central systolic blood pressure (cSBP) of -3.0 ± 1.4 mmHg for highest compared with lowest intake, medium arterial pressure was lowered by -2.3 ± 1.2 mmHg for highest compared with lowest intake, and pulse wave velocity was reduced by around 0.4 m/s for highest compared with lowest intake (Jennings et al., 2012).

However, not all the epidemiological studies have found a positive correlation between anthocyanins and decrease in the incidence of cardiovascular events (Cassidy et al., 2012; Hertog et al., 1997; Lin et al., 2007; Rimm et al., 1996). In a study with more than 38,000 healthy women followed up for 7 years, flavonoid intake was not associated with a reduction of cardiovascular risk (Sesso et al., 2003). Two studies prospectively evaluating health risks and diet in nearly 70,000 women in the Nurses' Health Study did not see an inverse association between flavonoid intake and risk of stroke (Cassidy et al., 2012) or coronary heart disease (Lin et al., 2007).

In summary, the evidence from these studies suggests that a diet rich in anthocyanins and rich in flavonoids might improve cardiovascular health. Even small amount of these substances might be beneficial although the results of the studies were not unanimous. The strengths of these studies are the large study population investigated, the length and the use of relevant hard clinical endpoints.

However, epidemiological studies have several limitations. They report associations and cannot establish causality. The evidence is based on dietary assessment, more specifically food frequency questionnaires, which are prone to bias and not very reliable. In addition, food composition databases are limited and do not take into account variations in polyphenol content due to agricultural practices or processing, for example. Randomized controlled trials (RCTs) complemented by analysis of reliable biomarkers of their intake in plasma, urine, or stool samples are needed to confirm these results (Rodriguez-Mateos, Heiss et al., 2014).

1.4.2 Evidence from randomized controlled trials (RCTs)

RCTs are the best way to prove cause and effect relationships (Cartwright, 2010; Schulz et al., 1995), however, in the context of nutritional interventions, usually due to time and economic constraints, biomarkers of CVD risk are used instead of hard clinical endpoints, which is a limitation. The most commonly used prognostically validated biomarkers of cardiovascular risk are endothelial function, arterial stiffness, blood pressure and blood lipids (Vasan et al., 2006;Laurent et al., 2006; Mitchell et al., 2010; Roman et al., 2007).

A total of 34 RCTs have investigated the effects of anthocyanin-rich food on those biomarkers (Table 1). Six of the studies used purified anthocyanins from bilberries and blackcurrant, 8 of

them investigated the effects of blueberry (6 used freeze-dried blueberries, 1 used frozen blueberries and 1 used fresh blueberries), 7 of them used cranberries (5 cranberry juice, 2 cranberry extract), 4 used strawberries (3 freeze-dried, 1 fresh strawberries), 2 used black currant juice, 2 used elderberry extract and 2 used whortleberry extract. Study populations were mixed, with 8 studies investigating healthy subjects, 24 studies including people at risk of CVD and 2 studies looking at patients with CVD.

1.4.3 Effects of anthocyanins on endothelial function

Eight RCTs have investigated the effects of berries and anthocyanins on endothelial function. Four of them observed an increase in endothelial function (Khan et al., 2014; Rodriguez-Mateos et al., 2013; Rodriguez-Mateos et al., 2016; Zhu et al., 2011), while the others did not show statistically significant effects on endothelial function (Del Bo et al., 2013; Dohadwala et al., 2011; Jin et al., 2011; Riso et al., 2013).

Zhu et al. showed that supplementation with 320 mg of purified anthocyanins isolated from blackcurrants and bilberries improve endothelium-dependent vasodilation measured as FMD in hypercholesterolemic individuals. Significant increases in FMD of 2.7 and 1.8% were found at 1 h and 2 h after anthocyanin consumption, which correlated with increases of plasma anthocyanin concentrations ($p < 0.05$). A sustained increase of 28.4% after 12 weeks daily supplementation was also shown (Zhu et al., 2011). An intake and time-dependent increase in FMD at 1, 2, and 6 hours post consumption of anthocyanin-rich freeze-dried blueberries was also reported in healthy young men. FMD increased dose dependently up to 766 mg total blueberry polyphenol intake containing 310 mg of anthocyanins (equivalent to 240 grams of fresh blueberries), after which FMD plateaued (Rodriguez-Mateos et al., 2013). A correlation was also found between improvements in FMD and plasma blueberry derived phenolic metabolites.

Another RCT in healthy individuals showed that a blackcurrant juice drink drunk four times a day for six weeks increased FMD by 1.1%, but the effect was only seen in the highest amount of black currant juice that contained 36 mg of anthocyanins (Khan et al., 2014).

A study investigating the effects of cranberry juice consumption on people with carotid artery disease (CAD) did not see any effects on FMD after daily consumption for 4 weeks (Dohadwala et al., 2011). However, an uncontrolled pilot study in the same population found an increase in FMD after 2-4 h post consumption. The levels of anthocyanins in the cranberry juice were very low though due to processing (94 mg of 835 mg total polyphenols) and it is likely that the effects were due to other polyphenols present in the drink.

Two studies using endo-PAT instead of FMD did not see any acute improvements in endothelial function after consumption of a blueberry drink (Del Bo et al., 2013; Riso et al., 2013) containing 348/375 mg of anthocyanins. Jin et al. 2011 used laser Doppler iontophoresis to measure microvascular endothelial function with no effects seen after blackcurrant juice consumption (Jin et al., 2011). It is likely that these techniques are not suitable or sensitive enough to detect improvements in macrovascular function due to anthocyanin consumption.

1.4.4 Effects of anthocyanins on arterial stiffness

A total of 7 studies investigated parameters of arterial stiffness. Carotid−femoral pulse wave velocity (PWV) is the gold standard as it is the most validate and reproducible method for measuring arterial stiffness (Mitchell et al., 2010).

In a study population of prehypertensive and hypertensive postmenopausal women, 22 grams of blueberry consumption (freeze-dried blueberry powder) containing 845mg total polyphenols

and 470mg of anthocyanins showed a significant decrease in PWV in brachial-ankle pulse wave velocity (1,401±122 cm/second; $p<0.01$; baseline levels were 1,498±179 cm/second) after 2 months of daily blueberry consumption (Johnson et al., 2015). McAnulty et al. also showed a decrease in arterial stiffness after six weeks blueberry consumption (250 g of blueberry, 38g of dehydrated powder) in healthy but sedentary individuals. A decrease in carotid-femoral PWV (8.3 ± 2.3 to 7.8 ±2.2 m/s) after cranberry juice consumption for 4 weeks was also found in CAD patients (Dohadwala et al., 2011). However, two studies using freeze-dried blueberries and two other studies using cranberry juice did not observe any benefit on arterial stiffness (Ruel et al. 2013; Riso et al., 2013; Rodriguez-Mateos et al., 2013; Rodriguez-Mateos et al., 2016). Those studies investigated only acute effects, so it is possible that daily consumption of anthocyanins over a certain period of time is necessary in order to see effects on arterial stiffness.

1.4.5 Effects of anthocyanins on blood pressure

Seven RCTs reported a decrease in blood pressure after anthocyanin consumption whereas 19 studies did not observe any effect. A 12 week supplementation with 320 mg of purified anthocyanins per day in hypercholesterolemics was found to decrease systolic blood pressure by 5% (Zhu et al., 2011). Other studies showed a 3 to 6% decrease in systolic and diastolic blood pressure after chronic intake of freeze-dried blueberries, mixed berries, chokeberry extract and cranberry juice (Basu et al., 2010; Erlund et al., 2008; Johnson et al., 2015; McAnulty et al., 2014; Naruszewicz et al., 2007; Novotny et al., 2015). The populations were people with CVD or CVD risk factors (hypertensives, metabolic syndrome, overweight).

It is important to note that those studies used office blood pressure and not 24h ambulatory blood pressure (ABP), which is the gold standard for measurement of blood pressure (Gelfer et al., 2015). Only one study measured 24 h ABP after consumption of purified anthocyanins and did not report significant changes in blood pressure (Hassellund et al., 2012).

1.4.6 Effects of anthocyanins on blood lipids

The effects of anthocyanins on blood lipids are perhaps the most consistent improvements observed in clinical studies. Fourteen RCTs showed improvements in the blood lipid profile. Among them, five studies investigated pure anthocyanins. Most of the studies had study populations with elevated blood lipids (Basu et al., 2014; Kianbakht et al., 2014; Qin et al., 2009; Soltani et al., 2014; Zhu et al., 2011; Zhu et al., 2013) or at increased risk for developing elevated blood lipids, e.g. obese volunteers. In those studies, purified anthocyanins increased HDL-cholesterol by 14 to 19% in dyslipidemics (Li et al., 2015; Qin et al., 2009; Zhu et al., 2011) and 4% in prehypertensives (Hassellund et al., 2013). A decrease in LDL-cholesterol was also observed (7-13%).

In contrast, 12 of the RCTs with berries did not see any effect on blood lipids. Only one study that used purified anthocyanins did not see an effect on blood lipids, but this was the only study with a healthy study population (Karlsen et al., 2007). Four more studies did not see an effect on blood lipids in healthy volunteers (Curtis et al., 2009; Duthie et al., 2006; Jin et al., 2011; Karlsen et al., 2007; Murkovic et al., 2004). Only one study observed improvements in blood lipids in healthy volunteers, with significantly lower total and LDL cholesterol (8.8% and 13.7%, respectively, $p<0.05$) and triglycerides (20.8%, $p<0.05$) after 30 days of fresh strawberry consumption (Alvarez-Suarez et al., 2014).

Table 1: Summary of randomized controlled trials on berries and surrogate markers of cardiovascular disease (modified from Rodriguez-Mateos et al. 2014)

Type of berry	Population	Duration (days)	BP	Lipids	Arterial stiffness	Endothelial function	Polyphenol content (amount of fresh juice or fruit)	Reference
bilberry/black currant ACN	prehypertens	28	=	+			640 mg ACN	Hassellund 2013
bilberry/black currant ACN	prehypertens	28	=				640 mg ACN	Hassellund 2012
bilberry/black currant ACN	healthy	21		=			300 mg ACN	Karlsen 2007
bilberry/black currant ACN	T2D	168		+			160 mg ACN	Li 2015
bilberry/black currant ACN	dyslipidemic	84	=	+			160 mg ACN	Qin 2009
bilberry/black currant ACN	hyperchol	90	+	+		+	320 mg ACN	Zhu 2011
bilberry/black currant ACN	hyperchol	168	=	+			320 mg ACN	Zhu 2013
blackcurrant juice	healthy	1	=	=		=	204 mg TP, 50 mg ACN (250 ml)	Jin 2011
blackcurrant juice	healthy,	42				+	nd (250 ml)	Khan 2014
blueberries, freeze-dried	obese, MS	56	+	=			1624 mg TP, 742 mg ACN (350 g)	Basu 2010
blueberries, freeze-dried	CVD risk	42	=	=	=	=	375 mg ACN, 127 mg CA (148 g)	Riso 2013
blueberries, freeze-dried	healthy	1	=		=	+	319-1791 mg TP, 129-724 mg ACN, (100-560 g)	Rodriguez-Mateos 2013
blueberries, freeze-dried	obese	45	=	=			1462 mg TP,668 mg ACN (≈280 g)	Stull 2010
blueberries, fresh	smokers	21	=				nd (250 ml)	McAnulty 2005
blueberries, frozen	healthy	1	=			=	727 mg TP,348 ACN, (300 g)	Del Bo 2013
blueberry powder, FD	(pre)hypertens	56	+		+		845 mg TP, 470 mg ACN (22 g)	Johnson 2015
blueberry powder, FD	sedentary	42	+		+		nd (38 g)	McAnulty 2014
chokeberry extract	MI	45	+	=			215 mg TP, 64 mg ACN	Naruszewicz 2007

cranberry extract	T2D	84		=			nd (240 ml)	Chambers 2003
cranberry extract	T2D, medicated	84	=	+			nd	Lee 2008
cranberry juice	MS	56	=	=			458 mg TP, 25 mg ACN (480 ml)	Basu 2011
cranberry juice	CAD	28	=	=	+	=	835 mg TP, 94 mg ACN (480 ml)	Dohadwala 2011
cranberry juice	healthy	14		=			2,1 mg ACN (750 ml)	Duthie 2006
cranberry juice	overweight/healthy	56	+	+			62-173 mg TP (240 ml)	Novotny 2015
cranberry juice	obese	28			=		400 mg TP, 21 mg ACN (500 ml)	Ruel 2013
cranberry juice	healthy	1	=		=	+	409 to 1910 mg TP	Rodriguez-Mateos 2016
elderberry extract	healthy, postmenopausal	56	=	=			500 mg ACN (25 g)	Curtis 2009
elderberry extract	healthy	14		=			40 mg ACN (5 ml)	Murkovic 2004
mixed berries, frozen	CVD risk	56	+	+			835 mg TP, 515 mg ACN (160 g)	Erlund 2008
strawberries, FD	MS	28	=	+			200 mg TP, 154 mg ACN (500 g)	Basu 2010
strawberries, FD	obese, dyslipidemic	84	=	+			1001-2005 mg TP, 78-155 mg ACN (25-50 g)	Basu 2014
strawberries, FD	obese	21	=	+			nd (320 g)	Zunino 2012
strawberries, fresh	healthy	30		+			1.13g TP, 308 mg ACN (500 g)	Alvarez-Suarez 2014
whortleberry extract	hyperlipidemics	60		+			7.35 mg ACN (1050 g)	Kianbakht 2014
whortleberry extract	hyperlipidemic	28		+			90 mg ACN (1000 g)	Soltani 2014

Abbreviations: “hyperchol”=hypercholesterolemics, “T2D”= type II diabetes mellitus, “MS”= metabolic syndrome, “MI”= myocardial infarction, “CAD”= coronary artery disease,, “postmen”=postmenopausal, "TP"= total polyphenols, "ACN"= anthocyanins, "nd"= not determined, “FD”=freeze-dried

1.5 Flavonoids as phytoestrogens

1.5.1 Female sex hormones and vascular function

Female sex hormones seem to be supportive factors for cardiovascular health. It is an established epidemiological observation that the incidence of atherosclerosis in premenopausal women is lower than in men (Lerner and Kannel, 1986). Women in their reproductive age have less cardiovascular risk factors and seldom suffer from cardiovascular disease compared to men of the same age (Rossi et al., 2002; Thom et al., 2006).

However and because of this, cardiovascular events in women are less often diagnosed than in men (Skafar et al., 1997). In addition, the cardiovascular risk increases in women after the menopause, with postmenopausal women having a higher fatality rate from coronary attacks than men (32% vs 27%). The risk that a myocardial infarction is unrecognized is higher in women than in men (34% vs 27%) (Lerner and Kannel, 1986). Over the whole span of life, more women than men die of cardiovascular disease (Lejskova et al., 2012).

Some evidence suggest that the female hormone cycle influences endothelial function. Endothelium-dependent vasodilation measured as FMD has been shown to fluctuate during the menstrual cycle, with higher oestradiol levels in the ovulation phase increasing FMD in premenopausal women (Hashimoto et al., 1995; Williams et al., 2001). Synthetic female sex hormones have also been shown to modulate endothelial function (Hurtado et al., 2016). For example, short-term oestrogen replacement therapy (1 or 2 mg of oestradiol daily for 9 weeks) improved FMD in postmenopausal women (Lieberman et al., 1994) and some studies suggest that oral contraception can modulate endothelial function in premenopausal women (Friedman et al., 2011; John et al., 2000).

1.5.2 Menopausal Rating Scale

Menopause is defined as the cessation of menstruation, and a woman is considered postmenopausal after one year has passed since her last period.

As most tissues contain oestrogen receptors the changes occurring in menopause are complex. Deprivation of female sex hormones produces many different symptoms such as vasomotor, musculoskeletal, urogenital and psychological complaints (Hauser et al., 1997). Oestrogen deprivation also has long-term effects for example in bones and the cardiovascular system (Krause et al., 2015). These effects are amplified by the aging process (Hauser et al., 1994).

The Menopause Rating Scale (MRS) is a health-related quality of life scale (HRQoL), which is used as a tool for assessing menopausal complaints, including the burden and the development of symptoms. Before the MRS was developed there was no scale to measure the severity of female aging-symptoms and the degree of suffering (Heinemann et al., 2004). MRS includes questions on psychological, somatic and urogenital complaints. It can help to decide if therapy is necessary but can be used as well to evaluate hormone therapy or other remedies. The Kupperman Index is another HRQoL which includes more symptoms such as paraesthesia, which is the sensation of "pins and needles" or of a limb "falling asleep" without apparent physical cause or formication which is a form of paraesthesia that is described as sensation that resembles that of small insects crawling on the skin (Schneider et al., 2000). There are several other symptoms in the Kupperman Index that are non-specific for menopause, such as headaches and vertigo. On the other hand, several major complaints are not featured in the Kupperman Index, e.g. “vaginal dryness”. The “alteration of libido”, which is attracting more attention today, is completely absent from the Kupperman index as well as dysuria (Schneider et al. 2000). The main difference between them is that MRS lays more

stress on the psychological symptoms and the psychometric quality is more elaborated than the Kupperman index one. (Schneider et al., 2000). In the last five years, more studies have used the MRS II (177) to assess menopausal complaints than the Kupperman Index (89).

1.5.3 Anthocyanins as phytoestrogens

The use of hormone replacement therapy (HRT) to help coping with postmenopausal symptoms is controversial. Some older studies suggested HRT led to an increased risk of cardiovascular disease (Hulley et al., 1998; Rossouw et al., 2002) and an increase in incidence of breast cancers (Olsson et al., 2003). Later the Women's Health Initiative (2002) and the Million Women Study (2003) reported similar effects. The Women's Health Initiative was a RCT with more than 16, 000 postmenopausal women aged 50 to 79 years who were randomly assigned to HRT (0.625 mg conjugated equine oestrogen plus 2.5mg medroxyprogesterone acetate per day (n = 8506)) or matching placebo (n = 8102) (Chlebowski et al., 2016). Results showed that HRT doesn't protect postmenopausal women without CAD from MI. The Million Women Study is a prospective epidemiological study in more than a million women from the UK aged 50-64 years, which provided information about their use of HRT among other factors that influence women's health. The main finding published in 2003 was that current use of HRT is associated with an increased risk of incident and fatal breast cancer (Beral et al., 2003). After those studies, HRT was mostly limited to low-dose, short-term treatment of vasomotor symptoms (Basaria et al., 2009;Krause et al., 2015;Lipovac et al., 2010).

After reviewing all large studies on HRT the Guideline Development Group in the United Kingdom came to the conclusion that there is no convincing evidence that HRT increases the risk of CVD in women aged under 60 years (Krause et al., 2015). There is evidence for both oestrogen and oestrogen plus progestogen preparations and is not influenced by the way of administration. They also found some evidence that HRT with oestrogen and progestogen may be associated with an increased risk of breast cancer. The increase is low and should be compared to a possible benefit and risk ratio in using HRT for treating menopausal symptoms. This risk is getting even lower or is lost when HRT is stopped, because past HRT users don't have a higher risk of breast cancer (Krause et al., 2015).

A popular alternative to HRT are phytoestrogens. Phytoestrogens resemble ovarian oestrogens in structure and in function although they are not steroids (Mackey and Eden, 1998). According to surveys, 15-17% of German postmenopausal women use over-the-counter natural products against menopausal symptoms, such as phytoestrogens and herbs. Most of these women have slight menopausal complaints (Daley et al., 2006; Aidelsburger et al., 2012).The three main classes of phytoestrogens that can be found in plants are isoflavones (present in soy beans and red clover), lignans (found widely in cereals, fruits and vegetables) and cumestanes (found in alfalfa and soy bean sprouts).

Isoflavones are the most wide-spread phytoestrogens and also seem to have the most significant oestrogenic effect. They are found mostly in leguminous plants in particular in soybean (Crozier et al., 2006), and the most abundant type of isoflavones are genistein and daidzein. Compared to ovarian oestrogens, isoflavones are not very potent as their binding affinity to oestrogen receptors is 1,000 times lower than that of oestrogen (Cancellieri et al., 2007). However, their efficacy has not yet been proved (Chen et al., 2015).

A total of 22 randomized controlled trials investigating the effects of isoflavones on menopausal complaints have been conducted (Table 2). In seven of the studies, isoflavones were given in form of genistein, daidzein and glycetin. In one study, genistein and daidzein were given together and in three studies only genistein was used (Albertazzi et al., 2005; D'Anna et al., 2009; Ferrari et al., 2009). In three additional studies, isoflavones were combined with other herbs, vitamins and berberine (Cancellieri et al., 2007; Cianci et al., 2012; Sammartino et al., 2006). Fifteen of the studies observed a positive effect on menopausal symptoms (Albertazzi

et al., 2005; Basaria et al., 2009; Cancellieri et al., 2007; Casini et al., 2006; Cianci et al., 2012; D'Anna et al., 2009; Ferrari et al., 2009; Garcia-Martin et al., 2012; Han et al., 2002; Lipovac et al., 2010; Petri Nahas et al., 2004; Riesco et al., 2011; Sammartino et al., 2006; Welty et al., 2007; Ye et al., 2012). Six studies observed a decrease in hot flushes (Albertazzi et al., 2005; Welty et al., 2007; D'Anna et al., 2009; Cancellieri et al., 2007; Ferrari et al., 2009; Riesco et al., 2011). Two studies showed positive effects on quality of life scales (Garcia-Martin et al., 2012; Cianci et al., 2012), two studies observed positive effects on mood and less depressive and anxiety symptoms (Casini et al., 2006; Lipovac et al., 2010), and six observed a decrease in Kupperman Index (Cancellieri et al., 2007; Ferrari, 2009; Han et al., 2002; Petri Nahas et al., 2004; Sammartino et al., 2006; Ye et al., 2012). Seven studies did not observe any effects on menopausal complaints (Amato et al., 2013; del Giorno et al., 2010; Kok et al., 2005; Levis et al., 2011; Lewis et al., 2006; Nikander et al., 2005; Steinberg et al., 2011).

Anthocyanins resemble isoflavones chemically (Fig. 5), and some evidence suggest that they have estrogenic properties (Schmitt and Stopper, 2001; Walker et al., 2001). It has been proposed that their mechanism of action may be via oestrogen receptors (Nanashima et al., 2015; Chalopin et al., 2010).

However, it is not clear whether anthocyanins can act as phytoestrogens and can improve menopausal symptoms. An advantage to use anthocyanins as phytoestrogens is that no adverse effects have been reported for anthocyanins so far, in contrast to isoflavones.

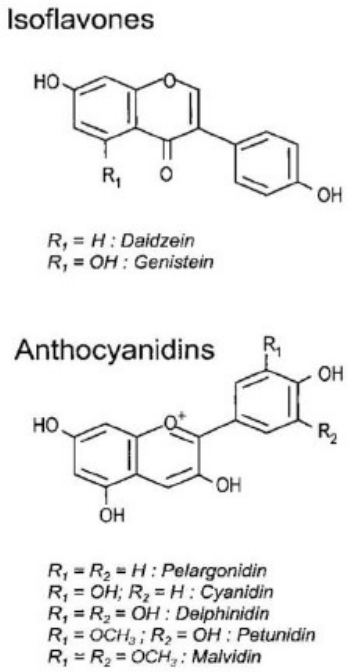


Fig. 5: Resemblance of isoflavones and anthocyanins in their chemical structure (Manach et al., 2004)

Table 2: Randomized controlled trials on the effects of isoflavones on menopausal complaints, modified after Aidelsburger et al. 2012

RCT study design	Isoflavones	Isoflavone content, intervention product	Duration (days)	Endpoint	Positive effects on menopausal symptoms	Reference
cross-over	genistein	90 mg	42	marker of bone metabolism, menopausal symptoms, adverse effects, compliance	+	Albertazzi 2005
parallel	aglycone hypocotyl soy isoflavone	80 or 120 mg/d	730	MENQoL	=	Amato 2013
cross-over	soy isoflavones	160 mg	84	MENQoL, neuropsychological tests	+	Basaria 2009
parallel	soy isoflavones and clover combined with black cohosh, monk's pepper tree, vit E	72 mg	180	Kupperman Index, BL, 60, 120, 180 d	+	Cancellieri 2007
cross-over	aglycone isoflavones	60 mg	180	DSM-IV cognitive performance, VAS, hot flushes per day	+	Casini 2006
parallel	isoflavones and berberine	60 mg + supplements	84	vasomotor symptoms, lipid profile, sweating, libido loss, vaginal dryness, serum lipid pattern (total cholesterol, HDL cholesterol, LDL cholesterol, TG), blood pressure, waist circumference, weight, transaminases, and creatine phosphokinase (CPK)	+	Cianci 2012
parallel	genistein, calcium carbonate, vit D	27 mg	730	hot flushes, thickness and maturation of endometrium	+	D'Anna 2009
parallel	trifolium praetense	40 mg Trifolium praetense	365	Kupperman index, Golombok Rust Inventory of Sexual Satisfaction (GRISS)	=	del Giorno 2010
parallel	isoflavones	60 mg of genistein	84	Kupperman Index, Hot flush frequncy	+	Ferrari 2009
parallel	isoflavones and milk products	50 mg/d	365	quality of life (Cervantes scale), markers of bone metabolism	+	Garcia-Martin 2012

parallel	soy isoflavones	100 mg	108	Kupperman Index, BL, 60, 120, 180 d	+	Han 2002
parallel	genistein, daidzein, glycitein	99 mg	365	bone density, cognitive performance, HRQoL, genistein plasma levels	=	Kok 2005
parallel	soy isoflavones	200 mg	730	bone density, menopausal symptoms, vaginal estrogenization, serum lipids, thyrotropin, and thyroid peroxidase autoantibodies	-	Levis 2011
parallel	lignans or soy isoflavones	42 g	112	MENQoL, hot flushes, excretion of phytoestrogens in urine for compliance assessment, dietary diary	=	Lewis 2006
parallel	isoflavones	80 mg	90	depression, anxiety state	+	Lipovac 2010
cross-over	isoflavones	114 mg	90	vaginal dryness, maturity and thickness of endometrium, histology	=	Nikander 2005
parallel	soy isoflavones	60 mg	168	Kupperman, blood lipids	+	Petri Nahas 2004
parallel	soy isoflavones, with and without exercise	25 mg	168	Kupperman Index, HRQoL	= (KI); + (hot flushes)	Riesco 2011
parallel	combination of isoflavones, lignans and Cimicifuga racemosa	Isoflavones 60 mg; lignans 100 mg	84	Kupperman Index	+	Sammartino 2006
parallel	daidzein,glycitein, genistein	100 mg/d or 120 mg/d (higher dose in second year)	730	bone density, safety, musculoskeletal complaints	=	Steinberg 2011
cross-over	special diet (TLC) + soybean	101 mg	56	number of hot flushes per day,MENQoL	+	Welty 2007
parallel	soy germ isoflavones	84 or 126 mg	168	Hot flush frequency, Kupperman Index, serum 17beta-estradiol, follicle-stimulating hormone, luteinizing hormone, and blood lipids	+	Ye 2012

MENQoL= Menopause-related quality of life questionnaire; DSM-IV= Diagnostic and Statistical Manual of Mental Disorders; HRQoL= Health-related quality of life; TLC= therapeutic lifestyle changes

1.6 Open questions and hypothesis

Although there have been some studies investigating the effects of anthocyanins on CVD risk during the last years, the evidence is limited. Even after thorough literature research no study could be found that has investigated whether pure anthocyanins can improve vascular function in healthy humans, and whether the effects are dose-dependent. This information is needed to understand whether berries and anthocyanin rich foods in our diet can be beneficial for the general population.

The hypothesis of the present thesis was that anthocyanin consumption can improve CVD risk markers in postmenopausal women. In this work, firstly a time-course and dose finding study was conducted in order to investigate whether pure anthocyanins can improve vascular function, and which is the optimal time and amount needed to obtain maximal effects. Secondly, the aim was to investigate whether pure anthocyanins can improve vascular function in healthy postmenopausal women after daily consumption for one month in a double-blind randomized controlled trial. As an exploratory endpoint it was also investigated whether anthocyanins could reduce menopausal symptoms. The optimal dosage of anthocyanins was chosen according to the results of the first study.

In the following the studies will be presented chronologically. At first the acute study will be presented with the materials and methods and the subsequent results. The chronic study which is based upon the first study and has a different study population, methods and focus will be presented afterwards also with a section on materials and methods and results.

2 Effects of anthocyanins on endothelial function: a dose response study

2.1 Materials and methods

2.1.1 Study participants and study design

The study was conducted at the Division of Cardiology, Pulmonology and Vascular Medicine of Düsseldorf University. Subjects were recruited via word of mouth from doctoral research students and personal acquaintances. The study population consisted of healthy young men aged 18-35 years. Women were excluded because of the complex hormonal changes during the menstrual cycle. Exclusion criteria were age over 35 years, an acute infection with a CRP >0.5 mg/dl and suffering from malignant diseases. Men with suspected or manifest vascular diseases including coronary heart disease, renal, peripheral or cerebrovascular occlusive diseases were also excluded. Other exclusion criteria were intolerance or allergies to berries, acute or chronic renal failure, as well as chronic heart failure and cardiac arrhythmia. Volunteers that took medication or dietary supplements were also excluded.

The study was a 6-armed randomized placebo-controlled double-blind crossover trial. Every volunteer took six different amounts of anthocyanins on each of the six study days. The study days were scheduled one week apart for wash out. One week prior to the study day the volunteers followed a special diet which excluded anthocyanin-rich food and beverages. The volunteers were asked to exclude all sorts of berries (strawberries, blueberries, raspberries, cranberries, etc. including fruits, juices, ice cream, cakes, marmalades, jam etc.), red oranges, red onions, red wine, aubergines, red cabbage and all other purple vegetables from their diet. The volunteers followed more extensive low polyphenol diet three days prior to the study day. They were also asked to excluded fruits and vegetables, chocolate/cocoa, tea (black, green etc.), coffee, fruit juices, jams and preserves, fruit teas and soy products from their diet. Twenty-four hours prior to the measurements they were not allowed to drink alcohol (wine, beer etc.) and were asked to fast at least twelve hours before the measurement. See Fig. 6 for the enrolment of the young healthy men.

On the study day, participants were asked to lie down for ten minutes prior to the examination and did not get up until the examination was completed. After the resting period, flow-mediated vasodilation (FMD) and blood pressure measurements were performed, followed by blood draws. The volunteer`s right arm was used for the FMD, whereas blood pressure was measured and blood was taken from the left arm. After the baseline measurement, the volunteers ingested the anthocyanin or control capsules with 500 ml of low nitrate water (Vio®). Measurements and blood draws were repeated at 2 and 6 hours post-consumption of the anthocyanin or control capsules.

Written informed consent was obtained from all volunteers before participating in the study. The studies were conducted following the guidelines laid down in the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the Heinrich-Heine-University (study number 4433R-2013091447-1).

Flow Diagram on the enrolment of young healthy men in the dose-dependency study

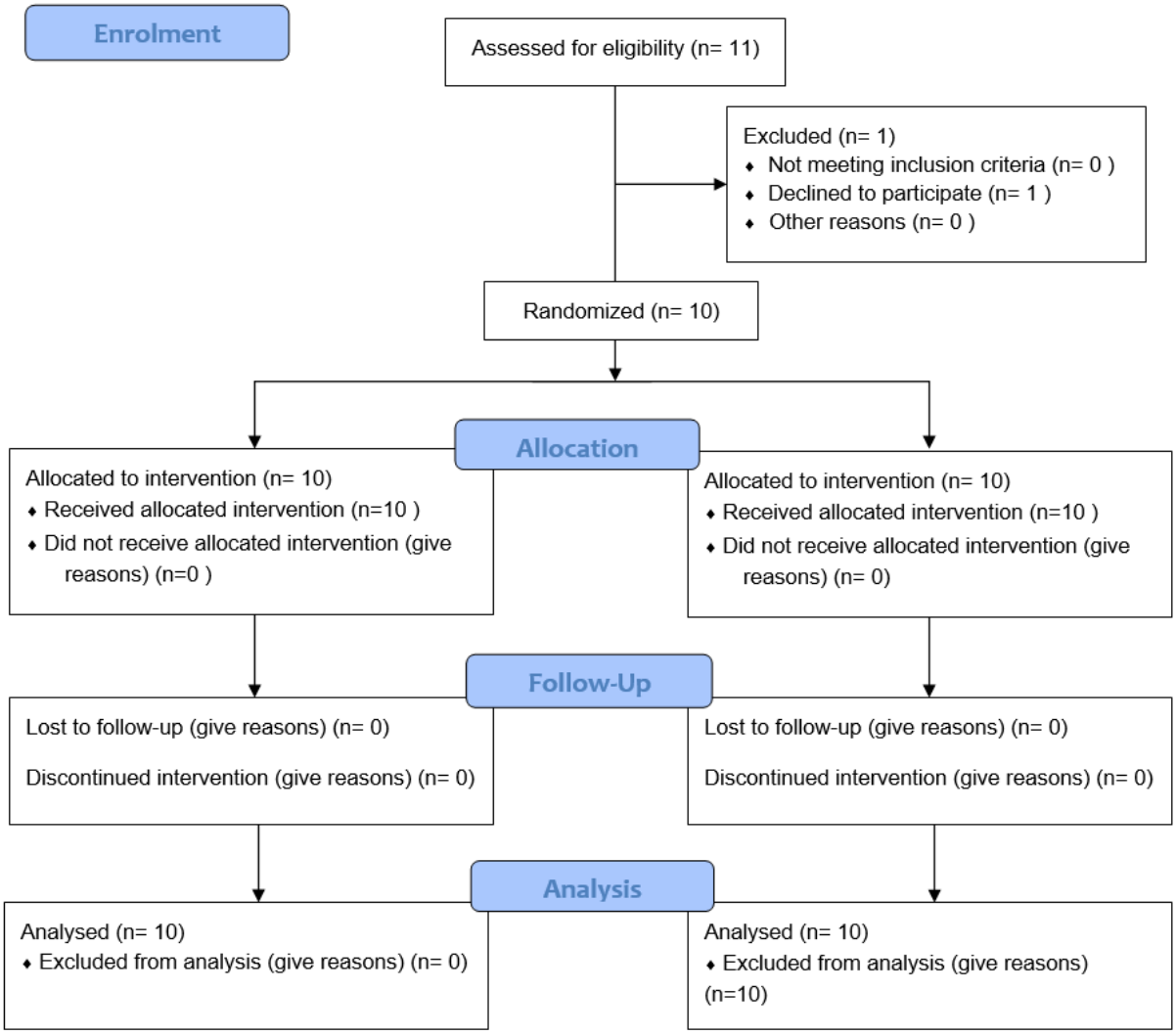


Fig.6: CONSORT Flow Diagram on the enrolment of young healthy men in the dose-dependency study

2.1.2 Test materials

Encapsulated purified anthocyanins were used. The capsules are commercially available and were provided by Medox® (Polyphenols AS, Sandnes, Norway). Each capsule contained 80 mg of pure anthocyanins derived from blackcurrants and bilberries. Beside anthocyanins (41%), each capsule contained 22,5% maltodextrin, 18,5% citric acid , 12,6% fructose, 5,1% lipids and 0,3% of other polyphenols (Qin et al., 2009, p. 486) (Table 3). The total amount of anthocyanins in the capsule consisted of 17 different kinds of anthocyanins: Each capsule contained 17 different types of anthocyanins, the most abundant being glucosides, galactosides and arabinosides of delphinidin (58%) and cyanidin (33%), followed by malvidin (3%), petunidin (2,5%) and peonidin (2,5%) derivatives. Delphinidin and cyanidin rhammnosides were also present in small amounts (1%).The placebo capsules looked identical to the anthocyanin capsules but they only contained pullulan and maltodextrin. The randomization was done via random sampling with opaque sealed envelopes. During every visit, volunteers took six capsules containing placebo or purified anthocyanins. There were six dosages: 0 mg anthocyanins (six placebo capsules), 80 mg anthocyanins (one Medox capsule, five placebo capsules), 160 mg (two Medox capsules, four placebo capsules), 240 mg (three Medox capsules, three placebo capsules), 320 mg (four Medox capsules, two placebo capsules) and 480 mg anthocyanins (six Medox capsules).

Table 3: Composition of the anthocyanin capsules provided by Medox (Polyphenols AS, Sandnes, Norway) with content of all compounds per dose

Capsule composition	**0 mg**	**80 mg**	**160 mg**	**240 mg**	**320 mg**	**480 mg**
Total weight (mg)	263	414	566	717	868	1170
Maltodextrin (mg)	263	263	263	263	263	263
Citric acid (mg)	0	36	72	108	144	216
Fruit sugars (mg)	0	25	49	74	98	147
Lipids (mg)	0	10	20	30	40	50
other polyphenols (mg)	0	0.6	1,2	0.7	42768	0.8
Anthocyanins (mg)	0	80	160	240	320	480
Composition of anthocyanins per dose (mg)						
Dp-3g, Dp-3gal, Dp3	0	46	93	139	186	278
Dp-3g, Dp-3gal, Cy-3a	0	26	53	79	106	158
Pe-3g, Pe-3gal, Pe-3a	0	2	4	6	8	12
Pn-3g, Pn-3gal, Pn-3a	0	2	4	6	8	12
Mv-3g, Mv-3gal, Mv-3a	0	2	5	7	10	14
Dp-3r, Cy-3r	0	0.8	1.6	2.4	3.2	4.8

Abbreviations.: Cy-3a, cyanidin-3-O-ß-arabinosides; Cy-3g, cyanidin-3-O-ß-glucosides; Cy-3gal, cyanidin-3-O-ß-galactosides; Cy-3r, cyaniding-3-O-rutinoside; Dp-3a, delphinidin-3-O-ß-arabinosides; Dp-3g, delphinidin-3-O-ß-glucosides; Dp-3gal, delphinidin-3-O-ß-galactosides; Dp-3r, delphinidin-3-O-rutinoside; Mv-3a, malvidin-3-O-ß-arabinosides; Mv-3g, malvidin-3-O-ß-glucosides; Mv-3gal, malvidin-3-O-ß- galactosides; Pe-3a, petunidin-3-O-ß-arabinosides; Pe-3g, petunidin-3-O-ß-glucosides; Pe-3gal, petunidin-3-O-ß-galactosides; Pn-3a, peonidin-3-O-ß-arabinosides; Pn-3g, peonidin-3-O-ß-glucosides; Pn-3gal, peonidin-3-O-ß-galactosides.

2.1.3 Flow-mediated vasodilation and power analysis of the primary end point

Flow-mediated vasodilation (FMD) measurements were conducted according to the protocols established in our work group in combination with an automated analysis system (Brachial analyser, Medical Imaging Applications, Iowa City, IA, USA). The volunteers rested in a supine position for 10 minutes before starting the measurement. All baseline measurements were conducted between 7:30 AM and 10:30 AM. The ultrasound machine used was a 10-MHz transducer (Vivid I, GE healthcare) coupled to a GE 12L-RS probe. Longitudinal images were taken of the brachial artery of the right arm above the cubital joint. The right arm was extended and supported comfortably. A standard blood pressure cuff (Boso clinicus, Jungingen, Germany) was attached to the volunteer's right forearm. ECG recordings were performed. A suitable measuring point with clearly visible intima with anatomic landmarks was chosen to help maintain the same position during the measurement as shown in Fig. 7. Afterwards the cuff was inflated to 50 mmHg above the systolic blood pressure (180 to 200 mmHg maximum). The same position was maintained for 5 minutes after which the occlusive cuff was released immediately. Doppler recordings confirmed artery location and measured blood flow. 2D ultrasound images for analysing FMD were stored at 20s, 40s, 60s, 80s after deflating the cuff deflation (Heiss, Sansone et al., 2015). Measurements were repeated exactly two hours and six hours after capsule intake. The volunteers did not eat anything between measurement and were only allowed to drink Vio® water ad libitum.

Images were analysed using the automatic edge-detection software (Brachial Analyzer, Medical Imaging Applications, Iowa City, IA, USA). Long segments of the vessel at baseline and 60 s were compared at the same point in time during the cardiac cycle at the end of the diastole (beginning of R-peak). The diameter chosen was the mean maximum value of three images. Percentage of FMD was calculated as maximal relative diameter gain compared to the baseline diameter : $(\text{diameter}_{max} - \text{diameter}_{baseline}) / \text{diameter}_{baseline} \times 100$ (Corretti et al., 2002).

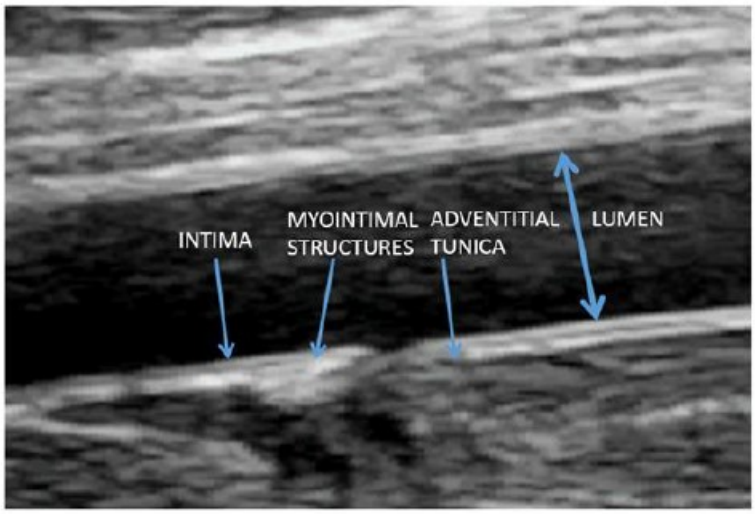


Fig. 7: Image detail, measuring point for FMD of brachial artery (longitudinally), 10-MHz transducer; Vivid I, GE

2.1.4 Blood pressure measurements

Systolic and diastolic blood pressure was measured at baseline, 2 and 6 hours post-consumption of the capsules, just before the FMD measurements. Office blood pressure (BP) measurements (OBPM) were performed using an electronic oscillometric device (Boso medicus PC2, Jungingen, Germany). The measurements were repeated three times on the non-dominant arm, and the first measurement was discarded. An average of two measurements was calculated.

2.1.5 Blood collection and biochemical measurements

Phlebotomy was performed on the left arm with a butterfly cannula immediately after the vascular measurements (at baseline, 2 and 6 hours post consumption of the test capsules). Blood samples were collected in EDTA or heparin tubes and were immediately processed after collection. The tubes were centrifuged at 1700*xg* for 15 min at 4°C. All samples were aliquoted and kept at -80°C until analysis. Samples for polyphenol analysis were spiked with 2% formic acid before storage.

All clinical chemistry parameters including total, LDL- and HDL-cholesterol, triglycerides, Hb_{A1c}, glucose, and whole blood count were analysed on baseline samples taken at the first visit of the last six volunteers by the Institute for Clinical Chemistry, University Hospital Düsseldorf, Germany, using standard techniques.

2.1.6 Diet questionnaires

A 24-hours dietary recall was carefully filled in by the volunteers to assess the compliance of the (poly)phenol-free diet.

2.1.7 Statistical methods

Power calculations were performed for the primary endpoint of the change in FMD response. In a crossover study, a total of 10 subjects will be necessary to detect a change of 0.73% in FMD, at a power of 0.8, significance level (adjusted for sidedness) of 0.025, and standard deviation within patients of 0.45.

This number is consistent with other studies with similar endpoints and study design (Rodriguez-Mateos et al., 2013). Moreover, the ‘‘repeated-measure’’ experimental design in which each subject act as their own control allows to conduct the experiment more accurately, thus reducing the error variance. Two factor repeated-measures ANOVA was fitted to analyse the data by using the GraphPad Prism version 6 software (GraphPad Software Inc). A post hoc analysis was carried out by using Tukey‘s test. Significance was defined at $p< 0.05$, with p values represented in figures as *$p < 0.01–0.05$, **$p < 0.001–0.01$.

2.1.8 Quantification of plasma phenolic metabolites

Plasma samples (600 µL were thawed on ice and centrifuged at 15000 x g for 15min at 4°C. Then, 350 µL of plasma was diluted 1:1 with 4% phosphoric acid and spiked with taxifolin (100nM). The samples were then loaded (600 µL) on Oasis® HLB µElution plates (2 mg sorbent per well, 30 mm) from Waters (Eschborn, Germany) for solid phase extraction. Samples were concentrated 5.8 times in HPLC grade 99.9% methanol purchased from Sigma-Aldrich Co. (Steinheim, Germany).

Quantification of plasma phenolic metabolites was performed using ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-q-TOF-MS) using a validated method previously described (Feliciano, Boeres, Massacessi, Istas, Ventura, Nunes Dos Santos et al., 2016; Feliciano, Mecha et al., 2016).

The following authentic standards were used: 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, isoferulic acid 3-O-sulfate disodium salt, isoferulic acid 3-O-β-D-glucuronide, dihydro isoferulic acid 3-O-sulfate disodium salt and dihydro isoferulic acid 3-O-ß-D-glucuronide were purchased from Toronto Research Chemicals (Toronto, Canada). 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 Claudia Nunes dos Santos and Dr Rita Ventura, and their synthesis has been described elsewhere (Pimpao et al., 2015). All the polyphenol and phenolic acid aglycones were obtained from Sigma-Aldrich Co. (Steinheim, Germany) and 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 mm) were from Waters (Eschborn, Germany). For the Milli-Q system (Merck KGaA, Darmstadt, Germany) ultra-pure water was used. Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich Co. (Steinheim, Germany).

2.1.9 Pharmacokinetic analysis

Area under the curve (AUC) values represent corrected AUC in the entire study. Correction was done by subtracting baseline plasma concentrations from the baseline, 2h and 6h plasma concentrations. Negative values were set to zero and corrected AUC was calculated (http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm377465.pdf).

2.2 Results

2.2.1 Baseline demographics and tolerance

Ten healthy Caucasian men aged 22 to 35 years were recruited. Baseline characteristics of the subjects were within normal limits. The characteristics are shown in Table 3. The median age was 23.5 years. The Framingham coronary heart disease risk score, that estimates the risk of heart attack in the next ten years, was within normal limits (less than 1%) (Wilson et al., 1998).

All 10 individuals completed the 6 visits. There were no significant differences in baseline characteristics between volunteers and the values in table 3 are within normal ranges for healthy individuals. The intervention capsules at all dosages were well tolerated by all subjects and they did not report any adverse events.

Table 4: Baseline characteristics of study population (n=10) and laboratory values of the last six volunteers

Characteristic	**mean±SD**
Age (years)	25 ±3.6
Height (cm)	182 ±5.7
Weight (kg)	79.5± 9.4
BMI (kg/m²)	24 ± 2
SBP (mmHg)	118.5±9.7
DBP (mmHg)	69±7
HR (bpm)	63±7
Total cholesterol (mg/dl)	170±30
LDL (mg/dl)	109±38
HDL (mg/dl)	54±7
Triglycerids (mg/dl)	91±53
Plasmaglucose (mg/dl)	90±4
CRP (mg/dl)	<0,1
Creatinine (mg/dl)	1±0.1
GFR (MDRD) (ml/min)	108±12

Abbreviations: CRP=c-reactive protein; GFR= Glomerular Filtration Rate estimated with Modification of Diet in Renal Disease (MDRD) formula

2.2.2 Flow-mediated vasodilation

Endothelial function, measured by flow-mediated vasodilation (FMD), was assessed in 10 individuals after intake of different anthocyanin doses. At 2 h after consumption, significant changes in FMD were observed for 240, 320 and 480 mg anthocyanins when compared with control (Fig. 7). The magnitude of the changes ranged between 0.2 and 1.3%. At 6 hours post-consumption, significant changes were found for 240 and 480 mg anthocyanins as compared to control (1.1 and 1.2 %, respectively, Fig. 8). No significant changes were seen after consumption of 80 or 160 mg of ACN. When compared with FMD baseline levels (within treatment), all anthocyanins capsules, except for placebo, increased significantly FMD at 2h post consumption (Rodriguez-Mateos et al., 2018). Capsules containing 160, 240, 320, and 480 mg anthocyanins increased FMD at 6h after consumption (Table 5).

The FMD reached a plateau at 240 mg and 320 mg. At the higher amount of 480 mg the FMD increased even further and reached the maximum FMD 2h after intake with a mean of 8.0±0.3%. The results show that anthocyanins increase FMD dose-dependently in young healthy male volunteers (Rodriguez-Mateos et al., 2018).

Table 5: FMD results. Statistical significance is shown for differences between baseline and FMD at 2 or 6h post-consumption for every dose. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test (mean ± SEM)

Dose (mg)	**0h**	**2h**		**6h**	
	FMD (%)	FMD (%)	**p-value**	FMD (%)	**p-value**
0	6.28 ± 0.71	6.51 ± 0.76	0.457	6.48 ± 0.74	0.562
80	6.39 ± 0.87	6.86 ± 0.69	0.048	6.68 ± 0.72	0.314
160	6.30 ± 0.72	7.00 ± 0.99	0.001	6.97 ± 0.84	0.002
240	6.20 ± 0.79	7.38 ± 0.90	<0.0001	7.25 ± 1.02	<0.0001
320	6.27 ± 0.97	7.42 ± 0.97	<0.0001	7.06 ± 0.93	0.0003
480	6.25 ± 0.74	7.55 ± 0.86	<0.0001	7.45 ± 0.93	<0.0001

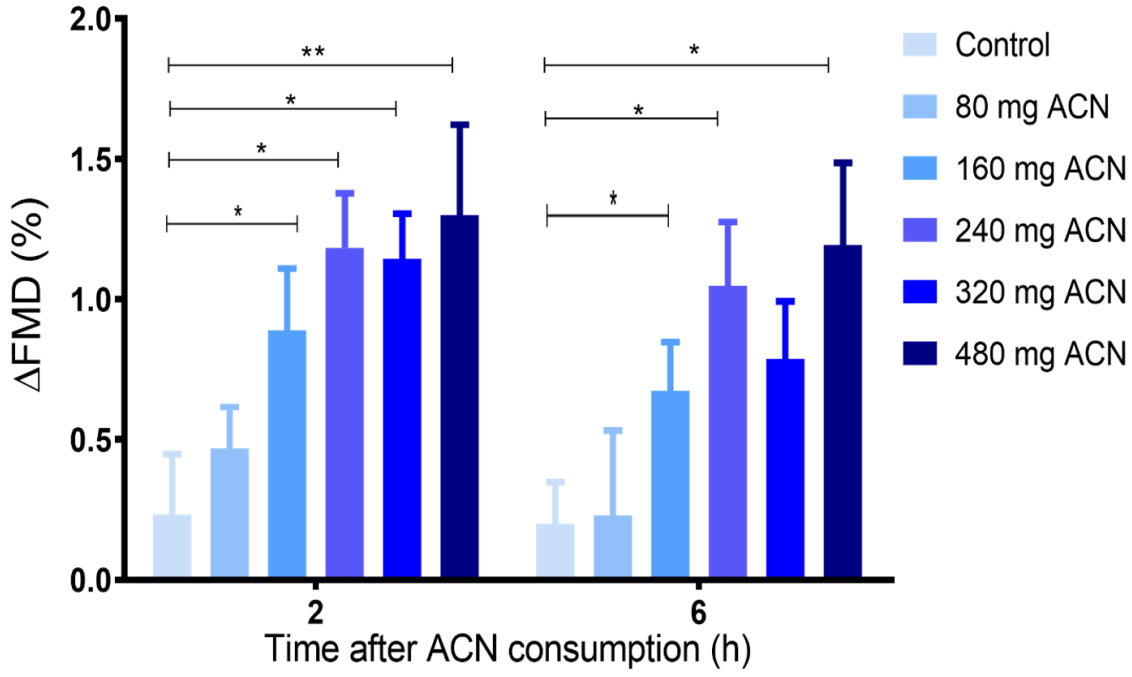


Fig. 8: Changes in % FMD values for every dose at 2h and at 6h after ACN consumption. Significant changes were observed for 240 mg (p=0.016), 320 mg (p=0.022) and 480 mg (p=0.005) ACN at 2h after consumption as compared to control. Six hours after consumption, a significant increase in FMD was observed for 240 mg (p= 0.038) and 480 mg (p= 0.010) ACN as compared to control.

2.2.3 Blood pressure

The blood pressure values were all within the normal limits for healthy young men. No significant changes in systolic or diastolic blood pressure were observed after consumption of anthocyanin or placebo capsules. There is no dose-dependent significant decrease in systolic or diastolic blood pressure (Fig. 9). The blood pressure remains stable throughout the intervention. No significant change in blood pressure compared to the baseline can be observed at any point in time (Fig. 10).

The mean systolic blood pressure before intake of treatment was not statistically relevant between visits, ranging from 114 to 117 mmHg, while the mean diastolic blood pressure at baseline ranged from 66 mmHg to 69 mmHg. Two and 6 hours after intake of anthocyanins no significant differences in blood pressure were found, when compared with placebo or when compared with baseline (Fig. 9). No significant differences in changes in blood pressure were also found between any treatment (Figure 10)(Rodriguez-Mateos et al., 2018).

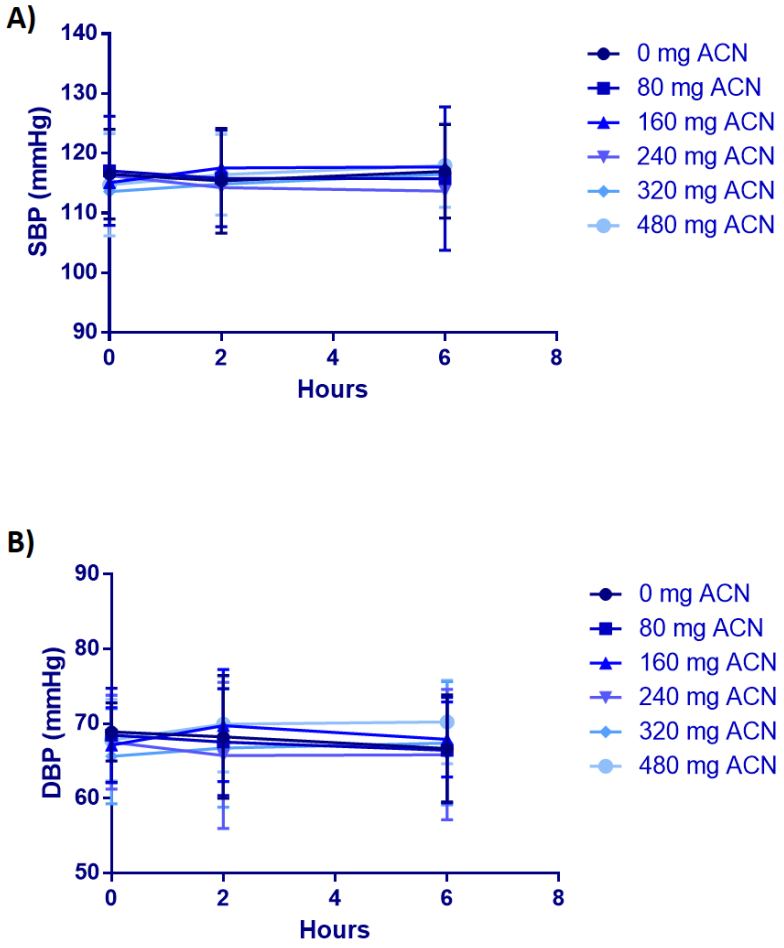


Fig. 9: Systolic A) and diastolic B) blood pressure after ACN consumption, all values are expressed as mean±SEM. No changes were observed in systolic blood pressure or diastolic blood pressure.

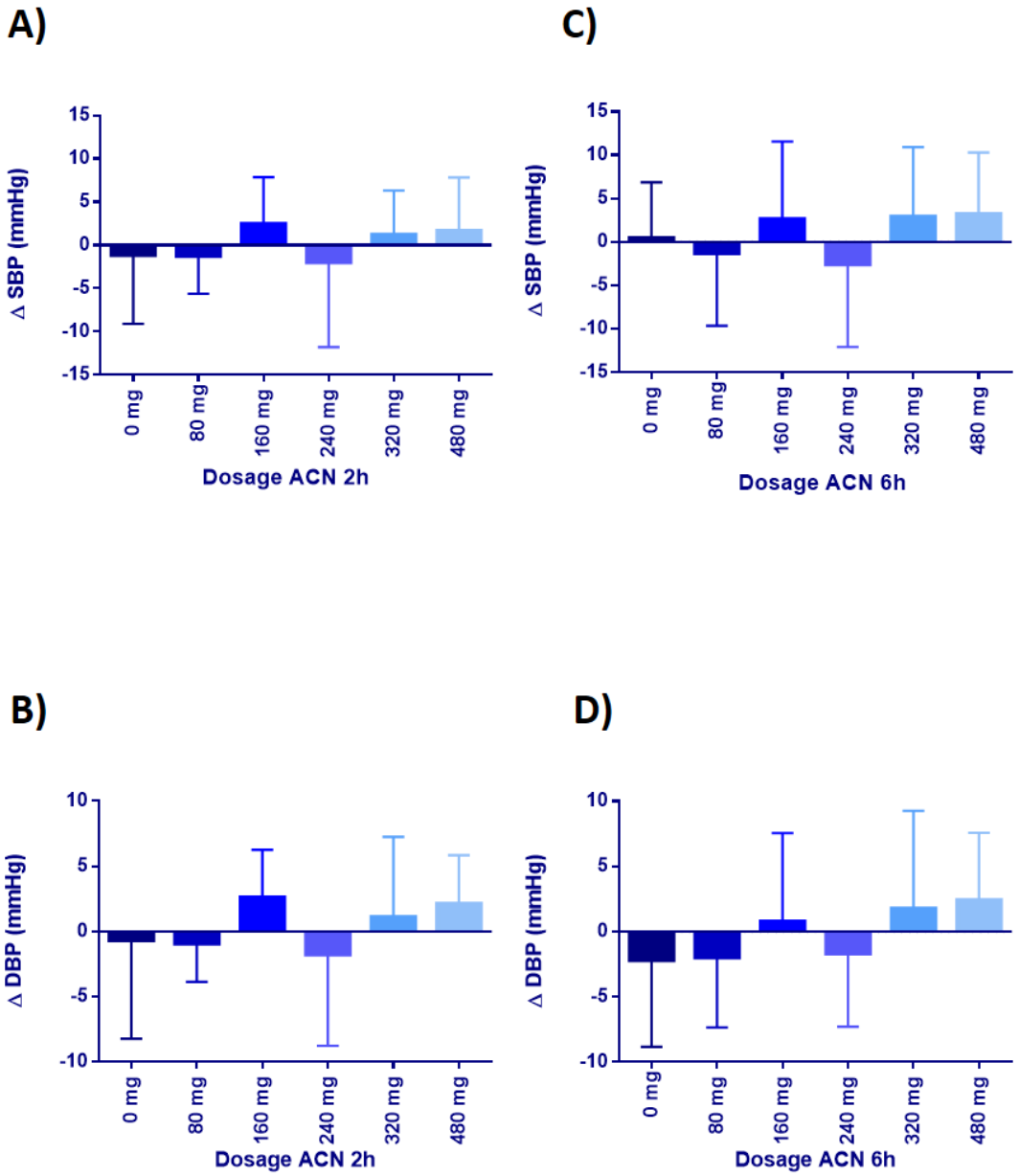


Fig. 10: Changes in systolic and diastolic blood pressure (SBP and DBP) compared to baseline blood pressure, expressed as mean changes ±SEM. A) SBP difference 0 to 2 hours, B) DBP difference 0 to 2 hours, C) SBP difference 0 to 6 hours, D) DBP difference 0 to 6 hours

2.2.4 Bioavailability and metabolism of anthocyanins

Using a validated UPLC-Q-TOF MS method with authentic standards (Feliciano, Boeres, Massacessi, Istas, Ventura, Nunes Dos Santos et al., 2016; Feliciano, Mecha et al., 2016), a total of 57 plasma-derived (poly)phenol metabolites were quantified at baseline, 2 and 6 h after consumption of 5 different doses of anthocyanins capsules and control. It was found that (R)-(+)-2-(4-hydroxyphenoxy)propionic acid, o-coumaric acid, sinapic acid, pyrogallol-O-1-sulfate, 2-methylpyrogallol-O-sulfate, 3-hydroxybenzaldehyde and 4-hydroxybenzoic acid did not

reach the method quantification limits and were therefore excluded. In total, 50 compounds were included in further analysis. These 50 (poly)phenols were then segregated into classes per molecular structure for a more comprehensive analysis (Rodriguez-Mateos et al., 2018).

Table 6 shows the area under the curve (AUC) concentrations of plasma (poly)phenols over 6 hours' time after consumption of control and 480 mg ACN capsules. Upon intake of 480 mg anthocyanins, the plasma AUC levels of 39 compounds increased whereas the AUC levels of 11 other compounds decreased. The total quantified amount of (poly)phenols (50 compounds) increased 1.7-fold in plasma after intake of the highest anthocyanins dose (480 mg). The highest fold changes (relative to baseline concentration) were observed for the pyrogallols with a total 14-fold increase after intake of 480 mg anthocyanins. The compound 1-methylpyrogallol-O-sulfate showed the highest (non-significant) increase ranging from control AUC values of 4 ± 2 nM*h to 480 mg ACN plasma AUC values of 182 ± 66 nM*h. Hippuric acids increased 5.7-fold with hippuric acid showing the highest relative increases in AUC from 682 ± 183 nM*h (control) to 7516 ± 2714 nM*h (480 mg ACN) as shown in table 6. A significant increase in plasma AUC levels was detected for 3-(4-Hydroxyphenyl)propionic acid, with an increase of 41991 nM*h ($p < 0.0001$). No other significant increases in plasma were detected, due to the high inter-individual variability.

2.2.4.1 Plasma anthocyanin metabolites correlate with improvements in FMD

A correlation analysis was performed between the changes in % FMD and the concentrations of plasma metabolites after 2 and 6h of anthocyanins consumption. Significant correlations were found for ferulic acid 4-O-sulfate, homovanillic acid, isoferulic acid, 4-hydroxyhippuric acid, isoferulic acid 3-O-β-D-glucuronide, dihydroferulic acid 4-O-sulfate, and for ferulic acid 4-O-β-D-glucuronide after 2h ACN intake (Table 7). Dihydroferulic acid 4-O-sulfate, dihydroferulic acid 4-O-β-D-glucuronide, 1-methylpyrogallol-O-sulfate, and dihydro caffeic acid 3-O-sulfate were significantly correlated with Δ% FMD 6h post anthocyanins consumption (Table 7) (Rodriguez-Mateos et al., 2018).

Table 6: Areas under the curve for plasma derived metabolites after consumption of anthocyanins-rich capsules (control and 480 mg) (n = 10 and results are shown in mean ± SEM). Relative changes were calculated by subtraction of AUC values of control from the 480 mg divided by control AUC values. Statistical analysis was done using t-test between control AUC and 480 mg AUC (* significant p-value < 0.0001)

	AUC (nM*h) ± SEM		**Relative Changes**
	Control	480 mg ACN	
Total (poly)phenols	**14,717 ± 3,792**	**39,874 ± 20,337**	**1.7**
Pyrogallols (total)	**11 ± 8**	**138 ± 81**	**14.0**
1-Methylpyrogallol-O-sulfate	4 ± 2	182 ± 46	**46.4**
Pyrogallol-O-2-sulfate	27 ± 17	253 ± 119	**8.5**
Hippuric acids (total)	**644 ± 223**	**4,858 ± 1,883**	**6.5**
Hippuric acid	682 ± 183	7,516 ± 2,714	**10.0**
3-Hydroxyhippuric acid	22 ± 8	109 ± 51	**4.0**
α-Hydroxyhippuric acid	672 ± 272	2,009 ± 1,325	**2.0**
4-Hydroxyhippuric acid	70 ± 24	125 ± 31	**0.8**

Catechols (total)	**183 ± 85**	**938 ± 547**	**3.5**
Catechol-O-sulfate	78 ± 27	1,125 ± 919	**13.4**
4-Methylcatechol-O-sulfate	228 ± 119	263 ± 50	**0.2**
Propionic acids* (total)	**12,424 ± 5,316**	**54,415 ± 33,295**	**3.4**
3-(4-Hydroxyphenyl)propionic acid*	12,424 ± 5,316	54,415 ± 33,295	**3.4**
Cinnamic acids (total)	**204 ± 52**	**630 ± 291**	**1.8**
Dihydroferulic Acid 4-O-sulfate	3 ± 2	32 ± 22	**8.5**
Dihydroferulic acid	103 ± 45	808 ± 610	**6.8**
Dihydroferulic Acid 4-O-β-D-glucuronide	5 ± 1	56 ± 47	**9.6**
Dihydrocaffeic Acid 3-O-sulfate	24 ± 19	152 ± 82	**5.4**
Caffeic acid 3-β-D-glucuronide	1 ± 0	4 ± 1	**4.6**
Dihydro isoferulic acid 3-O-β-D-glucuronide	4 ± 2	11 ± 4	**1.9**
Dihydrocaffeic Acid 3-O-β-D-glucuronide	1 ± 0	3 ± 1	**1.8**
Chlorogenic acid	2 ± 1	5 ± 2	**1.6**
Ferulic acid	67 ± 17	135 ± 62	**1.0**
Ferulic acid 4-O-β-D-Glucuronide	16 ± 7	24 ± 15	**0.5**
Dihydro isoferulic acid 3-O-sulfate	4 ± 3	6 ± 2	**0.5**
Dihydrocaffeic acid	53 ± 34	69 ± 19	**0.3**
p-Coumaric acid	8 ± 0	10 ± 3	**0.2**
m-Coumaric acid	26 ± 12	26 ± 15	**0.0**
Ferulic acid 4-O-sulfate	101 ± 47	93 ± 39	**-0.1**
Isoferulic acid 3-O-β-D-glucuronide	55 ± 25	48 ± 29	**-0.1**
Isoferulic acid	87 ± 42	74 ± 62	**-0.2**
Isoferulic acid 3-O-sulfate	5 ± 3	4 ± 2	**-0.2**
Caffeic acid 4-β-D-glucuronide	8 ± 5	4 ± 1	**-0.6**
Caffeic acid	3 ± 2	1 ± 0	**-0.6**
Phenylacetic acids (total)	**1,748 ± 864**	**3,393 ± 1,172**	**0.7**
4-Hydroxyphenyl acetic acid	217 ± 70	1,297 ± 724	**5.0**
Homovanillic acid	411 ± 108	1,621 ± 1,122	**2.9**
3-Hydroxyphenyl acetic acid	213 ± 107	287 ± 164	**0.3**
Phenylacetic acid	2,743 ± 1,364	3,617 ± 817	**0.3**
3.4-Dihydroxyphenyl acetic acid	59 ± 13	75 ± 21	**0.3**
Homovanillic acid sulfate	5 ± 3	3 ± 1	**-0.3**
Benzaldehydes (total)	**31 ± 14**	**48 ± 18**	**0.5**
4-Hydroxybenzaldehyde	23 ± 11	34 ± 19	**0.5**
3.4-Dihydroxybenzaldehyde	33 ± 12	30 ± 10	**-0.1**
Benzoic acids (total)	**2,735 ± 860**	**2,829 ± 722**	**-0.2**

2.3-Dihydroxybenzoic acid	0 ± 0	7 ± 2	**18.3**
2.5-Dihydroxybenzoic acid	18 ± 5	122 ± 61	**5.9**
2-Hydroxybenzoic acid	34 ± 14	205 ± 51	**5.0**
Gallic acid	5 ± 3	11 ± 4	**1.2**
Syringic acid	71 ± 20	154 ± 34	**1.2**
3.5-Dihydroxybenzoic acid	12 ± 4	19 ± 9	**0.6**
3-Hydroxybenzoic acid	11 ± 6	17 ± 4	**0.4**
4-Methylgallic-3-O-sulfate	228 ± 119	263 ± 50	**0.2**
Benzoic acid	1,752 ± 364	1,977 ± 783	**0.1**
Vanillic acid-4-O-sulfate	66 ± 35	70 ± 59	**0.1**
Protocatechuic acid	17 ± 12	14 ± 5	**-0.2**
Isovanillic acid	848 ± 610	365 ± 100	**-0.6**
Vanillic acid	2,335 ± 1,768	518 ± 286	**-0.8**

Table 7: Correlations between % FMD and plasma metabolite concentration. Data with equal and unequal variances was tested with Pearson and Spearman tests respectively. * < 0.05 and ** < 0.01

	Correlation	
Compound	**2h post consumption**	**6h post consumption**
Dihydro ferulic Acid 4-*O*-Sulfate	0.30 *	0.41**
Ferulic Acid 4-*O*-Sulfate	0.41**	-
Homovanillic acid	0.39**	-
Isoferulic acid	0.35 *	-
4-hydroxyhippuric acid	0.35 *	-
Isoferulic Acid 3-*O*-ß-D-Glucuronide	0.32 *	-
Ferulic acid 4-*O*-ß-D-Glucuronide	0.29 *	-
Dihydro Ferulic Acid 4-*O*-ß-D-Glucuronide	-	0.32 *
1-Methylpyrogallol-*O*-sulfate	-	0.32 *
Dihydro Caffeic Acid 3-*O*-Sulfate	-	0.31 *

2.2.4.2 Inter-individual variation

High interindividual variability was observed in the bioavailability of ACN-derived metabolites in plasma. Plasma concentrations of compounds grouped into subclasses showed a high coefficient of variation. Finally, variability between individuals was shown for every single compound by taking the log values of the AUC. AUC levels of benzoic acid, α-hydroxyhippuric acid and 4-hydroxyphenylacetic acid show the highest variability between the 10 participants (Fig. 11).

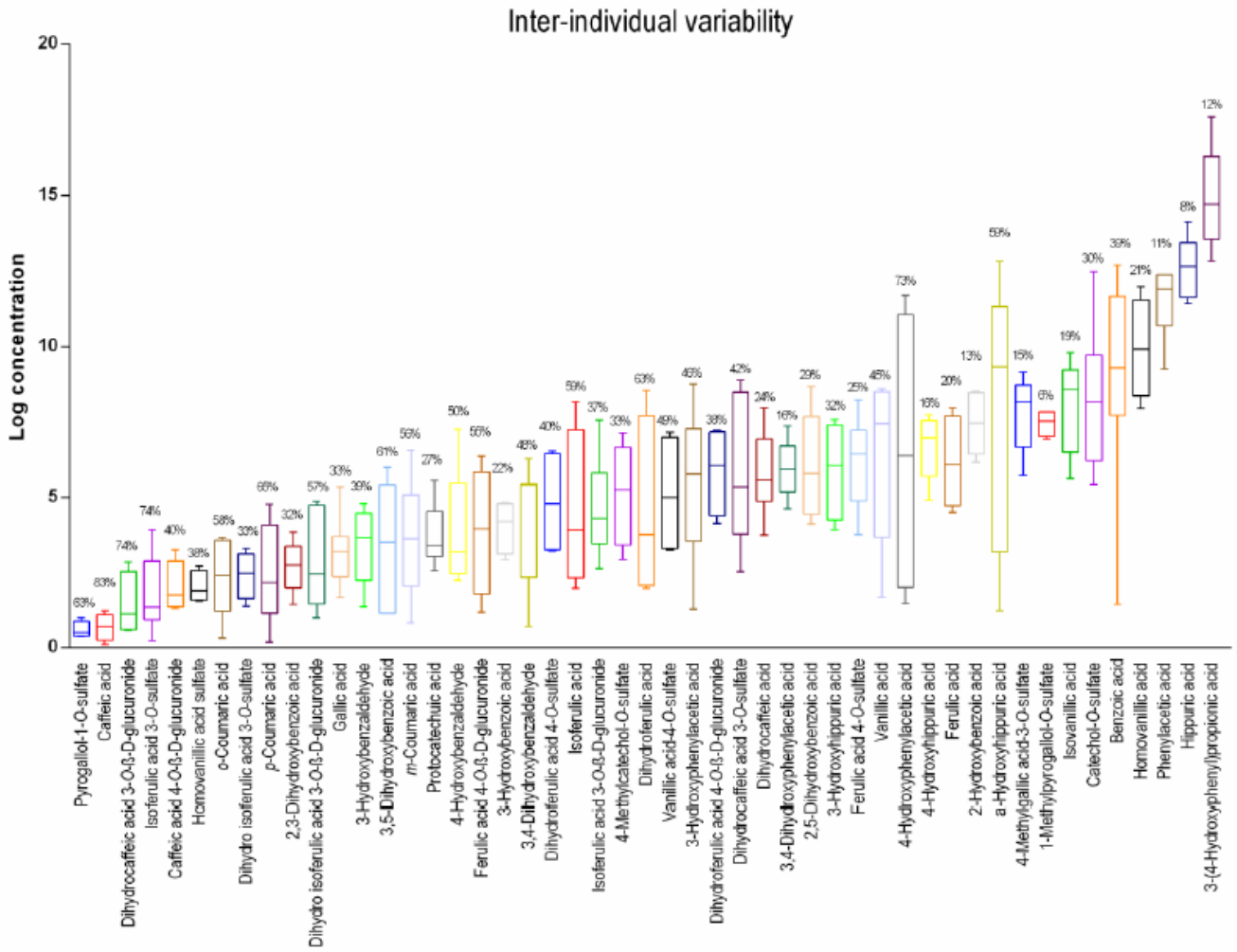


Fig.11: Interindividual variation per compound of anthocyanin metabolites (log values and % coefficient of variation)

3 Effects of daily anthocyanin supplementation on vascular function and menopausal complaints

3.1 Materials and methods

3.1.1 Study participants and study design

In order to investigate whether there was a chronic effect on vascular function and menopausal complaints with a dose of anthocyanins that showed acute vascular effects in young healthy men the study was performed in a group of 22 postmenopausal women. The double-blind randomised study took place in the same study rooms used for the preliminary study at the Division of Cardiology, Pulmonology and Vascular Medicine of Düsseldorf University between November 2014 and June 2015. The 22 female postmenopausal study participants were recruited via word of mouth. The volunteers were personal acquaintances, members of a local sport club for women or a local diving club. See Fig. 12 for the CONSORT Flow Chart. Menopause is defined as the last menstrual bleeding. When a woman older than 45 years has amenorrhea for twelve months one can assume that the menstruation has ceased permanently (Cochrane Library Definitions). So postmenopause is the time at least one year after the menopause. The exclusion criteria were an acute infection with an CRP >0.5 mg/dl and suffering from malignant diseases. Women with suspected or manifest vascular diseases including coronary heart disease, renal, peripheral or cerebrovascular occlusive diseases were excluded. Intolerance or allergies to berries was also an exclusion criterion. Volunteers with acute or chronic renal failure were excluded from the study, as well as volunteers with chronic heart failure and with cardiac arrhythmia. The study days were set 28 days apart (Fig. 13). One day before the study days the volunteers were asked not to eat berries and not to drink red wine and to fast at least 12 hours prior to the measurement. Otherwise the volunteers were asked to follow their usual diet and not to change their lifestyle. On the first study day they were asked to collect baseline urine and then rest in supine position for at least ten minutes. Office blood pressure was measured, followed by pulse wave velocity and FMD. A blood sample was also taken for the analysis of anthocyanins in plasma and for the clinical parameters including female sex hormones that were done by the Zentrallabor of the university medical centre in Düsseldorf.

The randomisation was done with sealed envelopes. The volunteers were asked to take two capsules with half a litre of Vio water. During the waiting period of 1.5 hours between taking the capsules and the second FMD measurement the volunteers were asked to fill in the questionnaires. After 15 min rest blood pressure, PWV, PWA and FMD were measured two hours after taking the capsules. Blood samples were also collected. Volunteers were asked to take two of the capsules in the morning and two twelve hours later in the evening with a glass of water. Adherence to the protocol was assessed by recalling the empty packages. Volunteers were also asked to collect 24-hour urine and wear a 24-hour ambulatory blood pressure device.

After an explanation and time for consideration written informed consent was obtained from all volunteers before participating in the study. The studies were conducted according to the guidelines laid down in the Declaration of Helsinki. The study protocol was approved by to the ethics committee of the Heinrich-Heine-University (study number 4433R-2013091447-1) (ClinicalTrials.gov identifier: NCT02517801)

Flow Diagram on the enrolment of healthy postmenopausal women in the study on chronic effects of anthocyanins on vascular function

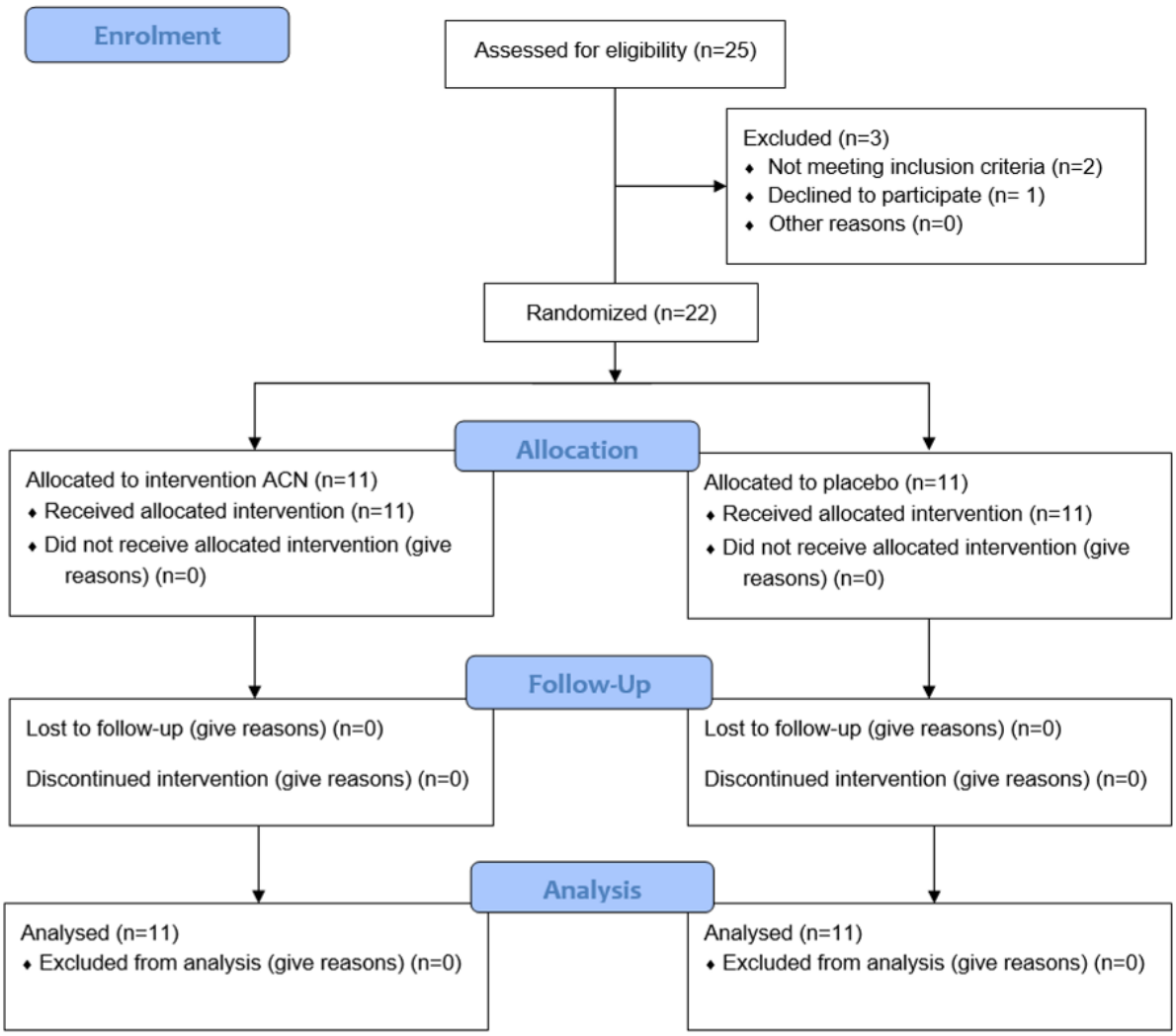


Fig. 12: CONSORT Flow Diagram on the enrolment of postmenopausal women in the study of chronic effects of anthocyanins on vascular function

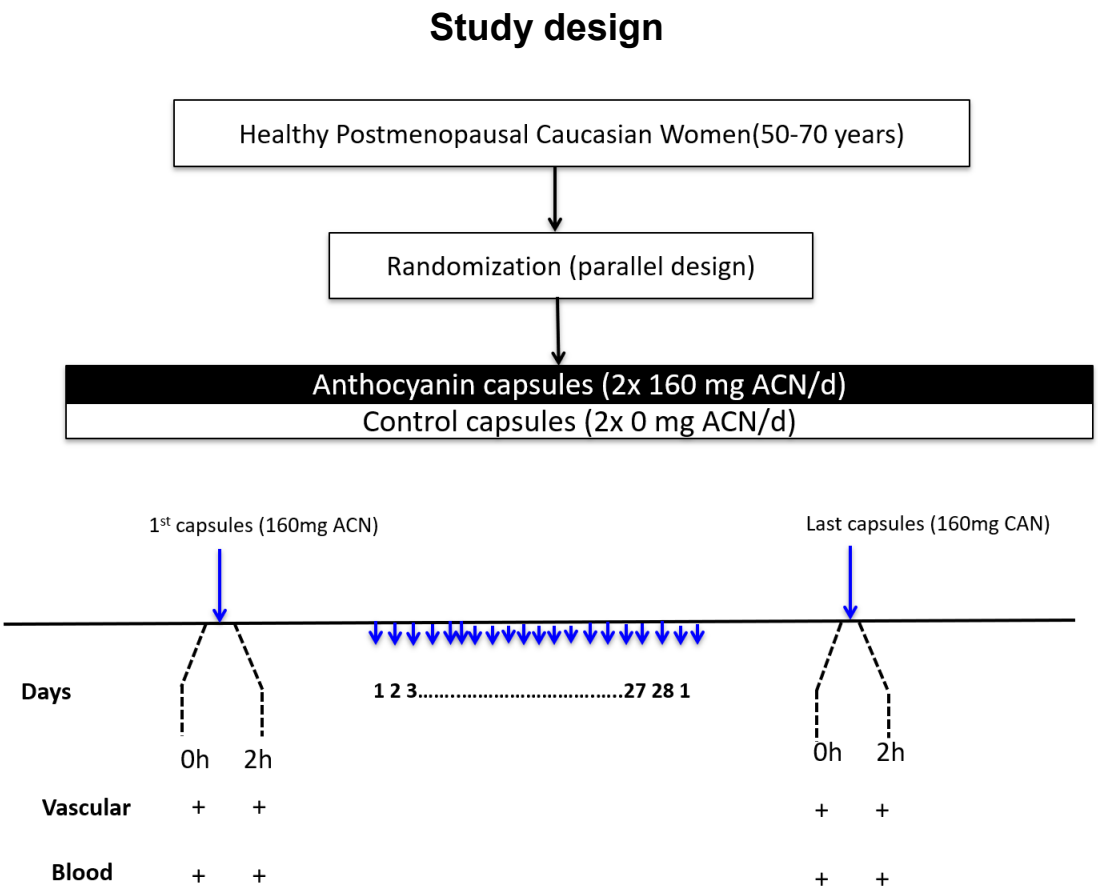


Fig.13: Study design and sequence of the measurements in the study on chronic effects of anthocyanins in postmenopausal women

3.1.2 Test material

Commercially available encapsulated purified anthocyanins provided by Medox®; Polyphenols AS, Sandnes, Norway, as described above in the dose dependency study, were the test material.

The placebo capsules that look identical were packed in identical blister packaging and weigh the same. They are made from pullulan and contain 45 mg maltodextrin. The chosen dosage was 160 mg of anthocyanins after the results of the dose-dependency study as the lowest dosage that showed a significant effect compared to baseline 2 and 6 hours post-consumption. The volunteers were randomised by drawing one of the 22 sealed envelopes. Throughout the intervention period no volunteer consuming anthocyanins or placebo reported any adverse effects.

3.1.3 Flow-mediated vasodilation and power analysis

FMD measurements were conducted according to the protocols existing in our work group in combination with an automated analysis system (brachial analyser, Medical Imaging Applications, Iowa City, IA, USA). The FMD procedure was identical to the procedure used for the men in the dose response study but there was no measurement after 6 hours. The environmental conditions that influence the way vessels react to FMD, the ultrasound probe used, the measuring points, the measurement process and the analysis of the pictures were the same as described above for the dose-dependency study.

The measurements were repeated exactly 2 hours after they took the capsules. The volunteers were allowed to drink Vio® water but not to eat, exercise or smoke.

3.1.4 Pulse wave velocity

In order to investigate effects on arterial stiffness pulse wave velocity and pulse wave analysis were measured with the SN SpygmoCor® system.

Central blood pressure parameters including augmentation index (AIX) were measured by applanation tonometry using the SphygmoCor® system. An inverse transfer function is used to reconstruct the pressure waveform of the ascending from the pulse of the radial artery. The augmentation index was also derived at the radial artery. After the resting period in supine position measurements started with the office blood pressure measurements as described above. The volunteers had three self-adhesive electrode pads for electrocardiogram. The gating of the pulse is needed to calculate the time lapse between pulse waves at the carotid and femoral sites. PWV was determined from measurements taken at the carotid and femoral artery (Heiss, Sansone et al., 2015; Sansone et al., 2015; van Bortel et al., 2012). A pencil-type probe was used for all measurements with the SphygmoCor®. The probe was placed at the base of the right side of the neck for the carotid artery and over the right femoral artery at the right groin. Recordings were taken when a reproducible signal was obtained with high-amplitude excursion and a steady curve shape. The distance between carotid and femoral sites, the distance from the jugular notch to carotid and the femoral pulse on the right side, were measured with a tape measure (Rodriguez-Mateos et al., 2013).

3.1.5 Pulse wave analysis

In pulse wave analysis the ascending aortic pressure wave is calculated from the arterial pressure pulse, in the present study measured non-invasively by applanation tonometry of the right radial artery. Blood pressure was measured as well. The arm was supported comfortably and the wrist was overextended. The measuring sensor was held at a 60° angle until the SpygmoCor® had collected sufficient data of good quality. The Operator index was considered the marker of good quality. Acceptable quality levels were defined as Operator Index ≥90.

3.1.6 Blood-pressure measurements

3.1.6.1 Office blood-pressure measurements

Office BP was measured with a calibrated and validated automatic oscillometric device (Boso medicus PC2, Germany), after 10 min of rest in a supine position, and under standardized conditions. Clinic BP values were calculated as the mean of two readings. Thereafter, 24-h ABPM was performed using the below mentioned ABPM device, programmed to register BP at 30-min intervals for the 24-h period. Appropriate cuff sizes were used. The ABPM recordings were performed on working days and the patients were instructed to maintain their usual activities, and to keep the arm extended and immobile at the time of each cuff inflation. Valid ABPM recordings had to fulfill a series of pre-established criteria, including successful recording of ≥80% of systolic BP and diastolic BP during both the daytime and nocturnal periods, and at least one BP measurement per hour. Daytime and nocturnal periods were defined according to the patient's self-reported data of going-to-bed and waking times (Gorostidi et al., 2015).

3.1.6.2 24-hour-blood pressure

The device used was a Tonoport V (PAR Medizintechnik, Germany). An appropriately sized cuff was put tightly around the non-dominant upper arm without limiting the mobility. The device was switched on between the baseline and the two-hour measurement to check the first reading and collect first data and the volunteers could get accustomed to the device. The blood pressure measurement was paused for the resting period and for the two-hour measurement. During the day the device recorded every 30 minutes and during the night every hour. Predefined timepoints were used to define night and day. Daytime was 8 AM to 10 PM and night from 10 PM to 8 AM. The volunteers were instructed to hold the arm extended, immobile and relaxed during the measurements especially at the time of the cuff inflation. To analyse the blood pressure measurements GE CardioSoft V6.71 was used (GE Healthcare, USA).

3.1.7 Cholesterol and clinical chemistry

Blood samples were taken from the left arm at baseline after the FMD measurements on the first study day and after a month. The following laboratory values were measured: electrolytes, creatinine, urea, urate, bilirubin, blood fats (cholesterol, HDL, LDL, triglycerides), total protein, CRP, TSH, complete blood count, differential blood count, blood clotting, glucose and sex hormones. All clinical chemistry measurements were done in the central laboratory of the University Hospital of Düsseldorf, according to standard procedures.

3.1.8 Diet questionnaires

In this study the same food-frequency questionnaire was used as in the dose-dependency study and the same questionnaire about the food eaten 24 hours prior to the study days, too.

This information was completed by three dietary recalls by our study nurse to confirm that the nutrient and energy intake of the participants did not change during the study.

3.1.9 Menopausal Rating Scale

In order to investigate whether anthocyanins act as phytoestrogens and improve menopausal symptoms a questionnaire was used. The Menopausal Rating Scale II (MRS II) is a tool for assessing menopausal complaints, the burden and development of symptoms. It can help to decide if therapy is necessary but can be used as well to evaluate hormone therapy or other remedies (Heinemann et al., 2004). The MRS II comprises of eleven symptoms and complaints that can be attached to three dimensions: psychological, somato-vegetative, and urogenital. The psychological symptoms consisted of depressed, anxious, irritable and exhausted. The maximum scoring that can be obtained is sixteen points. The somato-vegetative symptoms consisted of the following four symptoms: hot flushes and sweating, cardiac complaints, sleeping disorders and joint and muscle complaints. With the somato-vegetative symptoms the maximum scoring points is sixteen. The last dimension the urogenital subscale consisted of only three symptoms: sexual problems, urinary complaints and dryness of the vagina. The stronger the symptoms are, the higher the scoring point. The range is from (no complaints) to four (severe symptoms). The volunteers ticked the number that best described their symptoms (Heinemann et al., 2004). To analyse the score, one puts together the scoring points of the different symptoms of each dimension. The total score is the sum of all subscales (Fig. 14).

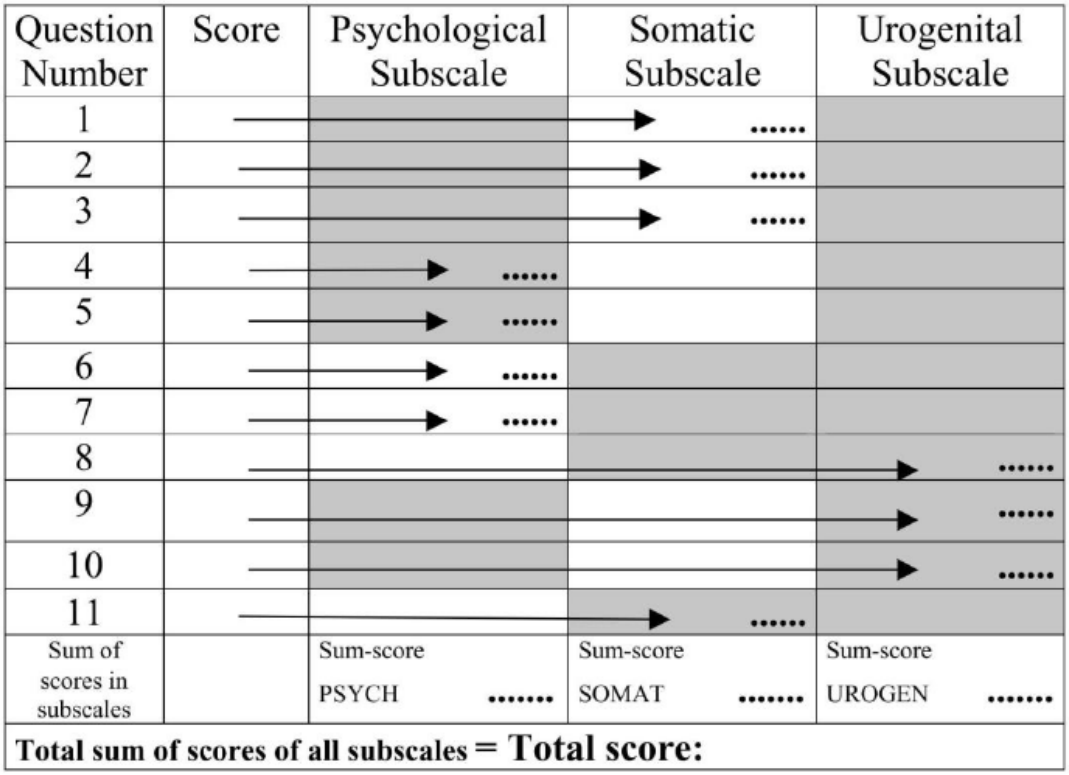


Fig. 14: How to analyse the sum-score of the MRS (http://www.biomedcentral.com/content/supplementary/1477-7525-1-28-s1.pdf 20-11-2015)

3.1.10 Statistical methods

Results are expressed as mean ± standard error of the mean (SEM). Baseline data represent data of first visit (day 1, 0 h). Power calculations were performed for the primary endpoint changes in FMD. Considering FMD as primary endpoint sample size has been calculated taking into account the expected variation. The power calculation was performed for the FMD measurement with the provided parameters based on the pilot study: significance level (adjusted for sidedness) (= 0.025), standard deviation (= 0.75), power (= 0.8), difference in means (= 1). The variable calculated was the total number of patients. The probability is 81 percent that a parallel study with 20 individuals will detect a treatment difference at a two-sided 0.05 significance level, if the true difference between treatments is 1.000 units. This is based on the assumption that the standard deviation of the response variable is 0.75 (http://hedwig.mgh.harvard.edu/sample_size/size.html).

The changes in the main outcomes after anthocyanin or placebo treatment were evaluated statistically by unpaired t-tests. The data was tested for outliers.

3.2 Results

3.2.1 Baseline characteristics, tolerance and compliance

Twenty-two postmenopausal women were recruited (Table 8). The age ranged from 50 to 70 years with an average age of 58.8±5.8 years. Their last menstruation was nearly 9 years ago in average with a range from 2 to 25 years since the last monthly bleeding. The sex hormone levels of all volunteers were within the range that is expected in women >1 year after menopause: progesterone 0.1-0.8 ng/ml, FSH 25.8-134.8 mIU/ml, LH 7.7-58.5 mIU/ml and oestradiol <138 pg/ml. They did not show any symptoms of chronic diseases. None of the volunteers had ever taken a postmenopausal hormone therapy. Three of the women took antihypertensive medication at a low dosage because of borderline hyertension. The two volunteers in the control group that took medication took Ramipril (ACE-inhibitor) and Hydrochlorothiazide (diuretic) in low dosages respectively. In the anthocyanin group one volunteer took bisoprolol (beta-blocker). Four of the volunteers took L-Thyroxin (thyroid hormone medication). Four volunteers are smokers, 18 are non-smokers (never smoked or quit smoking more than 15 years ago). Most volunteers were physically active every week. Four volunteers in the intervention group and eight in the control group do more than 1.5 hours exercise per week.

The baseline characteristics were within normal limits of postmenopausal women (Table 8). The Framingham Cardiovascular Risk Score was below the average of women in that age (average is 8-13% in the age cohort 50-69 years; https://www.framinghamheartstudy.org/risk-functions/coronary-heart-disease/10-year-risk.php). Total Cholesterol was significantly different between intervention and control group ($P=0.0194$ ($P<0.05$)).

All volunteers tolerated the treatments well and no adverse events were reported. It was confirmed that the volunteers did not eat anthocyanin-rich food 24h prior to the measurements.

The volunteers returned the remaining capsules after the intervention and the compliance was very good. In the mean the volunteers missed 2.41 timepoints over the course of one month with a standard deviation of 1.71 resulting in a compliance of about 96%.

Table 8: Baseline characteristics of postmenopausal study population

n=22	ACN (n=11)	Control (n=11)
Characteristics	**Mean ± SD**	**Mean ± SD**
Age (years)	58.36±5.51	59.18±6.24
BMI (kg/m²)	25.50±3.81	25.69±2.92
Total cholesterol (mg/dl)	243.55*±41.49	205.91*±26.27
LDL cholesterol (mg/dl)	151.45±30.51	128.09±25.51
HDL cholesterol (mg/dl)	73.64±24.74	64.00±10.78
Triglycerids (mg/dl)	108.73±97.31	79.27±18.34
Glucose (mg/dl)	93.00±7.56	90.55±5.61
HbA1c (%)	5.35±0.27	5.39±0.13
Creatinine (mg/dl)	0.82±0.13	0.81±0.13
Bilirubin (mg/dl)	0.37±0.1	0.50±0.13
Urea (mg/dl)	30.27±4.11	29.64±8.09
Leucocytes (1000/µl)	6.03±1.69	5.27±1.46
SBP (mmHg)	131.48±11.84	128.11±10.02
DBP (mmHg)	77.87±8.92	78.98±7.36
Last menstruation (y)	7.91±6.3	9.36±7.3
Smokers (n)	3	1
≥1.5h exercise/week (n)	4	8
Framingham Risk Score (%)	2.59±1.63	2.14±1.56

3.2.2 FMD

No significant changes in FMD were observed when the ACN was compared with the control group at all the tested timepoints neither after one month nor after 2h (Fig. 15). Comparing the anthocyanin and control group with baseline (within the group) no changes were observed neither.

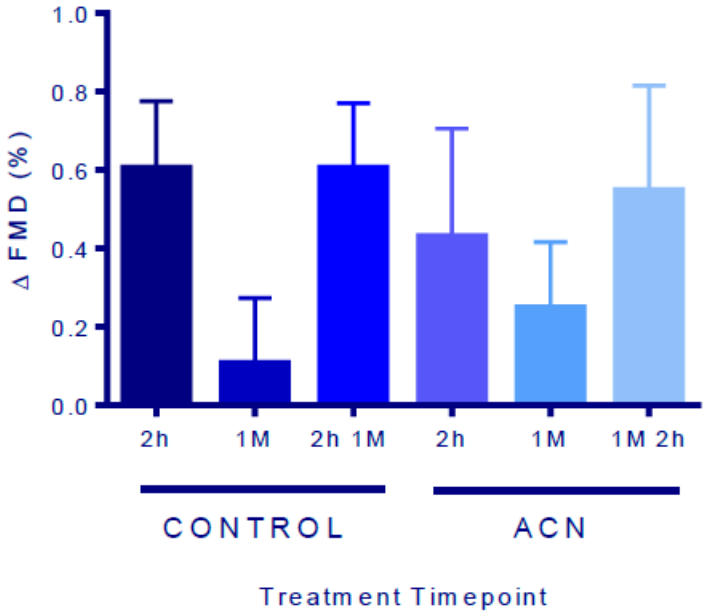


Fig. 15: % Changes in FMD at 2h, one month (1M) and 2 h and 1 month (1 M 2h) after daily consumption of 160 mg of anthocyanins or control capsules

3.2.3 PWV and PWA

Anthocyanin intake in postmenopausal women did not change significantly PWV acutely after 2h or chronically after one month compared with the control capsules or with baseline (Fig. 16).

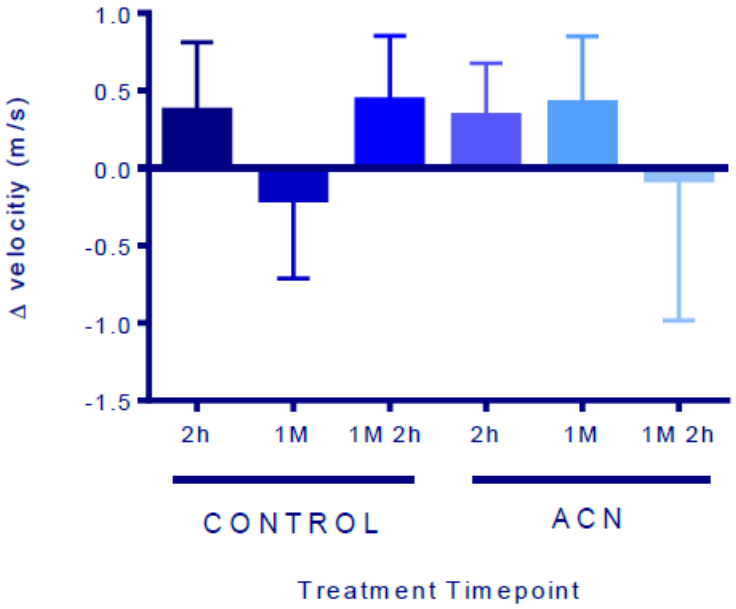


Fig. 16*:* Changes pulse wave velocity compared to control at different timepoints in control and treatment arm

No significant changes in AIX were found after intake of anthocyanins in postmenopausal women when compared to placebo or when compared with baseline (Fig. 17).

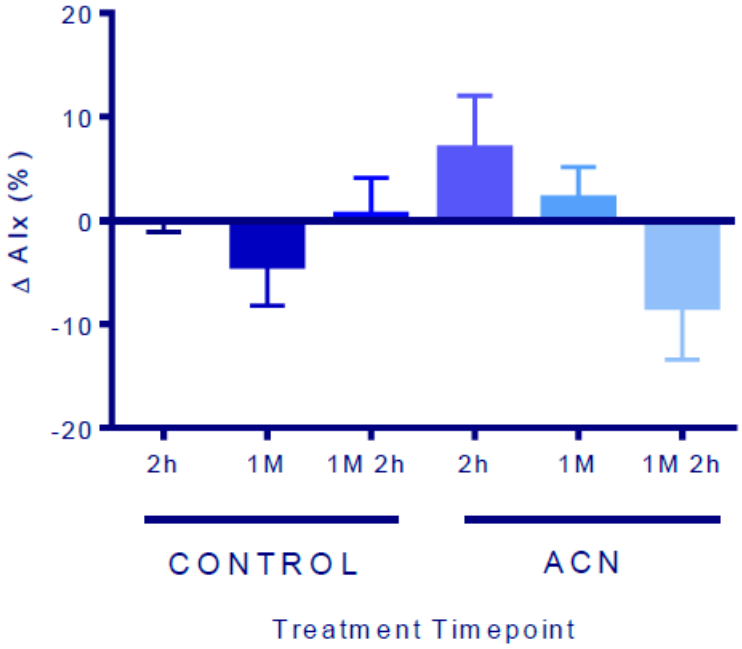


Fig. 17: % Changes Augmentation Index at the different timepoints in control and treatment arm

3.2.4 Blood pressure

In comparison with placebo and with baseline no differences were seen within and between interventions in 24-hour ambulatory, peripheral or central blood pressure (P=0.9506) (Fig. 18, 19, 20,21).

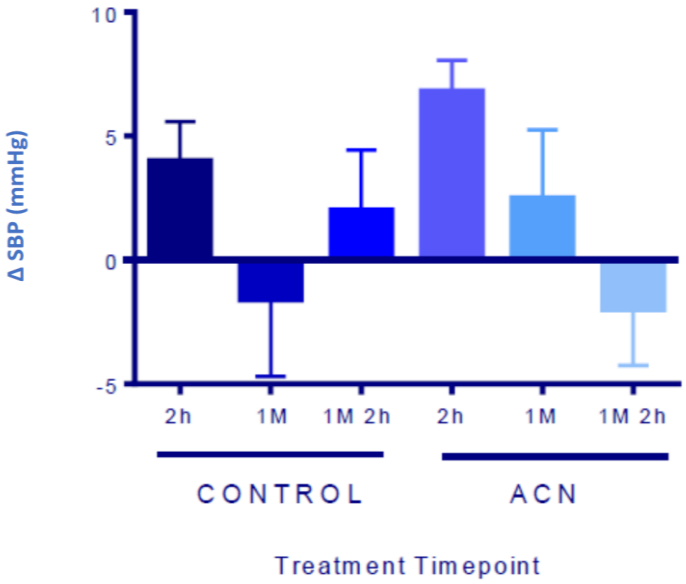


Fig. 18: Changes central systolic blood pressure at different timepoints in control and treatment arm

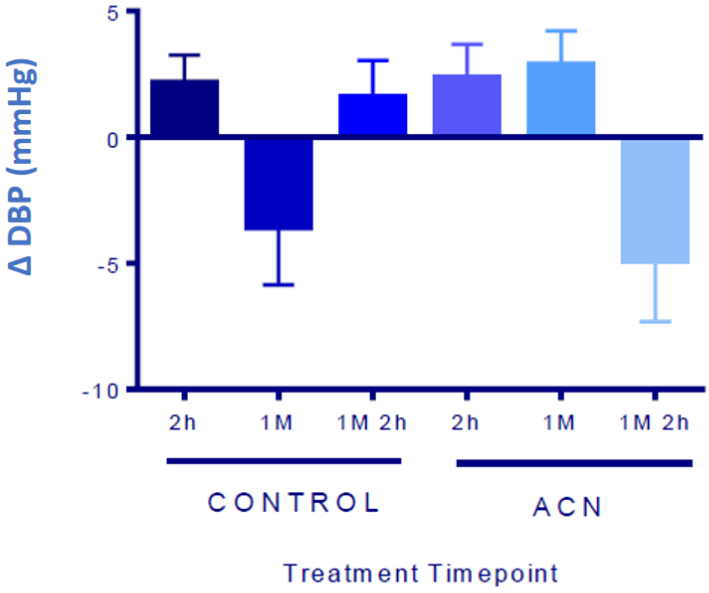


Fig. 19: Changes central diastolic blood pressure at different timepoints in control and treatment arm

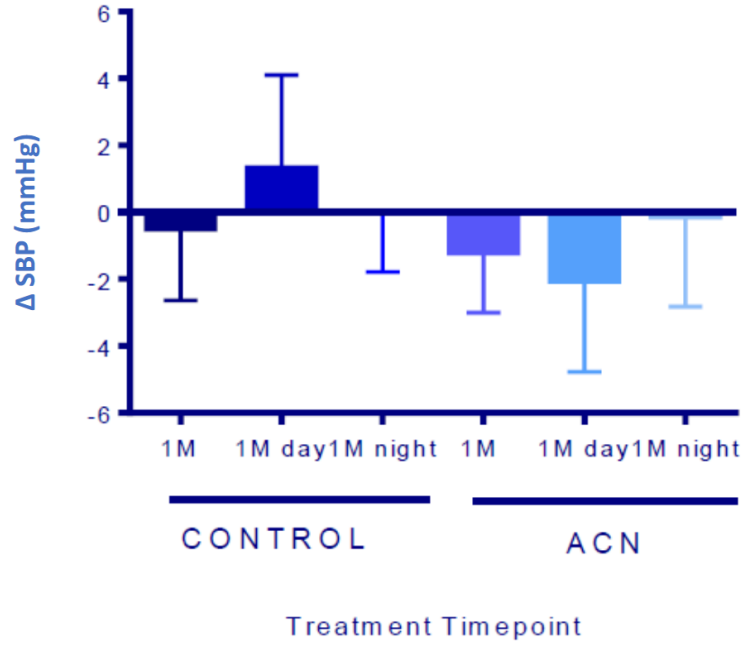


Fig. 20: Changes in the 24h ambulatory blood pressure measurements (systolic blood pressure) at different timepoints in control and treatment arm

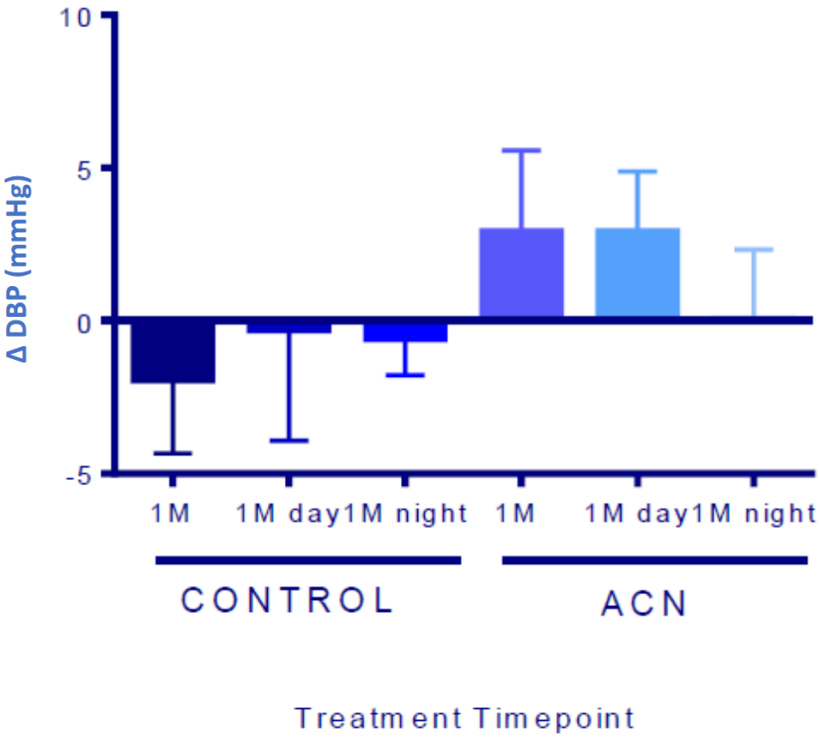


Fig. 21: Changes in the 24h ambulatory blood pressure measurement (diastolic blood pressure) at different timepoints in control and treatment arm

3.2.5 Clinical chemistry

No differences in total cholesterol (p=0.74), triglycerides (p=0.34), LDL cholesterol (p=0.8) or HDL cholesterol were observed between the ACN and the placebo group after one month. The HDL seemed to increase slightly after one month, but the increase is not statistically significant (p=0.23) (Fig. 22). No significant changes were seen within treatment neither (p>0.05).

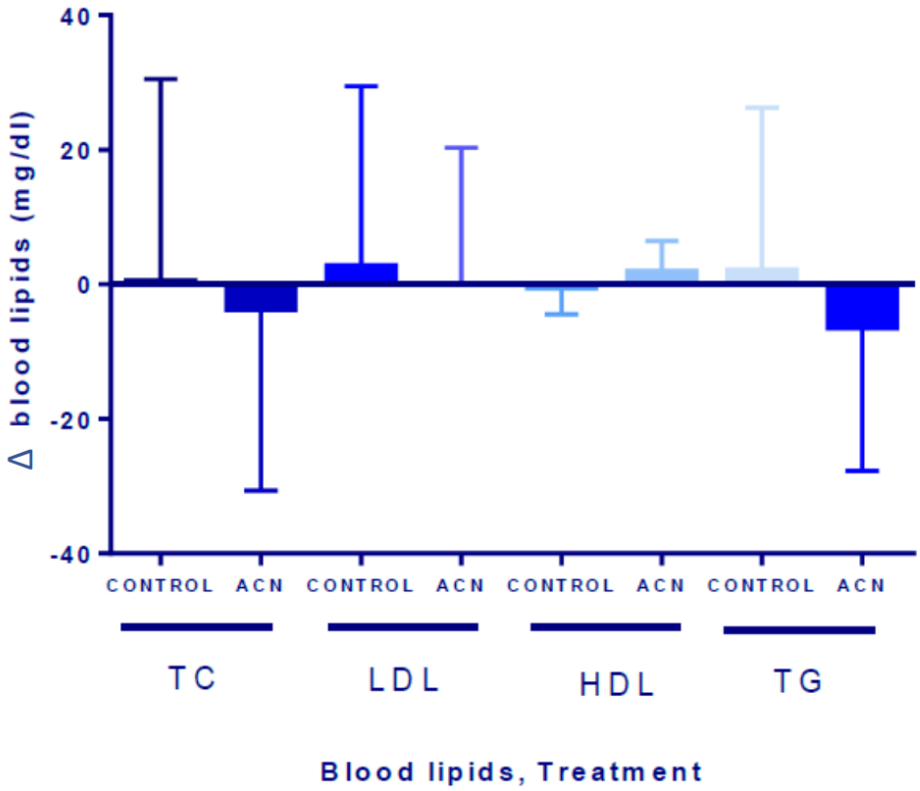


Fig. 22: Changes in blood lipids after the 4-week trial in control and treatment arm

There was no significant change in fasting glucose levels in healthy postmenopausal women after intake of anthocyanins for one month (p=0.7227) (Fig. 23). The baseline fasting glucose levels were normal (anthocyanin group 93 mg/dl ± 2.28 and placebo group 90.55 mg/dl ± 1.69) and the difference between means was small 1.09 ± 3.03.

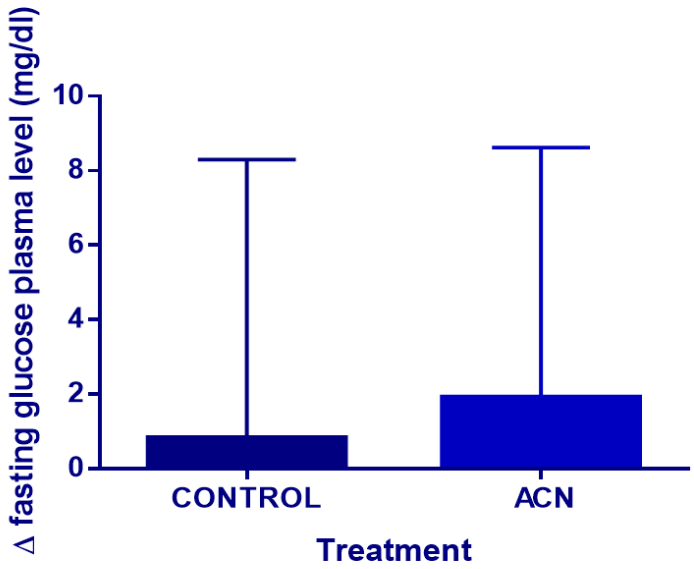


Fig. 23: Changes of fasting glucose plasma levels in control and treatment arm

No statistically significant changes in FSH or LH were observed (p=0.96; p=0.87 respectively). All observed values were within the normal range for postmenopausal women (Fig. 24).

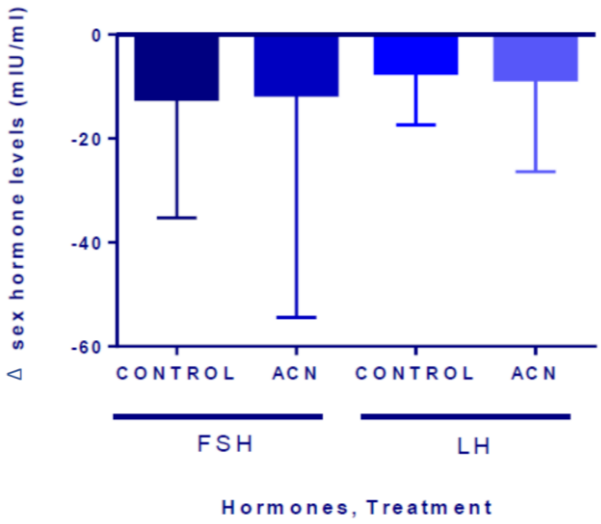


Fig. 24: Changes sex hormone levels after the 4-week trial in control and treatment arm; "FSH" = Follicle-stimulating hormone, "LH" =Luteinizing hormone

3.2.6 Menopausal rating scale

No significant changes were seen in the MRS. There was no subscale that changed significantly more than the other subscales of the MRS (Fig. 25 and 26). There were no significant changes in the MRS in the placebo group (p=0.73) (Fig. 26B). The differences among the means are not significant in the mean in the anthocyanins group either (p=0.46) (Fig. 25A). Comparing the changes of the somatic MRS subscale in the anthocyanins and the control group after one month there was no statistically significant effect due to the big SEM (Fig. 26A).

The somatic complaints consist of four subscales (Table 9). No effect on the “heart discomfort“ was observed comparing anthocyanins to placebo. The p value of the unpaired t-test was 0.0810. So significant according to α=10% but not to our definition of significance α=5%. The “sleep problems“ showed a decreasing tendency in the anthocyanin group after one month. But the decrease is not significant compared to placebo with a p value of 0.21 in the unpaired t-test.

Anthocyanins have a tendency of decreasing the urogenital MRS but with a p-value of 0.06 in the unpaired t-test that tendency is not statistically significant at a level of significance α=5% (Fig. 26B).

Concerning the three urogenital subscales of MRS there aren’t statistically significant effects of anthocyanins on any of them (Table 10). They seem to have a decreasing tendency concerning the subscale “sexual problems“ but with a p-value of 0.2068 it isn’t significant. The “bladder problems“ aren’t improved significantly either (p=0.088) although the effect would be significant for α=10%. Another urogenital complaint “dryness of vagina“ doesn’t improve significantly either. Comparing the complaints in the anthocyanins with the control group the P value of the unpaired t-test is 0.25. In the placebo group the complaint did not change at all. In the anthocyanin group the mean difference was -0.36 ± 0.31 (Fig. 27).

After comparing the total change in MRS score between anthocyanins and placebo groups after one month of intervention there is a decreasing tendency in the anthocyanin group without being significant with a p value of 0.095 (Fig. 26D). The mean value in the anthocyanin group is -1.36 ± 0.97, in the placebo group 0.909 ± 0.868. So, the difference between the means is -2.27 ± 1.3 and in 95% confidence interval is from -4.98 to 0.44 so the better part of the CI is in the opposite.

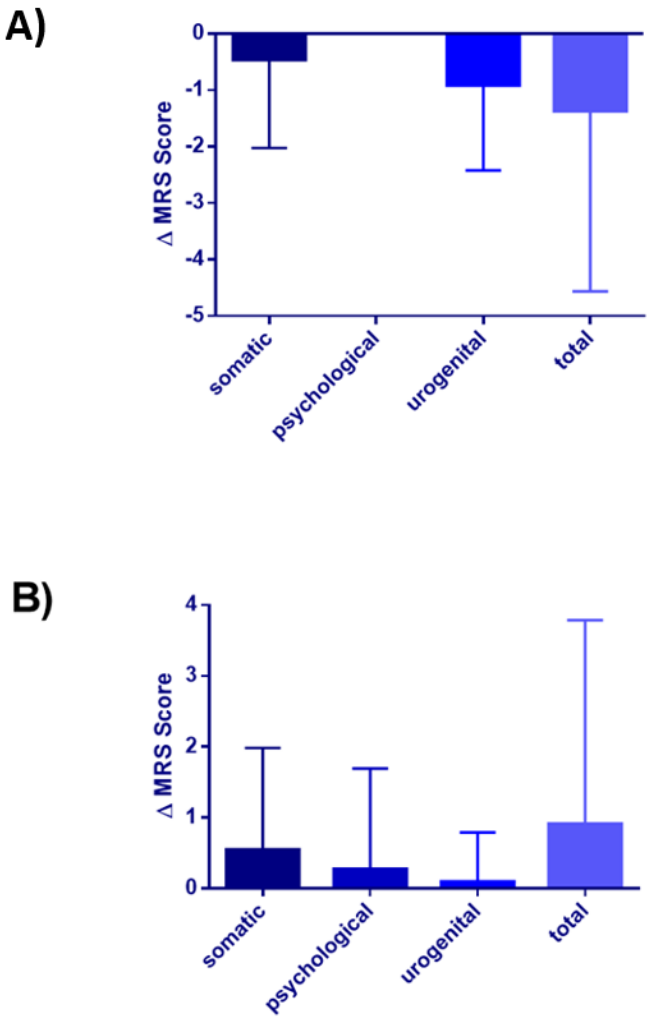


Fig. 25: Changes in Scores of Menopausal Rating Scale II; A) Anthocyanin group, B) Placebo group

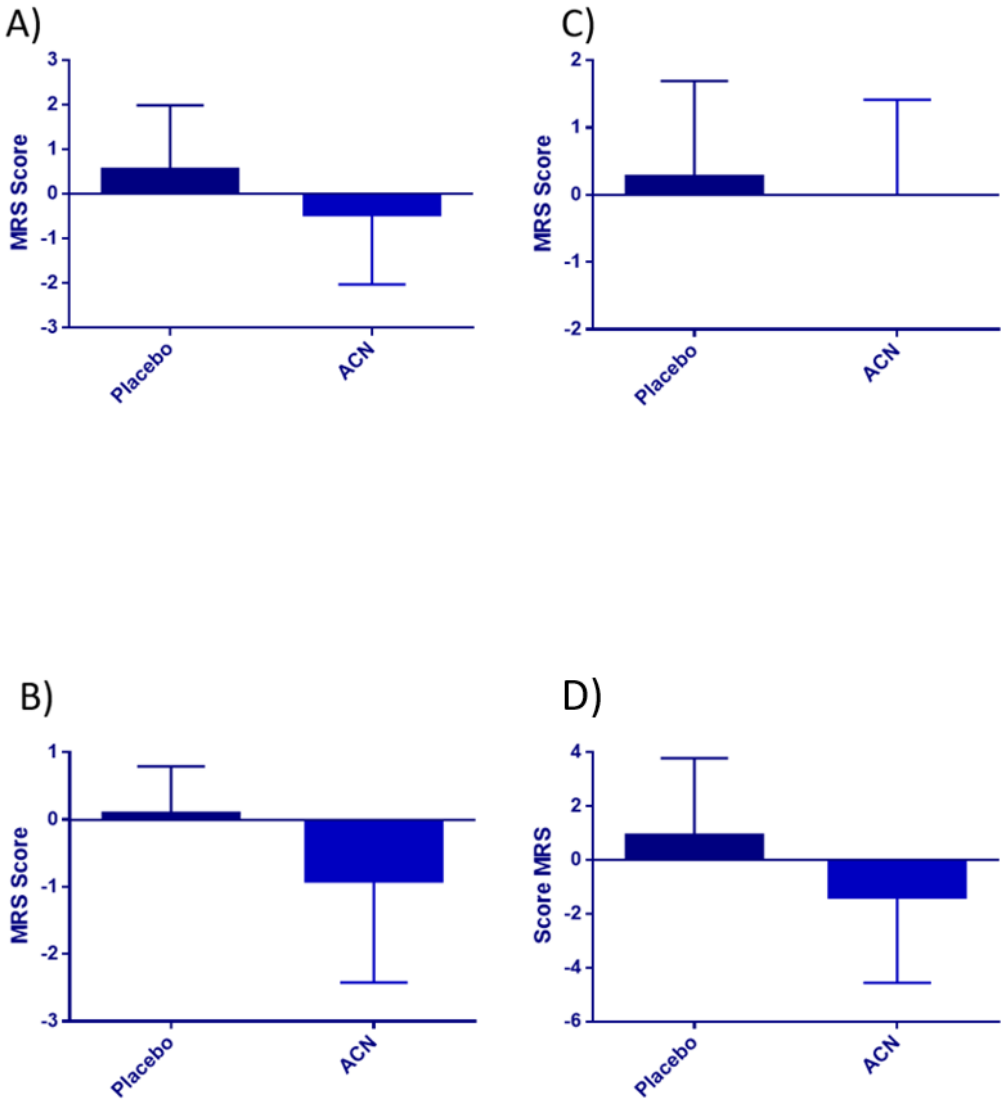


Fig. 26: Changes in the Menopausal Rating Scale II Scores after one month in control and treatment arm; A) Somatic Subscale, B) Urogenital Subscale, C) Psychological Subscale, D) Total MRSII Score

Table 9: Symptoms somatic subscale MRSII in mean ± SEM

Somatic subscales	ACN±SEM	Control±SEM	p-value
Sweating	-0.18±0.12	0.00±0.14	0.329
Heart discomfort	0.00±0.14	0.46±0.21	0.081
Sleep problems	-0.27±0.24	0.18±0.26	0.214
Joint and muscular discomfort	0.00±0.19	-0.09±0.16	0.721

Table 10: Symptoms of urogenital subscale MRSII in mean ± SEM

Urogenital subscales	ACN±SEM	Control±SEM	p-value
Sexual problems	-0.36±0.24	0.00±0.134	0.207
Bladder	-0.18±0.12	0.09±0.09	0.088
Dryness of vagina	-0.36±0.31	0.00	0.254

Table 11: Symptoms of psychological subscale of MRSII in mean ± SEM

Psychological subscales	ACN±SEM	Control±SEM	p-value
Depressive	0.09±0.16	0.00±0.19	0.721
Irratability	0.00±0.19	-0.09±0.21	0.723
Anxiety	0.09±0.25	0.27±0.2	0.573
Exhaustion	-0.18±0.18	0.09±0.16	0.277

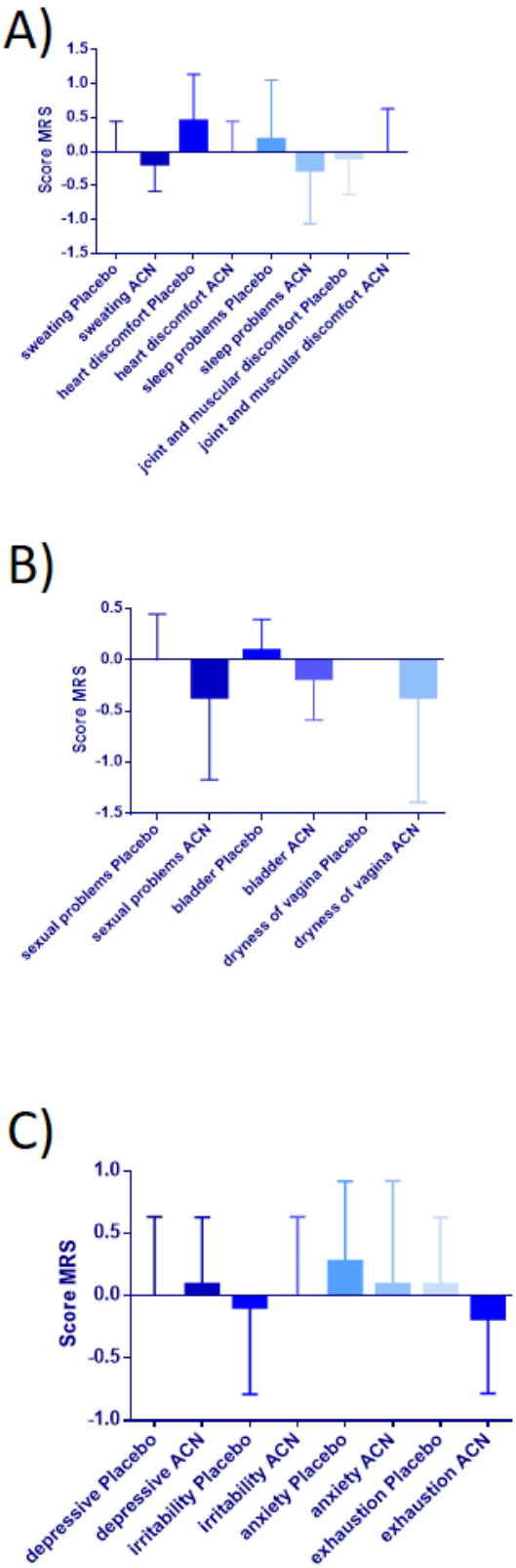


Fig. 27: Changes in the different symptoms of the MRS subscales after the 4-week trial in control and treatment arm. A) Somatic subscales, B) Urogenital subscales, C) Psychological subscales

4 Discussion

4.1 Summary of key findings and their importance in the context of previous studies

The present work investigated the cardiovascular health benefits of anthocyanin consumption in healthy individuals. The first study investigated whether acute anthocyanin consumption improves endothelial function, a biomarker of cardiovascular disease risk, in a group of young healthy men. The optimal time and optimal dosage of anthocyanins to induce maximal benefits was assessed in a double blind randomized controlled crossover trial. Then, in another double blind randomized trial, the aim was to investigate whether daily anthocyanin consumption could exert sustained health benefits in a group of postmenopausal women. The hypothesis was that anthocyanin consumption would improve endothelial function after acute consumption in a dose-dependent manner, and the effects would be sustained after daily consumption of anthocyanins.

Our first study showed for the first time a dose-dependent increase in endothelial function (measured as FMD) in healthy individuals after intake of purified anthocyanins. It was shown that 240, 320 and 480 mg of purified anthocyanins improved FMD at 2 and 6 h post-consumption when compared with control capsules. The changes in FMD were on the range of 1.1-1.3%, starting at 160 mg and with the highest dose of 480 mg of anthocyanins having the highest effect.

Our findings are in agreement with a previous clinical trial that was conducted using freeze-dried blueberry powder containing anthocyanin doses ranging from 129 to 724 mg (Rodriguez-Mateos et al., 2013). The maximum increase in FMD observed here (1.3 ± 1.0%) was obtained at 2h after consumption of 480 mg anthocyanins, which is much lower than the maximum FMD increase (2.4 ± 0.5%) shown after 2h post-consumption of a blueberry drink containing 310 mg of anthocyanins in a very similar study population (Rodriguez-Mateos et al., 2013). The blueberry powder used in the study contained not only anthocyanins, but also other (poly)phenols, fibres, vitamins and other micronutrients that may have contributed to the effect observed on FMD and therefore resulted in a nearly two-fold higher changes in FMD as compared to the purified anthocyanins. In the current study, linear increases in FMD were observed up to 480 mg anthocyanins, which is slightly different to the previous study where the maximum FMD was reached at 310 mg anthocyanins (766 mg (poly)phenols) and did not increase further after 517 and 724 mg of anthocyanins (1278 and 1791 mg of (poly)phenols) (Rodriguez-Mateos et al., 2013). The hypothesis is that this likely due to the contribution of other (poly)phenols to the improvements in FMD, which made the FMD plateau at lower concentrations of anthocyanins but higher concentrations of total (poly)phenols. Finally, the improved vascular function observed in the present study was sustained up to 6h after consumption, as previously reported for freeze-dried blueberries (Rodriguez-Mateos et al., 2013). This is likely due to colonic-derived metabolism of anthocyanins into phenolic metabolites which appeared in plasma at a later time than the metabolites absorbed in the small intestine (Czank et al., 2013; Selma et al., 2009).

Only one study has been published where FMD was measured after the consumption of an identical source of anthocyanins (Medox®, Sandnes, Norway). This study observed a significant increase in FMD at 1 and 2 h after consumption of 320 mg of anthocyanins in hypercholesterolemics (Zhu et al., 2011). The increase in FMD 2h post-consumption is comparable to our findings in healthy young men (ΔFMD of 1.8% vs 1.4 %, respectively).

Blood pressure did not change after the acute intervention with anthocyanins, which agrees with other studies conducted with purified anthocyanins (Hassellund et al., 2012; Hassellund

et al., 2013; Qin et al., 2009; Zhu et al., 2013) and anthocyanin-rich foods (Basu et al., 2014; Del Bo et al., 2013; Jin et al., 2011; Rodriguez-Mateos et al., 2013; Stull et al., 2010).

After elucidating the optimal time and dose of anthocyanins to exert improvements in vascular function the aim was to investigate whether anthocyanins could improve FMD after an overnight fast when ingested daily. A total of 20 healthy postmenopausal women had 160 mg of anthocyanins twice per day (320 mg/day) or placebo capsules over one month. No significant improvement in FMD, blood pressure, arterial stiffness, and blood lipids could be seen in comparison with placebo.

In hypercholesterolemics, Zhu et al. observed significant increases in FMD of 2% after 12-week consumption of the same amount of anthocyanins, 320 mg of anthocyanins per day (Zhu et al., 2011). The study duration was three times longer than our study, so this could be one reason for the discrepancies of findings. The study population was also different, and it is possible that our study population was too healthy to see effects.

The lack of chronic effects on the primary endpoint, FMD, was also found in CAD patients after one-month daily consumption of cranberry juice containing 835mg TP of which 94mg were anthocyanins. They also did not see an effect on blood pressure, but they saw a favourable effect on PWV, a parameter of arterial stiffness (Dohadwala et al., 2011). In our study with postmenopausal women no such effect on arterial stiffness was observed. An anthocyanin-rich elderberry extract was found to have a minor effect on blood lipids when taken over two weeks (Murkovic et al., 2004). Another study with postmenopausal women as study population agreed with our study as their intake of 500 mg/d of elderberry extract for 12 weeks did not change biomarkers of CVD risk in healthy postmenopausal women (Curtis et al., 2009).

In a recent meta-analysis of 12 randomized controlled trials investigating the effects of anthocyanins on markers of cardiovascular disease (Wallace et al., 2016), it was found that supplementation with anthocyanins significantly improved LDL cholesterol among people with elevated blood lipids. All 10 lipoprotein studies included reported a statistically relevant impact of anthocyanins on HDL. Six studies reported a significant increase in HDL with the anthocyanin intervention (Gurrola-Diaz et al., 2010; Hansen et al., 2005; Hassellund et al., 2013; Kianbakht et al., 2014; Qin et al., 2009; Zhu et al., 2013). Half of these studies with a statistically significant increase in HDL were conducted in dyslipidemic volunteers (Kianbakht et al., 2014; Qin et al., 2009; Zhu et al., 2013). The other three studies that showed an increase in HDL were conducted with healthy individuals (Hansen et al., 2005), with volunteers with metabolic syndrome (Karlsen et al., 2007), and pre-hypertensive volunteers (Hassellund et al., 2013). All ten studies in the review compared the effects of anthocyanins on blood cholesterol to placebo. In three of the 10 a significant improvement was seen (Gurrola-Diaz et al., 2010; Kianbakht et al., 2014; Soltani et al., 2014). In one study in subjects with metabolic syndrome total cholesterol increased in the control and the intervention groups, but the increase was smaller in the intervention group (Gurrola-Diaz et al., 2010). In the other two studies there was a decrease in LDL by 16.7% and 25.5% (Kianbakht et al., 2014; Soltani et al., 2014; Wallace et al., 2016).

However, anthocyanin supplementation did not significantly alter other markers of CVD in neither healthy individuals nor those with elevated markers. These findings agree with the lack of effects on blood lipids and blood pressure in our healthy study population. No adverse effects of anthocyanins were reported across studies at levels up to 640 mg/day (Wallace et al., 2016). The finding that anthocyanin supplementation is safe (Wallace et al., 2016), is supported by our study in which no adverse events were reported neither.

There is only one other study that used purified anthocyanins in a healthy study population consisting of 120 volunteers aged 40–74 years (61 women and 59 men) (Karlsen et al., 2007).

Measuring blood lipids after a three-week anthocyanin intervention (300 mg anthocyanins per day; consisting of two capsules 75 mg anthocyanins twice daily), they agree with our finding that anthocyanins don't improve blood lipids in healthy volunteers. Our results are inconsistent with the findings of all the other studies with purified anthocyanins that observed a decrease in blood lipids. The shortest of these studies lasted 28 days, like our study, but they used a much higher dosage, 640mg, four times our dosage (Hassellund et al., 2013). There were two studies using our dosage, but they were conducting a longer intervention 84 d (Qin et al., 2009) or 168 d (Li et al., 2015; Gurrola-Diaz et al., 2010). There are also several studies that did not observe any changes in blood lipids after intake of anthocyanins or anthocyanin-rich foods. Among the studies agreeing with ours was a study concentrating on insulin resistance also agreed with our study concerning other secondary markers of cardiovascular health. They did not see any difference in the inflammatory biomarkers, lipid profile, and blood pressure between the study group that drank a blueberry smoothie with 668 mg of anthocyanins and the placebo group in obese volunteers, although their primary endpoint, insulin resistance, decreased (Stull et al., 2010). Another study used cranberry juice with 458mg TP and 25mg anthocyanins in a population of women with metabolic syndrome and did not see any improvements neither in glucose and lipid profiles nor in blood pressure. The author attributed this to the relatively low anthocyanins of the treatment (Basu et al., 2011). Possible effects on blood lipids might depend on the study population and on the duration of the study. Anthocyanins might be more prone to improve blood lipids in dyslipidemic study populations, making our volunteers too healthy, to see possible effects on blood lipids.

Apart from the studies by Basu et al. (Basu et al., 2011; Basu et al., 2014) there are several other studies that agree with our studies anthocyanins don't lower blood pressure. For example a study also lasting one month with prehypertensive men that used the double dose of the identical anthocyanin capsules, could not see antihypertensive effects neither in office-BP nor in 24h-BP (Hassellund et al., 2012). So, these two studies agree that purified anthocyanins don't improve BP. These results are especially interesting as Hasselund et al. also used not only office-blood-pressure measurements but also measured 24h ambulatory BP. In a study with a longer duration and a little higher dosage of anthocyanins Riso et al. also couldn't see significant changes in blood pressure. In this 6-week study they used freeze-dried blueberries containing 375mg anthocyanins in a population at CVD risk. They only observed a non-significant effect of the wild blueberry drink on the modulation of peripheral arterial function (measured via endothelial-dependent vasodilation in the small finger arteries) that was attributed to the kinetics of wild blueberry anthocyanins and/or other (poly)phenols by the authors (Riso et al., 2013).

The only study using purified anthocyanins, which disagrees with our study concerning blood pressure, was conducted by Zhu`s working group in 2011 (Zhu et al., 2011). They observed a significant decrease in blood pressure. This contradicts another study also by the same working group, that used the same dosage and also hypercholesterolemic volunteers over an even longer period of time (168 d) and that did not see a decrease in blood pressure (Zhu et al., 2013). It has to be stated that in both studies by Zhu BP was measured as in-office BP, so unlike our study they did not use the gold standard, 24h ambulatory blood pressure. So, in conclusion anthocyanins (320 mg anthocyanins per day) might not be able to decrease blood pressure in healthy study populations over the course of one month.

The effect of anthocyanins on blood glucose levels is controversial. Two studies agree with our study that there is no change (Qin et al., 2009; Zhu et al., 2011) whereas two other studies disagree because they observed a change (Hassellund et al., 2013; Li et al., 2015).

4.2 Anthocyanin metabolites correlate with improvements in endothelial function

In order to better understand which of the circulating (poly)phenol metabolites correlate with vascular effects, blood plasma samples were collected at all time points in our dose-dependency study with young healthy men. A total of 50 (poly)phenols and phenolic acids were quantified in plasma, of which 39 increased after intake of anthocyanins. Most of these metabolites were also present in the plasma baseline, which makes more difficult to assess whether they are anthocyanin metabolites, endogenous compounds or coming from other food sources. Our data shows that mostly propionic, cinnamic and hippuric acids together with pyrogallols increased in plasma after anthocyanin intake. The compounds that showed the biggest increases in plasma were pyrogallol-2-*O*-sulfate, 1-methylpyrogallol-*O*-sulfate, catechol-O-sulfate, hippuric acid, dihydroferulic acid-4-O-sulfate and dihydroferulic acid 4-O-B-D-glucuronide. Likewise, cinnamic acids, phenylacetic and hippuric acid metabolites were shown to be the main degradation products of anthocyanins in humans using isotopically labelled cyanidin-3-glucoside (Czank et al., 2013). Catechol and pyrogallol metabolites have only recently been quantified for the first time in plasma after berry consumption (Pimpao et al., 2015). These compounds have not been extensively investigated as being anthocyanin-derived phenolic compounds and we suggest they might play a role in cardioprotective effects following anthocyanin consumption.

The bioactivity of (poly)phenols in humans is still poorly understood and even less information is available on the activity of specific (poly)phenol compounds with regard to endothelial function (Rangel-Huerta et al., 2015). To identify which compounds could be responsible for the observed vascular effects in our dose-dependency study, a correlation analysis between plasma (poly)phenols and FMD outcomes was performed. A total of 10 anthocyanin-derived metabolites were significantly correlated with improved endothelial function of which half of them were conjugated compounds. Most of them were cinnamic acid derivatives (7), with one hippuric acid, one phenylacetic acid and one pyrogallol metabolite. Remarkably, 3 of the correlated compounds overlapped with the ones found to correlate with FMD in a previously published cranberry juice intervention study (Rodriguez-Mateos et al., 2016), including ferulic acid 4-O-sulfate, isoferulic acid 3-*O*-β-D-glucuronide, and dihydroferulic acid 4-*O*-sulfate. Homovanillic acid and 4-hydroxyhippuric acid plasma levels were also correlated with FMD improvements after blueberry consumption (Rodriguez-Mateos et al., 2013). These results suggest that these degradation products might act synergistically as candidate bioactives with potential cardioprotective properties. Future studies will be necessary to identify bioactive compounds and to determine whether the synergistic effects are more important than the activity of single compounds. The mechanisms of action of (poly)phenols on the vascular endothelium are not fully understood but they most likely involve the increased availability of nitric oxide (NO) surrounding the vascular epithelium (Anter et al., 2004).

4.3 Can anthocyanins be used as hormone replacement therapy?

As an exploratory endpoint, the female sex hormones oestradiol, FSH and LH attracted attention and consequently the postmenopausal volunteers were asked to fill out an internationally recognised questionnaire on menopause symptoms, the Menopausal Rating Scale II (MRS II German version), and the scores were analysed.

No statistically significant changes of sex hormones were observed in the postmenopausal group after intake of anthocyanins. No changes in the MRS II were observed either. There was also no MRS II subscale that changed significantly more than the other subscales of the MRS. Although interestingly anthocyanins have a tendency of decreasing the urogenital MRS, but that tendency is not statistically significant.

No study has investigated the effects of anthocyanins on postmenopausal complaints, therefore a direct comparison with other studies is impossible. However, a significant amount of studies have investigated the effects of isoflavones, another group of flavonoids well known for their phytoestrogenic properties (Basaria et al., 2009; Murkies et al., 1998).

In a recent meta-analysis on the effects of phytoestrogens on menopausal complaints indicated that phytoestrogens improve the frequency of hot flushes in menopausal women, but no significant effect on the Kupperman Index (Chen et al., 2015).

There are two internationally used questionnaires on menopausal complaints, the Menopausal Rating Scale established in the 1990s that was modified and improved (MRS II) and the Kupperman Index that was established in 1953 (Heinemann et al., 2004; Tao et al., 2013). No study on the effects of isoflavones used the MRS II, but some used an older questionnaire the Kupperman Index, but the two questionnaires have a lot in common. A study that compared the results of menopausal women in both scales observed a correlation between total scores on these two menopausal symptom scales as well as on sub scores for the psychological and somatic domains (Tao et al., 2013). The MRS II (German version) was used for assessing menopausal complaints in this study for the following reasons: In the setting of this study the MRS II had several advantages over the Kupperman Index. The MRS was developed and validated in a random sample of German women, which is an advantage as our study was conducted in Germany. Another advantage is that the MRS defines the cut-off points between degrees of severity, as the spread of categories in the MRS is based on population. Whereas Kupperman index uses patients with severe symptoms as reference. The MRS differentiates more accurately the higher degrees of symptom severity than the Kupperman index. The scales are not directly comparable (Schneider et al., 2000). The MRS seems to have a better discriminatory power (Tao et al., 2013). The calculation of the total score of the MRS II is simpler than working with the Kupperman Index, as there is no weighting and the MRS II doesn't use multiplication factors (Schneider et al., 2000). It is also very efficient that the MRS II is filled out by the participating women themselves. It has been shown that the MRS II can be utilized as an age- and condition-specific measure of quality of life (Schneider et al., 2000).

There are seven studies that used the Kupperman Index to measure menopausal complaints (Cancellieri et al., 2007; del Giorno et al., 2010; Ferrari et al., 2009; Han et al., 2002; Petri Nahas et al., 2004; Riesco et al., 2011; Sammartino et al., 2006). Three of those studies showed a significant reduction of KI in the phytoestrogen group when compared with control (Cancellieri et al., 2007; Han et al., 2002; Sammartino et al., 2006). In contrary, four other studies reported no significance in the treatment arm (del Giorno et al., 2010; Ferrari, 2009; Petri Nahas et al., 2004; Riesco et al., 2011) while some of them saw a favourable effect on the hot flush frequency (Chen et al., 2015; del Giorno et al., 2010; Ferrari, 2009; Petri Nahas et al., 2004).

To our knowledge no study has investigated the effects of anthocyanins on menopausal complaints. Our study showed a favourable tendency for less menopausal complaints in the anthocyanin treatment arm, but it did not reach statistical significance ($P>0.05$). Our study with 22 participants had not enough power to detect differences. In the study by Petri Nahas with 50 volunteers that detected an improvement in KI, the minimal detectable difference in means was approximately 0.937 (Petri Nahas et al., 2004). Using those power calculations at least 50 volunteers would have been needed for our study as well, if the MRS II had been our primary endpoint in our parallel-design study. Several of the studies investigating effects of isoflavones on quality of life outcomes had more than 100 volunteers (Albertazzi et al., 2005; Cancellieri et al., 2007; D'Anna et al., 2009). The smallest study population consisted of 50 women (Petri Nahas et al., 2004) and the biggest of 389 (D'Anna et al., 2009). In the mean the women that participated in the isoflavone studies was aged 48-56 years, so they were younger than our

study population that was nearly 59 years old in the mean (58.77 years). In the mean the women participating in our study had been postmenopausal for nearly 9 years (8.64 years), whereas the other study populations consisted of women in early postmenopause (Ye et al., 2012) up to 6 years of amenorrhea (Casini et al., 2006). A bigger and younger study population would be needed in order to better investigate possible effects on menopausal complaints. Women in our study were already quite long in postmenopause and did not have many symptoms. Changes in FSH and oestradiol after the final menstrual period have good evidence for staging postmenopause based on random population data (Harlow et al., 2012). According to that definition by Executive summary of the Stages of Reproductive Aging Workshop + 10 our volunteers were in the postmenopause stage +1c to +2. In common usage Stage +1c represents the period of stabilization of high FSH levels and low oestradiol values that is estimated to last 3 to 6 years. According to this definition the early postmenopause lasts approximately 5 to 8 years. The stage +2 is defined by increasing somatic aging problems especially symptoms of vaginal dryness and urogenital atrophy become increasingly prevalent at this time (Harlow et al., 2012). According to this staging our volunteers were in late menopause (postmenopause stage +1c to +2 in common usage stage or senium). Our study population had only slight menopausal complaints, which makes them more difficult to compare.

On the other hand, our study population who were women over 50 years old, non-smokers, sportive, with slight complaints are the target group for dietary supplements.

It was important to measure sex hormone levels in our study for two reasons: to assess the eligibility of the volunteers for the study that requires postmenopausal women and because possible effects of anthocyanins as a phytoestrogen might trigger negative feedback on the sex hormones of the pituary gland, FSH and LH. In contrast most of the studies on changes of menopausal complaints with isoflavones did not measure hormone levels. The three studies that did, couldn't see any relevant hormonal changes even if they observed positive effects of isoflavones on menopausal symptoms (Basaria et al., 2009; Ye et al., 2012). Steinberg et al. neither observed a positive effect on menopausal complaints nor a change in sex hormones but a decrease in blood lipids (Steinberg et al., 2011). So, these studies agree with our observations as our volunteers showed low oestradiol and higher FSH levels that remained stable for the duration of the study. Slight non-significant changes in sex hormones are most likely due to the kind of hormonal cycle that preserves for many years even in postmenopause. This finding is supported by a study investigating the effects of soy germ isoflavones that observed no significant difference in the changes in oestradiol, FSH and LH (Ye et al., 2012).

4.4 Limitations

The most profound and most important limitations of RCTs were avoided such as lack of blinding, absence of proper controls, and lack of detailed compositional analysis of the foods that were tested. A suitable control intervention that was indistinguishable from the intervention capsules was used. Apart from the anthocyanins no other bioactive compounds were given. The placebo did not contain anthocyanins and was free from bioactive compounds. Metabolites of anthocyanins are known to enter the circulatory system. As the understanding of (poly)phenol bioavailability is not yet complete, blood samples were collected (Rodriguez-Mateos, Heiss et al., 2014). The volunteers were asked to return the remaining capsules after the intervention and the compliance was good (in average two missed time points over the course of one month).

There are some limitations that should be considered when interpreting the present data. First, the volunteers' diet was error-prone. The postmenopausal volunteers were not required to follow a low-(poly)phenol diet but not to change their normal diet and lifestyle. The reason was

to investigate realistic effects of anthocyanins integrated in the normal diet of volunteers. Although the volunteers filled in dietary questionnaires, there are still inadequacies in the ability of commonly used dietary intake questionnaires and food composition tables to accurately assess flavonoid intake in human populations. So, it is likely that possible chronic effects of anthocyanins on FMD in postmenopausal women are disguised by effects of (poly)phenols from their everyday diet. As our study population was healthy and very interested in healthy lifestyle and diet, they have most likely a high baseline flavonoid intake. It would be necessary to collect plasma, urine, or stool samples in order to assess flavonoid intake and perform an analysis of reliable biomarkers (Rodriguez-Mateos, Heiss et al., 2014). Evaluation of baseline status of flavonoid and/or (poly)phenol intakes may improve the consistency between small clinical interventions.

Second, another limitation is the relatively low number of participants. The study was powered for the primary endpoint FMD. These power calculations were based on previous studies from our working group. These studies were powered for FMD and included 20 volunteers, e.g. in the Flaviola Age study in which 22 young and 20 elderly healthy participants were included and their response to cocoa-flavanol intake was compared (Heiss, Sansone et al., 2015). As several techniques of measurement were combined to observe a potential impact on endothelial function, arterial stiffness, blood pressure, blood markers of cardiovascular risk factors, menopausal complaints and sex hormones, the focus was on a small number of participants. For several investigations such as possible effects of anthocyanins as a phytoestrogen (see above) and changes in blood lipids a larger number of participants and a longer intervention period would be needed.

Third, the FMD protocol could theoretically be improved. FMD measurements were performed according to the FMD protocol of our working group that meets most of the criteria demanded by guidelines (Schnabel et al., 2011). However, no automated mathematical algorithms were used to calculate the peak diameter but 40 and 60s images were analysed as the expected time points after cuff-deflation for the maximum FMD, but the peak FMD might have been missed in rare cases (Arrebola-Moreno et al., 2012). Neither a stereotactic apparatus was used (Arrebola-Moreno et al., 2012).

These would be possible ideas to improve the quality of the measurements even further. In addition, there are limitations that are due to the techniques itself. FMD of the brachial artery has been validated comparing it with invasive methods and is the best non-invasive method to measure vascular function but it still is a surrogate parameter. The central aortic pressures are reconstructed from the radial pulse wave using inverse transfer functions.

Fourth, there is another limitation on the investigation on possible phytoestrogenic properties of anthocyanins. This problem emerges from the fact that there are still small changes in the sex hormones the menstrual cycle after the menstrual cycle has ceased. The longer a woman is in the menopause the smaller is usually the effect of these residual hormonal changes making vascular effects unlikely. This is why volunteers in late menopause were chosen (postmenopause stage +1c to +2 in common usage stage or senium). On the other hand, the aim was to observe if anthocyanins improve postmenopausal complaints, and the present study population hardly showed severe menopausal complaints, e.g. the vasomotor symptoms that are typical for early menopause and postmenopause.

Concerning the plasma metabolite measurements of the dose-dependency study the high interindividual variability in plasma (poly)phenol bioavailability is a major limitation. Interindividual variation in (poly)phenol bioavailability depends on various factors like body weight, sex, age, diet, genetic factors, gut microbiota and more (Manach et al., 2016). In the future, well-designed intervention trials with anthocyanin containing foods and integrated omics methods are necessary to stratify individuals based on plasma (poly)phenol bioavailability and

responsiveness to anthocyanins. Another limitation of the present work is the high levels of (poly)phenol metabolites in plasma found at baseline, despite the strict dietary restrictions applied to the young men before each study day. As most phenolic metabolites come from different food sources and from endogenous metabolites, it is extremely difficult to reach low baseline levels even after a (poly)phenol low diet.

4.5 Conclusion

In conclusion, anthocyanins increase vascular function acutely in young healthy individuals with low baseline polyphenol intake, while there seem to be no significant chronic effects in healthy women with a higher baseline polyphenol consumption.

It would be interesting to investigate whether anthocyanins have a chronic effect on the vascular function of people with an unbalanced diet. Cardiovascular disease is the number one killer worldwide and there are many people with a relatively low baseline polyphenol due to unbalanced diet. These people might benefit from the intake of an easily achievable dosage of anthocyanins as used in the present studies that might significantly reduce cardiovascular risk. In the present studies it was shown for the first time that not only diseased people benefit from anthocyanins but also healthy individuals. These findings stress the importance of anthocyanins as an integral element of healthy diet as primary prevention of cardiovascular disease. Anthocyanins might increase the good health and subjective well-being of many individuals. Bigger study populations would be needed in order to investigate possible effects of anthocyanins as phytoestrogens that might be a promising lead to improve the well-being of women during the menopause with the diet and polyphenols that have not shown any adverse effects.

On top of primary prevention and the known effects of modifying cardiovascular risk factors such as dyslipidaemia patients suffering e.g. from myocardial infarction might even benefit from the vascular effects of anthocyanins.

Integrating anthocyanins is a very promising approach for preventing cardiovascular disease. In order to comprehend possible chronic effects of anthocyanins better further studies with more participants and different diets and a longer study duration will be necessary.

5 References

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