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# Interactions between cocoa flavanols and inorganic nitrate: Additive effects on endothelial function at achievable dietary amounts

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*For my family*

*Annem ve Babam için...*

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# Summary

Epidemiological studies have shown that diet is an important modifiable risk factor for the prevention of cardiovascular disease (CVD). The consumption of diets rich in fruit and vegetables are associated with a lower risk of CVD. Dietary intervention studies indicate that cocoa flavanols (CF) and inorganic nitrate can improve endothelial function, suggesting that these two bioactives may be responsible for beneficial health effects. The aim of this thesis is to study interactions between CF and nitrate focusing on absorption, bioavailability, excretion and efficacy to increase vascular function. In a double-blind randomized, dose-response cross-over study, flow-mediated dilation (FMD) was measured in 15 healthy subjects before and at 1, 2, 3, and 4h following ingestion of low and increasing amounts of CF or nitrate. In order to study flavanol-nitrate interactions, an additional intervention trial was performed with nitrate and CF taken together at low and high amounts. FMD was measured before and at 1h after ingestion of nitrate or water. Then subjects received a CF drink or a micro- and macronutrient matched CF-free drink. FMD was measured again at 1, 2, and 4h thereafter. Saliva, blood and urine samples were also taken at different times to investigate the bioavailability of flavanols and nitrate. The intragastric formation of NO after CF and nitrate consumption was also investigated. The main findings of the present study can be summarized as follows:

- Flavanols and nitrate acutely improved endothelial function. They both individually led to an acute and intake-dependent increase in FMD.
- At low intake amounts, additive effects were observed, but at the high amount tested, no further improvement in FMD was observed when CF and nitrate are consumed together.
- CF in combination with nitrate decreased plasma nitrite levels and increase NO formation in the stomach.
- The absorption, metabolism, and excretion of CF were not affected by nitrate consumption.

In conclusion, an additive effect on FMD was evident after combined intake of low dietary achievable amounts of these bioactives suggesting that, alone or combined, flavanol- and nitrate-rich foods may exert beneficial cardiovascular effects.

# Zusammenfassung

Epidemiologische Studien haben gezeigt, dass Ernährung ein wichtiger modifizierbarer Faktor in der Prävention kardiovaskulärer Erkankungen ist. Die Aufnahme von gemüse- und obstreicher Ernährung ist mit einem niedrigen kardiovaskulärem Risiko assoziiert. Studien zu Ernährungsinterventionen zeigen, dass Flavanole und Nitrat die Endothelfunktion verbessern und dass diese zwei bioaktive Substanzen für gesundheitsfördernde Wirkungen verantwortlich sein können. Das Ziel dieser Arbeit war, die Interaktion zwischen Kakaoflavanolen und Nitrat, in Bezug auf ihre Absorption, Bioverfügbarkeit, Ausscheidung und ihre positiven Effekte auf die Gefäßfunktion zu untersuchen. In einer doppelblind, randomisiert, kontrollierten Cross-over Studie wurde die Dosisabhängigkeit der beiden Testsubstanzen bezüglich der Endothelfunktion untersucht. Mit Hilfe der FMD-Untersuchung wurden bei 15 Probanden vor und 1, 2, 3 und 4h nach Einnahme von Flavanolen oder Nitrat in steigender Menge, Messungen durchgeführt. Außerdem wurde die kombinierte Einnahme von Flavanolen und Nitrat in niedrigen und hohen Mengen nach additiven Effekten untersucht. Die Ergebnisse der folgenden Untersuchung lassen sich wie folgt zusammenfassen:

- Flavanole und Nitrat verbessern akut die Endothelfunktion und führen einzeln zu einer dosisabhängigen Steigerung der Endothelfunktion.
- Additive Effekte konnten bei Gabe niedriger Mengen von Flavanolen und Nitrat beobachtet werden, wobei es bei hohen Mengen zu keiner weiteren Besserung der Endothelfunktion kam.
- Flavanole und Nitrat führten zusammen zu einem Abfall vom Plasma-Nitrit-Spiegel und steigerten die Bildung von NO im Magen.
- Die Absorption, der Metabolismus und die Ausscheidung von Flavanolen wurden nicht durch die Einnahme von Nitrat beeinflusst.

Daraus ergab sich die Schlussfolgerung, dass die kombinierte Einnahme von Flavanolen und Nitrat in niedrigen ernährungsrelevanten Mengen eine additive Wirkung auf die Endothelfunktion aufweisen. Die alleinige oder die kombinierte Einnahme flavanol- und nitratreicher Ernährung kann einen positiven Einfluss auf das Herz-Keislauf-Sytem haben.

# Abbreviations

| | |
|---|---|
| **5C-RFM** | 5-carbon ring fission metabolites |
| **3/1C-RFM** | 3- and 1-carbon-side chain ring fission metabolites |
| **$BH_4$** | Tetrahydrobiopterin |
| **BD** | Blood draw |
| **BJ** | Beetroot juice |
| **BP** | Blood pressure |
| **CAD** | Coronary arterial disease |
| **CF** | Cocoa flavanols |
| **cGMP** | Cyclic guanylate monophosphate |
| **CVD** | Cardiovascular disease |
| **CVR** | Cardiovascular risk |
| **DBP** | Diastolic blood pressure |
| **DC** | Darc chocolate |
| **eNOS** | Endothelial nitric oxide synthase |
| **ESRD** | End stage renal disease |
| **FMD** | Flow-mediated dilation |
| **FMN** | Flavin mononucleotide |
| **HPLC** | High Performance Liquid Chromatography |
| **HR** | Heart rate |
| **IR** | Ischemia-reperfusion |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate |
| **ND** | Not detected |
| **NiR** | Nitrite reductase |
| **NO** | Nitric oxide |
| **$NO_2^-$** | Nitrite |
| **$NO_3^-$** | Nitrate |
| **NOS** | Nitric oxide synthase |
| **iNOS** | Inducible nitric oxide synthase |
| **nNOS** | Neuronal nitric oxide synthase |
| **$O_2$** | Oxygen |
| **PMP** | Postmenopausal |
| **RBC** | Red blood cell |
| **RCT** | Randomized controlled trial |
| **ROS** | Reactive oxygen species |
| **S** | Saliva samples |
| **SBP** | Systolic blood pressure |
| **sGC** | Soluble guanylyl cyclase |
| **SREM** | Structurally related (−)-epicatechin metabolites. |
| **T2DM** | Type 2 diabetes mellitus |
| **WHO** | World Health Organization |

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# 1 Introduction

## 1.1 Bioactives in food: cocoa flavanols and nitrate

According to the World Health Organization (WHO) approximately 17,5 million people die each year because of CVD worldwide. Diet has long been thought and recognised to be one of the major modifiable risk factors for CVD (WHO, 2003). Indeed, the most important behavioural risk factors for heart disease and stroke are unhealthy diets (Mozaffarian et al. 2016). On the other hand, epidemiological evidence has shown that consumption of diets rich in fruit and vegetables is associated with a decrease in the risk of CVD (Joshipura et al. 2001, Dauchet et al. 2006, He et al. 2007). However, it is still not clear which compounds present in fruits and vegetables are responsible for those beneficial effects. The high content in fibers, minerals, and vitamins are likely to be responsible for some effects (Thurnham and Northrop-Clewes 2016), but in recent years research has focused in other types of food bioactives present in fruits and vegetables that may also have health benefits.

Among these bioactives, flavanols and inorganic nitrate have become a focus of public attention because of their potential to improve vascular function and decrease blood pressure (BP), as shown in several randomized controlled trials (Hooper et al. 2012, Bondonno et al. 2015). Whereas cocoa, red wine, berries, and tea are rich in flavanols, nitrate is found in beetroot, spinach, lettuce, and other green leafy vegetables (Hammerstone et al. 2000, Bondonno et al. 2015). For half a century it was believed that inorganic nitrate, as a toxic constituent in our diet, was involved in the development of gastric cancer and other malignancies (Tannenbaum and Correa 1985, Mirvish 1995). This is the reason why the nitrate levels have been strictly regulated in drink water and food. However, in recent years nitrate and flavanols have gained attention because of their potential beneficial effects on cardiovascular health. Therefore, one could argue that the cardiovascular health benefits of diets rich in certain fruits and vegetables are in part related to the presence of nitrate.

## 1.2 Sources of nitrite and nitrate

Systemic levels of nitrate in blood and tissues originate from two major sources, namely oxidation of endogenously produced nitric oxide (NO) and our diet (Moncada and Higgs 1993). Thus circulating nitrate is dependent on the activity of the NO synthases and the type of diet. The L-arginine-NOS system is the origin of endogenous nitrite an nitrate production, which is the classical pathway for the generation of NO, and contributes to around 70% of plasma nitrite (Moncada and Higgs 1993, Rhodes et al. 1995, Lundberg et al. 2008) (Figure 1). In the presence of molecular oxygen ($O_2$), the nitric oxide synthase (NOS) enzymes with several cofactors synthesize NO from the amino acid L-arginine (Moncada and Higgs 1993, Rhodes et al. 1995, Lundberg et al. 2008). Nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide, tetrahydrobiopterin ($BH_4$), calmodulin, and calcium are the cofactors. From the three NOS isoforms (endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS)) the eNOS is the major source of NO, which plays an important role in regulating vascular tone (Moncada and Higgs 1993). In addition to the L-arginine-nitric oxide pathway, the other major source of nitrate and nitrite is our diet (nitrate-nitrite-nitric oxide pathway, Fig. 1). Vegetables are the main source of dietary nitrate (80-95% of the total), in particular leafy green vegetables, whereas most dietary nitrite comes from cured meats and baked goods and cereals (Bryan 2009, Hord et al. 2009). Tab. 1 shows examples of the nitrate content of common vegetables.

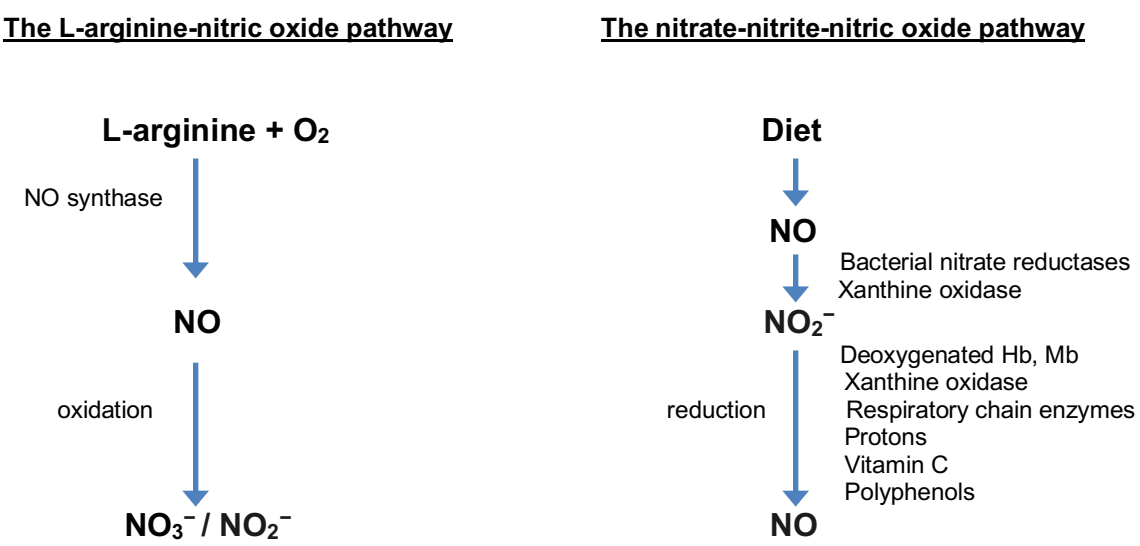



**Figure 1:** Two parallel pathways for NO formation in mammals. NO synthases (NOS) catalyse the formation of NO from the substrates L-arginine and molecular oxygen. NO is rapidly oxidized to form nitrite $NO_2^-$ and nitrate $NO_3^-$. Nitrate reduction to nitrite is mainly carried out by commensal bacteria in the oral cavity and also by mammalian enzymes in tissues (xanthine oxidase). Once nitrite is formed, several pathways exist with the capacity to further metabolize nitrite to NO and other biologically active nitrogen oxides. Most of these pathways are greatly accelerated under hypoxic conditions. Thus, nitrite reduction represents an alternative to the classical NOS pathway for the generation of NO (Lundberg et al. 2011).

**Table 1:** Examples of nitrate concentration in common vegetables. (*5th and 95th percentiles), (Hobbs et al. 2013)

| Vegetable | Sample size (n) | Median (mmol/kg) | Mean (mmol/kg) | Range* |
|---|---|---|---|---|
| *High* | | | | |
| Rocket | 1943 | 77 | 75 | 25-118 |
| Beetroot | 1013 | 18 | 22 | 2-59 |
| Lettuce | 7749 | 15 | 21 | 0,9-59 |
| Celery | 387 | 11 | 18 | 0,3-54 |
| Spinach | 6657 | 13 | 17 | 1-49 |
| *Medium* | | | | |
| Kale | 169 | 4 | 9 | 0,3-30 |
| Beans | 48 | 7 | 6 | 0,1-13 |
| Aubergine | 182 | 5 | 5 | 0,5-9 |
| Cabbage | 1198 | 4 | 5 | 0,8-2 |
| Carrot | 2383 | 2 | 5 | 0,2-5 |
| Broccoli | 227 | 3 | 5 | 0,3-12 |
| Cucumber | 989 | 2,5 | 3 | 0,4-7 |
| Potato | 2795 | 1,7 | 3 | 0,2-55 |
| Onions | 230 | 1 | 3 | 0,02-10 |
| Cauliflower | 289 | 2 | 2 | 0,1-6 |
| *Low* | | | | |
| Garlic | 13 | 1 | 1 | 0,1-3 |
| Mushroom | 12 | 0,7 | 1 | 0,5-2 |
| Tomato | 856 | 0,4 | 0,7 | 0,02-2 |
| Peas | 407 | 0,02 | 0,5 | 0,02-2 |

### 1.2.1 Metabolism of inorganic nitrate

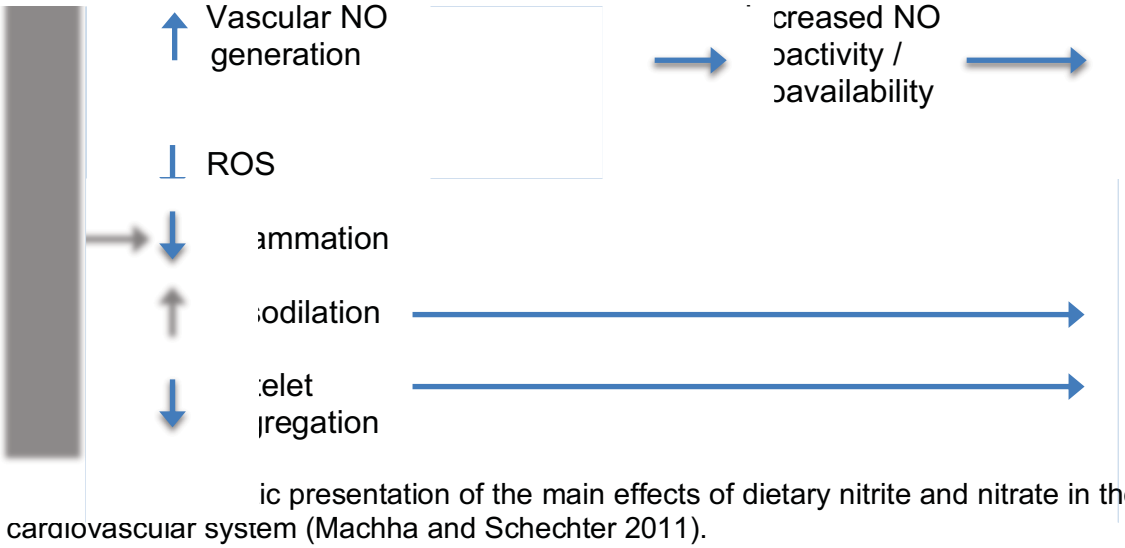
After ingestion of inorganic nitrate, nitrate is rapidly and efficiently absorbed in the upper gastrointestinal tract and mixes with nitrate from endogenous sources (Spiegelhalder et al. 1976, Duncan et al. 1995). The nitrite levels in plasma also increase after nitrate ingestion. The plasma half-life of nitrate is 5-6 hours (van Velzen et al. 2008), with a plasma level peak of 15-30 minutes after ingestion. About 75% of ingested nitrate is excreted via the kidney in the urine and the rest (25%) is actively reabsorbed and concentrated in the salivary glands, which is called enterosalivary cycle (Spiegelhalder et al. 1976, Tannenbaum et al. 1978, Duncan et al. 1995). There is an active uptake of circulating nitrate by a factor 10-20 in saliva (Spiegelhalder et al. 1976, Lundberg and Govoni 2004). This represents a mechanism of reutilization of nitrate by the body. In the oral cavity, nitrate is reduced to nitrite by commensal facultative bacteria. This action of nitrate reductase enzymes may contribute to high salivary levels of nitrite.

Under hypoxic conditions the bacteria, which are on the dorsal surface on the tongue, use nitrate as an alternative electron acceptor to the oxygen during respiration to produce ATP (Duncan et al. 1995, Doel et al. 2005). When swallowed nitrite reaches the acidic environment of the stomach, it is metabolized non-enzymatically to NO and other reactive nitrogen oxides after formation of nitrous acid ($HNO_2$) (Benjamin et al. 1994, Lundberg et al. 1994). Lundberg and colleagues showed for the first time NOS-independent NO generation from inorganic nitrate and nitrite in humans (Lundberg et al. 1994). A significant amount of nitrite survives the gastric passage and reaches the systemic circulation (Lundberg and Govoni 2004), where it is transformed by a number of enzymes and proteins to NO. The reduction to NO and other reactive nitrogen species occur in several pathways in blood and tissue. These include deoxihaemoglobin (Cosby et al. 2003), deoxymyoglobin (Shiva et al. 2007), cytoglobin, neuroglobin (Petersen et al. 2008), xanthine oxidoreductase (Millar et al. 1998), cytochrome P450 (Li et al. 2006), aldehyde oxidase (Li et al. 2008), carbonic anhydrase (Aamand et al. 2009), and endothelial NOS (Gautier et al. 2006).

### 1.2.2 Cardiovascular effects of inorganic nitrate

In the last years, evidence is accumulating on the cardiovascular health benefits of inorganic nitrate. Several studies have shown that the ingestion of dietary nitrate reduces BP (Lampe and Chang 2007, Larsen et al. 2007, Kapil et al. 2010) inhibits platelet function, protects against experimentally-induced endothelial ischaemia reperfusion (IR) injury (Webb et al. 2008) and improves endothelial function (Heiss et al. 2012). It has also been shown that the amount of cardiovascular risk factors correlates inversely with plasma nitrite concentration (Kleinbongard et al. 2006), and that there is a reduced bioavailability of NO in patients with cardiovascular risk factors including hypertension (Panza et al. 1993), hypercholesterolaemia (Chowienczyk et al. 1992) and endothelial dysfunction. There is increasing evidence that dietary nitrate can improve NO bioavailability (Machha and Schechter 2011), inhibit mitochondrial reactive oxygen species (ROS) production (Shiva and Gladwin 2009), inflammation (Kubes et al.

1991), platelet aggregation (Richardson et al. 2002), and exert vasodilation (Fig. 2) (Palmer et al. 1987, Loscalzo 2001, Machha and Schechter 2011).




ic presentation of the main effects of dietary nitrite and nitrate in the cardiovascular system (Machha and Schechter 2011).

Diffusion of endothelium-derived NO into vascular smooth cells causes vascular relaxation by the classical NO-cyclic guanylate monophosphate (cGMP)-protein kinase G signaling pathway. The inhibition of platelet adhesion is thought to be partly through a cGMP-mediated mechanism (Palmer et al. 1987, Loscalzo 2001). NO also may be an important endogenous modulator of leukocyte adhesion and emigration, which is characteristic of acute inflammation (Kubes et al. 1991).These beneficial effects accentuate the obvious therapeutic potential of NO and NO donors (Herman and Moncada 2005). The $NO_3^-$-$NO_2^-$-NO pathway can be manipulated to boost NO activity *in vivo* with demonstrated beneficial effects on CVD, exercise capacity and metabolism, which suggest a potentially easy and cheap way to improve both cardiovascular health and disease (Kapil et al. 2014). It is known that mitochondria are the source of ROS over production in CVD (Ballinger 2005) and it was shown that dietary nitrate and nitrite may have the potential to inhibit mitochondrial ROS production (Shiva and Gladwin 2009). Thus increased NO bioavailability by dietary nitrate intake may reduce ROS overproduction and prevent against CVD (Hobbs et al. 2013).

### 1.2.3 Effects on blood pressure

Hypertension, which is defined as a BP of >140/90 mmHg, is a major risk factor for CVD and an important public health challenge worldwide (MacMahon et al. 1990, McCormack et al. 2012). A number of human intervention studies have investigated the acute and chronic effects of dietary nitrate ingestion on BP using potassium nitrate, sodium nitrate salts, or with natural sources of nitrate like beetroot juice or spinach.

The acute effects of dietary nitrate on BP were shown by many authors. Most of them have demostrated decreases on BP in particular systolic blood pressure (SBP), without changes in diastolic blood pressure (DBP) (Kapil et al. 2010, Bahra et al. 2012, Bondonno et al. 2012, Coles and Clifton 2012). Webb et al. reported in a study with fourteen healthy volunteers that an ingestion of a single dose of 500 ml beetroot juice reduced SBP by 10 and DBP by 8 mmHg, (Webb et al. 2008). In a subsequent study with a lower dose of beetroot juice (250 ml), a decrease of 5 mmHg was found (Kapil et al. 2010). In older subjects diagnosed with peripheral arterial disease and hypertensives, similar effects were described (Kenjale et al. 2011, Ghosh et al. 2013). Hobbs et al. demonstrated the dose-dependent effects with three doses of beetroot juice (100, 250, and 500 ml) on BP. All the doses showed a significant decrease in SBP and DBP (AUC 0-24h). (Hobbs et al. 2012).

There are also few studies that have investigated the effects of other nitrate-rich foods, such as spinach and apple on endothelial function and BP in healthy participants. The study showed a significant reduction in SBP when spinach and apples were given separately, but not when they were given simultaneously (Bondonno et al. 2012). Both interventions didn't show effects on DBP. In a different study Liu et al. presented the acute effects of a nitrate-rich meal (220 mg of nitrate derived from spinach) on arterial stiffness and BP in twenty-six healthy volunteers aged 38-69 years. They observed a significant reduction in SBP by 7,5 mmHg at 120 min post meal (Liu et al. 2013). The majority of human intervention studies have confirmed the blood pressure-lowering effects of dietary nitrate, mostly in healthy young subjects (Webb et al. 2008, Kapil et al. 2010, Sobko et al. 2010, Vanhatalo et al. 2010, Coles and Clifton 2012, Hobbs et al. 2012), although there are also some conflicting results that show no effects on

SBP (Kenjale et al. 2011, Hobbs et al. 2012) and on both SBP and DBP (Lidder et al. 2011).

Several studies have investigated longer term effects of nitrate consumption on BP. For example, daily consumption of 500 ml of beetroot juice for 6 days lowered SBP by 8 mmHg (Bailey et al. 2009). In 2006, Larsen et al. showed that short-term dietary supplementation with sodium nitrate at a dose of 0,1 mmol/kg/day for 3 days lowered DBP by 3,7 mmHg in young healthy volunteers (Larsen et al. 2006). Rammos et al. investigated the effects of sodium nitrate intake (150 $\mu$mol/kg/day) for 4 weeks in older adults with moderate CVD risk. They observed one day after the last intake of nitrate a reduction in SBP from 137 to 129 mmHg in older adults with mild hypertension, but DBP values were unchanged (Rammos et al. 2014). Sobko et al. concluded that Japanese traditional diet, rich in nitrate compared with control diet for 10 days reduces DBP of 5 mmHg in healthy volunteers (Sobko et al. 2010). These findings suggest an important role for dietary nitrate in the management of hypertension. There is also a long term study with no effect on BP (Bondonno et al. 2015) for example Gilchrist et al. showed that the chronic ingestion of nitrate-rich beetroot juice did not lower SBP and DBP in type 2 diabetics (Gilchrist et al. 2013). In a meta-analysis of 4 randomized controlled trials (RCT), chronic supplementation of beetroot juice was not associated with significant changes in systolic and diastolic 24 h ambulatory blood pressure (Siervo et al. 2015).

### 1.2.4 Effects on endothelial function

The effects of dietary nitrate on endothelial function have been investigated in a limited number of human intervention studies. In a study by Webb et al., endothelial function was assesed by FMD in 10 healthy young subjects. They demonstrated that an acute intake of 500 ml beetroot juice protect against endothelial dysfunction induced by ischaemic-reperfusion injury of the forearm and preserved the FMD response (Webb et al. 2008), whereas Gilchrist et al. did not find an improvement (Gilchrist et al. 2013, Joris and Mensink 2013). However, Bahra et al. suggested that a dose of 0,5 g potassium nitrate did not affect the FMD but improves vascular function by reducing the pulse wave velocity (Bahra et al. 2012). Bondonno et al. also showed that nitrate-rich spinach in-

creased FMD by 0,5% at 2 h after intervention (Bondonno et al. 2012). Another human intervention study by Heiss et al. presented the improvement in FMD after ingestion of sodium nitrate (12,7 mg/kg), without change in endothelium-independent vasodilation after nitroglycerin (Heiss et al. 2012). More recently in a randomized, placebo-controlled study in healthy, older adults with moderately increased cardiovascular risk, a daily intake of sodium nitrate (9,3 mg/kg) for 4 weeks improved endothelial function modestly (Rammos et al. 2014). The chronic intake of nitrate-rich beetroot juice improves endothelial function in hypertensives (Kapil et al. 2015) and hypercholesterolemics (Velmurugan et al. 2016). The mechanism by which dietary nitrate improves endothelial function is unclear and needs further investigation, but the available evidence indicates beneficial effects of dietary nitrate on endothelial function. Tab. 2 shows a summary of the trials.

**Table 2:** Randomised, placebo-controlled trials investigating the effects of dietary nitrate on endothelial function.

| Reference | Duration & Duration | Treatment | FMD change (%) |
|---|---|---|---|
| (Velmurugan et al. 2016) | 6 weeks, hypercholesterolemics | 372 mg/d nitrate (0,25 l BJ) | +1,1 |
| (Kapil et al. 2015) | 4 weeks, hypertensives | 397 mg/d nitrate (0,25 l BJ) | +1,0 |
| (Rammos et al. 2014) | 4 weeks, CVR | 9,3 mg/kg bw sodium nitrate | +0,5 |
| (Gilchrist et al. 2013) | 2 weeks, T2DM | 465 mg/d nitrate (0,25 l BJ) | NS |
| (Joris and Mensink 2013) | 0-2 h, overweight elderly | 496 mg nitrate (140 ml BJ) | +0,4 |
| (Heiss et al. 2012) | 0-1,5 h, healthy young | 9,3 mg/kg bw sodium nitrate | Increased |
| (Bahra et al. 2012) | 0-3 h, healthy young | 496 mg potassium nitrate | NS |
| (Bondonno et al. 2012) | 0-2 h, healthy elderly | 186 mg nitrate (200 g spinach) | +0,5 |
| (Webb et al. 2008) | 0-2 h, healthy young | 1,4 g nitrate (0,5 l BJ) | Protection against IR |

## 1.3 Cocoa flavanols

Polyphenols are a group of phytochemicals very abundant in our diet, in particular in fruits and vegetables, coffee, cocoa, tea, olive oil, and soy products (Manach et al. 2004). In recent years polyphenols are getting increased atten-

tion due to epidemiological and clinical studies suggesting they have cardiovascular health benefits (Del Rio et al. 2013). Flavonoids are the biggest class of polyphenols and the ones that have been more investigated regarding health benefits (Del Rio et al. 2013). The main subclasses of flavonoids are flavonols, anthocyanins, flavanols, isoflavones, flavanones, and flavones (Scalbert and Williamson 2000) (Fig. 4).

The main sources of flavanols in our diet are cocoa, red wine, tea and several fruits and vegetables like apple or grapefruit (Hammerstone et al. 1999, Hammerstone et al. 2000). Flavanols are very abundant in cocoa, and include the monomers (-)-epicatechin and (+)-catechin (Fig. 3) and their oligomers, also known as procyanidins (Richelle et al. 1999, Holt et al. 2002). Many factors like maturity, growing, fermentation, and also post-harvest handling and processing as well as food preparation play an important role in the flavanol content of foods (Beecher 2003, Erdman et al. 2007). Tab. 3 shows the total flavanol content of some foods.

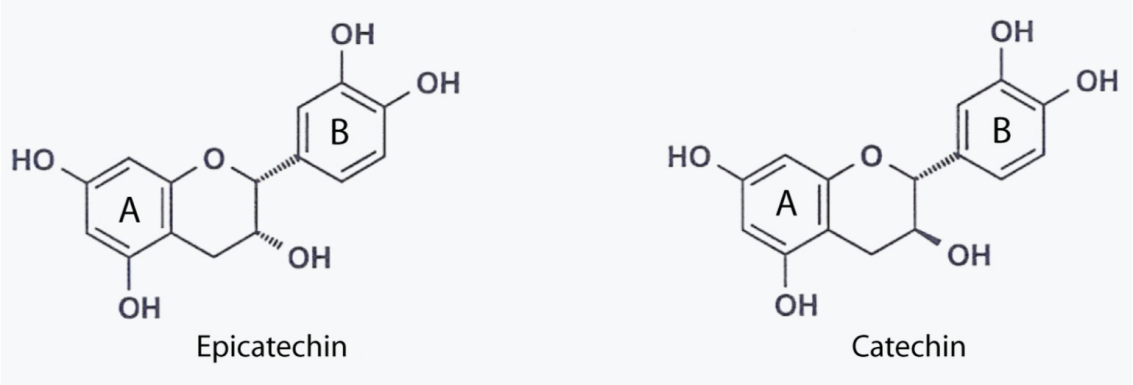



**Figure 3:** Chemical structures of catechin and epicatechin

**Table 3:** Relative contribution of food groups and some main foods to the intake of total and groups of flavan-3-ols by European region (Vogiatzoglou et al. 2014).

| Foodstuff | Southern (%) | Central (%) | Northern (%) |
|---|---|---|---|
| Grain and –grain based products | 4,9 | 3,2 | 3,8 |
| Vegetables and vegetable product | 14,1 | 6,3 | 2,4 |
| Tea and herbs for infusion | 1,5 | 4,0 | 0 |
| Cocoa beans and cocoa products | 12,6 | 2,3 | 2,4 |
| Legumes, nuts and oilseeds | 3,4 | 2,1 | 1,1 |
| Fruit and fruit products | 37,9 | 18,5 | 24,4 |
| Citrus fruits | 1,3 | 0,4 | 1,2 |
| Pome fruits | 19,1 | 10,8 | 13,0 |
| Stone fruits | 11,9 | 2,1 | 2,0 |
| Berries and small fruits | 4,0 | 3,4 | 6,8 |
| Miscellaneous fruits | 0,6 | 0,3 | 0,6 |
| Sugar and confectionery | 1,9 | 2,3 | 3,2 |
| Fruit and vegetable juice | 1,0 | 0,9 | 2,0 |
| Non alcoholic beverages | 22,7 | 61,8 | 56,4 |
| Tea (infusion) | 22,4 | 61,5 | 55,9 |
| Coffee (beverage) | 0 | 0 | 0,2 |
| Cocoa (beverage) | 0,2 | 0,2 | 0,4 |
| Alcoholic beverages | 5,1 | 2,7 | 4,2 |
| Wine | 4,8 | 2,0 | 3,6 |
| Beer and cider | 0,2 | 0,6 | 0,6 |
| Herbs, spices and condiments | 7,2 | 1,0 | 0,1 |
| Composite foods | 0,1 | 0,5 | 0,7 |

### 1.3.1 Metabolism and bioavailability of cocoa flavanols

After ingestion CF are absorbed and enter the circulation rapidly. Flavanol monomers and oligomers pass the stomach without being degraded to the upper intestinal tract (Rios et al. 2002). Only the flavanol monomers and to a very small extent the dimers are absorbed in the small intestine where they are metabolized by the phase II enzymes into glucuronides, sulfates, and methylated compounds (Richelle et al. 1999, Schroeter et al. 2006). Large flavanol oligomers that are not absorbed in the small intestine, undergo a metabolization by colonic microflora in the large intestine (Rios et al. 2003, Kwik-Uribe and Bektash 2008) leading to smaller phenolic metabolites such as valerolactones and phenolic acids (Ottaviani et al. 2016) (Fig. 4). Factors such as food matrix, processing, age, sex, genetic polymorphisms, dietary background, and drug-flavanol interactions can affect the bioavailability and kinetics of absorption, metabolism, and excretion (Cifuentes-Gomez et al. 2015).

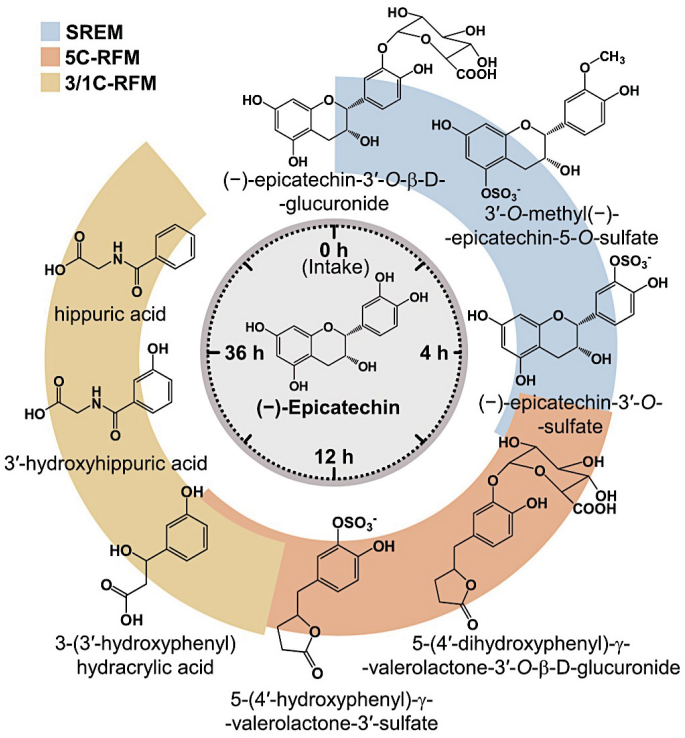



**Figure 4:** Schematic representation of (−)-epicatechin metabolism in humans as a function of time post-oral intake. SREM= structurally related (−)-epicatechin metabolites. 5C-RFM: 5-carbon ring fission metabolites. 3/1C-RFM= 3- and 1-carbon-side chain ring fission metabolites. The structures of the most abundant (−)-epicatechin metabolites present in the systemic circulation and in urine are depicted (with permission from Ottaviani et al. 2016).

## 1.3.2 Cardiovascular effects of cocoa flavanols

The relationship between the risk of CVD and flavonoid intake has been the subject of many epidemiological trials. For a number of flavanol-rich foods such as chocolate (Khawaja et al. 2011), tea (Ivey et al. 2013), red wine, and apples (Mink et al. 2007), positive effects on CVD were observed. In a recent meta-analysis of 14 prospective cohort studies, Wang et al. showed that higher consumption of flavanols was associated with a relative reduction of 0,87 (95% CI 0,80, 0,95; p=0,002) in the risk of CVD, when comparing the highest category of intake with the lowest category (Wang et al. 2014). Another meta-analysis of 7 studies showed that higher consumption of chocolate and cocoa decreased the risk of CVD and stroke by 37% and 29% respectively (Buitrago-Lopez et al. 2011). Several controlled human intervention studies with flavanols or flavanol-rich foods have demonstrated positive effects on BP (Desideri et al. 2012, Ried et al. 2012) and endothelial function (Engler et al. 2004, Schroeter et al. 2006,

Heiss et al. 2007, Balzer et al. 2008, Heiss et al. 2010) and these findings were corroborated by meta-analyses (Shrime et al. 2011, Hooper et al. 2012). A correlation was found between these positive effects on vascular function and changes in plasma flavanol metabolites, and similar vascular effects was also found after the intake of the main cocoa flavanol monomer, (-)-epicatechin, in its pure form (Schroeter et al 2006). Furthermore, a cause and effect relationship was shown between vascular function improvements and consumption of CF (Schroeter et al. 2006, Schroeter et al. 2010). Although the mechanisms of actions have not been fully elucidated yet, there is some evidence on the modulation of NO bioavailability in the cardiovascular system by flavanols, either via upregulation of endothelial nitric oxide synthase (eNOS) activity or via inhibition of NADPH oxidases (Steffen et al. 2007, Steffen et al. 2008).

### 1.3.3 Effects on blood pressure

Several studies showed that the ingestion of flavanol rich foods may lower SBP and DPB in hypertensive (Grassi et al. 2005, Grassi et al. 2008), but also in healthy people (Grassi et al. 2005). In a meta-analysis of 20 studies mainly in healthy volunteers, Ried et al. reported a decrease in SBP of 2,8 mmHg and DBP of 2,2 mmHg. BP lowering effect was greater for trials that compared flavanol-rich product to a flavanol-free control product (SBP –3,7 mm Hg; DBP – 2,7 mmHg. But most of the investigations, which used flavanol free placebo products were not double blinded and poorly controlled. Ten of the studies in the meta-analysis did not show significant changes in BP (SBP − 0.7 mmHg, DBP – 0,8 mmHg) where blinding was accomplished by matching a low-flavanol control product to a high-flavanol product Compared to the flavanol free control, low flavanol control did not show a significant reduction in BP (Ried et al. 2012). Hooper et al. also suggest that doses of 50 mg epicatechin or higher per day resulted in greater effects on SBP and DBP (Hooper et al. 2012). Flavanol-rich cocoa induced vasodilation via activation of the nitric oxide system (Fisher et al. 2003), suggesting that this vasodilative agent is responsible for the reducing effects on BP.

### 1.3.4 Effects on endothelial function

Several clinical studies have shown that flavanol-rich foods improve endothelial function, measured as FMD, and this is associated with increasing plasma flavanol metabolites (Balzer et al. 2008, Heiss et al. 2015, Sansone et al. 2015). Hooper et al. demonstrated in a meta-analysis that the acute intake of cocoa or chocolate improved the FMD at 2 h post-consumption by 4.0% and chronic intake by 1,5% (Hooper et al. 2012). Investigations showed that after ingestion of cocoa or chocolate, flavanol metabolites appear in plasma and led to a time- (Balzer et al. 2008) and dose-dependent increase in FMD with peak effects at 2 hours post-consumption (Heiss et al. 2005). Table 4 shows a summary of trials investigating the effects on endothelial function. The mechanisms underlying these beneficial effects have not been clarified yet and are still under investigation. It is believed that the improvements in FMD are likely due to the effects of flavanols on increased nitric oxide bioactivity (Heiss et al. 2003, Balzer et al. 2006). Schewe et al. investigated the bioavailability of NO in the vascular endothelium. They showed that exposure of human endothelial cells to (−)-epicatechin raises the cellular levels of NO and cyclic GMP and protects against oxidative stress elicited by proinflammatory agonists. Reduced elimination of NO through inhibition of NADPH oxidase leads to promotion of bioavailability and bioactivity of NO. Therefore, they suggest that endothelial NO metabolism rather than general antioxidant activity is a major target of dietary flavanols and that NADPH oxidase activity is the essential site of action (Schewe et al. 2008). Short-term effects could be explained by reduced NO elimination thorough NADPH oxidase as caused by *O*-methylated (−)-epicatechin metabolites, whereas long-term effects may additionally include increased generation of NO as a result of a higher cellular level of active eNOS protein (Schewe et al. 2008). However, less than 1% of ingested (-)-epicatechin is present in circulation with 99% being metabolized into glucuronides, sulfates, and methylated compounds (Ottaviani et al. 2012). Therefore, this hypothesis needs to be confirmed with the structurally related epicatechin metabolites, which are the most likely compounds to be responsible for the beneficial effects

**Table 4:** Randomised placebo-controlled trials investigating the effects of flavanols on endothelial function

| Reference | Duration &Subjects | Treatment | FMD change (%) |
|---|---|---|---|
| (Heiss et al. 2015) | 2 weeks, healthy young & elderly men | 450 mg CF drink | young: +1,3<br>elderly: +1,3 |
| (Sansone et al. 2015) | 1 month, healthy middle-aged | 450 mg CF drink | +1,2 |
| (Rassaf et al. 2016) | 0-2 h, ESRD | 900 mg CF drink | 3,2 ± 0,6<br>to 4,8 ± 0,9 |
| (Esser et al. 2014) | 4 weeks, healthy overweight men | 70 g DC | +1,0 |
| (West et al. 2014) | 4 weeks, healthy overweight | 37 g DC and 814 mg CF drink | NS |
| (Mogollon et al. 2013) | 12 weeks, healthy young pregnant women | 20 g chocolate (400 mg flavanol) | NS |
| (Grassi et al. 2012) | 3 days, healthy young | 100 g DC | 7,9 ± 0,7<br>to 8,5 ± 0,7 |
| (Heiss et al. 2010) | 1 month, elderly with CAD | 750 mg CF drink | 4,6 ± 0,5<br>to 8,4 ± 0,8 |
| (Balzer et al. 2008) | 0-2, T2DM | 963 mg CF drink | 3,8 ± 0,3<br>to 5,5 ± 0,4 |
| (Balzer et al. 2008) | 1 month; 0-2 h T2DM | 963 mg CF drink | 4,3 ± 1,2<br>to 5,8 ± 1,6 |
| (Davison et al. 2008) | 0-2 h,<br>12 weeks,<br>healthy obese | 451 mg CF drink<br>902 mg CF drink | +2,4<br>+1,6 |
| (Heiss et al. 2007) | 1 week, healthy young men | 918 mg CF drink | 3,7 ± 0,4<br>to 6.6 ± 0.5 |
| (Farouque et al. 2006) | 6 weeks, elderly men with CAD | 444 mg flavanols | NS |
| (Hermann et al. 2006) | 0-2 h, smokers healthy men | 40 g DC | 4,4 ± 0,9<br>to 7,0 ± 0,7 |
| (Schroeter et al. 2006) | 0-2 h, healthy young male | 917 mg CF drink | increased |
| (Wang-Polagruto et al. 2006) | 6 weeks, PMP hypercholesterolemic women | 446 mg CF drink | +2,0 |
| (Grassi et al. 2005) | 15 days, hypertensives | 100 g DC | 7,4 ± 1,4<br>to 8,9 ± 1,4 |
| (Heiss et al. 2005) | 0-2 h, smokers | 100 ml CF Drink (176-185 mg) | 4,5 ± 0,8<br>to 6,9 ± 0,9 |
| (Vlachopoulos et al. 2005) | 0-1 h, healthy young | 100 g DC | +1,4 |
| (Engler et al. 2004) | 2 weeks, healthy | 46 g DC | 1,3 ± 0,7 |
| (Heiss et al. 2003) | 2 days, CVR | 176 mg CF drink | 3,4 to 6,3 |

## 1.4 Aims of this thesis

Clinical and epidemiological studies suggest that both flavanols and nitrate are food bioactive compounds present in the diet and have the potential to improve vascular function. However, these bioactives are often consumed together, and it is currently unknown whether interactions exist between them. Thus, the aim of this thesis is to investigate whether there are interactions between CF and nitrate focusing on the absorption, metabolism, excretion, and the efficacy to increase endothelial function. Our hypothesis is that CF and nitrate interact with each other and improve endothelial function in a synergistic way, when ingested together. We first conducted dose-response studies to investigate the effects of nitrate and flavanols (ingested separately) on endothelial function and BP in healthy subjects. Then we will investigate the interactions between nitrate and flavanols when ingested together and the effects on CF absorption, metabolism, excretion, and efficacy to improve endothelial function.

# 2 Materials and Methods

## 2.1 Statement of personal contributions

Based on the study design prepared by Dr. Rodriguez-Mateos and Prof. Heiss, I, Hilal Aydin, have performed the following tasks: Scheduling of study subjects, performance of vascular exams and blood draws, analysis of vascular measurements, blood sample preparation and storage for flavanol and NO parameter analyses, participated in statistical analysis of results. Plasma flavanol anaylses were carried out at Reading University, whereas nitrate and nitrite analyses were accomplished at Karolinska Institute in Sweden**.** Biochemical analysis of blood draws were measured in the central laboratory of the University Hospital of Düsseldorf.

## 2.2 Materials

All individual flavonoid and phenolic acid standards were obtained from Sigma-Aldrich Co. Ltd (Poole, UK) or Extrasynthese (Genay, France). β-glucuronidase and sulfatase (Helix pomatia, Type H1) was purchased from Sigma-Aldrich Co Ltd (Poole, UK). Water, methanol, acetic acid and acetonitrile (high performance liquid chromatography (HPLC) grade) were purchased from Fisher Scientific (Loughborough, UK). HPLC columns were from Hichrom (Reading, UK). Oasis HLB solid phase extraction cartridges were purchased from Waters (Elstree, UK). Unless stated otherwise, all chemicals and reagents were obtained from Sigma-Aldrich Co Ltd (Poole, UK) or Fisher Scientific (Loughborough, UK).

## 2.3 Intervention study subjects

In this study, healthy young male were recruited. They were contacted through adverts placed at the university and word of mouth. Before starting the study, volunteers were investigated for good general health and were screened by their responses to a standard medical questionnaire. Inclusion criteria were: age 18–35 years, a signed consent form, good general health assessed by their responses to a standard medical questionnaire and blood results (normal hemoglobin, hematocrit, liver enzymes and leukocyte counts), absence of CVD

(e.g. hypertension, heart disease, stroke, disease of the circulation or suffering from any illness that may affect the ability of the blood to clot), absence of anemia, gall bladder problems, and diabetes mellitus. The exclusion criteria included: evidence of cardiovascular diseases known to affect vasomotion such as arterial hypertension, hypercholesterolemia, chronic heart failure, diabetes, disease of the circulation or suffering from any illness that may affect the ability of the blood to clot or stroke, taking antibiotics, prebiotics, probiotics, synbiotics or anti-inflammatory or BP lowering medication within a 2-month period prior to the study. Participants were given verbal and written description of all procedures, purpose and risks involved in the study prior to participation and written informed consent was obtained.

## 2.4 Study Design

A total of 4 RCTs were conducted (Fig. 5). Two of the studies were double-blind randomized intake-response investigations with a crossover design. The dose dependency of nitrate and CF, respectively and the interactions between flavanols and nitrate were investigated. In all the studies, FMD of the brachial artery was the primary outcome. BP was the secondary outcome. Saliva, blood and urine samples were taken for analysis of CF metabolites, nitrite, and nitrate. All measurements were obtained between 8:00 am and 1:00 pm and each participant was required to lie quietly in an air-conditioned and temperature controlled room for more than 10 min before the first scan. There was a washout period of one week between all study days.

Volunteers were asked not to alter their usual dietary or fluid intake within the study period. They were also asked to follow a low polyphenol and nitrate diet for 24 h before and during each intervention day. They were instructed not to eat any polyphenol-rich food including, vegetables, fruits, coffee, cocoa, chocolate, tea and wine, and also not to ingest nitrate rich foods like leafy green vegetables and beetroot. Volunteers were asked to fill a 48 hour diet diary to ensure they complied with the diet. They were also asked to avoid any strenuous exercise (> 3 x 20 min/week) and consuming alcohol from 24 h prior to, and during the study.

The volunteers and researchers involved with assessing study outcomes were blinded with regard to the allocation of study subjects throughout the study. The random allocation to treatment sequence by using a Williams design were performed by an independent researcher. This present study was conducted according to the guidelines laid down in the Declaration of Helsinki. In the ethics committee of Düsseldorf the study is conducted under the number 3657. The studies were also registered with the National Institutes of Health (NIH)-randomized trial records held on the NIH ClinicalTrials.gov website (NCT01262521 and NCT01799005). The study was conducted from May 2012 to May 2014.

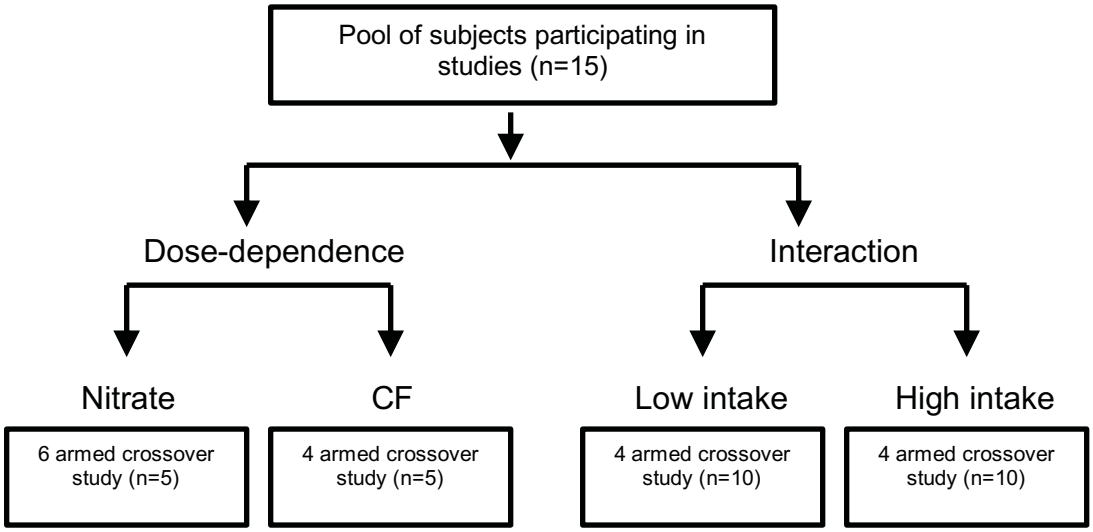



**Figure 5:** Study design

## 2.4.1 Dose-dependency nitrate

For the dose-dependence of nitrate, study participants received each intervention day another dose of inorganic nitrate (0,1, 0,3, 1,0, 3,0, 8,5, and 10,0 mg/kg bw). So that all participants got different amounts of nitrate on different days (Williams Design). FMD was measured before and at 1, 2, 3, and 4 hours post-consumption of nitrate (Fig. 6). BP was recorded and saliva samples (S) were collected after each FMD measurement. Blood samples were collected before (0 h) and at 1 h post ingestion of nitrate.

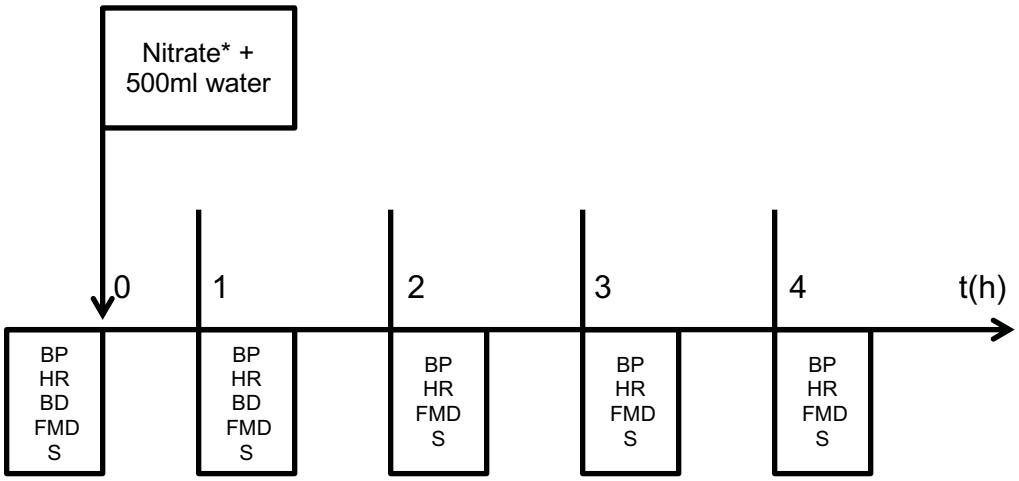



**Figure 6:** Study design of nitrate dose dependency. (* different nitrate doses (0,1-10,0 mg/kg bw for each participant and each visit)

## 2.4.2 Dose-dependency CF

The dose-dependence of CF were accomplished similar to the dose-dependence study of nitrate. Study participants ingested each visit different doses of CF (1,4, 2,7, 5,5, and 10,9 mg/kg body weight [bw]). FMD and BP measurements were performed before (0h) and at 1, 2, 3, and 4 hours postconsumption of CF. Blood samples were collected before and 1h post CF ingestion (Fig. 7).

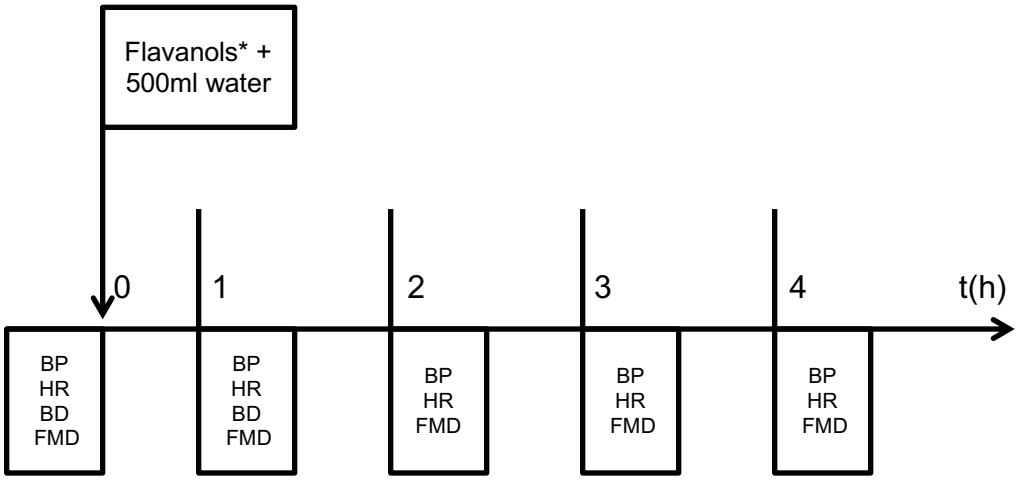



**Figure 7:** Study design of CF dose-dependence study. (*different CF doses (1,4-10,9 mg/kg bw for each participant and each visit)

### 2.4.3 Interactions CF and nitrate

In order to study interactions between CF and nitrate, two four-armed double blind RCTs were carried out with nitrate and CF taken together at low and high doses (Fig. 5). FMD was measured before (0h) and at 1h after ingestion of nitrate (3,0 or 8,5 mg/kg bw) or water. After the second FMD measurement, the subjects ingested a CF drink (2,7 or 10,9 mg/kg bw) or a micro- and macronutrient matched CF-free drink. FMD and BP were measured before (0h) and at 1, 2, and 4 h thereafter (Fig. 8). Saliva was collected after each BP measurement. Prior to the ingestion of the drink and at 1, 2, and 4 hours post-ingestion blood samples were taken from the volunteers. Urine samples were collected as baseline morning urine and for 24 h after ingestion of nitrate.

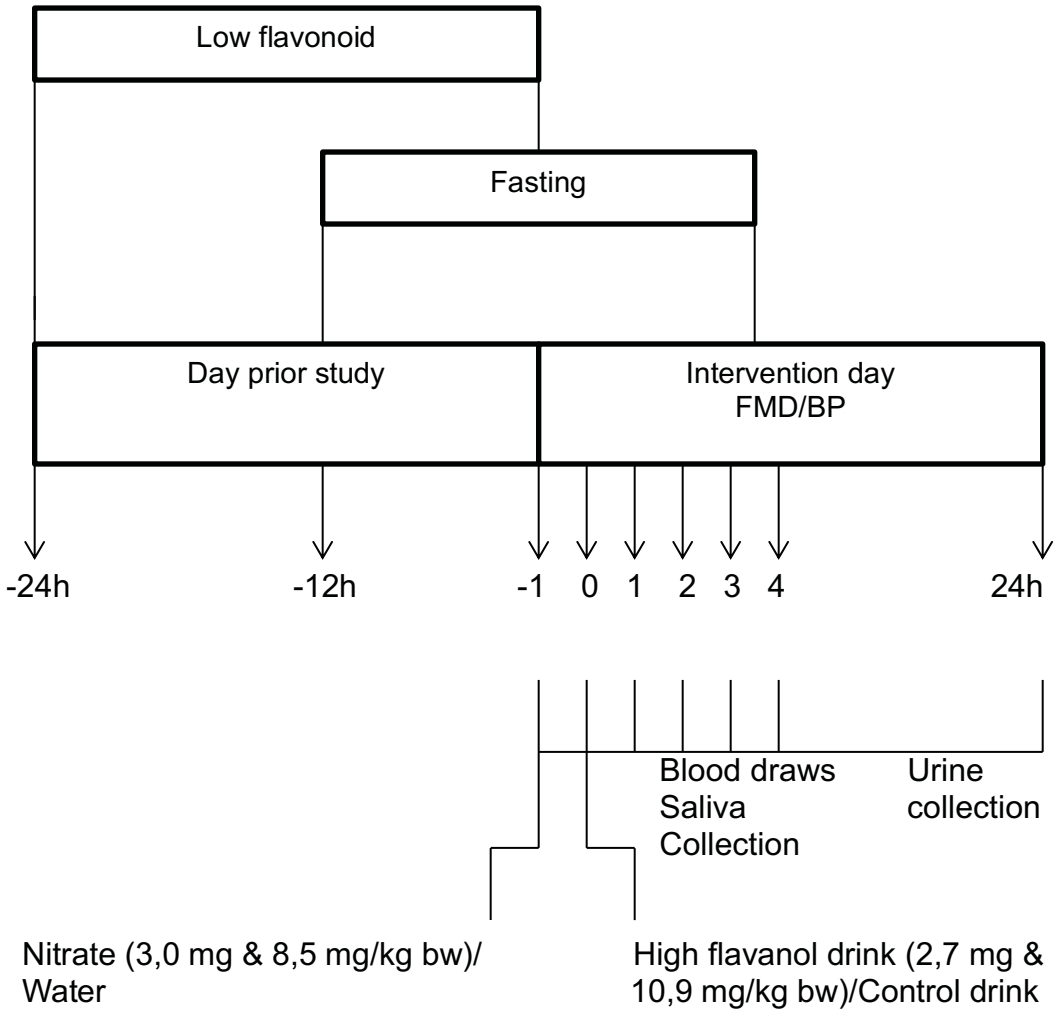



**Figure 8:** Scheme of four-arm double blind RCT designed to assess CF-nitrate interactions.

## 2.5 Test materials

CF and the control powder were supplied as dry beverage mixes by Mars Incorporated, which were prepared for consumption by mixing with 500 ml of low nitrate water by mixing. The test drinks were similar in taste with the same color and packaging. Table 5a shows the macro- and micronutrient matched and isocaloric drinks with high and no flavanol content. Table 5b shows the amounts of CF and control drink powder used in the CF intake-response and in the CF/nitrate interaction study. Food grade sodium nitrate (mg/kg bw) was dissolved in 500 ml of low nitrate water and. The volunteers drank the water nitrate solution immediately. The control of the studies using nitrate was 500 ml of water.

**Table 5a:** Composition of test drinks (per kg of body weight)

| | Flavanol drink | Control drink |
|---|---|---|
| Total flavanols (mg | 10,9 | ND |
| Monomers (mg) | 1,8 | ND |
| (−)-Epicatechin (mg) | 1,5 | ND |
| Dimers (mg) | 0,1 | ND |
| Dimers-Decamers (mg) | 1,8 | ND |
| Theobromine (mg) | 8,3 | ND |
| Caffeine (g) | 0,2 | 0,1 |
| Fat (g) | 0 | 0 |
| Carbohydrates (g) | 0,2 | 0,2 |
| Protein (mg) | 0 | 0 |
| Energy (kcal) | 0,7 | 0,7 |
| Sodium (mg) | 0,1 | 0,1 |
| Potassium (mg) | 2,9 | 2,2 |

**Table 5b:** Amounts of CF and control drink powder used in the studies

| | Intake of preparation amounts | | | | |
|---|---|---|---|---|---|
| **Target CF intake amount (mg/ 75kg bw)** | **0,0** | **102** | **205** | **410** | **820** |
| **Target CF intake amount (mg/kg bw)** | **0,0** | **1,4** | **2,7** | **5,5** | **10,9** |
| **CF drink powder (g/75 kg bw)** | **0,0** | **1,8** | **3,5** | **7,0** | **14,0** |
| **Control drink powder (g/75 kg bw)** | **14,0** | **12,3** | **10,5** | **7,0** | **0,0** |
| **Amount of water (ml)** | **500** | **500** | **500** | **500** | **500** |
| | **Use of test drink** | | | | |
| **In CF intake-response study** | **–** | **+** | **+** | **+** | **+** |
| **In CF/nitrate interaction study 1** | **+** | **–** | **+** | **–** | **–** |
| **In CF/nitrate interaction study 2** | **+** | **–** | **–** | **–** | **+** |

## 2.6 Flow-mediated dilation

The endothelial function of the healthy subjects was assessed by measuring noninvasively the FMD of the brachial artery by ultrasound (General Electrics, Vivid 7) by the same investigator in combination with an automated analysis system (Brachial Analyzer, Medical Imaging Applications, Iowa City, IO) in a temperature conditioned room as previously described (Rodriguez-Mateos et al. 2013). Before starting with the first scan the participants lied 10 min quietly to make sure that all of them were investigated under same conditions. A blood-pressure tourniquet located at the proximal forearm distal to the antecubital fossa was inflated to 250 mmHg for 5 min. Brachial artery dilation was recorded at baseline before and immediately after release of cuff, at 20, 40, 60, and 80 sec. The FMD was calculated from the difference post ischaemia and baseline artery diameter (maximum dilation) divided by baseline and multiplied by 100. Vasodilation results were presented as percentage change. All ultrasound scans were performed and analized by the same operator using the same equipment, who was blinded to the measurement details.

## 2.7 Blood pressure

BP and heart rate were recorded before and 1, 2, 3, and 4 h after ingestion of the test drink as the mean of the three measurements using an Omron MX2

automatic digital upper arm BP monitor according to established guidelines (Williams et al. 2004) (Omron Healthcare UK Ltd, Milton Keynes, UK). All BP measurements were performed in seated position to make sure that all participants were measured under same conditions.

## 2.8 Plasma flavanol analysis

Plasma samples were drawn into EDTA-containing vials, supplemented with 2% formic acid and immediately stored at -80˚C until analysis. The analysis of flavanol in plasma and urine was accomplished by using enzymatic hydrolysis, which consists of ß-glucuronidase and sulfatase, as described previously (Rodriguez-Mateos et al. 2012). Metabolites of (−)-epicatechin were reduced into non-methylated (−)-epicatechin, 3′-*O*-methylated (−)-epicatechin, and 4′-*O*-methylated (−)-epicatechin. Samples were analyzed by HPLC with fluorescence detection using authentic standards at Reading University.

## 2.9 Plasma, saliva, and urine nitrite and nitrate analysis

Plasma samples were also drawn into EDTA-containing vials, supplemented with 2% formic acid and immediately stored at -80˚C until analysis. Plasma nitrite and nitrate were determined by chemiluminescence, as previously described (Govoni et al. 2008).

Nitrite measurements: Air tight micro-reaction vessel were prepared with a 45mM potassium iodide, 10mM iodine and glacial acetic acid solution and purged with inert nitrogen gas to inject them with plasma. Before transfer into the chemiluminescence reader (CLD 77 EcoPhysics, Switzerland) the reaction vessel is stored at 60°C, which is coupled with a condenser. A continuous flow of cold and heat water controlled the temperature of the condenser and a trap, which is filled with sodium hydroxide protects against contamination. By using Azur 5.0 (Le Touvet, France) the chemiluminescence data was collected and analyzed. Results were analyzed by correlation to a fresh standard curve of sodium nitrite dissolved in ultrapure water.

Nitrate measurements: Plasma was deproteinized by 1:3 dilution with ice cold ethanol followed by centrifugation. Plasma nitrate was analyzed through reduc-

tion to NO in a solution of 50 mM vanadium(III) chloride dissolved in 1 N hydrochloric acid heated to 94°C in the same apparatus as described above. Nitrate concentrations were obtained by correlation to a fresh standard curve of sodium nitrate dissolved in ultrapure water followed by subtraction of the measured nitrite from the NO value.

Salivary NOX (sum of nitrite and nitrate) and urinary nitrate were analyzed by HPLC (ENO-20) and autosampler (840, EiCom, Kyoto, Japan) as described before (Govoni et al. 2008). Samples were initially diluted in 10% methanol. Nitrate and nitrite were isolated by reverse phase/ion exchange chromatography followed by nitrate reduction to nitrite by cadmium and reduced copper. The nitrite was then derivatized using Griess reagent to form diazo compounds and analyzed by detection at 540 nm. The salivary nitrite and nitrate was correlated to the protein content of the samples as measured by protein assay reagent (Bio-Rad, Hercules, CA, USA).

## 2.10 Expelled stomach NO

The intragastric formation of nitric oxide (NO) after CF and nitrate consumption was investigated by collecting saliva before and at 1, 2, 3, and 4 h after consumption of nitrate or CF and nitrate together. As with the other trials, fasting volunteers ingested nitrate or water followed by flavanol (10,9 mg/kg bw) or placebo ingestion an hour later. The release of gastric NO was induced by ingestion of 150 ml carbonated water (LOKA, Sweden), collected in an air-tight bag and immediately measured in parts per billion using chemiluminescence at baseline, 1 h after nitrate and 15 and 30 minutes after flavanol ingestion. Highest expulsion value was used and results are presented as a ratio to baseline.

## 2.11 Biochemical analysis

All clinical parameters including plasma levels of glucose, triacylglycerol (TAG), total cholesterol, HDL cholesterol, LDL cholesterol were measured in the central laboratory of the University Hospital of Düsseldorf with using standard procedures. The blood samples, which were collected in lithium/heparin tubes were centrifugated (1,700x*g*; 10 min; 4ºC) as quick as possible after taking blood from the volunteers. Such blood samples, which were also collected in serum

separation tubes, should be centrifugated (1,300x*g*; 10 min; 21ºC) in the next 30 min and shouldn't stay longer. All samples, including blood, urine, and saliva, were aliquoted and frozen at -80°C until analysis.

## 2.12 Statistical methods

Power calculations were performed for the primary endpoint, change in FMD response. Power was based on the intra-individual variability of the operator that performed the FMD analysis (5% CV, SD=0.3, based on previous studies where the same subjects were measured on 4 different occasions at the same time of day). According to CONSORT guideline SD was used for baseline characteristics and SEM was used for changes and results. At 0.8 power, a 0.05 significance level and a mean FMD of 7.2%, the number of subjects required to detect a difference of 0.3% in the response of matched pairs in a crossover study is 10. This number is consistent with other studies carried out with similar endpoints and study design (Heiss et al. 2005, Schroeter et al. 2006). The primary test for an effect was two-way repeated measurements analysis of variance. The data was computed with the SAS version 9.1 software package (SAS Institute, Cary, NC, US) and GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, US). Post-hoc analysis was accomplished by using the Bonferroni test. P values $< 0.05$ were regarded as statistically significant. With using the trapezoidal method the calculation of the area under the plasma concentration versus time curve (AUC) was performed. Correlations were calculated by using Pearson's coefficient.

# 3 Results

## 3.1 Baseline characteristics of study population

A total of 21 subjects were screened for eligibility, but only 15 of them met the inclusion criteria and were included in the study (Fig. 9). The study population consisted of young healthy male volunteers between 18–35 years old. As indicated in Tab. 6, the baseline characteristics of the subjects, including BP, heart rate and clinical chemistry parameters were all within normal values. They all showed normal hemoglobin, hematocrit, leukocyte counts and liver enzymes in their blood results. None of the subjects were on medication, and none of them revealed any present or past evidence of cardiovascular diseases known to affect vasomotion such as arterial hypertension, hypercholesterolemia, chronic heart failure, diabetes, disease of the circulation or suffering from any illness that may affect the ability of the blood to clot or stroke. They were all life-long non-smokers. The intervention drinks were well tolerated by all participants and no adverse events were reported. All participants signed a consent form before starting the study.

**Table 6:** Baseline clinical characteristics study population (n=10, mean ± SD).

| **Baseline characteristics** | |
|---|---|
| **Age (years)** | **25 ± 1** |
| **BMI (kg/m$^2$)** | **24.1 ± 0.7** |
| **GFR (ml/min)** | **98 ± 5** |
| **Cholesterol (mg/dl)** | **186 ± 9** |
| **Triglycerides (mg/dl)** | **136 ± 17** |
| **Glucose (mg/dl)** | **80 ± 2** |
| **Creatinine (mg/dl)** | **0.9 ± 0.04** |
| **Bilirubin (mg/dl)** | **0.5 ± 0.04** |
| **Urea (mg/dl)** | **35 ± 6** |
| **SBP (mmHg)** | **119 ± 2** |
| **DBP (mmHg)** | **68 ± 1** |
| **Heart rate (bpm)** | **56 ± 3** |

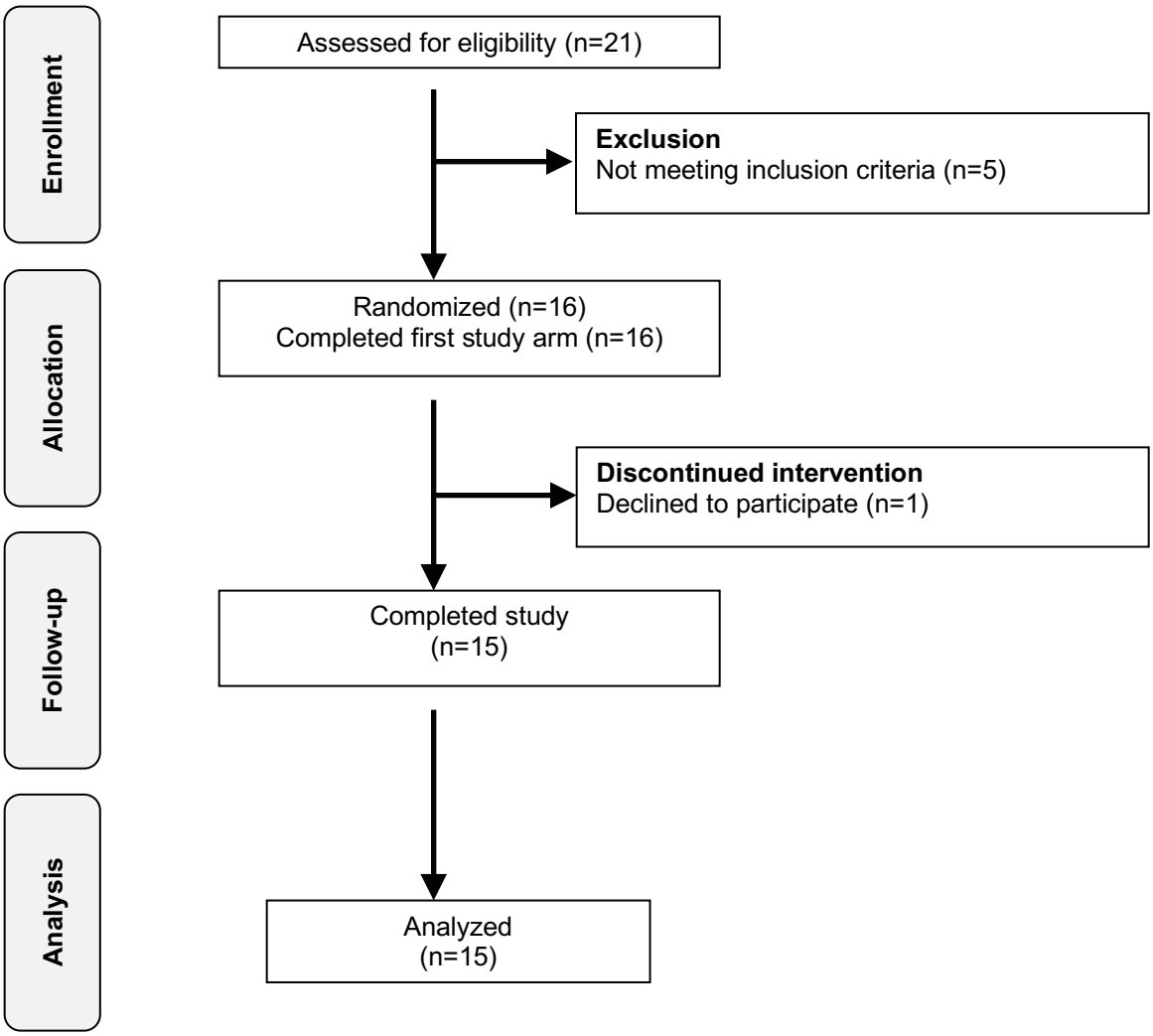



**Figure 9:** Study flow of intervention study subjects

# 3.2 Intake-dependent increase in FMD

## 3.2.1 Intake-dependent increase in FMD by CF

In a double blind, crossover, RCT, whether CF improve FMD in an intake-dependent manner was investigated. Study participants received 1,4, 2,7, 5,5 and 10,9 mg CF/kg bw on different days with a washout period of one week. FMD was measured before and at 1, 2, 3, and 4h after consumption of CF. No significant differences in baseline FMD values for each participant on different days was observed. An intake-dependent increase in FMD was shown after CF consumption (Fig. 10A). Changes in FMD at 1 h post-consumption with respect to baseline were 0,3 ± 0,1% for 1,4 mg/kg, 0,6 ± 0,01% for 2,7 mg/kg and 1,1 ± 0.1% for the 5.5 mg/kg CF dose. The maximum increase (2,5 ± 0,2%) was obtained for the 10,9 mg/kg CF dose. The ED50, i.e. the amount necessary to induce a half maximal increase in FMD, was 548 mg for CF (7,3 mg/kg bw). The

minimum amount of CF to elicit a significant increase in FMD was 202 mg (2,7 mg/kg bw).

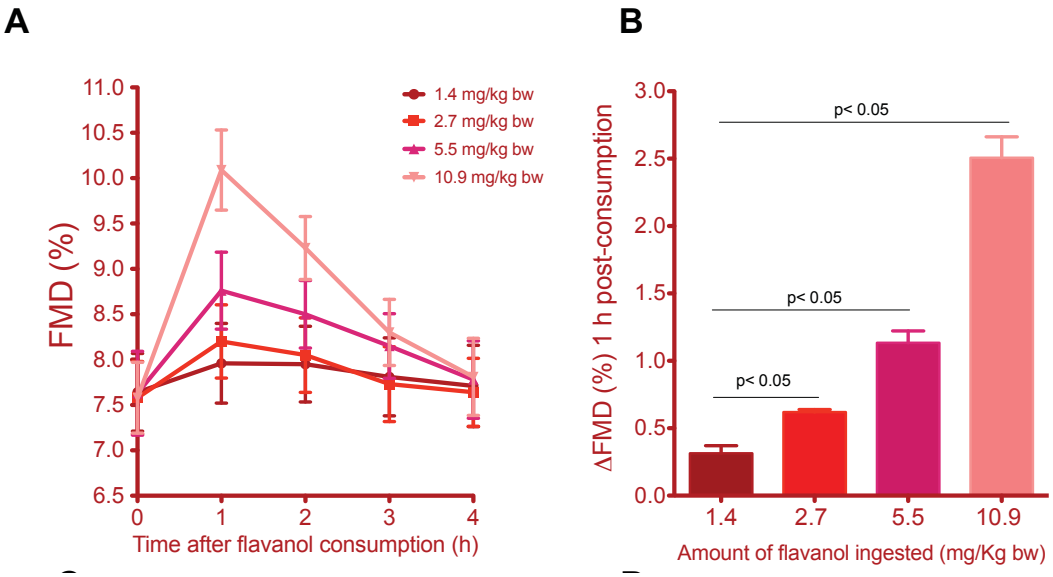



**Figure 10:** Intake dependence of cocoa flavanols on FMD in healthy individuals. Results are expressed as the mean ± SEM.

### 3.2.1 Intake-dependent increase in FMD by inorganic nitrate

In order to investigate the intake-dependent increase in FMD after nitrate consumption, amounts of 0,1, 0,3, 1, 3, 8,5 and 10 mg of nitrate/kg bw were given to volunteers in separate days and FMD was measured before and at 1, 2, 3 and 4h post-consumption. After a wash-out period of 1 week between study days no significant differences in baseline FMD values were detected. An acute intake-dependent increase in FMD was found (Fig. 11). The minimum increase (0,2 ± 0,1%) was achieved with the 0,1 mg/kg and the maximum increase (2,0 ± 0,2%) with the 10 mg/kg nitrate dose. The FMD increases with respect to baseline were 0,2 ± 0,1 % for 0,3 mg/kg, 0,7 ± 0,1% for 1 mg/kg, 1,1 ± 0,2% for 3 mg/kg, and 1,8 ± 0,2% for the 8.5 mg/kg nitrate dose (Fig. 11B). The ED50, i.e. the amount necessary to exert a half-maximal increase in FMD, was 371 mg for nitrate (4,9 mg/kg bw) and the minimum amount of nitrate to elicit a significant increase in FMD was 75 mg (1 mg/kg bw). As depicted in Figure 10 and 11, the time courses of FMD following ingestion of CF and nitrate were very similar with maximal values being achieved at 1 h post ingestion and FMD values gradually decreasing thereafter reaching baseline values at 4 h.

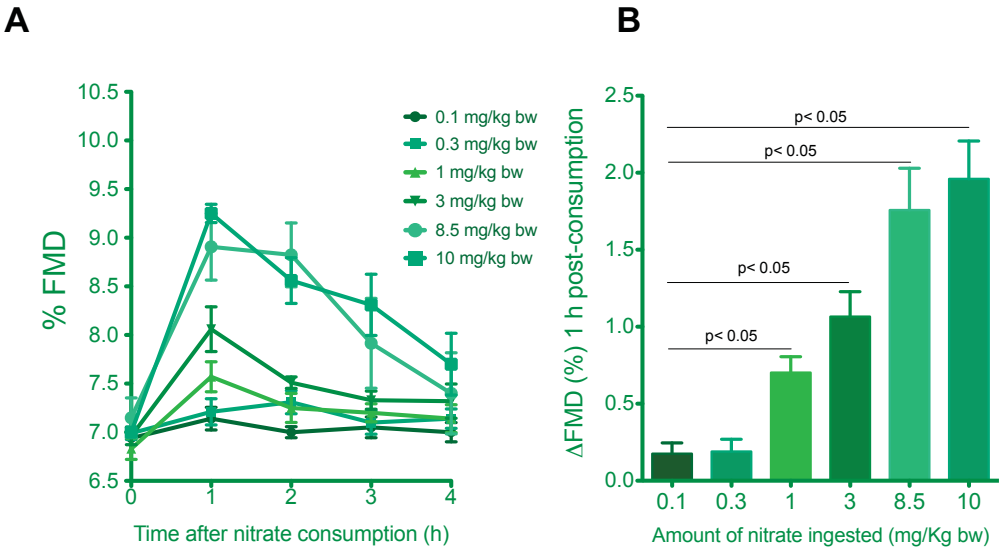



**Figure 11:** Intake dependence of nitrate on FMD in healthy individuals. Results are expressed as the mean ± SEM.

## 3.3 Salivary nitrite and nitrate of the dose-response study of nitrate

Saliva was collected before and at 1, 2, 3, and 4 h post-consumption of nitrate. As shown in Fig. 12, the salivary nitrite and nitrate values were higher for the highest intake of nitrate and lower for the lowest dose of nitrate. Values were gradually decreasing thereafter nearly reaching baseline values at 4 h post-consumption. As expected, salivary nitrate values were around 10 times higher than nitrite values, in the order of 5-15 mM for nitrate and 0,5-1,5 mM for nitrite.

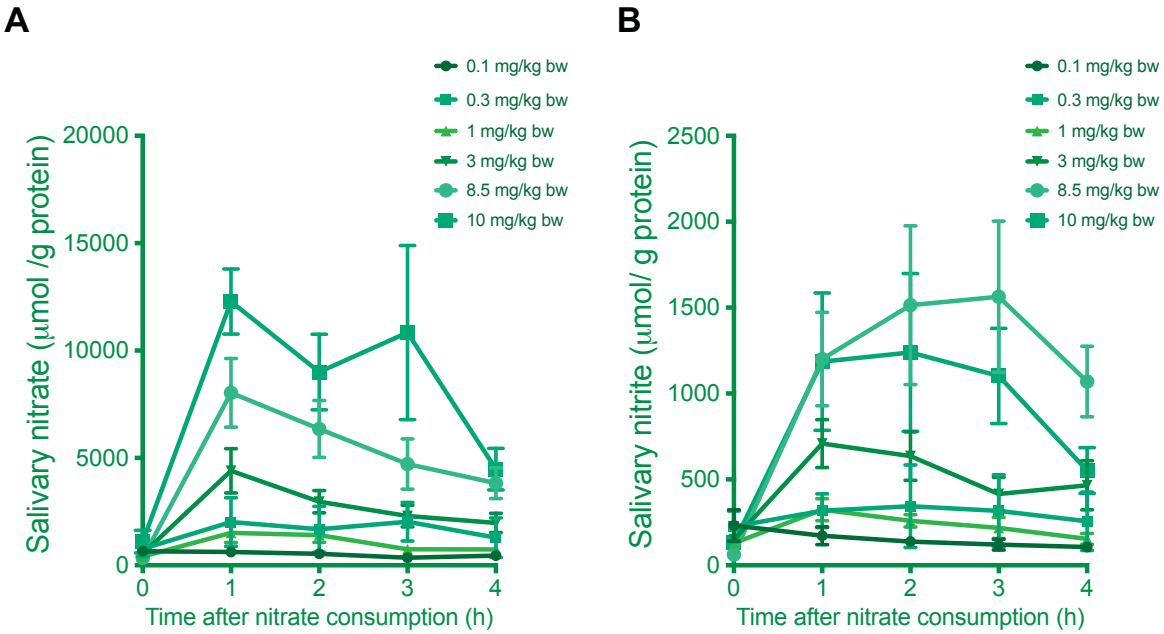



**Figure 12:** A) Saliva nitrate and B) saliva nitrate. Results are expressed as Mean ± SEM.

## 3.4 Additive effects of flavanols and nitrate on endothelial function

When CF and nitrate were taken sequentially together at the amounts of 2,7 mg/kg bw of CF and 3 mg/kg bw of nitrate (equivalent to 200 mg CF and 225 mg of nitrate per 75 kg bw), an increase in the maximum % FMD response was observed when compared with nitrate or CF consumption individually (1% change in FMD, $p<0,05$, Fig. 13A and 13C), however at the higher intake amounts tested (8,5 mg/kg bw nitrate and 10,9 mg/kg bw CF) no difference was observed in the maximal FMD response when nitrate and/or CF were consumed alone or in combination (Fig. 13B and 13C). This shows that the effects of flavanols and nitrate when consumed together are additive at the lower amounts but not at the higher amounts tested suggesting a saturation of effects that my be due to similar mechanisms of action.

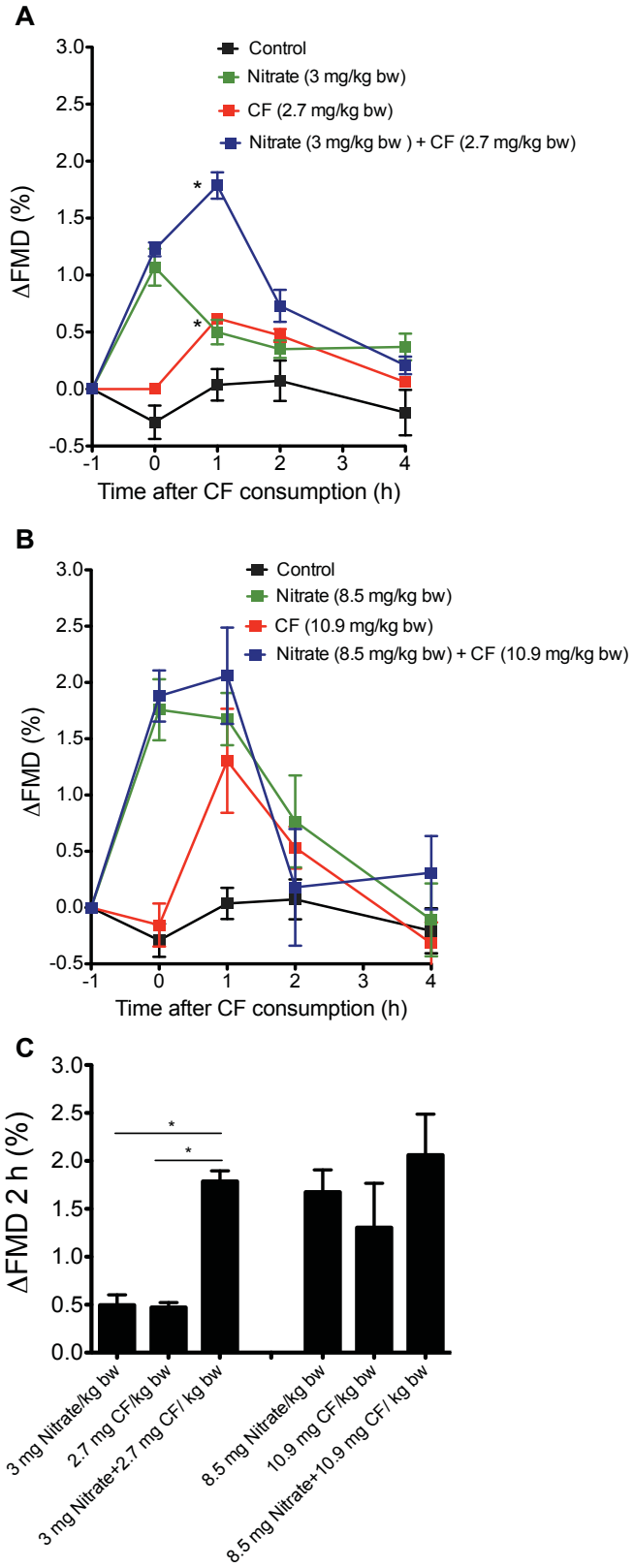



**Figure 13:** Additive effects on FMD between flavanols and nitrate at A) low intake, B) high intake and C) changes in FMD 1 h post-consumption of CF (2 h post-consumption of nitrate) in healthy individuals. Results are expressed as Mean ± SEM. (*p<0.05 significant differences between treatments)

## 3.5 The bioavailability of nitrate is affected by flavanol consumption

Flavanols and nitrate taken together led to a lower plasma nitrite and nitrate level, in comparison when nitrate was taken alone (Fig. 14). The plasma nitrite levels when flavanols were taken alone were higher than control and plasma nitrate levels. Fig. 14A and 14D present the plasma nitrite levels with the Plasma AUC between 0 – 5h. The plasma nitrate levels are depicted in Fig. 14B and 14C.

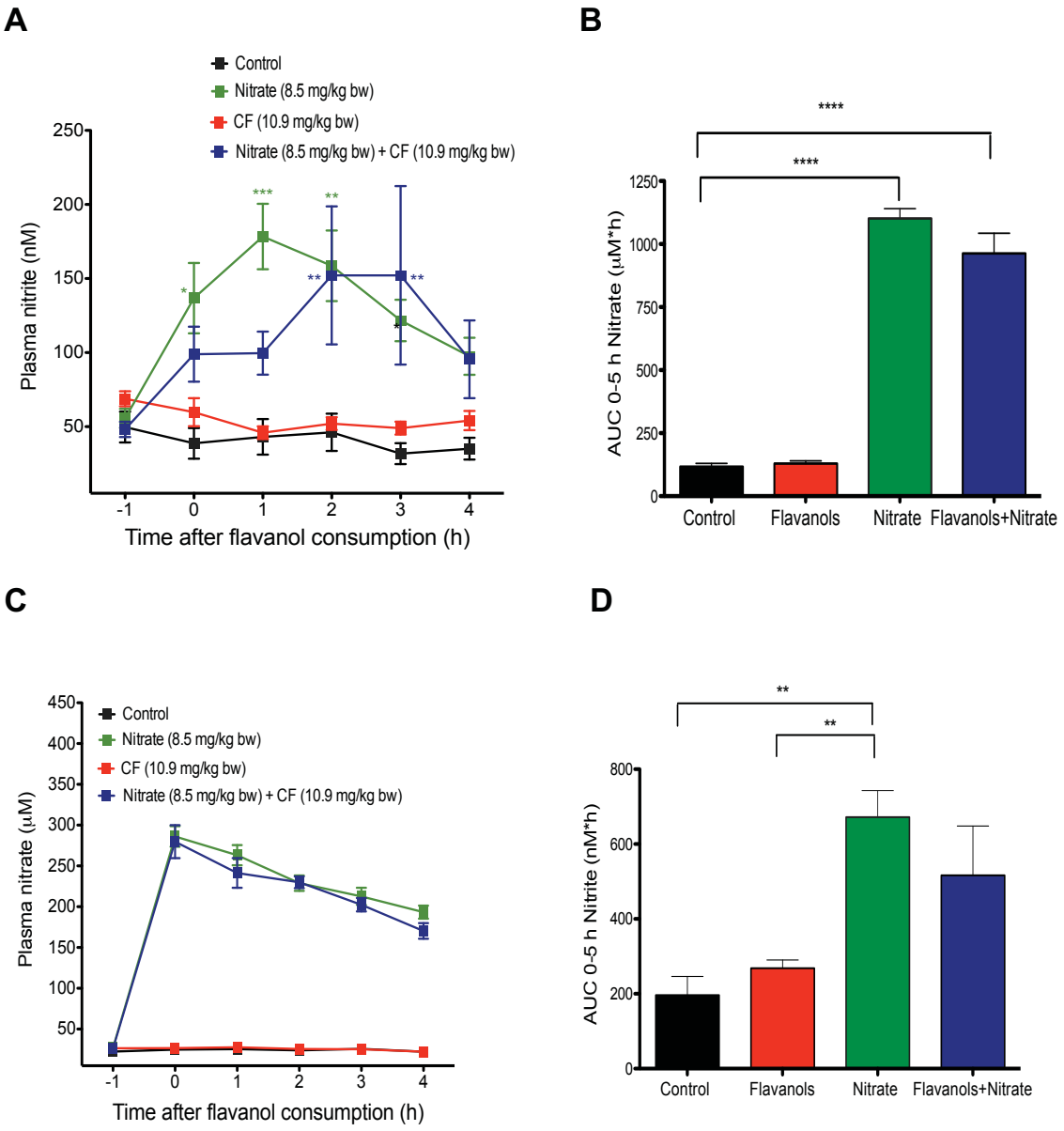



**Figure 14:** Interactions between CF and nitrate in high doses: A) plasma nitrite, B) plasma AUC 0-5h nitrate, C) plasma nitrate and D) plasma AUC 0 – 5h nitrite. Results are expressed as Mean ± SEM.

## 3.6 Flavanols in combination with nitrate decrease plasma nitrite levels and increase NO formation in the stomach

When flavanols were consumed together with nitrate, plasma levels of nitrite were significantly lower than when nitrate was consumed alone (Fig. 15B). However, plasma and urinary nitrate concentrations were unchanged (Fig. 15A and 15C). In addition, NO in expelled air from the stomach after consumption of nitrate and flavanols was higher than when flavanols or nitrate were consumed alone (Fig. 15D). CF attenuated the increase in plasma nitrite after nitrate intake and enhanced nitrate-related gastric NO formation.

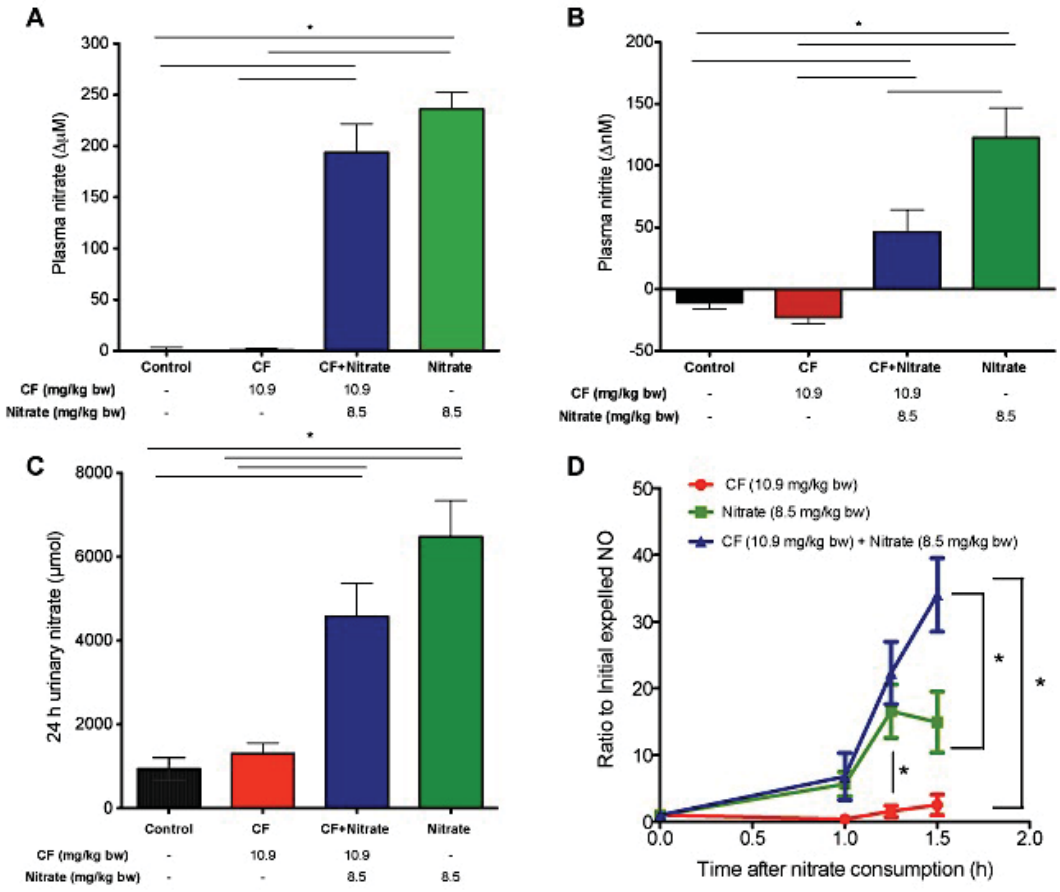



**Figure 15:** Interactions between CF and nitrate: A) plasma nitrate, B) plasma nitrite, C) 24 h urinary levels, D) intragastric expelled NO, after consumption of a control, CF, nitrate or a combination of CF and nitrate drink in healthy individuals. Results are expressed as Mean ± SEM.

## 3.7 The bioavailability of CF is not affected by nitrate consumption

Total plasma and urinary levels of CF metabolites were not significantly different when CF was taken alone or together with nitrate (Fig. 16). Plasma AUC between 0 – 5 hours were not as different as the urinary excretion and plasma flavanol levels. The individual levels of non-methylated (-)-epicatechin, 3'-O-methylated (-)-epicatechin, and 4'-O-methylated (-)-epicatechin metabolites were not significantly different between treatments ($p < 0,05$). For example, at 2 hour post consumption, non-methylated (-)-epicatechin, 3'-O-methylated (-)-epicatechin, and 4'-O-methylated (-)-epicatechin metabolite levels were 1,317 ± 177 nM, 276 ± 52 nM, and 120 ± 23 nM after consumption of CF alone 1,150 ± 271 nM, 213 ± 66 nM, and 131 ± 19 nM after consumption of CF + nitrate, respectively.

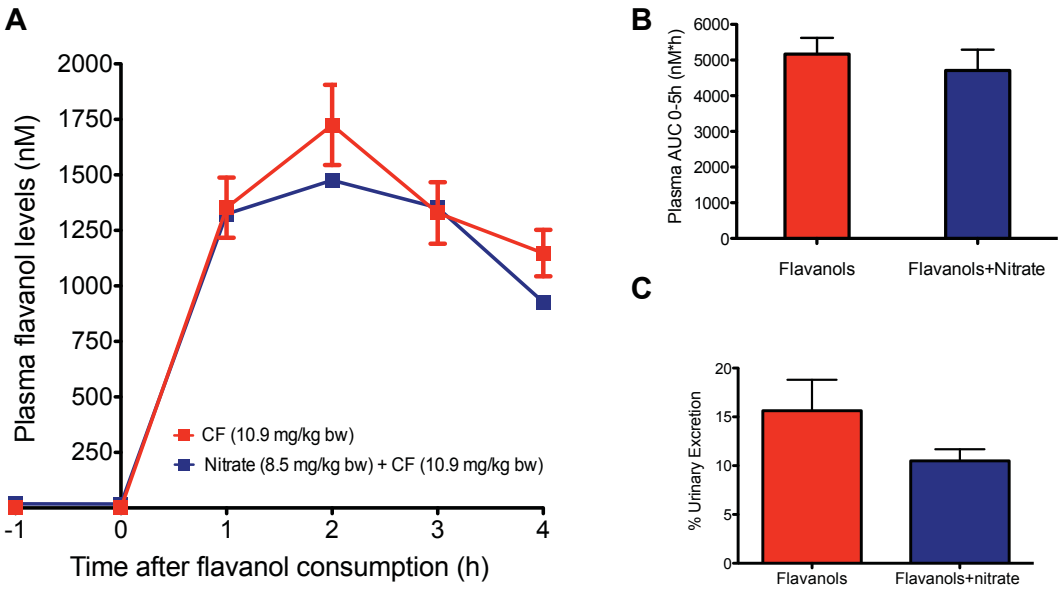



**Figure 16:** A) Plasma, B) Plasma AUC 0-5h (nM*h) and C) 24 h urinary levels of CF metabolites with and without concomitant nitrate consumption. Results are expressed as Mean ± SEM.

## 3.8 Blood pressure is not affected by the consumption of nitrate and CF

The mean SBP and DBP before and at 1, 2, 3, and 4h after ingestion the different doses of nitrate (0,1-10 mg/kg bw) and CF (1,4-10,9 mg/kg bw) are presented in Figures 17 and 18, respectively. Systolic and diastolic BP were not affected by the acute consumption of the test drinks. Figures 19 and 20 show

the systolic and diastolic BP values for nitrate (8,5 mg/kg bw / 3,0 mg/kg bw) and CF (10,9 mg/Kg bw / 2,7 mg/kg bw) in high and low doses, respectively. There was no effect on systolic or diastolic BP by any of the treatments.

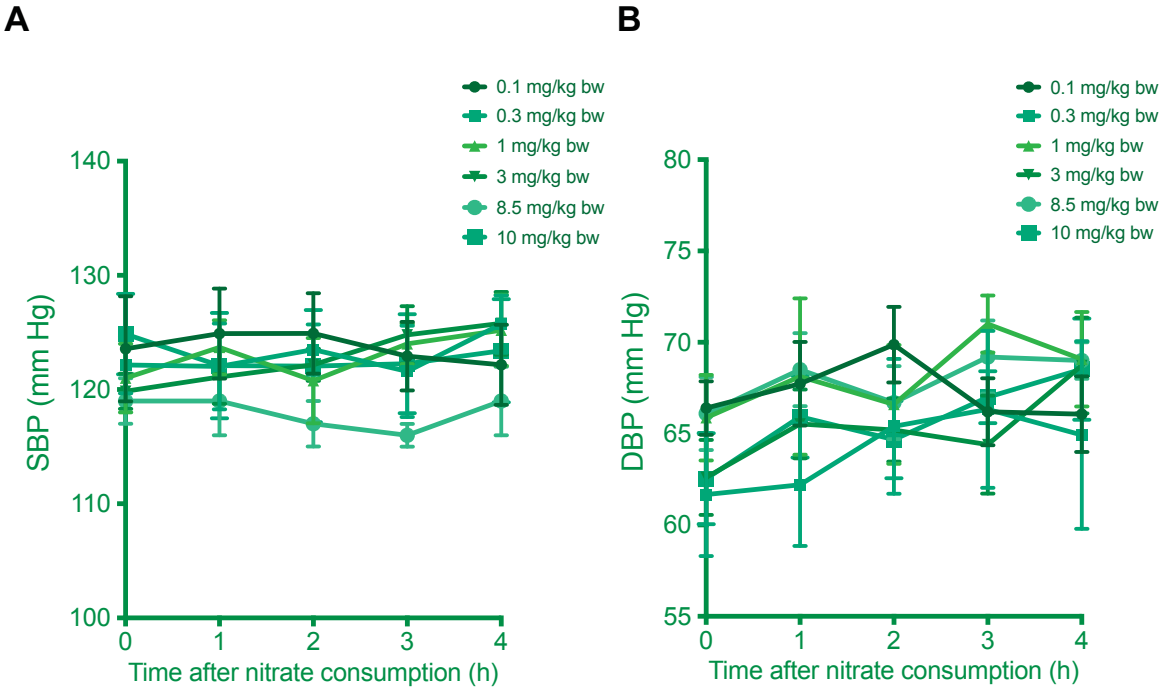



**Figure 17:** A) SBP, B) DBP of the dose-response study of nitrate. Results are expressed as Mean ± SEM.

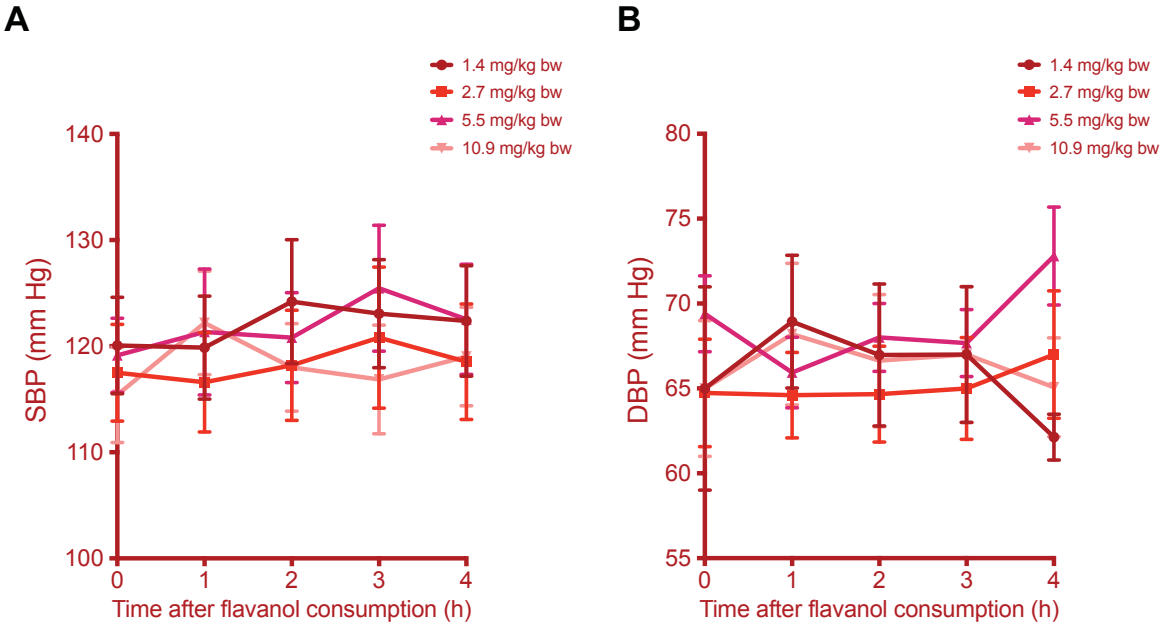



**Figure 18:** A) SBP and B) DBP of the dose-response study of CF. Results are expressed as Mean ± SEM.

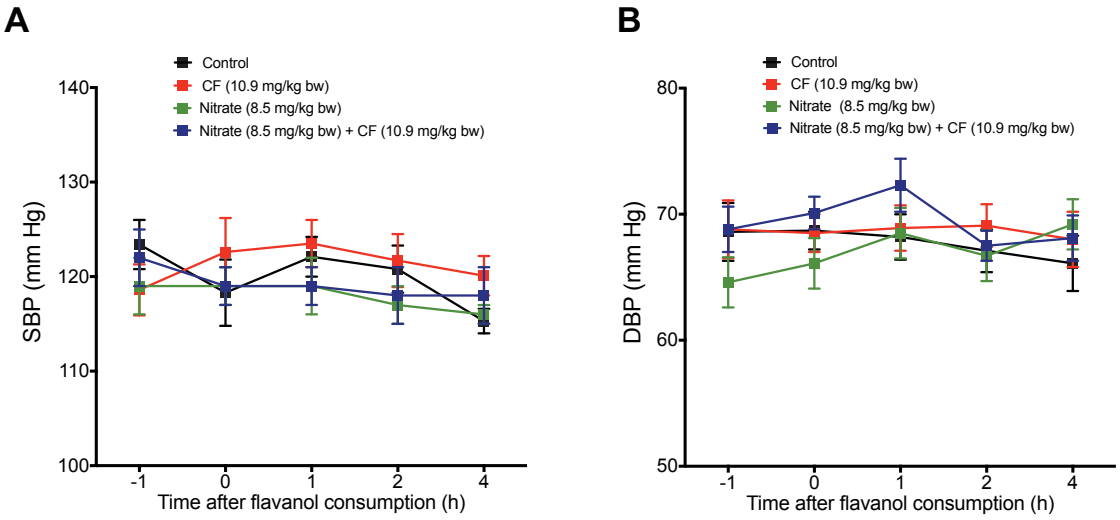



**Figure 19:** A) SBP and B) DBP of nitrate (8,5 mg/Kg bw) and CF (10,9 mg/Kg bw) in high doses. Results are expressed as Mean ± SEM.

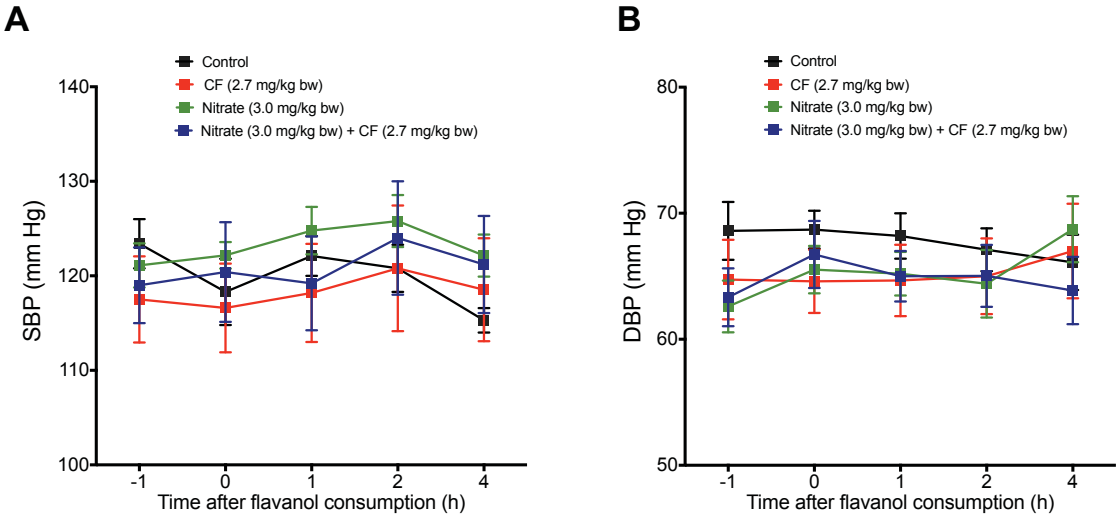



**Figure 20:** A) SBP and B) DBP of nitrate (3,0 mg/Kg bw) and CF (2,7 mg/Kg bw) in low doses. Results are expressed as Mean ± SEM.

# 4 Discussion

The aim of this thesis was to investigate whether interactions between CF and nitrate exist, focusing on the absorption, metabolism, excretion, and efficacy to increase endothelial function. The hypothesis was that CF and nitrate interact with each other and improve endothelial function in a synergistic way, when ingested together. In order to investigate this, several randomized controlled trials were conducted, with the main outcome being endothelial function using FMD. Plasma and urine were also collected to investigate the absorption, metabolism and excretion of flavanols and nitrate.

The main results of the presented dissertation can be summarized as follows:

1. Flavanols improved FMD in an intake-dependent manner, with improvements in FMD of 0,3 – 2,5% at 1 h after consumption of 1,4 – 10,9 mg CF.
2. Nitrate improved FMD in an intake-dependent manner, with improvements in FMD of 0,2 – 2,0% at 1 h after consumption when 0,1 - 10 mg nitrate.
3. When consumed together, the effects of flavanols and nitrate on endothelial function were additive at low amounts, but not at higher amounts and saturated effects.
4. Consumption of CF in combination with nitrate decreased plasma nitrite levels and increased NO formation in the stomach, when compared with CF and nitrate taken individually.
5. The absorption, metabolism and excretion of CF was not affected by nitrate consumption.

In the next sections, the methodological limitations of the present work will be first discussed (4.1.), followed by a discussion on the main results obtained here in the context of the current knowledge in the field (4.2 & 4.3). Then, the potential mechanisms of the interactions between CF and nitrate will be discussed, together with the relevance of the findings in the context of clinical

practice (4.4.). Finally, open gaps and recommendations for future work and conclusions will be outlined (4.5).

# 4.1 Methodological limitations

## 4.1.1 Assessment of endothelial function using FMD

In the present work endothelial function was measured non-invasively using high resolution ultrasound of the brachial artery, with a technique called FMD, which is considered the non-invasive gold standard for measuring endothelial function (Deanfield et al. 2007). For this technique, a blood-pressure tourniquet is used to induce ischaemia in the forearm. This leads to vasodilation of the resistance vessels and consecutively increases blood flow in the afferent conductance vessel after release of the cuff. It is believed that blood flow-induced increases in shear stress endothelial cells of arterial vessel walls leads to an influx of calcium ions which cause an increase in activity of eNOS by phosphorylation (Busse et al. 1993, Corson et al. 1996, Fleming and Busse 2003). This in turn would lead to an increase of NO synthesis and thereby dilation of the brachial artery (Corson et al. 1996, Corretti et al. 2002), which can be measured non-invasively by ultrasound. Investigations show that the FMD measurements of peripheral conductance vessels are NO dependent, because the intra-arterial injection of NG-monomethyl L-arginine, a nitric oxidase inhibitor, could completely suppress the FMD response (Joannides et al. 1995).

Epidemiological studies have demonstrated that all previously known CVD risk factors including hypercholesterolemia, hypertension, smoking (Celermajer et al. 1993), diabetes mellitus (Kawano et al. 1999), male gender (Celermajer et al. 1994), positive family history (Clarkson et al. 1997) and old age (Celermajer et al. 1994, Corretti et al. 1995, Grundy et al. 1998) are accompanied with a reduction of FMD. Furthermore it is known that the circadian rhythm affects FMD values with a maximum in the late-afternoon (Etsuda et al. 1999). The fluctuation of female sex hormones estrogen and progesterone during the menstrual cycle has also been shown to affect FMD (Hashimoto et al. 1995). Moreover, diet and lifestyle factors are known to modulate endothelial function. For example, acute hyperglycaemia (Kawano et al. 1999) and a fat-rich diet (Vogel

et al. 1997) led to a significant decrease in endothelium-dependent dilation. Acute mental stress (Ghiadoni et al. 2000) and smoking a single cigarette (Lekakis et al. 1997) have also a negative acute effect on the FMD. To keep these influencing factors constant, in this work FMD measurements were assessed always at the same time of the day, between 8:00 (first measurement) and 13:00 (last measurement). Subjects underwent the measurements with an empty stomach, after fasting for 10 hours. All measurements were obtained in the same air conditioned and quiet room after lying relaxed for 10 min, to make sure all participants were investigated under the same conditions. All participants were non-smokers, young and only male, so the influence of female menstrual cycle could be avoided.

Besides biological influencing factors, there are other possible sources for methodological variation:

1. Size of the ischaemic area
2. Time of ischaemia
3. Measuring points of the brachial artery diameter
4. Timepoint of the measurement after release of cuff
5. Timepoint of the measurement within one heart cycle
6. Technical equipment
7. Investigator

In this work a blood-pressure tourniquet located at the proximal forearm distal to the antecubital fossa was inflated to 250 mmHg for 5 min and induced in the area of the brachial artery an ischaemia. The ultrasound scans were made 20, 40, 60 and 80 sec after release of the cuff. Comparative investigations indicated that the position of the blood-pressure tourniquet and size of the ischaemic area play an important role for the FMD measurements. While some studies demonstrated that blood-pressure tourniquet located at the upper arm proximal to the antecubital fossa (Berry et al. 2000, Vogel et al. 2000) leads to higher FMD values, other studies with occlusion of the forearm achieved higher values of FMD (Corretti et al. 1995). Currently there is no consensus on the best and preferred method performing the FMD measurements (Berry et al. 2000, Corretti et al. 2002). In this work the blood pressure tourniquet was located at the proximal forearm distal to the antecubital fossa to avoid influences like direct occlusion and ischaemia of the investigated area of the brachial artery (1.). The results of

the FMD measurements depend on the duration of the ischaemia. An ischaemia of 4.5 min leads to maximal dilation, which means that longer ischaemia times can't increase FMD significantly (Corretti et al. 2002). Therefore, 5 min is a good and tolerable time (2.). Most working groups performed the measurements of the brachial artery diameter from the anterior to posterior m-line at fixed anatomical markers at end-diastole (3.). Maximal dilation of the brachial artery was measured 60 sec after release of cuff. More recent investigations indicate that the maximal dilation, achieved for both children and adults, may differ considerably from each other. Because of this, in the present work measurements were performed as well at 40 and 80 sec after releasing of cuff, so the maximal dilation could be achieved more precisely. Nevertheless it cannot be excluded, that in some cases measured FMD underestimates the real value of the maximal endothelial dilation (4.). All measurements were carried out under continuous pulse and electrocardiographic control at four cardiac cycles and average to one mean value, because the diameter of the brachial artery is dependent on pulsatile changes of the cardiac cycle. In summary, the investigation protocol complied with the recommended standards of the guidelines (5.) (Corretti et al. 2002). The main sources of variability in the ultrasound scans are dependent on investigators experience. For example, technical equipment and investigators experience in ultrasound are important determinants for having precise sonographic measurements. Technical requirements for non-invasive measurements of endothelial function consisted of linear transducers with a frequency of about 7 to 12 MHz, which were the current standard at the time of the investigation (Corretti et al. 2002). In the present work a transducer with a frequency of 15 MHz was used. The physical resolution for a 15 MHz transducer and an assumed mean sound velocity of 1500 m/s in tissue book amounts to 0,1 mm, whereas a transducer with a frequency of about 7,5 MHz achieves 0,2 mm. That means, the resolution could be doubled by using higher frequencies (6.). Only well-trained and experienced investigators are able to see differences of 0,1 mm in diameter (Corretti et al. 2002). Differences in diameter of endothelium dependent and independent dilation of an artery with an initial diameter of 3,8 mm are between 0,1 - 0,6 mm (2,7% - 16,7%). Sorensen et al. assessed the accuracy of detecting small changes in vessel diameter by using phantom arteries. These phantom arteries were constructed by moulding agar around

metal cylinders of known diameter. This study has shown that differences in vessel diameters of 0,1 - 0,2 mm were correctly identified, which makes the values obtained very reliable and precise (Sorensen et al. 1995). In this work, prior to conduct the studies the operator measured the diameter of the brachial artery on various days at various times on different people to make sure that the coefficient of variation was below 1%. Besides the accuracy of the ultrasound and measurement itself, the variability of the diameters of the vessels also depends on the data analysis. There are differences, which are occurred by the evaluator (within-observer variability), differences in the analysis which is performed by two different persons (between-observer-difference) and investigations operated on different days (day-to-day-difference). The day-to-day-variability also depends on the biological variation of the vascular function and the variability through investigators. Data analyses were performed by an automated computer based analysis system of the vessel diameter, the Brachial Analyzer (Medical Imaging Applications, Iowa City, IO), which avoids and keeps sources of errors at a minimum and saves time in contrast to manual readers (Preik et al. 2000). Within a user-defined region of interest, the Brachial Analyzer system is able to determine the artery diameter by averaging a large number of local vessel diameters (Preik et al. 2000). All the measurements were performed and analysed by the same operator using the same equipment to avoid inter-observer variability (7.).

### 4.1.2 Quantitative analysis of circulating NO and flavanol metabolites

Plasma nitrite and nitrate were analyzed by methods previously described (Govoni et al. 2008). In this work NO was not determined directly in blood. Instead of that NO metabolites nitrate and nitrite were quantified, which is a limitation of the work. However, currently no accurate method exist to measure NO in blood (Bryan and Grisham 2007). Because of the short half-life in whole blood, direct quantification of NO is difficult (Lauer et al. 2001). Nitrate and nitrite measurements were determined with the chemiluminescence analyzer and with the eno-analyzer, which is an HPLC based method which are both considered good, accurate and reliable methods for the analysis of nitrate and nitrite in biological samples (Bryan and Grisham 2007). Blood samples were centrifugated

immediately and stored at -80°C after blood collecting, to avoid the degradation of NO. Based on current literature, plasma values in the micromolar range for nitrate and nanomolar range for nitrite were expected (Rhodes et al. 1995, Kelm 1999, Lauer et al. 2001), which fitted very well with our results (20-280 µmol/l for nitrate and 30-180 nM for nitrite). The values obtained in saliva and urine also agreed well with previously published papers (Bahra et al. 2012, Bondonno et al. 2015, Bondonno et al. 2015).

In plasma, CF metabolites, are found as glucuronide, sulfate and/or methylated conjugates. As these metabolites are not commercially available to use as analytical standards for quantification, enzymatic treatment with glucuronidase and sulfatase (to convert the sulfated and glucuronidated forms of flavonoids to their aglycone form) is commonly used for quantification (Rodriguez-Mateos et al. 2012). Metabolites of (−)-epicatechin were reduced into non-methylated (−)-epicatechin, 3′-*O*-methylated (−)-epicatechin, and 4′-*O*-methylated (−)-epicatechin. Samples were analyzed by HPLC with fluorescence detection using authentic standards. Although this is considered a good method of analysis, there are some limitations, such as incomplete hydrolysis of the conjugates, specially the sulfates, which can be resistant to hydrolysis, therefore flavanol bioavailability can be underestimated by this method (Saha et al. 2012). However when the right conditions and type of enzyme are used, as in this work, this method is stil pretty accurate, with percentages of hydrolysis close to 100% (Ottaviani et al. 2012).

## 4.2 Influence of flavanols and nitrate on endothelial function

In this work, whether the effects of CF and nitrate on endothelial function are dose-dependent were investigated. Both CF and nitrate when consumed separately induced a similar intake-dependent increase in FMD. FMD measurements were performed before and 1, 2, 3, and 4h after ingestion of different doses CF. Maximal values of FMD were achieved at 1h post consumption of the test drinks, and then gradually decreased to reach baseline values after 4h. Saliva nitrate and nitrite values also showed an intake-dependent increase, which could also explain intake-dependent increases in FMD. It is clear that the reduc-

tion of nitrate to nitrite by commensal facultative bacteria on the tongue is essential for these vascular benefits. An interruption of the enterosalivary conversion of nitrate to nitrite prevents the rise in plasma nitrite and inhibits the decrease in BP following increased nitrate intake (Webb et al. 2008). Some investigations on the intake-dependency of CF have been conducted before, however the study populations consisted of healthy older adults, diabetics and smokers (Heiss et al. 2005, Heiss et al. 2007, Balzer et al. 2008, Monahan et al. 2011), and not young healthy men as in the present study. A single ingestion of CF was dose dependently associated with increases in FMD. In these studies, the FMD response increased with increased CF intake but the magnitude of the changes were different, with FMD increases ranging from 1 to 7% after acute intakes of 100 to 1000 mg of CF. Diabetics seem to have the lowest improvements (Balzer et al. 2008) and smokers the highest (Heiss et al. 2005, Heiss et al. 2007). Our results are similar to the ones obtained in healthy older adults (Monahan et al. 2011), with changes between 0,3 to 2,5 % from 1,4 mg to 10,9 mg/kg bw CF tested. Monahan et al. used experimental test drinks with cocoa contents of 0 (placebo), 2, 5, 13 or 26 g cocoa containing 9,3, 25,8, 66,6 and 146 mg of flavanols. The largest increase in FMD was observed after the highest quantity of cocoa, and also total plasma epicatechin correlated with increases in the FMD, which was measured 1 and 2 h post-ingestion (Monahan et al. 2011).

Few studies have investigated the effects of nitrate on FMD after acute or chronic intake (Bahra et al. 2012, Bondonno et al. 2012, Heiss et al. 2012, Gilchrist et al. 2013, Rammos et al. 2013) with conflicting results. Bahra et al. and Gilchrist et al. did not find any changes in the FMD response after either acute 8 mmol of potassium nitrate (Bahra et al. 2012) or chronic nitrate-rich BJ containing 8 mmol of nitrate (Gilchrist et al. 2013) in healthy volunteers or type 2 diabetics. In contrast, three studies found improvements in FMD after acute and chronic consumption in healthy young and elderly individuals (Bondonno et al. 2012, Heiss et al. 2012, Rammos et al. 2013). Here we show for the first time an intake-dependent increase in FMD after nitrate supplementation. The effects of inorganic nitrate on FMD, increased with higher intake of nitrate, from 0,1 mg to 10 mg. Importantly, even at intake amounts as low as 1 mg/kg bw (75

mg of nitrate), nitrate produced a significant increase in FMD (Fig. 11A and B). Since this amount of nitrate is similar to what is found in a small serving of a green leafy vegetable such as lettuce, spinach or beetroot and is also within the range of the average daily intake of nitrate in Europe and the US (Mensinga et al. 2003), the findings are very relevant as they are easily achieved with a normal diet.

In previous work Bondonno et al. investigated the acute effects of the ingestion of apples and spinach separately and together as sources of flavonoids and nitrate (184 mg of quercetin, 180 mg of (−)-epicatechin, and 182 mg nitrate), respectively. Both flavonoid-rich apple and nitrate-rich spinach resulted in augmented NO status separately (Bondonno et al. 2012) but they did not have an additive effect on endothelial function after acute consumption. In contrast to our own observations, no significant decreases in plasma nitrite were shown in the combination versus isolated intervention treatments. An important difference when compared to the present study is that the participants and investigators were not blinded during the intervention and that both spinach and apples were given at the same time, whereas the subjects of the present study received the nitrate treatment 1h before the flavanol test drink. The reasoning behind nitrate dosing 1h before the flavanols was to allow enough time for the nitrate to enter the entero-salivary circulation thereby maximizing salivary nitrite levels at the time of flavanol ingestion. In addition, apples have a different phenolic composition than CF, which may explain the different results obtained. If we assume that (-)-epicatechin in cocoa was responsible of the effects seen in FMD, the amounts given in the present study were lower in epicatechin and higher in nitrate than in Bondonno et al. Spinach has also other bioactives and components beside nitrate, which can also result in different effects on vascular function.

## 4.3 Influence of flavanols and nitrate on BP

BP measurements was recorded before and 1, 2, 3 and 4 hours after ingestion of the test drink as the mean of three measurements using an Omron MX2 automatic digital upper arm BP monitor according to established guidelines (Williams et al. 2004). All BP measurements were performed in seated position to make sure that all participants were measured under same conditions. In this work, SBP and DBP were not affected by the acute ingestion of nitrate and CF separately and when they were consumed together in low and high amounts. The results on CF are in agreement with a meta-analysis of 42 randomized controlled trials that indicated that the acute intake of CF did not lead to a decrease in SBP and DBP, whereas significant reductions in DBP were observed after chronic intake of CF (Hooper et al. 2012). It has been shown that short-term trials of 2-18 weeks with daily ingestion of CF leads to a decrease in BP (Ried et al. 2012) not only in hypertensive patients, but also in healthy people (Grassi et al. 2005).

We did not see any changes in BP after acute intake of nitrate. Most of the authors demonstrated decreases on BP in particular SBP, without changes in DBP after acute (Kapil et al. 2010, Bahra et al. 2012, Bondonno et al. 2012, Coles and Clifton 2012, Liu et al. 2013) and chronic (Vanhatalo et al. 2010, Rammos et al. 2014, Ashworth et al. 2015) ingestion of dietary nitrate. One reason why several studies have seen a decrease in BP could be, that the volunteers start with greater than 120 mmHg SBP and 70 mmHg DBP (Kapil et al. 2010, Coles and Clifton 2012, Hobbs et al. 2013). In this work volunteers had a baseline SBP of 119 mmHg and DBP of 68 mmHg. There are several studies with BP lowering effects of acute and chronic intake of dietary nitrate in hypertensives (Kapil et al. 2015) or patients with cardiovascular risk factors (Kenjale et al. 2011, Rammos et al. 2014). But this study has investigated healthy and active volunteers without diseases or risk factors, which could explain the results in this work. In agreement with our findings several studies also showed that acute (Lidder et al. 2011) or chronic (Bondonno et al. 2014, Bondonno et al. 2015) ingestion of dietary nitrate did not lower BP. The mechanisms of blood pressure-lowering effects of nitrate have not been fully explained.

In the study of Bondonno et al., a significant reduction in SBP was observed when spinach and apples were given separately, but not when they were given simultaneously (Bondonno et al. 2012). As previously mentioned, other bioactives present in spinach and apples different than flavanols and nitrate may be responsible for the effects, with different mechanisms.

## 4.4 Potential mechanisms of the interaction between nitrate and flavanols

In order to study the interactions between CF and nitrate, FMD was measured at baseline and 1 hour after nitrate intake or water. The third FMD measurement was performed after ingestion of CF drink or a micro- and macronutrient matched CF-free drink. The effects on endothelial function were additive at lower intake levels (3 mg nitrate & 2,7 mg CF /kg bw) whereas CF (10,9 mg/kg bw) did not further increase FMD after high nitrate (8.5 mg/kg bw) intake (Fig. 13). The results also show here that CF is able to amplify the increase in NO generated in the stomach after consumption of nitrate (Figure 15D). This increase may be correlated to the decrease in plasma nitrite observed when CF and nitrate were consumed together (Fig. 15B), which indicates that the bioavailability of nitrate is affected by CF consumption. Less gastric nitrite may have escaped conversion to NO leading to lower systemic nitrite levels. The decrease in plasma nitrite could also be explained by redistribution to other nitrogen species when it was combined with CF as CF and other polyphenols rather enhance the generation of NO and other bioactive nitrogen species including S-nitrosothiols from nitrite or a greater systemic consumption of nitrite. Another potential possibility for the observed plasma nitrite decrease could be the formation of nitroso-flavanols from gastric interaction of NO and flavanols. However nitroso-flavanols were not detected in plasma and might not be absorbed according to previous findings, that is why more NO is formed in the stomach due to the presence of flavanols, hence the decrease in plasma nitrite. In a pre-clinical rat model nitroso-flavanols were not absorbed from the jejunum, in contrast to flavanols (Lee et al. 2006). In this work no difference was found in the plasma concentration of flavanols 4 hours after ingestion, therefore this latter is less likely. Yet, another explanation could be that CF scavenges oxygen radicals such as superoxide thereby increasing the bioavailability of the NO produced

from nitrate. But it must be added that total plasma and urinary levels of CF metabolites were not significantly different when CF were taken alone or together with nitrate (Fig. 16), which indicates that the bioavailability of CF is not affected by nitrate. Plasma and urinary concentrations were also unchanged, hence it can be said that absorption and excretion do not seem to be influenced of the combined flavanol-nitrate intake (Fig 15C). The non-additive effects of high intake levels on FMD could be due to the fact that the threshold of increase in FMD was reached due to the high doses of nitrate and flavanols used in the study, therefore not possible to assess whether there is an additive effect.

## 4.5 Conclusion

Diet has been recognised as an important factor influencing CVD, and epidemiological evidence has pointed out that the consumption of diets rich in fruit and vegetables is associated with a lower risk of CVD (Joshipura et al. 2001, Dauchet et al. 2006, He et al. 2007). For this reason, flavanols and inorganic nitrate, as potential bioactives present in many fruits and vegetables may be at least partially responsible for such positive effects. This study has investigated the interactions between flavanols and nitrate and their additive effects on vascular function. There are several reasons for performing intake-response experiments with CF and nitrate and to investigate their interactions. CF and nitrate are common in our everyday diet and both have been considered to possess beneficial cardiovascular effects. By investigating the intake-response effects on FMD, an established marker of vascular function, it allows for a better discussion on their beneficial role in our normal diet. Moreover, there are mechanistic reasons to study interactions between CF and nitrate. Not only do they separately affect NO bioavailability by different mechanisms but polyphenols also have the capacity to enhance the reduction of nitrite to NO (Gago et al. 2007, Rocha et al. 2009, Rocha et al. 2010), as also shown in this study with the marked increase in stomach NO by the combination of CF and nitrate. Both CF and inorganic nitrate, in amounts easily achievable with a normal diet, can dose-dependently improve vascular function in healthy subjects. An additive effect on FMD was evident after combined intake of low dietary amounts of these bioactives suggesting that, alone or combined, flavanol- and nitrate-rich

foods may interact with the uptake and bioactivation of nitrate and nitrite and exert beneficial cardiovascular effects.

The average daily intake of nitrate in Europe has been estimated to be 50-140 mg/day (Mensinga et al. 2003) and for CF 105 mg/day (Vogiatzoglou et al. 2014). The low flavanol and nitrate intake combination tested here is easy to achieve from a normal diet, corresponding to an intake of 100 g lettuce or 30 g of spinach for nitrate (Hord et al. 2009) and to 10 g of high-flavanol dark chocolate or 2,5 g of high-flavanol cocoa powder (EFSA 2012). It is known that vegetables are believed to be protective against CVD and in several studies green leafy vegetables stand out as particularly protective against type 2 diabetes and stroke (Joshipura et al. 1999, Bazzano et al. 2008, Joshipura et al. 2009, Carter et al. 2010). It is tempting to speculate that nitrate participates in promoting this protective effect, since this subgroup of vegetables is particularly high in nitrate. Further studies are clearly needed to prove whether or not this assumption is correct.

Whether consumed alone or together, both CF and nitrate have the capacity to increase endothelial function. Significant interactions does not appear to exist on the levels of absorption or excretion between both bioactives. Local interactions between nitrite and CF in the acidic gastric environment warrant further study. Further experiments should include lower doses of flavanol-nitrate and perform dose dependent curves of CF and nitrate (separately) at lower and higher doses than the ones used to determine the maximal FMD increase. A long-term study should be conducted to demonstrate that the effects are persistant after daily consumption. The exact mechanism of the interaction between CF and nitrate need to be investigated further.

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