Cocoa flavanols, exercise and the brain

Lieselot Decroix

Doctoral dissertation submitted in fulfilment of the requirements for the degree of Doctor in Rehabilitation Sciences and Physiotherapy - 2018

Thèse présentée pour obtenir le grade de docteur de l'Université de Lille - Discipline : Sciences et Techniques des Activités Physiques et Sportives - Option Physiologie

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Abbreviations

ΔHbO_2	change in oxyhemoglobin
ΔHb_{tot}	change in total hemoglobin
ΔHHb	change in deoxyhemoglobin
ABTS	2,2'-azinobis(3-ethylbenzothiazoline 6-sulphonate)
ACC	anterior cingulate cortex
ACE	angiotensin-converting enzyme
ADMA	asymmetric dimethyl-arginine
AMPK	AMP-activated protein kinase
Arg	arginine
ASL	arterial spin labelling
BA	brodmann area
BBB	blood-brain barrier
BCAA	branched chained amino acids
BDNF	brain-derived neurotrophic factor
BH_4	tetrahydrobiopterin
BH_2	dihydrobiopterin
BMI	body mass index
BOLD	blood oxygenation level dependent
BP	blood pressure
Ca^{2+}	calcium
CaO_2	arterial concentration of O_2
CBF	cerebral blood flow
CBV	cerebral blood volume
CF	cocoa flavanols
Citr	citrulline
CK	creatine kinase
COX-2	cyclo-oxygenase 2
$CMRO_2$	cerebral metabolic rate of oxygen
CNS	central nervous system
CT	cognitive task
CVD	cardiovascular disease
EEG	electroencephalography
EFSA	European Food Safety Authority
eNOS	endothelial NO synthase
EPR	electron paramagnetic resonance
ET-1	endothelin-1
ERP	event-related potentials
FFA	free fatty acids
FMD	flow-mediated dilation
F_IO_2	fraction of inspired oxygen
fMRI	functional Magnetic Resonance Imaging
fNIRS	functional Near Infrared Spectroscopy
FOXO1	forkhead box protein O1
GC	guanylate cyclase
GLUT	glucose transporter
GPX	glutathione peroxidase
GRX	glutaredoxin
GSH	glutathione
GSSG	glutathione disulfide
H	hypoxia
H_2O_2	hydrogen peroxide
HBA1c	haemoglobin-A1c
HDL	high density lipoprotein
HPLC	high pressure liquid chromatography
HR	heart rate

ICA	independent component analysis
IL	interleukin
iNOS	inducible NO synthase
LDL	low density lipoprotein
LKB1	liver kinase B1
L-NMMA	L-NG-monomethyl Arginine acetate
MAP	mean arterial pressure
MDA	malondialdehyde
Min	minutes
MPV	mean platelet volume
N	normoxia
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal NO synthase
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NVC	neurovascular coupling
O_2	oxygen
$O_2^{\bullet -}$	superoxide
$^{\bullet}OH$	hydroxyl radical
$^{\bullet}ONOO-$	peroxynitrite
PaO_2	arterial pressure of O_2
PC	procyanidins
PDW	platelet distribution width
PFC	prefrontal cortex
PGC-1α	proliferator-activated receptor-gamma coactivator 1 alpha
PKG	protein Kinase G
PL	placebo
RER	respiraoty exchange ratio
ROS	reactive Oxygen species
RPE	rating of perceived exertion
RPM	rounds per minute
RNS	reactive nitrogen species
RT	Reaction time
SaO_2	arterial saturation of O_2
SD	standard deviation
SDMA	symmetric dimethylarginine
SIRT	sirtuin
sLORETA	standardized low-resolution brain electromagnetic tomography
SOD	superoxide dismutase
SpO_2	arterial O_2 saturation
SS	steady state
TAC	total antioxidant capacity
TBARS	thiobarbituric acid reactive substances
TEAC	trolox equivalent antioxidative capacity
TNF-α	tumor necrosis factor-α
TOI	tissue oxygenation index
TRX	thioredoxin
TSI	tissue saturation index
TT	time trial
TTE	time to exhaustion
UA	uric acid
VEGF	vascular endothelial factor
VO_{2max}	maximal oxygen consumption

Chapter 1. General introduction

1.1 Food supplements

Under normal circumstances, a varied and well-balanced diet is sufficient to provide all necessary nutrients for a healthy life. However, in some cases and/or in some specific populations, it may be advisable to increase the intake of specific nutrients by taking food supplements. A food supplement, also called nutritional or dietary supplement, is defined as a product intended to supplement the normal diet, containing a concentrated source of nutrients or other substances with a physiological effect (EC 2002). Food supplements can be produced as capsules, pastilles, tablets, pills, sachets of powder, ampoules of liquids, drop dispensing bottles (Dwyer et al. 2018). The last decade, the use of food supplements has become tremendously popular; in 1994 only 4000 supplements were on the US market, while 20 years later (in 2014), over 85 000 food supplements were on the US market (Wallace 2015).

According to European legislation, a food supplement should be safe and bear adequate labelling, but there is no high quality scientific evidence required to support the product claim. The type and amount of evidence needed to demonstrate their efficacy remains a hotly debated issue (Dwyer et al. 2018). While *in vitro* and animal studies may show promising results for some supplements, this is not always translated into the same conclusions in human studies. Moreover, large differences in the exact compositions and doses of tested supplements used in diverse studies contribute to equivocal results. Thus, more and better clinical studies examining the efficacy of many food supplements on health outcomes, cognitive and physical performance and the underlying mechanisms are warranted (Dwyer et al. 2018).

Amongst food supplement consumers, athletes form a large subpopulation as they constantly search for ways to improve physical and cognitive performance. The "2016 supplement business report" showed that retail sales of "sports nutritional supplements" accounted for 13.8% or 5.67 billion dollar from the total 41.16 billion dollar total sales for dietary supplements (Nutrition Business Journal 2016). Tscholl et al. reported the use of food supplement in 3887 top-level athletes, and found a total consumption of 6523 supplements (1.7 per athlete). They showed that supplements were used more than twice as much in endurance athletes compared to soccer and multilevel sports (Tscholl et al. 2010).

Athletes might justify the use of supplements as *(i)* prevention or treatment of nutrient deficiencies, especially when requirements for a specific nutrient are elevated due to their exercise program, *(ii)* a more convenient form of nutrients in situations where the required nutrients from normal food intake are not available, or *(iii)* as potential ergogenic aid (Castell et al. 2015). Despite their popularity, there is still relatively little scientific evidence on the beneficial effects of many of these supplements. It is now generally known that some supplements can offer benefits to the elite athlete if used appropriately, but that some may be detrimental for performance and health. Scientists continue to call for an evidence-based approach in prescribing nutritional supplements to athletes in order to protect their health and

prevent them from positive doping tests. Indeed, a significant amount (5-20%) of nutritional supplements contains prohibited substances (Tscholl et al. 2010), and prolonged and/or exaggerated intake of certain supplements (e.g. antioxidants such as vitamin C) is likely to be harmful (Braakhuis and Hopkins 2015). Thus, it is necessary to educate both athletes, coaches and health professionals about the use of supplements, including the associated risks and benefits and to conduct further research to unravel the efficacy of food supplements in sports performance. The urgency and importance of this matter were also recognized by the International Olympic Committee (IOC). In 2018, the IOC consensus statement on "Dietary supplements and the high-performance athlete" was published, presenting the conclusions of the IOC medical and scientific expert group meeting in May 2017. It was the goal of this IOC consensus statement to provide information on the use of supplements and to assist high-performance athletes and their support team in making informed decisions (Maughan et al. 2018).

Nutritional supplements can, or claim to, affect exercise performance in several ways; by improving strength or endurance, exercise efficiency, exercise tolerance or by postponing fatigue and enhancing recovery (Castell et al. 2015). Athletes may also use nutritional supplements to improve focus, attention and clarity of cognition during sports performance (Baker et al. 2014). Thus, nutritional supplements as potential ergogenic aids not only have an impact on muscle, but also on the brain.

1.2 Nutritional supplements and the brain in sports performance (partly based on: Nutritional supplements and the brain. Meeusen and Decroix 2018)

It is clear that nutrition plays an important role in optimal brain functioning. Nutrition provides the proper building blocks for the brain to create and maintain neuronal connections, which is critical for proper cognitive performance. Dietary factors have a broad and positive action on neuronal function and plasticity. Brain function is certainly dependent on adequate nutrition, and short-term variations in the amount and composition of nutrient intake in healthy individuals influence measures of cognitive function (Meeusen 2014).

There is increasing interest in examining the possible effect of nutritional supplements on exercise performance. While sports performance largely depends on physical factors, cognitive functioning also plays a great role, as performance in many sports involves decision making and skill accuracy. Motor control, decision making, coordination, reaction time, and other cognitive tasks are essential during several sports, including team sports. Besides the crucial role of cognitive functioning in exercise performance, the brain is also involved in the development of fatigue. Fatigue does not only occur at the peripheral level, but according to the 'central' fatigue hypothesis, it also involves brain mechanisms (Meeusen and Roelands 2018). While different neurotransmitter systems are involved in the development of central fatigue (Meeusen and Roelands 2018), it can also be elicited by low brain oxygenation, caused by insufficient O_2 delivery to the brain under some experimental conditions (Amann and Calbet 2008).

From the above, it seems clear that "the brain" plays an important role in exercise performance, and evidence exists that brain functioning can be influenced by nutrition and/or nutritional supplements (Meeusen 2014). Table 1 summarizes the effects of several dietary supplements on cognitive performance and exercise performance. For a more detailed overview, we refer to the review of Meeusen and Decroix 2018 on nutritional supplements and the brain (Meeusen and Decroix 2018).

Table 1. Nutritional supplements for cognitive performance during exercise and exercise performance, according to their level of evidence. *Based on Meeusen and Decroix 2018.*

Level of evidence	**Supplement**	**Exercise performance**	**Cognitive performance**	
				Mechanism
High	Carbohydrates	+	+	↓ perception of effort ↑ substrate delivery to active muscles + brain
	Caffeine	+	+	↓ perception of effort ↑ brain activity in reward centres of the brain
Moderate	Polyphenols	+	+	↑ regional cerebral perfusion ↓ oxidative stress
	Quercetin	+		
	Cocoa flavanol		+	↑ regional cerebral perfusion
	Beetroot juice	+	+	↑ regional cerebral perfusion
Low	Ginseng		↔	
	Ginkgo Biloba		↔	
	Rhodiola Rosea		/	
	Sage		+	
	BCAA	/		
	Tyrosine	/	/	

1.3 Polyphenols

As shown in Table 1, there is a moderate level of evidence that polyphenols have beneficial effects on cognitive performance. This is supported by a number of epidemiological and experimental studies which examined the effects of polyphenols on brain health and cognitive function (Vauzour 2012). Polyphenols are abundant micronutrients in plant-derived foods and are powerful antioxidants. Fruits and beverages such as tea, red wine, cocoa, and coffee are major dietary sources of polyphenols. The largest group of polyphenols is the flavonoids. There are six dietary groups of flavonoids: flavones (e.g. apigenin, luteolin), which are found in parsley and celery; flavanones/flavanonols (e.g. hesperetin, naringenin/astilbin, engeletin), which are mainly found in citrus fruit, herbs (oregano), and wine; isoflavones (e.g. daidzein, genistein), which are mainly found in soy and soy products; flavonols (e.g. kaempferol, quercetin), which are found in onions, leeks, and broccoli; anthocyanidins (e.g. pelargonidin, cyanidin, and malvidin), whose sources include red wine and berry fruits and flavanols [e.g. (+)-catechin, (−)-epicatechin, epigallocatechin, and epigallocatechin gallate], which are abundant in green tea, red wine, and cocoa. The non-flavonoid group of polyphenols may be separated into two different classes: the phenolic acids (including the hydroxybenzoic acids (C1–C3 skeleton) and hydroxycinnamic acids (C3–C6 skeleton)) and the stilbenes (C6–C2–C6 skeleton). Caffeic acid is generally the most abundant phenolic acid, and is mainly found as the quinic ester, chlorogenic acid, in blueberries, kiwis, plums, and apples. Resveratrol, the main stilbene, can be found in the cis or trans configurations, either glucosylated (piceid) or in lower concentrations as the parent molecule of a family of polymers such as viniferins, pallidol, or ampelopsin A. Resveratrol dietary sources include grapes, wine, and peanuts. While polyphenol intake can be increased by conscious dietary choices of foods with high content (juices, tea infusions, chocolate etc.), the concentration of active substances is much higher in supplements.

Polyphenols have consistently been associated with a reduced risk of developing dementia, improved cognitive performance in normal aging, and improved cognitive evolution (Vauzour 2012). Emerging evidence suggests that flavonoids may be beneficial to attention, working memory, and psychomotor processing speed in a general population (Socci et al. 2017). The evidence also points towards a dose-dependent effect of flavonoids. While many of the mechanisms underpinning their beneficial effects remain to be elucidated, it has become clear that they involve decreasing oxidative/inflammatory stress signalling, increasing protective signalling, upregulating gene expression encoding for antioxidant enzymes, phase-2 enzymes, neurotrophic factors and cytoprotective proteins and improving cerebrovascular function (Lamport et al. 2015). Overall, there is encouraging evidence that flavonoid supplementation can benefit cognitive outcomes within an acute time frame of 0-6 hours. Nonetheless larger studies, combining cognitive and physiological measures, are needed to strengthen the evidence base.

Besides the potential of polyphenols to improve cognitive performance, a recent meta-analysis suggests that polyphenol supplementation is also associated with a clear moderate improvement of athletic performance with no adverse effects reported (Somerville et al. 2017). Still, the use of polyphenols is nowadays controversial. Because polyphenols are strong antioxidants, they may dampen training adaptations as small levels of exercise-induced reactive oxygen species (ROS) are a cornerstone in the adaptive response to exercise training (Pingitore et al. 2015). In contrast, it is believed that polyphenol intake will reduce muscle damage, by counteracting the excess of free radicals produced during exhaustive exercise (Braakhuis and Hopkins 2015). Furthermore, evidence shows that various polyphenols can increase flow-mediated dilatation (FMD) and endothelial function in humans by increasing endothelial nitric oxide (NO) synthesis and NO availability. This is believed to enhance blood flow and oxygen (O_2) and nutrient delivery to the active muscles, thereby improving exercise tolerance and exercise recovery (Somerville et al. 2017).

As previously mentioned, flavanols are a subgroup of polyphenols which are found in large amounts in cocoa. While several beneficial effects of cocoa flavanols (CF) have been demonstrated in specific populations (Heiss et al. 2005; Sarria et al. 2014; Ramos et al. 2017), very little is known about their effects on cognitive function in an exercise setting and on exercise performance.

1.4 Cocoa flavanols

1.4.1 Origin and components

Cacao, designated as Theobroma cacao in the 18th century, was the Aztec word for chocolate and was originally named as "Food of the Gods". This shows that the Mayan people already recognized its multiple health benefits centuries ago (Verna 2013). In the 16th century, cocoa was brought to Europe by Columbus and Cortez, but it was not until the 17th century that chocolate became popular in Europe and until the 18th century that the chocolate industry started to expand. Several modifications of the original cocoa bean took place over the centuries and included fermentation, drying, toasting, grinding the cocoa seeds and mixing with several other ingredients.

The term "cocoa" refers to the cocoa powder, which is the result of grinding cocoa seeds and the removal of cocoa butter from the cocoa solids. Cocoa contains more than 300 different constituents, with minerals (magnesium, potassium, iron and zinc), methylxanithines (theobromine and caffeine) and flavanols being the main components (Araujo et al. 2016). The main flavanols, also called flavan-3-ols, found in cocoa are the monomeric (−)-epicatechin (hereafter referred to as epicatechin) and, to a lesser extent(+)-catechin (hereafter referred to as catechin), and polymeric proanthocyanidins (Figure 1 for structures of the main monomeric CF). The natural cocoa bean contains high levels of CF (12-48% of dry weight of the cocoa bean), but many of the manufacturing processes, such as fermentation, drying, roasting and certainly alkalizing, destroy most of these CF (Araujo et al. 2016). Therefore, most chocolate products only contain very little CF (Badrie et al. 2013). With the genetic background of the cocoa plant, the manufacturing process and the final cacao content influencing the CF content, it is clear that CF contents of chocolate-products are highly variable (Badrie et al. 2013). However, through optimized and controlled sourcing and processing, large amounts of CF can be preserved in the final cocoa powder, resulting in cocoa powder with high CF content (Badrie et al. 2013).

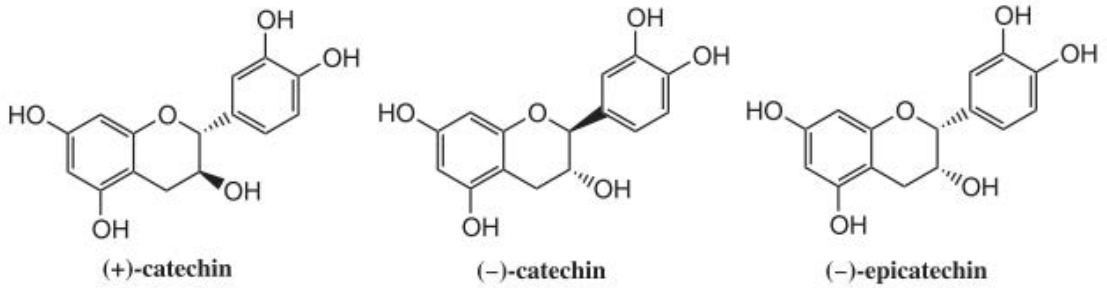

Figure 1. Structures of the predominant monomeric cocoa flavanols.

1.4.2 Bioavailability

A large number of *in vitro* studies has shown that CF have several advantageous effects, but whether CF can also exert their beneficial action *in vivo* depends on their bioavailability and bioactivity (Spencer et al. 2000). The average oral absorption of CF varies from 1 to 50% of the ingested dose. The large variability in bioavailability can be ascribed to the complexity of the *in vivo* gastro-intestinal system involving absorption, metabolism, distribution and elimination.

After intake, CF appear in plasma after 30-60 minutes (min), with epicatechin appearing in a larger concentration than catechin. This increase takes place in a dose-dependent way. The peak concentration is obtained 2 hours post-ingestion and CF are still detectable after 8 hours (Schramm et al. 2003). The rate and extent of absorption and metabolism can however be influenced by components in the food matrix of the chocolate product and co-ingested macronutrients. It was shown that concomitant intake of CF with a carbohydrate-rich meal increases flavanol bioavailability, while co-ingestion of proteins and lipids has little effect on flavanol bioavailability (Schramm et al. 2003).

While Spencer et al. showed that procyanidins (trimers to hexamers) are hydrolysed to monomeric flavanols in the gastric milieu, it appears that both monomeric and oligomeric CF remain intact during gastric transit (Spencer et al. 2000; Strat et al. 2016). Monomeric epicatechins and catechins are efficiently and rapidly absorbed and metabolized to O-methyl-, glucuronide- and O-methylglucuronide-conjugates by the enterocytes in the small intestine. Further phase 1 (oxidation, reduction and hydrolysis) and phase 2 (conjugation: glucuronidation, methylation, sulfation or combination) biotransformation takes place in the liver, as these metabolites and any remaining native epicatechins are transported to the liver via the portal vein. The oligomeric proanthocyanidins are not absorbed in the small intestine and proceed to the colon where they undergo transformation by colonic microbiota before being absorbed (Scalbert and Williamson 2000). It has been suggested that about 5-10% of CF are absorbed in the small intestine, while 90-95% proceed to the colon (Cardona et al. 2013).

From the above, it seems clear that the composition of the gut microbiota influences the transformation of CF and therefore also the bioavailability and effects of CF. The metabolism of proanthocyanidins by the colonic microbiota involves cleavage of glycosidic linkage and breakdown of heterocyclic backbone, resulting in production of lactones and aromatic and phenolic acids having different side-chain lengths and hydroxylation patterns (Manach et al. 2005). These metabolites are then absorbed, pass through the portal vein to the liver and are there subjected to phase 2 biotransformation. From the liver, CF metabolites enter the systemic circulation and are distributed to the organs where they can interact with receptors. It is noteworthy that the *in vivo* efficacy is still dependent on binding of the CF metabolites to serum albumin or cellular proteins and the accumulation in the target cells. Finally, they undergo renal excretion to be excreted in urine. Alternatively after metabolism in the liver, CF can re-enter the

gastro-intestinal tract through biliary excretion and can undergo phase 2 metabolism by colonocytes in the colon (Cardona et al. 2013). Also, it must be noted that a portion of CF will undergo faecal elimination without absorption (Figure 2).

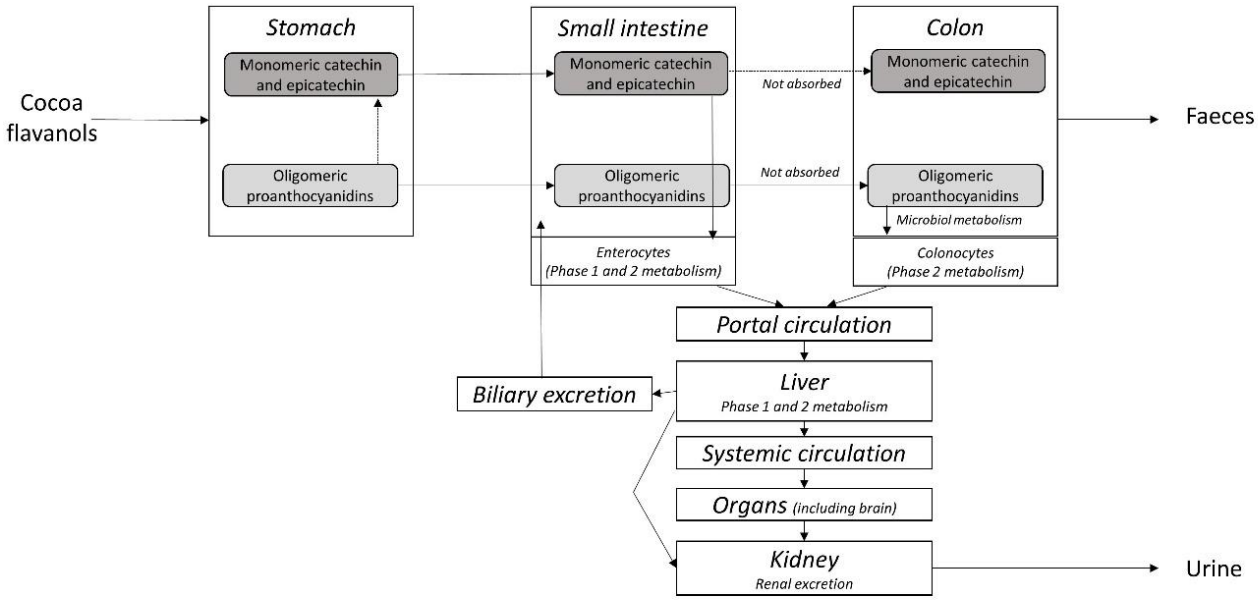

Figure 2. Routes for cocoa flavanols and metabolites through the gastro-intestinal tract to reach the organs where they can exert their function. *Based on Cardona et al. 2013.*

1.4.3 General health benefits

1.4.3.1 Promoting cardiovascular health

The first proofs of the beneficial effects of CF on cardiovascular health came from the Kuna Indians, who consumed large amounts of CF on a daily base. Compared to other Pan-American populations, reduced blood pressure, lower frequencies of stroke, type 2 diabetes and myocardial infarct and lower mortality due to cardiovascular disease (CVD) were reported (Hollenberg et al. 2009). Since then, epidemiological and interventional studies provided further evidence that regular intake of CF is associated with lower risk of CVD (Corti et al. 2009). The beneficial impact of CF on cardiovascular health is mainly caused by improved vascular function and reduced blood pressure, but also involves reduction of oxidative stress and inflammation, modulation of lipid and glucose metabolism, and reduction of platelet aggregation (Ludovici et al. 2017). It has been shown that most of the physiological effects of CF on cardiovascular health are mediated by increased NO availability and reduced oxidative stress (Petyaev and Bashmakov 2017). Therefore, we will first briefly explain the role of NO in vascular function and the process of oxidative stress.

Nitric oxide

In the endothelium, NO is synthesized by endothelial NO synthase (eNOS) from the precursor L-arginine in the presence of the cofactor tetrahydrobiopterin (BH_4) and O_2. Activity of eNOS can be increased in a calcium (Ca^{2+})-dependent way, mediated through the PI3-kinase/Akt pathway, or by post-translational modification, namely phosporylation. Several signals can modulate the release of NO, including hormones (e.g. catecholamines, oestrogen), biological factors (e.g. vascular endothelial growth factor (VEGF)) and physical signals such as a decreased temperature and shear stress (Vanhoutte et al. 2017).

NO induces relaxation of vascular smooth muscle cells by stimulating guanylate cyclase (GC), increasing intracellular cGMP concentrations, which in turn activates cGMP-dependent protein kinase or Protein Kinase G (PKG) and leads to the decrease of intracellular Ca^{2+}, thus interrupting contraction of the smooth muscle cell (Vanhoutte et al. 2017). In addition to its crucial role in vasodilation, NO also prevents leukocyte adhesion and migration, smooth muscle cell proliferation, platelet adhesion and aggregation, and stimulates mitochondrial respiration and biogenesis, glucose uptake and sarcoplasmic reticulum Ca^{2+} handling (Förstermann 2010).

In the brain, NO is also produced by neurons by neuronal NOS (nNOS), where it plays a key role in neurovascular coupling (NVC), which is the close relationship between neuronal activity and regional cerebral blood flow (CBF) (Calabrese et al. 2007).

The eNOS-NO system can become dysfunctional with aging, dietary unbalances (e.g. vitamin D deficiency), exposure to air pollution, and under pathological conditions (e.g. diabetes, hypertension, obesity, atherosclerosis) that are often associated with increased ROS production.

Reactive oxygen species

Free radicals are atoms containing unpaired electrons and are therefore highly reactive and unstable. Reactive oxygen and nitrogen species (ROS/RNS) are free radicals formed by the incomplete reduction of O_2 and nitrogen, which leads to the production of superoxide ($O_2^{\cdot -}$) and NO. $O_2^{\cdot -}$ is further converted to hydrogen peroxide (H_2O_2) and the hydroxyl radical (·OH) (Pingitore et al. 2015). Ninety percent of ROS are generated in the mitochondria, as electrons escape from the mitochondrial electron transport chain. The other 10% of ROS are produced by electron chain transport in the endoplasmic reticulum, plasmatic and nuclear membranes and by oxidases, located in cell membranes, such as xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. While it was initially believed that mitochondria in muscle fibres were the main source of ROS production, emerging evidence reveals that this is not the case (Powers et al. 2011). RNS can be produced from NO and can also contribute to the free radical production in the electron transport chain. NO can bind to cytochrome C oxidase, ultimately leading to $O_2^{\cdot -}$ production. NO can also combine with $O_2^{\cdot -}$ to form peryoxynitrite (·ONOO-), which is one of the more destructive radicals.

ROS/RNS play an important role in various cell signalling pathways and contribute to homeostasis. For instance, controlled production of ROS contributes to mitochondrial biogenesis, angiogenesis, immune function and muscle adaptation. However, excessive amounts of ROS/RNS are deleterious for human health and therefore, ROS/RNS are removed or neutralised by various antioxidant defence systems leading to an optimal ROS/RNS steady state level. Antioxidants are defined as substances, when present in small concentrations, that can inhibit oxidation of a substrate. Enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), glutaredoxin (GRX) and thioredoxin (TRX), which are mainly located in the cytosol and are produced endogenously. Non-enzymatic antioxidants include vitamins (vitamin C, E, β carotene), polyphenols, proteins as thiols, and various other compounds as ubiquinone and uric acid (UA), which are mainly located in cell membranes and are derived from nutrition. Antioxidants can exert their function by 1) prevention of formation of ROS/RNS (1st line defence), 2) scavenging/ removing active species before they cause molecular damage (2nd line defence), 3) repairing damage (3rd line defence) or 4) upregulating antioxidant transcription and translation (Niki 2010).

When excessive amounts of ROS/RNS are produced and the body's antioxidant defence system cannot sufficiently eliminate ROS/RNS, the large amounts of ROS/RNS result in oxidative stress. Oxidative stress refers to the imbalance between oxidants/ free radicals and antioxidants in favour of the oxidants,

leading to a disruption of redox signalling and/or molecular damage. Large amounts of ROS/RNS will interact with cellular proteins (protein oxidation), lipids (lipid peroxidation) and DNA (oxidative DNA damage), leading to cellular damage and even cell death. Oxidative stress is involved in many pathologies including neurodegenerative diseases (such as Parkinson's disease, Alzheimer's disease), cardiovascular disease, cancer, chronic fatigue syndrome and diabetes (Corti et al. 2009). Besides, oxidative stress is also elicited during exhaustive exercise. Although the human body has an efficient defence system against oxidative stress, supplemental protection against free radicals by nutritional antioxidants can be warranted when the endogenous production of free radicals is augmented or during exposure to exogenous ROS (e.g. produced from pollutants, tobacco) (Alam et al. 2013; He et al. 2016).

Besides the detrimental effects of ROS on lipids, proteins and DNA (oxidative stress), the presence of ROS can also decrease NO availability by decreasing eNOS activity and increasing NO disposition. Elevated levels of ROS will reduce BH_4 levels, the essential cofactor of eNOS and disrupt the dimeric structure of eNOS, leading to eNOS uncoupling which will form O_2^- instead of NO. Also, increased ROS production *(i)* causes a higher breakdown of L-arginine by arginase, thereby lowering substrate availability of eNOS, *(ii)* accelerates the production of asymmetric dimethyl-arginine (ADMA), which acts as an endogenous eNOS inhibitor, *(iii)* inhibits eNOS gene transcription, thus reducing protein presence of eNOS. In conditions of elevated ROS production, NO can react with $O_2^{\bullet-}$ to form $^{\bullet}ONOO^-$, thus degrading NO and lowering NO availability (Förstermann 2010). An overview of the production of NO in endothelial cells, the interplay with ROS and the regulation of vascular tone by endothelium-derived NO is shown in Figure 3.

Effects of CF on NO metabolism and oxidative stress

It has been suggested that NO production and availability can be stimulated by dietary supplements containing NO precursors such as nitrate and L-arginine, but the efficacy of their use remains controversial in most scenarios. Moreover, it has been proposed that reducing ROS production by antioxidants intake has can alleviate the decrease in NO availability caused by ROS (Vanhoutte et al. 2017).

Accordingly, for CF, *in vitro* work revealed that CF can improve NO availability by several molecular mechanisms. These include, but are not limited to: *(i)* Ca^{2+}-independent eNOS phosphorylation and activation (Karim, et al. 2000); *(ii)* inhibition of arginase, thereby increasing L-arginine which is the precursor of NO (Schnorr et al. 2008); *(iii)* inhibition of NADPH oxidase, thus reducing the generation of $O_2^{\bullet-}$-radicals, and in turn reducing the elimination of NO by formation of $^{\bullet}ONOO^-$ (Schewe et al. 2008) and iv) decreasing levels of oxidative stress by direct scavenging of free radicals and upregulation of endogenous antioxidants (Ruijters et al. 2013; Ramirez-Sanchez et al. 2013) (Figure 4). It has been proposed that *in vivo*, the short-term effect of CF intake is caused by diminished NO elimination through

inhibition of NADPH oxidase, while long term CF effects are additionally mediated by altered gene expression and protein synthesis of eNOS (Corti et al. 2009).

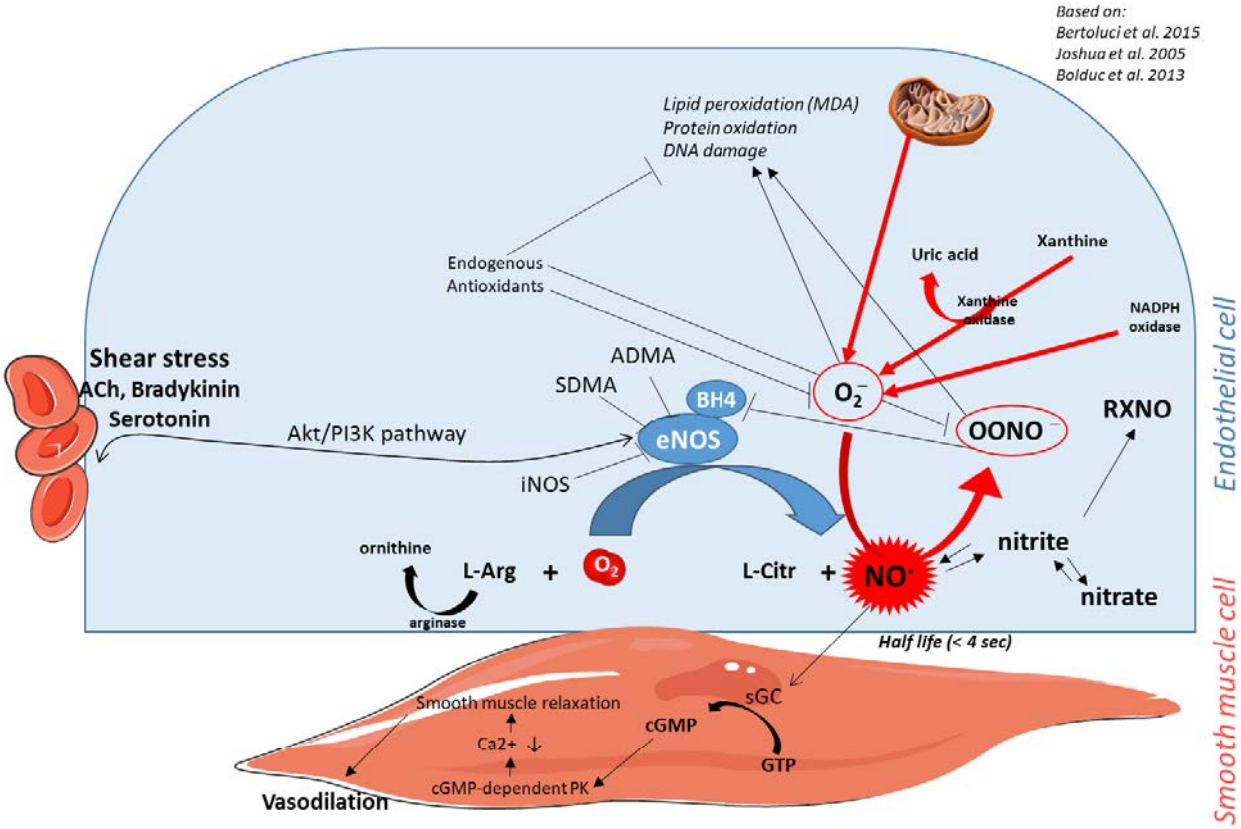

Figure 3. Production of nitric oxide (NO) in endothelial cells, the interplay with reactive oxygen species (ROS) and the regulation of vascular tone by endothelium-derived NO. *NO, together with L-citrulline (L-citr), is formed by endothelial NO synthase (eNOS) from the precursor L-arginine (L-arg) in the presence of the cofactor tetrahydrobiopterin (BH4) and oxygen (O_2). NO diffuses away and can stimulate guanyl cyclase (sGC) to convert guanosine triphosphate (GTP) in cyclic guanosine monophosphate (cGMP) in smooth muscle cells. This results in activation of cGMP-dependent protein kinase (PK) and leads to the decrease of intracellular calcium (Ca^{2+}), thus interrupting contraction of the smooth muscle cell and vasodilation. NO can also be oxidized to nitrite and further to nitrate, and in reverse, nitrate can be reduced to nitrite and NO as well. eNOS is stimulated by shear stress, but also by factors such as acetylcholine (Ach), bradykinin and seronin through activation of the Akt/PI3K pathway. eNOS-dependent NO production is inhibited by inducible NOS (iNOS), symmetric dimethylarginine (SDMA), asymmetric dimethylarginine (ADMA) and ROS. ROS, such as superoxide ($O_2^{\cdot}$-), are generated in the mitochondria and xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the cell membrane. The endogenous antioxidants act together to reduce ROS and prevent the harmful reaction of ROS with proteins, DNA and lipids. The latter is called lipid peroxidation and results in formation of malondialdehyde (MDA), which one of the many biomarkers of oxidative stress. Together with exogenous antioxidants, the endogenous hydrophilic antioxidants contribute to the total antioxidant capacity of a substance which is regularly assessed in plasma. $O_2^{\cdot -}$ also reacts with NO to form peroxynitrite ($ONOO^-$), thus degrading NO and lowering NO availability. Figure based on Bertoluci et al. 2015, Joshua et al. 2005 and Bolduc et al. 2013.*

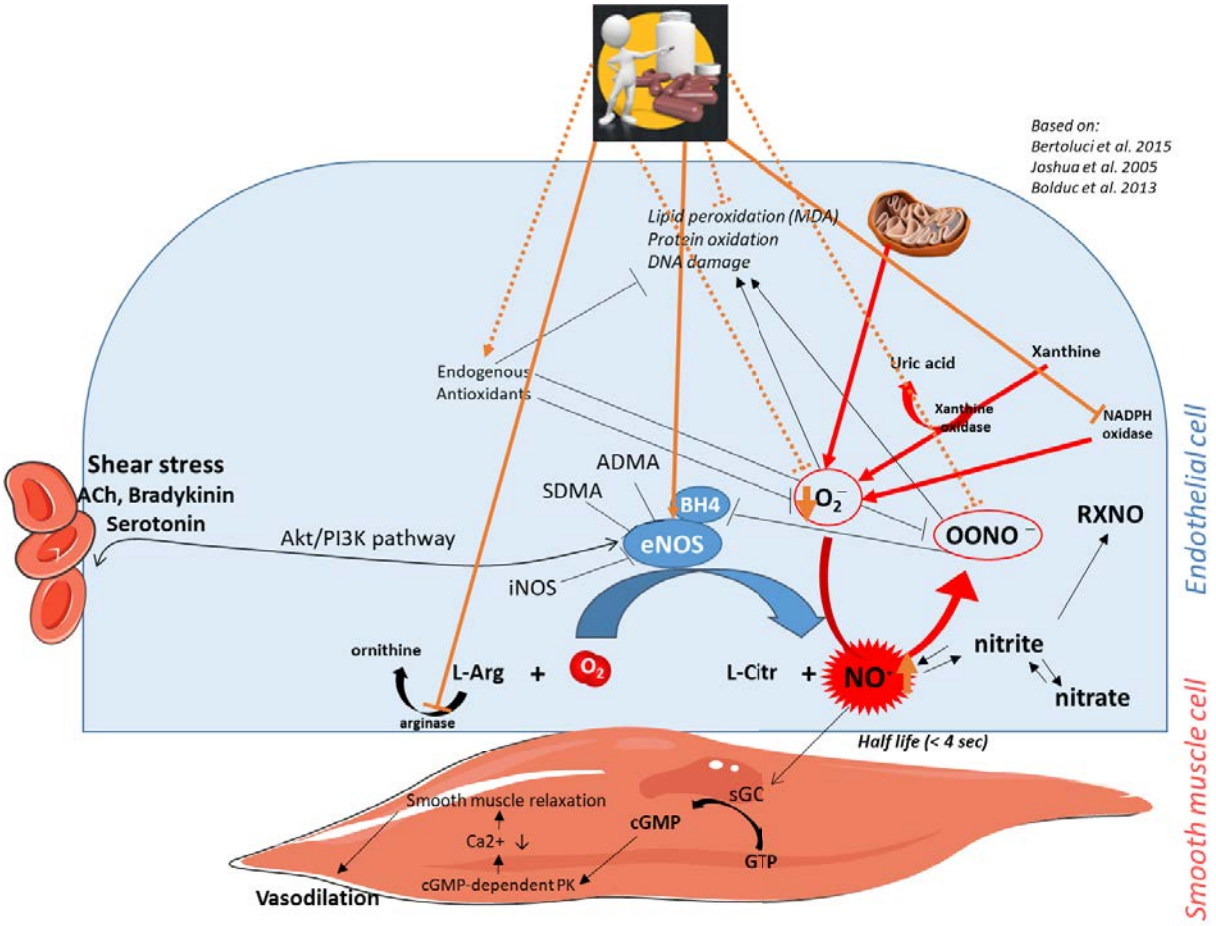

Figure 4. Putative mechanisms of how cocoa flavanols (CF) intake can increase nitric oxide (NO) availability and reduce oxidative stress. *CF can i) activate eNOS, ii) inhibit arginase, thereby increasing L-arginine which is the precursor of NO, iii) inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, thus reducing the generation of superoxide ($O_2^{\cdot -}$), and in turn reducing the elimination of NO by formation of peroxynitrite ($^{\cdot}ONOO^-$) and iv) decreasing levels of oxidative stress by direct scavenging of free radicals and upregulation of endogenous antioxidants. We refer to figure 3 for all abbreviations.*

1. Antioxidant properties

It has been shown both *in vitro* and *in vivo* that CF and their metabolites are strong antioxidants (Nabavi et al. 2015). Initially, it was believed that CF acted as direct scavengers of free radicals but recent *in vitro* works suggest a more complicated way of action. It seems that CF and their metabolites mainly reduce oxidative stress by inhibiting pro-oxidant enzymes such as NADPH oxidase, lipoxygenase and myeloperoxidase and by inducing upregulation of endogenous antioxidant enzymes (Schewe et al. 2008; Rodriguez-Ramiro et al. 2011). The latter is mediated by stimulation of nuclear translocation and activation of sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor-gamma coactivator (PGC) 1- α and Forkhead box protein O1 (FOXO1). These are key transcriptional modulators of SOD2 and catalase, two endogenous antioxidants (Ramirez-Sanchez et al. 2013). Moreover, the increased SOD activity leads to less $O_2^{\bullet-}$-radicals, yielding reduced peroxynitrite formation and reduced nitrosylation of enzymes such as SOD2 and catalase, thereby increasing their enzyme activity (Ramirez-Sanchez et al. 2013). In humans, CF intake has been reported to decrease lipid peroxidation and plasma F2-isoprostane-levels and increase total antioxidant capacity in several diseased populations (Nabavi et al. 2015).

2. Endothelial function

CF improves endothelial function by stimulating NO-mediated vasodilation, resulting in reduced systemic blood pressure and decreased arterial stiffness (Ried et al. 2012; West et al. 2014; Sansone et al. 2015). Inhibition of NO synthase has been shown to abrogate the observed improvements in endothelial function after CF intake, pointing out a crucial role for NO in mediating the beneficial effects of CF (Fisher et al. 2003). However, other mechanisms inducing vasodilation may also be involved, including the inhibition of endothelin-1 (ET-1) production (Loke et al. 2008a) and the decrease of angiotensin 2 through inhibition of angiotensin-converting enzyme (ACE) (Ludovici et al. 2017).

Improvements in endothelial function and blood pressure have been observed in both healthy and diseased populations, but the effects were larger in older (> 50 years) adults and people with restricted vascular function than in healthy, young subjects (Fisher et al. 2012). The magnitude of the observed effects also reflects the dose and duration of CF intake. Notably, the effects of CF on endothelial function have been mimicked by epicatechin intake, pointing out that this main monomeric compound of CF mediates the changes in NO availability (Schroeter et al. 2006). Also the glucuronidated metabolites, and in a lesser extent the methylated metabolites of epicatechin have been shown to be directly and causally involved in the improved vascular function (Schroeter et al. 2006).

In 2013, the European Commission approved for the first time a health claim for CF-rich cocoa powder and dark chocolate, stating that “daily intake of 200 mg CF contributes to normal blood circulation by helping to maintain the elasticity of the blood vessels” (EFSA 2012). This amount could be provided by

2.5 g of high CF powder or 10 g of high CF dark chocolate. Following the advice of the European Food Safety Authority (EFSA) who recognized a "cause and effect" relationship between CF intake and maintenance of normal endothelium-dependent vasodilation, the health claim was extended and expanded to applications of CF in the form of capsules and tablets in 2015 (EFSA 2014). A recent study of Grassi et al. (2015) showed that CF intake dose-dependently improves endothelial function and that even 80 mg CF intake/day increased endothelial function.

3. Reducing blood pressure

A meta-analysis showed that 2-week CF intake results in a small, but significant reduction in mean systolic and diastolic blood pressure of 4.7 and 2.8 mm Hg, respectively. These effect sizes lay in the same range of currently used antihypertensive drugs (Taubert et al. 2007). A more recent meta-analysis included 40 studies examining the effects of CF treatment, involving 1804 mainly healthy participants. This study revealed a small, but significant reducing effect of 2 to 18-week CF intake on mean systolic and diastolic blood pressure of 1.7 and 1.8 mm Hg, respectively. However, the magnitude of the effect seems to depend on the baseline blood pressure (Ried et al. 2017). Still, larger interventional studies are warranted before clinical use.

4. Anti-inflammatory properties

Low-grade inflammatory processes are known to contribute to the pathogenesis of CVD and are involved in platelet activation. Besides, these inflammatory processes involve upregulation of pro-inflammatory enzymes such as xanthine oxidase and NADPH-oxidase and thus also results in higher production of ROS (Ellinger and Stehle 2016).

In vitro and animal studies have unequivocally demonstrated that CF reduce the release of pro-inflammatory cytokines (e.g. interleukin (IL)-1b and IL-2) and increase production of anti-inflammatory cytokines (e.g. IL-4 and IL-5) (Mao et al. 2002). CF can inhibit inflammatory mediators cyclo-oxygenase 2 (COX-2) and inducible NO-synthase (iNOS) (Lee et al. 2006). Moreover, CF can decrease adhesion molecules, downregulate leukocyte activation and reduce biosynthesis of leukotrienes and increased production of prostacyclin (Schramm et al. 2001; Monagas et al. 2009; Katz et al. 2011; Ellinger and Stehle 2016), thereby reducing vascular inflammation and oxidative stress in patients at risk for CVD. However, it must be noted that many of the above findings were observed in *in vitro* studies using pharmacological doses of CF, which cannot be attained *in vivo*. Still, some interventional trials in (hypercholesterolemic, overweight and hypertensive) humans support the anti-inflammatory effect of CF. Future research is required to further evaluate the possible clinical applications and should also focus on the effects of CF in healthy subjects exposed to increased inflammatory burden (Ellinger and Stehle 2016). For a detailed overview of the anti-inflammatory properties of CF, we refer to the review of Goya et al. (2016).

5. Platelet function

In vivo studies have shown that CF reduce platelet aggregation and adhesion in healthy subjects and smokers (Innes et al. 2003; Hermann et al. 2006). These platelet-inhibitory properties have been ascribed to reduced expression of glycoprotein IIb/IIIa surface proteins (Loffredo et al. 2016).

6. Glucose metabolism

By improving endothelial function, CF can reduce insulin resistance, especially in overweight and/or diabetic subjects (Grassi et al. 2005a). Other mechanisms include inhibition of lipid accumulation in β cells, and enhancement of glucose-stimulated insulin secretion (β cell function).

In vitro studies have shown that CF may slow down carbohydrate digestion and absorption in the gut, by interacting with several digestive enzymes (e.g. α-amylase) in an inhibitory fashion. Besides, CF can enhance the incretin response (i.e. hormones secreted after a meal), thereby improving glucose disposal, slowing gastric emptying and reducing appetite as well (Strat et al. 2016). While CF are present in the gut in large quantities, the relatively low concentrations of CF and metabolites in the systemic circulation may prevent the inhibitory effect on glucose transporters in other tissues *in vivo*. In conclusion, *in vitro* work showed many beneficial effects of CF on glucose homeostasis, but further research is needed to pinpoint the cellular mechanisms behind these effects in order to enable therapeutic targeting and to confirm these findings in large human clinical trials (Strat et al. 2016).

7. Blood lipids

Both in healthy subjects and hypertensive patients, 2-3 week CF intake leads to significant reductions of serum low density lipoprotein (LDL) cholesterol levels, increases in high density lipoprotein (HDL) cholesterol level and inhibition of lipid peroxidation and LDL oxidation (Grassi et al. 2005b; Baba et al. 2007). However, some other studies found no beneficial effects, suggesting that larger well-controlled studies are needed. Importantly, it must be noted that processed (normal) chocolate contains high amount of fat, and therefore may be less favourable on blood lipids (Corti et al. 2009).

1.4.3.2 Neurological effects

It has been shown *in vitro* and in animals that CF can cross the blood-brain barrier (BBB) to enter the brain, thus opening the possibility to exert beneficial effects in the brain (Abd El Mohsen et al. 2002). The majority of human interventional studies assessing the effects of CF on cognitive function, observed dose-dependent beneficial effects on several cognitive domains including working memory, executive function and attention, after both acute and chronic CF intake (Crews Jr et al. 2013; Socci et al. 2017). It seems that especially elderly and patients at risk for neurocognitive disorders and CVD can benefit from CF supplementation (Socci et al. 2017). A longitudinal prospective study in elderly showed that chocolate consumption was associated with a lower risk of cognitive decline over a 4-year period (Moreira et al. 2016). Daily intake of CF during 8 weeks improved cognitive function in elderly with and without mild cognitive decline (Desideri et al. 2012; Mastroiacovo et al. 2015).

The exact mechanisms of action responsible for the beneficial effect of CF on cognitive function are not yet fully understood, but improved CBF certainly plays a crucial role in an "indirect manner" (Figure 5). CF can enhance NO-mediated vascular function, not only in the periphery but also in the cerebral circulation and hence improve brain perfusion. *In vivo* studies reported increased blood flow velocity in the middle cerebral artery measured by transcranial Doppler ultrasound (Sorond et al. 2008), enhanced global perfusion at rest measured by Arterial Spin Labeling (ASL) functional Magnetic Resonance Imaging (fMRI) (Lamport et al. 2015), and increased blood oxygenation level dependent response (BOLD) in specific brain regions during cognitive tasks in response to acute and short-term CF consumption in healthy subjects (Francis et al. 2006; Field et al. 2011). *In vitro* and animal studies point towards the involvement of other direct mechanisms as well. CF are believed to interact with cellular cascades resulting in upregulation in the expression of neuroprotective and neuromodulatory proteins, promoting neurogenesis, neuronal survival and synaptic plasticity and/or inhibiting neuronal apoptosis induced by ROS (Sokolov et al. 2013). Thus, CF have the potential to protect the brain against oxidative stress, neural inflammation, neurotoxins and amyloid β protein-induced neurotoxicity and to lower the risk of stroke and Alzheimer's disease in humans (Sokolov et al. 2013). Besides, it is noteworthy that chocolate consumption also improves mood state and makes people feel good, thereby possibly protecting against depression (Strandberg et al. 2008).

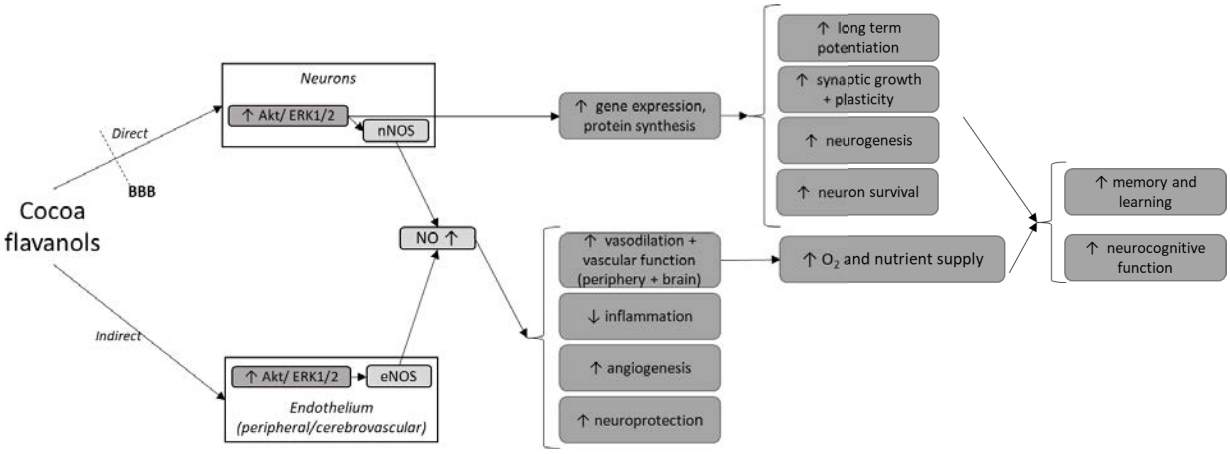

Figure 5. Putative mechanisms of action of CF in the brain. *Based on Rendeiro et al. 2015.*

1.4.3.3 Other functions

It has been suggested that CF can improve the gut barrier function, thereby reducing systemic endotoxin exposure. As stated previously, CF are metabolized by gut microbiota, but CF can also modulate the gut microbiome as they have prebiotic properties. CF and their metabolites can inhibit growth of pathogen bacteria and stimulate the growth of beneficial bacteria, thereby promoting gut health (Cardona et al. 2013).

Many animal studies have shown that CF intake can prevent or slow down the initiation and progression of different types of cancer. The cellular mechanisms behind the anti-carcinogenic properties of CF include modulation of redox state and signalling pathways related with cell proliferation, differentiation, apoptosis, inflammation and metastasis. The anti-carcinogenic properties of CF, as well as the optimal dose and route of administration, still need to be evaluated by well-controlled clinical trials. However, based on the existing literature, it is suggested that CF intake in combination with polyphenol-rich food might have the potential to optimize general health, including the efficient prevention of cancers with minimal toxicity (Martin et al. 2013).

1.4.4 Hypothetical benefits for sports performance

CF may affect sports performance because of their *(i)* ability to improve cognitive function, *(ii)* ability to promote NO production and availability, and *(iii)* antioxidant capacities. CF intake may enhance sport performance by boosting cognitive function since it has been shown that CF intake can improve executive function (decision making) and motor control, two important factors influencing sports performance. An abundance of research has been published reporting beneficial effects of CF on vascular function via modulation of NO. Thus, it has been proposed that enhanced vascular function and increased O_2 and nutrient tissue delivery may lead to improved exercise performance. Moreover, NO is also involved in mitochondrial biogenesis and efficiency. While NO production and availability are upregulated through repeated exposure to shear stress during regular exercise training, it has been hypothesized that increasing NO availability by NO-related supplements, such as CF, may support or enhance exercise performance (Jones 2014) (Figure 6).

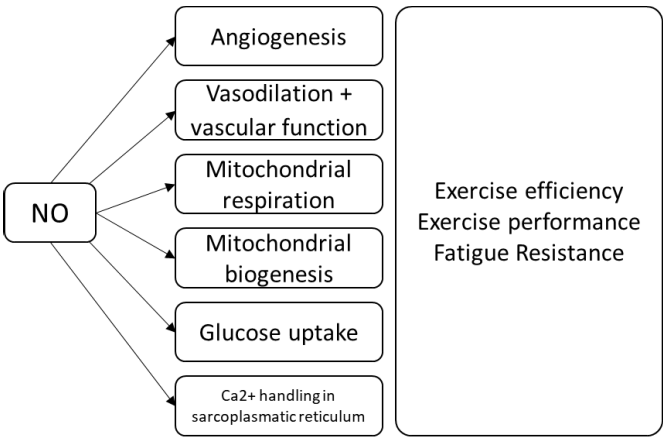

Figure 6. Importance of NO in physiological processes that may support exercise performance.

Because of their antioxidant capacities, CF can reduce ROS formation and oxidative stress. As mentioned previously, heavy exercise can lead to increased (mitochondrial) formation of ROS (Bentley et al. 2012). Although moderate levels of ROS initiate and promote adaptation to exercise training (Peternelj and Coombes 2011), excessive levels of ROS have detrimental effects on cell structure and function by interacting with lipids, proteins, DNA and may cause oxidative damage to the mitochondria and muscle contractile proteins. This contributes to muscular damage and fatigue after exercise (Bentley et al. 2015). Hence, CF intake might also improve exercise performance and recovery through increasing antioxidant capacity and reducing oxidative stress.

Still, evidence on how CF intake can affect exercise performance and cognitive performance is relatively scarce.

1.5 Purpose of the PhD

The purpose of this PhD is to identify the putative effects of CF on physical and cognitive performance, and to examine the underlying mechanisms.

In the general introduction (**chapter 1**), we aimed to provide a context for the research problem.

Chapter 2 consists of a systematic review, summarizing the known effects of CF and exercise, answering the following research question: can CF intake alter *(i)* exercise performance and recovery and *(ii)* acute and chronic exercise-induced changes in vascular function, cognitive function, oxidative stress, inflammation and carbohydrate and lipoprotein metabolism?

Based on the results of this review, some research questions remain unanswered. This leads us to the experimental studies of this PhD, which aim to:

1) Investigate the effects of acute CF intake on cognitive performance and underlying mechanisms by answering the following research questions:
   - Can acute CF intake improve executive function in combination with exercise in well-trained athletes? Are cerebral hemodynamic changes and/or changes in Brain Derived Neurotrophic Factor (BDNF) altered in response to acute CF intake and exercise? (Chapter 3)
   - Can acute CF intake improve cognitive performance at (simulated) altitude in young, healthy subjects? Are cerebral hemodynamic changes and/or brain activity during cognitive performance in moderate hypoxia altered by CF intake? (Chapter 4)
   - What is the effect of acute CF intake on cognitive performance and the BOLD response (cerebral hemodynamic change) in patients with type 1-diabetes (and healthy controls)? (Chapter 5)

Thus, chapters 3 - 5 focus on the effects of CF intake on cognitive performance. In **chapter 3**, we describe the results of a randomized, double-blind, crossover interventional trial conducted in healthy, well-trained men. We assessed the effects of acute CF in combination with exercise, on prefrontal oxygenation, BDNF levels and cognitive performance.

Many sports events take place at high altitude where tissue O_2 delivery is compromised, resulting in decreased tissue oxygenation and increased levels of oxidative stress (Subudhi et al. 2007; McGinnis et al. 2014). As a consequence, cognitive performance is deteriorated at increasing altitude. We assessed if hemodynamic changes and brain activity during cognitive performance are altered in hypoxia and whether CF intake can modify these changes in healthy, young subjects in **chapter 4**.

Given the excellent vascular function (and endogenous antioxidant system) of healthy, well-trained athletes, we recognized prior to the start of this PhD that the beneficial effect of CF intake might be

minimal and negligible compared to the effects of (daily) exercise training. The beneficial effects of CF intake have been shown in elderly and populations *at risk* for cardiovascular or cerebrovascular disease, such as hypertensive, obese or smoking subjects, as well as patients with type 2 diabetes. However, while type 1 diabetes is also a disease prompting endothelial dysfunction and cognitive decline (Tonoli et al. 2014), the effects of CF in this population have not yet been assessed. Therefore, in **chapter 5**, we investigated the effects of CF intake on cognitive performance and the cerebrovascular response in patients with type 1 diabetes.

2) Investigate the effects of acute and 1-week CF intake on exercise performance and recovery, and underlying mechanisms, by answering the following research questions:
   - Can acute CF intake increase exercise-induced NO production and/or reduce oxidative stress and inflammation? What are the implications of acute CF intake for exercise performance and recovery in well-trained athletes? (Chapter 6)
   - Are NO production, oxidative stress and tissue oxygenation during exercise in hypoxia altered in response to 1-week CF intake in well-trained athletes? Are there implications of 1-week CF intake for exercise performance in hypoxia? (Chapter 7)

Thus, chapters 6 - 7 focus on the effects of CF intake on oxidative stress, NO availability, tissue oxygenation and exercise performance. In **chapter 6**, we examined whether acute CF could improve NO production and reduce oxidative stress and inflammation during exhaustive exercise and whether this could enhance exercise performance and/or recovery in well-trained athletes by performing a randomized, double-blind, crossover interventional trial. Given the negative impact of hypoxia on exercise performance, tissue oxygenation and oxidative stress, we aimed to evaluate whether CF could partially counteract the effects of hypoxia on oxidative stress, prefrontal and muscle oxygenation and exercise performance in well-trained athletes in **chapter 7**.

To conclude, **chapter 8** contains a general discussion of the results from the different studies in this research project. We will highlight the most important findings and try to address practical implications and formulate recommendations. We will also identify some limitations and provide guidelines for future research.

1.6 General methods

Figure 7 shows an overview of the different methods used in the studies of this PhD. The effects of 1 day and 1-week CF intake on exercise and cognitive performance in normoxic and hypoxic environments were studied in well-trained athletes, young, healthy subjects and patients with type 1 diabetes. Near-Infrared spectroscopy (NIRS) was used to assess oxygenation changes in the M. vastus lateralis and prefrontal cortex during exercise, in response to CF intake. Functional NIRS (fNIRS) was used to assess the NVC in the prefrontal cortex during cognitive tasks. Electroencephalography (EEG) and BOLD-fMRI were used to measure direct brain activity and the neurovascular response, respectively. Several parameters were measured in plasma or serum, including markers of oxidative stress, antioxidant capacity, inflammation, NO metabolism and BDNF. During exercise, several parameters including heart rate, blood lactate, rating of perceived exertion (RPE), blood glucose and saturation, were measured as well. While each of these methods will be described in detail in the corresponding chapter, we briefly introduce the different methods in the following section.

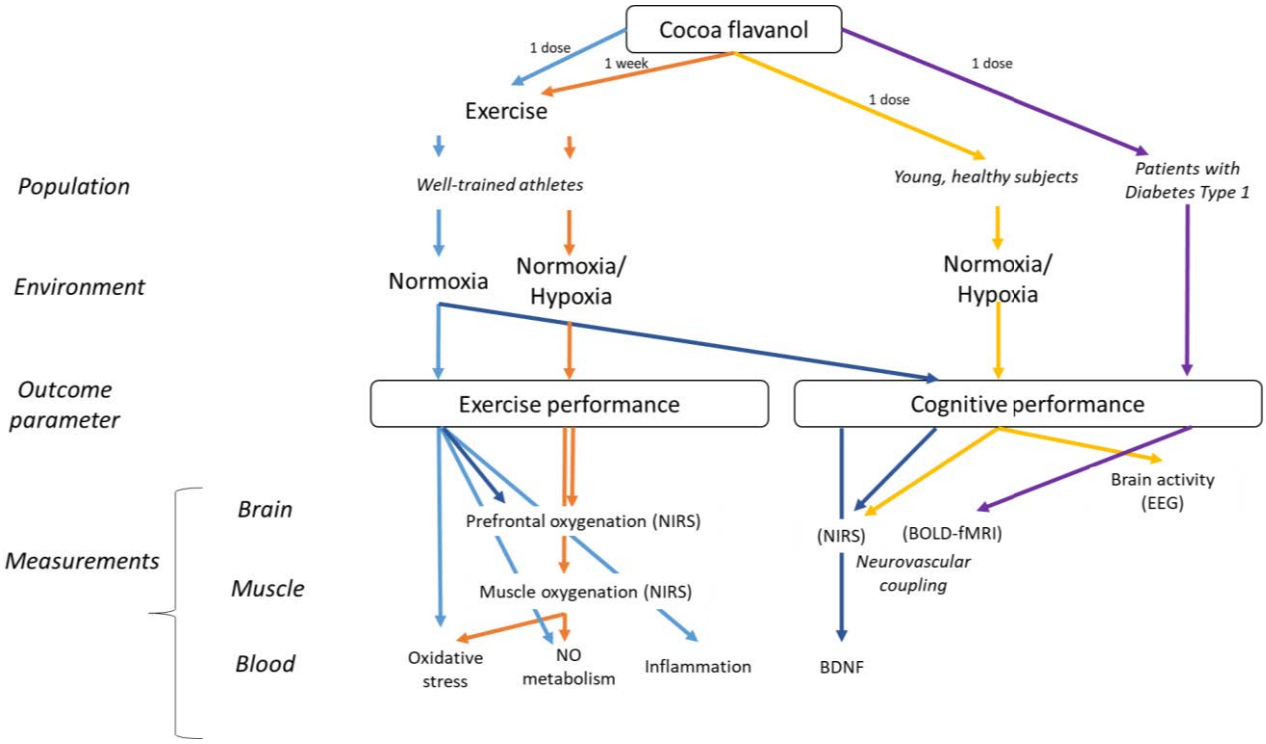

Figure 7. Overview of the methods used in the different studies in this doctoral thesis. *Light blue: study described in chapter 6, dark blue: study described in chapter 3, orange: study described in chapter 7, yellow: study described in chapter 4, purple: study described in chapter 5. NIRS: near infrared spectroscopy, BOLD-fMRI: Blood oxygenation level dependent response functional magnetic resonance, EEG: electroencephalography.*

1.6.1 Cocoa flavanol intervention

A large diversity in CF interventions exists in published research and the effects of CF seem to depend on the timing and dosage of the CF consumption. The doses of CF used in this dissertation are based on the outcomes of several studies, presented in Table 2.

Schramm et al. showed that CF intake leads to increase in plasma epicatechin concentrations, peaking 2 h post ingestion (Schramm et al. 2003). It has been shown that acute CF intake (450 mg CF, 494 mg CF) can improve CBF 2 h post-ingestion (Francis et al. 2006; Lamport et al. 2015). The administration of an acute dose of 917 mg CF elevated levels of circulating NO species, enhanced the FMD response and augmented microcirculation 2 h post-ingestion, and these effects were mimicked by ingestion of pure epicatechin, with maximum FMD effects occurring at 2 h after single-dose consumption (Schroeter et al. 2006). Acute intake of epicatechin (200 mg) increased plasma nitrite concentrations in healthy humans (Loke et al. 2008a). Moreover, acute CF intake (187 mg CF) lowered *in vivo* lipid peroxidation (F2-isoprostanes) after strenuous exercise (Wiswedel et al. 2004). More recently, Vlachojannis et al. performed a meta-analysis and found that CF intake with 100 mg epicatechin can reliably increase FMD (Vlachojannis et al. 2016). Based on these previous findings, we chose to administer a **single dose of 900 mg CF with 196 mg epicatechin** in the studies described in chapter 3, 5 and 6. In the study described in Chapter 4, we chose to use a **single dose of CF with 100 mg epicatechin**.

Table 2. Overview of studies on which we based our choice to administer a single dose of 900 mg CF with 196 mg epicatechin, a single dose of CF with 100 mg epicatechin, and 1 week of CF with 100 mg epicatechin.

Reference	CF intervention	Population	Outcome measure
Francis et al. 2006	Acute 450 mg CF	Healthy young subjects	Cerebral blood flow increased
Lamport et al. 2015	Acute 494 mg CF	Healthy older subjects	Cerebral blood flow increased
Schroeter et al. 2006	Acute 917 mg CF	Healthy young subjects	FMD increased NO species increased
Loke et al. 2008	Acute 200 mg epicatechin	Healthy men	Plasma nitrite increased
Vlachojannis et al. 2016	Acute 100 mg epicatechin Acute 900 mg CF	Review with healthy and diseased populations	FMD increased Blood pressure decreased
Wiswedel et al. 2004	Acute 187 mg CF	Non-smoking, untrained, healthy subjects	Lipid peroxidation decreased
Heiss et al. 2007	One week 918 mg CF	Smoking subjects	FMD increased Plasma nitrite increased
Grassi et al. 2015	One week 500 mg CF with 100 mg epicatechin	Healthy, non-smoking subjects	FMD increased Pulse wave velocity decreased Blood pressure decreased

In the pilot study of Heiss et al., one week CF intake (918 mg CF, 180 mg epicatechin) resulted in improved FMD and increased plasma nitrite concentrations (Heiss et al. 2007). Grassi et al. showed that 1 week CF dose-dependently improved FMD, decreased pulse wave velocity and blood pressure, and lowered ET-1 levels. Significant improvements in vascular function were already observed at a dose of 80 mg CF (16 mg epicatechin) per day. The maximal effects were seen at a dose of 500 mg CF with 100 mg epicatechin, while a higher dose of CF (800 mg CF with 168 mg epicatechin) per day did not add further improvements (Grassi et al. 2015). Therefore, we chose a 1 week CF intervention with 100 mg epicatechin in the study described in Chapter 7.

1.6.2 Environmental stress: hypoxia

When athletes train or compete at high altitude, the decreased arterial pressure of O_2 (PaO_2) and arterial saturation of O_2 (SaO_2) compromise tissue O_2 delivery. This places an extra burden on physical and cognitive performance (Petrassi et al. 2012). Therefore, there is a scientific and general interest in whether nutritional supplement intake could reduce the effect of acute hypoxia on cognitive and physical performance.

Since brain function and brain integrity are dependent on continuous O_2 supply, brain desaturation may result in impaired cognitive function in hypoxia (Ando et al. 2013). The severity of the impairment is related to the extent of high altitude, with at 3000 m (=14.3% O_2;=71% of O_2 available at sea level) psychomotor impairments starting to be visible (Yan 2014). Cerebral O_2 delivery is dependent on both the arterial O_2 concentration (CaO_2) and CBF, with the former directly influencing cerebral oxygenation and the latter being influenced by the complex sum of the opposing drives of cerebral vasodilation and vasoconstriction (Fan et al. 2013). Cerebral oxygenation, as measured by concentrations of oxyhemoglobin (ΔHbO_2), is lowered in hypoxia, with a concomitant increase in deoxyhemoglobin (ΔHHb). These parameters can be measured by NIRS, a non-invasive technique allowing for the continuous measure of ΔHbO_2 and ΔHHb concentrations in various tissues (Derosière et al. 2013).

During exercise in hypoxic conditions, exercise and hypoxemia both lead to cerebral vasodilation, but when hyperventilation occurs, the induced hypocapnia can lead to cerebral vasoconstriction. As a result of the opposing vasodilation and vasoconstriction, similar blood velocities at (sub)maximal exercise have been observed in the middle cerebral artery in normoxia and acute hypoxia (Subudhi et al. 2011). Since hypoxia does not only impair O_2 delivery to brain tissue, but also to muscle tissue, the decreased O_2 supply to and impaired oxidative energy production in the exercising muscle negatively affects exercise performance (Masschelein et al. 2012; Van Cutsem et al. 2015). Muscle tissue oxygenation seems to be lowered in hypoxia and exercise-induced drops in ΔHbO_2 and tissue oxygenation index (TOI) in the M. vastus lateralis are larger in hypoxia compared to normoxia during maximal cycling efforts (Masschelein et al. 2012; Bourdillon et al. 2014). Moreover, hypoxia-induced reductions in

cerebral oxygenation may favour central fatigue, i.e. the failure of the central nervous system to excite the motor neurons adequately, hence impairing exercise performance in hypoxic conditions (Amann and Calbet 2008).

Besides the aforementioned effects of altitude on O_2 delivery, hypoxia also results in increased levels of oxidative stress (Bakonyi and Radak 2004). Since it was previously shown that CF intake can reduce oxidative stress and can increase CBF and cerebral oxygenation, we aimed to investigate the potential of CF to reduce the impact of acute hypoxia. Young, healthy athletes have an optimal antioxidant defence system and vascular function. Exercise and/or cognitive performance under acute hypoxic stress might create a situation where the effects of CF could be more pronounced than in a normobaric environment. As athletes regularly train and compete at high altitude, examining the potential of CF to minimize the impact of hypoxia on cognitive and exercise performance is highly relevant as well (MacLeod et al. 2015).

1.6.3 Methods to measure exercise performance

Measuring endurance exercise performance should ideally simulate real-world conditions. However, to do so in a scientific controlled way is rather difficult and open for discussion. Time-to-exhaustion (TTE) and time trials (TT) are two traditional tests to measure exercise performance. In TTE, a subject has to continue a specific effort as long as possible, until exhaustion, without the need for pacing. However, the reliability and sensitivity of a TTE is rather low (with a high coefficient of variation (10%)). A TT, where athletes need to cover a certain amount of work as fast as possible (or perform as much work as possible in a certain time) and thus need to choose their pacing as well, has a lower coefficient of variation (1-5%) (Currell and Jeukendrup 2008). Therefore, we chose to use a TT as mode to measure exercise performance in well-trained athletes in this dissertation.

1.6.4 Methods to assess cognitive performance

Currently, a large number of tests evaluating different domains of cognitive functioning is available. Cognitive function categories include, but are not limited to, (long-and short term; verbal and visual) memory, reaction time, attention, information processing, executive function and crystallized intelligence (Chang et al. 2012). When designing experiments, it is of utmost importance to choose the most relevant cognitive test to answer the specific research question. It has been shown that especially executive function is positively affected by exercise and that exercise performance, on its turn, is dependent on executive function (Chang et al. 2012; Baker et al. 2014). Therefore, we used the Stroop task and Flanker test, both assessing executive function, in the studies of this dissertation. Moreover, as reaction time is another important factor influencing exercise performance, we used a simple reaction time test in studies where exercise and CF intake were combined. As we aimed to evaluate the effect of

CF intake on several aspects of cognitive performance during hypoxic exposure in young subjects, we chose to use a cognitive test battery, called Cognition, which was designed for a high-performing astronaut population and showed high sensitivity to multiple cognitive domains (Basner et al. 2015).

1.6.5 Methods to study the brain

Figure 8 shows the setup of the different methods to study the brain, used in this dissertation. Today, several non-invasive techniques exist to investigate human brain functioning. Electrophysiological neuroimaging modalities such as EEG, measure functional brain activity *directly* by detecting variations in electrical field, produced by neuronal activity across the scalp. These electrical signals arise from the simultaneous (post-synaptic) firing of neurons in response to stimuli. While EEG has a high temporal resolution, its spatial resolution is poor and restricted to the cortical layers under the scalp. Current brain imaging techniques, such as standardized low-resolution brain electromagnetic tomography (sLORETA), enable researchers to investigate electrocortical alterations within the entire brain. Moreover, EEG during a cognitive test also allows the measurement and interpretation of event-related potentials (ERP), i.e. latency and amplitude. ERPs are neuro-electric activation patterns that record specific events or cognitive processes and involve high temporal resolution and separated components allowing the investigation of intrinsic and distinct cognitive processes. The high sensitivity to motion-artefacts is one of the major drawbacks of classic EEG recording (Thompson et al. 2008).

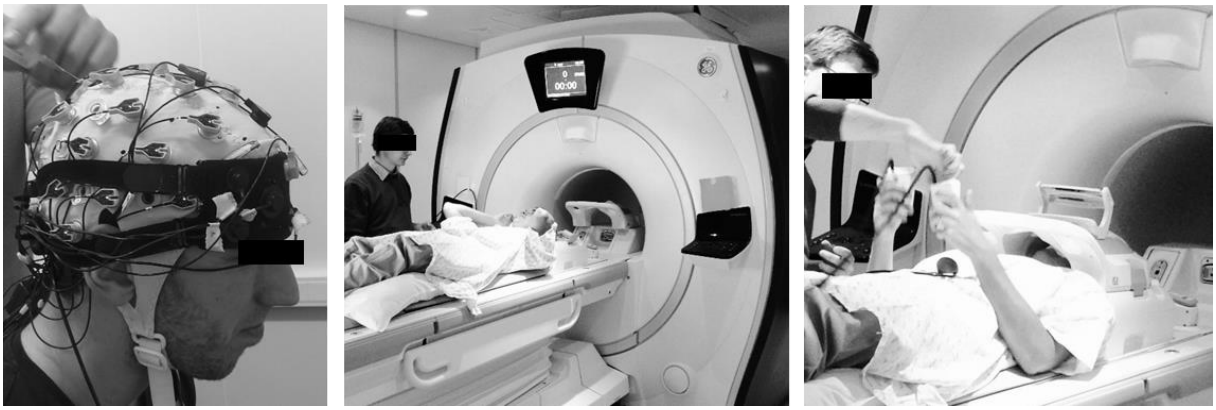

Figure 8. Methods to study the brain. *Left: Set-up to measure brain activity by EEG, concomitant with hemodynamic changes in the prefrontal cortex by fNIRS. Middle and right: Functional Magnetic Resonance Imaging (fMRI). Subjects enter the fMRI scanner in supine position, with their head "fixed" and are requested to lay still during the test. Subjects hold a button in each hand which they need to press in response to the stimuli during the cognitive test.*

Hemodynamic neuroimaging techniques, such as fNIRS and BOLD-fMRI measure changes in neuronal activation *indirectly* by assessing local increases in CBF subsequent to increased neuronal activity, the

so-called NVC. In response to neuronal activity, CBF is locally increased to ensure adequate delivery of O_2 and glucose to commensurate to the energy needs of the neurons. This NVC involves the interaction of neurons, glia and vascular cells; neurons and glia cells release several vasoactive factors, such as NO, prostacyclin and ET-1, initiating vasodilation. The vascular cells (endothelial cells, smooth muscle cells and pericytes) transduce this signal into carefully orchestrated vasodilation in the activated area during the period of activation (Girouard and Iadecola 2018). It is beyond the scope of this dissertation to discuss the exact mechanisms of NVC, but we refer to the review of Girouard and Iadecola (Girouard and Iadecola 2018) for a detailed overview on the regulation of the cerebral NVC. The importance of NVC for brain health has been demonstrated by numerous studies revealing that NVC is disrupted in several neurological pathologies, where CBF is no longer matched to the metabolic needs of the activated neurons. This neurovascular dysfunction is mostly due to deleterious actions of ROS, created by NADPH oxidase, on cerebral blood vessels (Girouard and Iadecola 2018).

Upon neuronal activity, the increase in local blood flow and O_2 delivery, or so-called hemodynamic response, strongly exceeds the cerebral metabolic demand of O_2. As a consequence, the concentration of oxygenated blood increases and concentration of de-oxygenated blood decreases in response to neuronal activity. These hemodynamic changes form the basis of the BOLD response in fMRI and changes in ΔHHb and ΔHbO_2 in fNIRS (Figure 9). In fNIRS, near-infrared light is introduced through the skull. HbO_2 and HHb absorb light at slightly different wavelengths (800–940 nm and 600–750 nm, respectively), allowing the measurement of their relative concentrations in the cerebral capillary blood (Perrey 2008). Whereas ΔHbO_2 and ΔHHb reflect the balance of O_2 delivery and extraction, the sum of both, total haemoglobin (ΔHb_{tot}) is an index of changes in regional blood volume. The BOLD-fMRI response is constituted by concentrations of Hb_{tot} and HHb. It is important to keep in mind that both the BOLD-fMRI response and fNIRS measure the hemodynamic response, which reflects the interplay between regional CBF, blood volume, and metabolic rate of O_2 and results from alterations in neuronal activity (Steinbrink et al. 2006).

fNIRS and fMRI both have low temporal resolution, since they are based on the “slower” haemodynamic changes in response to neuronal activity, and high spatial resolution. While fNIRS is restricted to cortical layers under the scalp, fMRI covers the entire brain with millimetre-level precision. However, fMRI requires the subject to lay still inside the scanner, which makes it almost impossible to use this technique in a real-life exercise-setting. In contrast, fNIRS is a relatively low-cost, non-invasive neuroimaging technique which is robust to motion and can therefore easily be applied during sports performance.

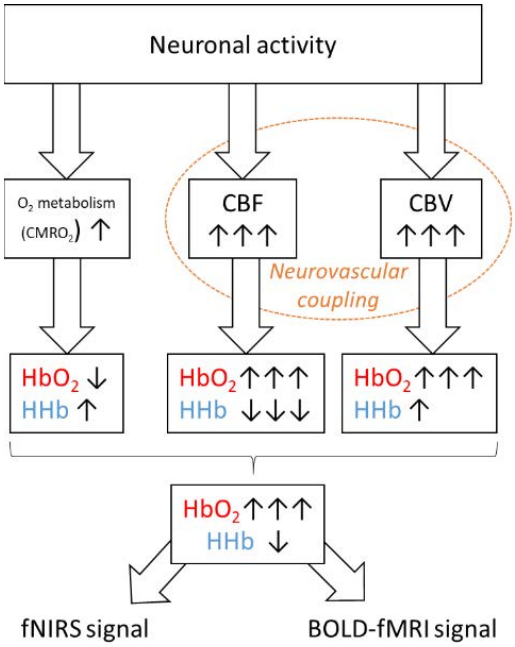

Figure 9. Physiology of the hemodynamic response during increased neuronal activity. *$CMRO_2$: cerebral metabolic rate of oxygen, CBF: cerebral blood flow, CBV: cerebral blood volume, HbO_2: oxy-hemoglobin concentration, HHb: deoxy-hemoglobin concentration, fNIRS: functional near infrared spectroscopy, BOLD: blood oxygenation level dependent, fMRI: functional magnetic resonance imaging. Based on Lindauer 2010.*

It is clear that each technique has its strengths and limitations and that the preferred method to be used in a study depends on the specific research question and research setting. However, by employing a multimodal neuroimaging approach of combining fNIRS, fMRI and/or EEG, further advances can be made in the understanding of precise functional specialization and inter-areal coupling contributing to better insights in the human brain function (Muthalib et al. 2013). The practical details on the installation and set-up, data acquisition and data analysis of EEG, fNIRS and BOLD-fMRI are provided in the chapters where these techniques are employed.

1.6.6 Methods to measure oxidative stress and NO metabolism

In this dissertation, we aim to measure the potential of CF to reduce ROS formation and oxidative stress elicited during exercise and hypoxic exposure. Free radicals are highly reactive and have a relative short half-life, making it extremely difficult to measure them directly *in vivo*. The golden standard of free radical assessment is electron spin resonance spectroscopy with spin traps, but this method is expensive and requires a lot of expertise. As a result, indirect measures, being oxidation products generated by the reaction of free radicals with biomolecules, are more commonly used to study the effects of antioxidants *in vivo*. The main molecular targets of free radicals are proteins, DNA and lipids (Greilberger et al. 2015).

A countless number of different redox biomarkers exists, but biomarkers may not always respond to the oxidative pathway of a specific oxidant stimulus. Therefore, and because no “ideal biomarker” exists, it

is advisable to measure not just one biomarker, but to choose a set of redox biomarkers, based on the hypothesis and experimental setup of the study (Vassalle et al. 2015). The multistep reaction of free radicals with lipids found in cell membranes (mainly polyunsaturated fatty acids) but also in cytosol (triacylglycerides), is called lipid peroxidation and includes initiation, propagation and termination steps. The two most popular biomarkers for the detection of lipid peroxidation are thiobarbituric acid (TBA)-reactive substances (TBARS) and isoprostanes. While TBARS *in vivo* are not always related to lipid peroxidation, the measurement of TBA_2-malonaldehydes (MDA) in plasma by means of high pressure liquid chromatography (HPLC) is highly specific. MDA is formed by β scission of peroxidised, mainly arachidonic acids (Lepage et al. 1991). F2-isoprostanes are the most commonly measured isoprostanes and can be measured by HPLC and by immunoassay kits, but the 2 methods do not measure the same compounds. The drawback of isoprostanes is their instability in plasma with a short half-life of only 20 min. Therefore, we chose to use MDA as biomarker of lipid peroxidation in plasma samples.

Antioxidants play an important role in preventing oxidative stress, and knowing the antioxidant capacity of a nutritional antioxidant *in vivo* is important for dietary intervention recommendations. One of the most frequently used methods for the detection of the antioxidant capacity is the trolox equivalent antioxidative capacity (TEAC) assay. In this assay, 2,2'-azinobis(3-ethylbenzothiazoline 6-sulphonate) (ABTS) is incubated with peroxidase and H_2O_2. The suppression of the absorbance of radical cations of ABTS by antioxidants in the sample is measured, as antioxidants quench ABTS radicals in a non-linear dose-response way (Greilberger et al. 2015). UA, a strong scavenger of free radicals, accounts for most of the antioxidant ability in plasma and contributes to more than 60% of the total plasma antioxidant capacity. UA is also upregulated by exercise, as a result of an elevated energy-rich purine phosphate catabolism. Hence, UA plasma concentrations were determined as well and TEAC was corrected for UA (Jówko et al. 2014).

NO is another free radical with a half-life of only a few seconds, which functions as an intracellular messenger. NO does not only act locally, but its spatial distribution in tissue is large. Because of its high solubility in hydrophobic environments, it diffuses freely across biological membranes and can signal many cell diameters away from where it is generated (Vanhoutte et al. 2017). NO can be directly measured in isolated tissue preparations and cell suspensions by electron paramagnetic resonance spectroscopy with spin traps. However, in plasma, NO is rapidly oxidized to nitrite and nitrate through reactions with oxyheme proteins such as oxyhemoglobin. NO also transfers into erythrocytes, which controls plasma availability and diffusion distance of endothelial-derived NO (Rochette et al. 2013). Given the extremely short half-life, biochemical quantification of endothelial NO formation in plasma is hampered so far. Still, two direct methods claimed to provide real-time estimates of the *in vivo* NO concentrations by the use of a protoporphyrinic probe (electrochemical microsensor) which was inserted in a blood vessel in the hand (Siervo et al. 2011), and a microgas analyser (using direct wet scrubbing

and fluorescence detection) measuring NO in breath (Toda et al. 2009). Indirect methods measure other molecules which are correlated with NO bioavailability. These indirect methods include isotopic methods, using intravenous or oral administration of stable isotope tracers, laboratory analyses of nitrite, nitrate, L-arginine or cGMP in serum or plasma, and clinical methods. It has been shown that serum nitrite sensitively reflects changes in endothelial NO formation in human forearm circulation (Kelm et al. 1999). Currently, clinical methods such as FMD still seem to be the most accurate methods for the assessment of NO bioactivity (and endothelial function) (Siervo et al. 2011). During FMD, the diameter of the brachial artery is measured using ultrasound before and after a transient period of forearm ischemia, which induces shear stress and endothelium-dependent dilation. In this dissertation, both a clinical method (FMD) and laboratory analyses of serum/plasma nitrite, nitrate, ADMA, arginine and citrulline were used to assess NO availability and vasodilation after CF intake.

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Chapter 2. Cocoa flavanol supplementation and exercise: a systematic review

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SYSTEMATIC REVIEW

Cocoa Flavanol Supplementation and Exercise: A Systematic Review

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The manuscript published in Sports Medicine was slightly adapted for this chapter. The manuscript of Decroix et al. 2017 was excluded from the results, as this research will be presented as a separate chapter (Chapter 3) in this dissertation.

2.1 Abstract

Background: Cocoa flavanols (CF) have antioxidant and anti-inflammatory capacities and can improve vascular function. It has recently been suggested that CF intake may improve exercise performance and recovery. This systematic review aimed to evaluate the literature on the effects of CF intake on exercise performance and recovery and exercise-induced changes in vascular function, cognitive function, oxidative stress, inflammation and metabolic parameters.

Methods: Two electronic databases (Pubmed and Web of Science) were searched for studies examining the combination of CF intake and exercise in humans (until March 28th 2017). Articles were included when exact amount of CF were mentioned. The methodological quality and level of bias of the 13 included studies was assessed by the checklist for randomized controlled trials from the Dutch Cochrane centre.

Results: Acute, sub-chronic (2 weeks) and chronic (3 months) CF intake reduced exercise-induced oxidative stress. Evidence on the effect of CF on exercise-induced inflammation and platelet activation was scarce. Acute CF intake reduced and tempered the exercise-induced increase in blood pressure in obese participants. Acute and sub-chronic CF intake altered fat and carbohydrate metabolism during exercise. Acute and sub-chronic CF intake did not have ergogenic effects in athletes, while chronic CF intake improved mitochondrial efficiency in untrained participants. While combining sub-chronic CF intake and exercise training improved cardiovascular risk factors and vascular function, evidence on the synergistic effects of CF and exercise training on oxidative stress, inflammation and fat and glucose metabolism was lacking.

Conclusion: CF intake may improve vascular function, reduce exercise-induced oxidative stress and alter fat and carbohydrate utilization during exercise, but without affecting exercise performance. There is a strong need for future studies examining the synergetic effect of chronic CF intake and exercise training.

Key points

- Acute and sub-chronic CF intake can reduce exercise-induced oxidative stress and alter carbohydrate and fat metabolism during exercise in trained participants, but does not improve exercise performance
- Combining sub-chronic CF intake and exercise training improves cardiovascular risk factors and vascular function in both healthy and overweight participants
- There is a strong need for future studies examining the synergetic effect of chronic CF intake and exercise training on oxidative stress, inflammation and fat and glucose metabolism

2.2 Introduction

Athletes believe that nutritional supplements can enhance performance and recovery by reducing muscle damage, immune dysfunction, oxidative stress and fatigue (Pingitore et al. 2015). Nevertheless, much debate remains on the effectiveness of most supplements, including polyphenols (Myburgh 2014). Polyphenols are plant-based micronutrients, characterized by the presence of many phenol structural units with potential health benefits. Flavonoids are polyphenols consisting of 2 phenyl rings and a heterocyclic ring and include several subgroups as flavanols, flavonols, isoflavones, flavones and anthocyanidins. Recently, a large body of research has focussed on the beneficial health effects of flavanols, which are present in many types of food including cocoa, wine, tea, fruits and vegetables. Cocoa is derived from seeds of the fruit of the Theobroma cacao tree and certain cocoas can be manufactured to be extra rich in flavanols (Holt et al. 2002). It is known that cocoa flavanols (CF) stimulate nitric oxide (NO) production resulting in improved vasodilation and endothelial function and reduced blood pressure (BP). Moreover, CF can reduce platelet activity and aggregation (Ferri et al. 2015). In addition, CF protect against oxidative stress and inflammation. It has also been shown that CF can improve insulin sensitivity and lipid profiles in participants with or without cardiovascular risks (Heiss et al. 2005; Davison et al. 2008; Berry et al. 2010; Katz et al. 2011; Flammer et al. 2012). Furthermore, CF can cross the blood brain barrier (Wu et al. 2012) and increase cerebral blood flow in healthy young participants (Francis et al. 2006). Cocoa contains the monomeric CF epicatechin and catechin, and oligomeric procyanidins. (−)-Epicatechin, the most commonly found CF monomer, seems primarily responsible for all of these above mentioned beneficial effects (Schroeter et al. 2006). Indeed, it has been shown that ingestion of pure (−)-epicatechin mimics vascular effects observed after CF consumption (Schroeter et al. 2006), that (−)-epicatechin, and not catechin, is capable of mediating vasodilatation *in vivo* (Ottaviani et al. 2011) and that (−)-epicatechin increases NO production (Ramirez-Sanchez et al. 2010).

Not surprisingly, CF gained the attention of exercise physiologists, because endurance-type exercise also exerts beneficial effects on the same parameters that are influenced by CF intake. Thus, combining physical activity with CF intake might be a successful strategy to prevent cardiovascular diseases, insulin resistance and other age-related disorders. Nevertheless, whether CF intake and exercise have additive effects on oxidative stress, inflammation and vascular function is not yet clear. In mice, (−)- epicatechin-rich cocoa supplementation increased exercise performance and muscle fatigue resistance, which was paralleled by increased oxidative phosphorylation-complex proteins and capillarity (Nogueira et al. 2011). The question rises whether CF intake also has ergogenic effects in humans. This hypothesis is mainly based on the capacity of CF to increase NO bioavailability, mediated through elevated NO generation as a consequence of higher cellular levels of active endothelial NO

synthase (eNOS) and diminished NO elimination via peroxynitrite formation, caused by the inhibition of NADPH-oxidase (Schewe et al. 2008). NO is a major vasodilator and thus, an increase (physiological) level of NO is expected to increase blood flow to the muscle, allowing for improved nutrient and oxygen delivery and waste removal (Jobgen et al. 2006). Moreover, NO is involved in muscle contractility and mitochondrial biogenesis and respiration through upregulation of 5' AMP-activated protein kinase (AMPK) and downstream peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Jones 2014). Thus, augmenting NO bioavailability by CF intake is expected to lead to enhanced mitochondrial efficiency and enhance muscle function (Bescós et al. 2012). Besides, CF intake can improve executive function (decision making) and motor control through its beneficial vascular effect and thus increase cerebrovascular blood flow (acute intake), and through interaction with critical protein and lipid kinase signalling cascades in the brain, leading to promotion of neuronal survival and synaptic plasticity (chronic intake) (Vauzour et al. 2008). It is clear that improving executive function and motor control are two important factors influencing sports performance (Baker et al. 2014).

Because of their antioxidant capacities, CF can reduce reactive oxygen species (ROS) formation (e.g. superoxide) and repress the formation of peroxynitrite. Heavy exercise can lead to increased mitochondrial formation of reactive oxygen species (ROS) (Bentley et al. 2012) and increased levels of inflammation (Malaguti et al. 2013). Although moderate levels of ROS initiate and promote adaptation to exercise training (Peternelj and Coombes 2011), excessive levels of ROS have detrimental effects of cell structure and function. When increased levels of ROS exceed the antioxidant capacity of our body, the body's homeostatic balance is disturbed, and as a result oxidative stress occurs. The large amount of ROS can then interact with lipids, proteins, and DNA and may cause oxidative damage to the mitochondria and muscle contractile proteins. It seems plausible that this also results in muscular damage and fatigue after exercise (Bentley et al. 2015). Hence, CF intake might improve exercise performance and recovery through increasing antioxidant capacity and reducing oxidative stress. Nevertheless, equivocal results have been reported on the effects of CF to modulate exercise-induced oxidative stress and/or exercise performance (Wiswedel et al. 2004; Fraga et al. 2005; Allgrove et al. 2011; Davison et al. 2012; Stellingwerff et al. 2014; Patel et al. 2015; González-Garrido et al. 2015; Taub et al. 2016)(Wiswedel et al. 2004; Fraga et al. 2005; Allgrove et al. 2011a; Davison et al. 2012; Stellingwerff et al. 2014; Patel et al. 2015; González-Garrido et al. 2015; Taub et al. 2016).

Because of the recent growing body of literature describing the effects of CF in an exercise setting, the aims of this systematic review were to examine the effect of CF intake on *(i)* exercise performance and recovery and *(ii)* acute and chronic exercise-induced changes in vascular function, cognitive function, oxidative stress, inflammation and carbohydrate and lipoprotein metabolism.

2.3 Materials & Methods

This systematic review was written according to the guidelines of the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement, which is an evidence-based protocol describing a set of items for reporting in systematic reviews and meta-analyses (Liberati et al. 2009).

Data sources and search strategy. The overall goal of the current systematic review was to examine the effects of CF intake (intervention) and exercise (intervention) on a variety of outcome parameters, such as: exercise and cognitive performance, markers of oxidative stress and inflammation, and metabolic and (cardio) vascular parameters (outcome) in humans (participants). Therefore, two electronic databases were consulted: *Pubmed* and *ISI Web of Knowledge*. Key terms (and synonyms searched through the MeSH database) that were included and combined were: 'exercise', 'exercise performance', 'polyphenols', 'flavanols', 'epicatechin' and 'cocoa'. The final search was carried out on 28 March 2017. The search strategies were combined, and duplicates were removed by Endnote and manually by two of the authors, LD and CT.

Study selection process. Studies in this systematic review needed to fulfill the following inclusion criteria: 1) research conducted with human participants, 2) original data on CF supplementation *with* an acute or long-term exercise intervention (RCT, case control, cross-over studies), 3) no severe methodological deficiencies and 4) published before March 2017. Exclusion criteria were: 1) studies not mentioning the exact amount of CF (e.g. carbohydrate (CHO)/protein ratio chocolate recovery milk drinks), 2) studies written in languages other than English, 3) animals studies, 4) congress or workshop publications, 5) studies in which no exercise was performed, 6) studies in which no supplementation was given. No limits were used concerning the year of publication. Inclusion or exclusion of articles was performed by applying the above criteria on the title and abstract in a first screening and on full texts in a second screening. Case studies and reviews were excluded, although the bibliographies of the latter were consulted. The university's library, hand searches, electronical databases and contact with the authors (by mail) were used for the extraction the manuscripts.

Data extraction, synthesis and report. All data concerning exercise performance (heart rate (HR), rating of perceived exertion (RPE), maximal oxygen uptake (VO_{2max}), respiratory exchange ratio (RER), time to exhaustion (TTE), time trial (TT) performance), oxidative stress (total antioxidant capacity (TAC), vitamin C, vitamin E, Uric Acid (UA), malondialdehyde (MDA), thiobarbituric acid-reactive substances (TBARS), plasma free F2-isoprostane), inflammatory markers (cytokines: interleukin (IL) 6, 10, 1r and creatine kinase (CK)) and measurements of cognitive performance (reaction times, accuracy) were extracted out of the research papers and are shown in Table 4 to 7. Moreover, we calculated Hedge's effect sizes (g) of some parameters reported in Tables 4 to 7 to highlight the practical significance of the effects and to compare the results of the different studies. Hedge's effect sizes were only calculated for

parameters for which the numerical values were presented in the original investigation. Hedge's effect sizes were considered small when <0.2, moderate when <0.8 and large when >0.8.

Methodological quality assessment of the individual studies. To assess the methodological quality of the selected studies, the Dutch Cochrane Centre (CBO)-checklist for randomized controlled trials from the Dutch Cochrane center was used (NICE 2009). Each paper was assessed twice by 2 independent reviewers (CT and LD) with oral discussions if disagreements were obtained. A positive answer was given 1 point, a negative answer resulted in no points and a '?' was ascribed if unknown; resulting in a maximum score of 14. A score of ≥75% indicated strong quality and very low risk of bias, a score of 55–75% indicated moderate quality and low risk of bias, and a score≤55% indicated weak quality and a high risk of bias. According to the guidelines of the National Institute for Health and Clinical Excellence, a level of evidence was assigned for each paper based on the study design and risk of bias (National Institute for Health and Clinical Excellence 2006). Moreover, we screened whether the included articles used an a priori power analysis (with minimal level of power=0.8) to calculate the sample size needed to observe a statistically significant result with a desired likelihood.

2.4 Results

Twelve studies, including a total of 228 participants (70 overweight untrained, 35 untrained, 123 well-trained), fulfilled the in-and exclusion criteria and examined either the effects of CF intake on exercise performance and/or exercise-induced changes in oxidative stress, inflammation, vascular function, metabolic factors and cognitive function. All studies used a significance level of $p<0.05$.

2.4.1 Study selection and characteristics

Figure 10 shows the development of the literature screening and the reasons for inclusion or exclusion. An initial raw screening using the listed search terms resulted in a selection of 490 published studies. Removal of duplicates and a more detailed screening of titles, abstracts and full-texts led to a selection of 15 studies. Two additional studies were excluded because insufficient data were presented on the exact amount of flavanols included in the supplementation. The study of Decroix et al. (2016) was excluded in this chapter as it will be presented as a separate chapter further in this dissertation. Twelve studies ultimately met our inclusion and exclusion criteria for determining the effects of CF supplementation and exercise on a variety of outcome measurements. According to the guidelines of the National Institute for Health and Clinical Excellence, levels of evidence were assigned to all the included studies based on the study design and risk of bias (National Institute for Health and Clinical Excellence 2006). All included studies were randomized controlled trials, indicating the highest level of evidence (National Institute for Health and Clinical Excellence 2006). The quality assessment scores, assessing risk of selection, performance, attrition and detection bias, ranged from 9 to 13 out of 14 (Table 3). This indicated that 5 studies (Fraga et al. 2005; Allgrove et al. 2011; Stellingwerff et al. 2014; Peschek et al. 2014; Patel et al. 2015) were of moderate quality with a low risk of bias (level of evidence 1+) and 7 studies (Wiswedel et al. 2004; Singh et al. 2006; Davison et al. 2008, 2012; Berry et al. 2010; Taub et al. 2012; Soleimani et al. 2013) were of high quality with a very low risk of bias (level of evidence 1++) (National Institute for Health and Clinical Excellence 2006). Only 2 of the 12 studies performed an a priori power calculation to calculate the sample size (Table 3). All included studies declared that there were no conflicts of interest.

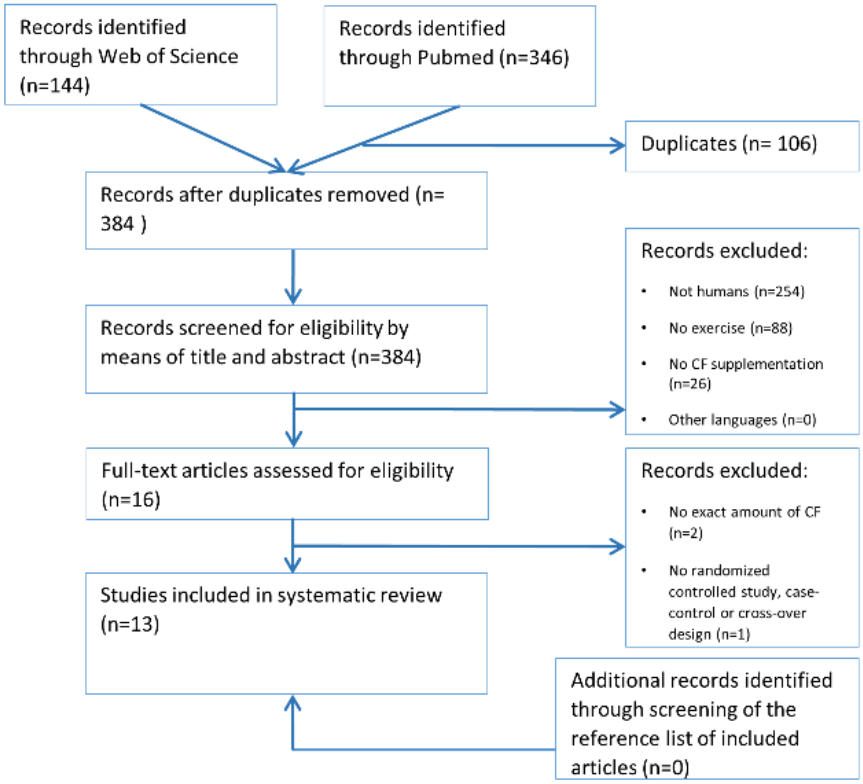

Figure 10. Selection process for research articles (n=13) included in this systematic review. *Adapted version of the recommendations in the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) statement (Liberati et al. 2009). (In this chapter, only 12/13 included studies were discussed, as the 13th study is described as a separate chapter in this dissertation.)*

Table 3. Quality assessment of the included studies according to the Methodology checklist for randomized controlled trials of the National Institute for Health and Clinical Excellence.

Reference	**Quotation score**															**A priori power analysis**
	Selection bias		Performance bias				Attrition bias			Detection bias						
	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3	D4	D5	Rating (/14)*	
(Berry et al. 2010)	1	?	1	1	1	1	1	1	1	1	1	1	1	1	13 strong	No
(Davison et al. 2008)	1	?	1	1	1	1	1	1	1	1	1	1	1	1	13 strong	No
(Fraga et al. 2005)	1	?	1	1	0	0	1	1	1	1	1	1	0	0	9 moderate	No
(Patel et al. 2015)	1	?	1	1	1	0	1	1	1	1	1	1	0	0	10 moderate	No
(Singh et al. 2006)	1	?	1	1	1	1	1	1	1	1	1	1	1	1	13 strong	No
(Soleimani et al. 2013)	1	?	1	1	1	1	1	1	1	1	1	1	1	1	13 strong	No
(Allgrove et al. 2011)	1	?	1	1	1	0	1	1	1	1	1	1	0	0	10 moderate	No
(Davison et al. 2012)	1	1	1	1	0	1	1	1	1	1	1	1	1	1	13 strong	No
(Wiswedel et al. 2004)	1	?	1	1	1	1	1	1	1	1	1	1	1	1	13 strong	No
(Taub et al. 2016)	1	?	1	1	1	1	1	1	1	1	1	1	1	1	13	Yes
(Stellingwerff et al. 2014)	1	?	1	1	0	0	1	1	1	1	1	1	0	0	9 moderate	No
(Peschek et al. 2014)	1	?	1	1	1	0	1	1	1	1	1	?	0	0	9	Yes
	Low bias (2,1/3)		Low bias (2,4/3)				Low bias (3/3)			Low bias (4,2/5)						

*Quality scores: ≥75% strong, 55–75%: moderate, ≤55% weak

Six studies displayed the effects of CF and exercise on vascular function, 6 studies demonstrated the effects on oxidative stress and inflammation and 5 studies gave information on metabolic factors. Seven studies examined effects on physical performance and one study on cognitive performance. Details from these selected studies are shown in tables 4 to 7.

2.4.2 CF and exercise-induced oxidative stress and inflammation

A total of 6 studies looked into the effects of CF supplementation and exercise on parameters of oxidative stress (Wiswedel et al. 2004; Fraga et al. 2005; Singh et al. 2006; Allgrove et al. 2011; Davison et al. 2012; Taub et al. 2016). Two studies analysed the effects of acute intake of CF, 2 h prior to an exercise bout. Davison et al. (2012) found that a single supplementation of 248 mg CF (39.1 mg epicatechin) resulted in increased total antioxidant status (TAS) pre-exercise compared to baseline or to PL in trained men. Moreover, the increase in free F2-isoprostanes after prolonged steady state (SS) exercise (2.5 h cycling at 60% VO_{2max}) tended to be lower due to CF intake. The exercise-induced increase in vitamin C was not different between the two trials. Wiswedel et al. (2004) found that the acute intake of 186 mg CF (no details on epicatechin) tempered the increase in F2-isoprostane and relative MDA after 29 min cycling in healthy untrained men, while they did not observe any differences in TAC. Allgrove et al. (2011) studied the effects of a 2-week intake of 40 g of dark chocolate (38.7 mg epicatechin) or PL in 20 male participants. On the testing day, participants consumed a 'double dose' 2 h before exercise. Exercise consisted of a SS cycling exercise at 60% VO_{2max} for 1.5 hr, in which every 10 min the exercise intensity increased until 90% VO_2max for 3s, followed by a time-to-exhaustion trial (90% VO_{2max}). CF intake caused a significant smaller increase in (free) F2-isoprostane, oxidized low-density lipoproteins (LDLs) and F2-isoprostane after the time-to-exhaustion trial and 1 h post-recovery compared to PL. Singh et al. (2006) examined the effects of 7-day CF (240 mg CF) or PL supplementation in 8 healthy trained and 8 healthy untrained participants. They did not find any differences between CF and PL in TAS, before 1 h of cycling exercise at 70% VO_{2max}. Fraga et al. (2005) examined the combined effects of 2-week CF (or PL) intake (186 mg CF or <5 mg CF) and exercise training (3x football/week) in 28 healthy male football players on oxidative stress. This study demonstrated a 12% decrease in MDA after 14 days of CF intake compared to baseline, in contrast with a 10% increase in MDA in the PL trial. Moreover, after 2 week CF intake, urate was decreased and vitamin E/cholesterol and β-carotene were increased, while these parameters did not change after PL intake. However, no difference between PL and CF was found for plasma TAC, vitamin E, lycopene and co-enzyme Q10. In the study of Taub et al. (2016), 3 months of CF intake (175 mg CF, 26 mg epicatechin) resulted in an increased ratio of reduced vs. oxidized gluthathione (GSH:GSSG) and decreased protein carbonylation in the M. vastus lateralis compared to PL in untrained men at rest. Only the results of the study of Taub et al. showed a large effect size, while all other reported improvements had small effects.

Only 2 studies examined the effect of CF intake on exercise-induced inflammation. Neither the study of Allgrove et al. (2011) nor the study of Davison et al. (2012), found effects of (acute nor sub-chronic) CF supplementation on exercise-induced increases in leukocyte count, neutrophil count, plasma IL-6 and IL-10 concentrations.

2.4.3 CF, exercise and vascular function

Six studies examined the effects of CF and exercise on vascular function using different techniques in obese and healthy participants: *(i)* BP (Fraga et al. 2005; Davison et al. 2008; Berry et al. 2010; Patel et al. 2015), *(ii)* Flow mediated dilation (FMD) (Davison et al. 2008; Berry et al. 2010) and *(iii)* platelet count and volume (Singh et al. 2006; Soleimani et al. 2013).

The acute intake of CF (701 mg, 139 mg epicatechin) increased FMD and tempered the exercise-induced increase in mean BP (by 14%) and diastolic BP (by 68%) compared to PL in 21 overweight or obese men and women (Berry et al. 2010). Davison et al. (2008) looked at the concomitant effects of 12-week exercise training and CF supplementation on FMD and BP in overweight or obese participants. The four interventional groups included in this study were *(i)* CF (=902 mg), *(ii)* CF + exercise (12 weeks, 3 x 45 min at 75% of the predicted HR_{max}), *(iii)* PL (=36 mg); and (iv) PL + exercise. Exercise alone neither improved vascular function nor decreased BP. In contrast, 12-week CF intake resulted in a decreased diastolic BP and mean BP and enhanced vascular function (increase in FMD (1.6%)), while exercise training did not augment this effect.

The acute intake of 5 mg/kg CF (~360 mg) 2 h prior to an exhaustive 'Bruce aerobic exercise test' decreased blood platelets, mean platelet volume (MPV) and platelet distribution width (PDW) in healthy, young, male soccer players compared to PL (Soleimani et al. 2013). On the other hand, no beneficial effects of 7-day CF intake (240 mg) on acute exercise-induced changes in total platelet count and MPV were found in 16 healthy men (8 trained; 8 untrained) (Singh et al. 2006).

The effect sizes of the beneficial influence of CF intake on BP, FMD, cerebral blood flow and platelet function reported in the studies of Berry, Davison, Fraga and Soleimani were large.

2.4.4 CF, exercise and carbohydrate and lipoprotein metabolism

Five studies examined lipid and/or carbohydrate metabolism in response to exercise and CF intake. Two studies examined the acute and sub-chronic effects of CF on carbohydrate and fat metabolism during exercise (Allgrove et al. 2011; Stellingwerff et al. 2014). Two studies examined the effects of a combined intervention of CF intake and exercise training on body composition and carbohydrate metabolism at rest in well-trained and untrained participants (Fraga et al. 2005; Davison et al. 2008).

The study of Taub et al. (2012) examined the effect of 3-month CF intake on skeletal muscle metabolic changes at rest (prior to a cycling test).

Stellingwerff et al. (2014) found significant changes in carbohydrate and fat metabolism during an acute exercise, consisting of a cycling SS (2.5 h) and 15' TT, after acute CF intake (262 mg CF, 89 mg epicatechin) compared to low CF intake. These authors demonstrated an increase in glucose concentration and decrease in glucagon during exercise, with a concomitant increase in insulin concentration during recovery. However, the effect sizes were small. Allgrove et al. (2011) found a 21% increase in free fatty acids (FFA) during exercise after a 2-week CF intake (98.7 mg CF, 77.4 mg epicatechin) compared to PL in healthy participants. Levels of glucose, insulin, glucagon or cortisol were not affected.

Two studies examined the effects of sub-chronic CF intake in combination with exercise training. Fraga et al. (2005) found that 2-week CF intake (186 mg) in combination with football training decreased cholesterol and LDL cholesterol, while this effect was not evident after PL intake. However, the effect size was small. In a population of overweight participants, Davison et al. (2012) found that 2-week CF supplementation in combination with or without exercise training (3 x 45 min per week) improved insulin resistance. Exercise alone led to increased fat oxidation and decreased abdominal body fat, while CF intake did not augment these effects and did not affect these parameters in the absence of exercise. In contrast, insulin resistance was lowered by CF intake, but this improvement was not altered by concomitant exercise training.

Taub et al. (2016) aimed to investigate the effects of 3-month CF intake, without exercise training, on cardio metabolic changes, skeletal muscle metabolic changes and exercise performance in sedentary untrained participants. Three-month CF intake (175 mg CF, 26 mg epicatechin) resulted in increased high density lipoprotein (HDL) cholesterol and decreased triglycerides (TG), while glucose, cholesterol, LDL, CRP and Hemoglobin-A1c (HBA1c) were not altered (Taub et al. 2016). Moreover, the authors found that the expression and activation of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and its upstream regulators AMP-activated protein kinase α (AMPK) and Liver kinase B1 (LKB-1) in the muscle were upregulated by CF intake. CF intake did not affect mitochondrial volume, but increased citrate synthase, a marker of mitochondrial function. These results had a large effect size.

2.4.5 CF and exercise performance and recovery

Seven studies examined the effect of acute (2 h pre-exercise), sub-chronic (2 weeks) or chronic (3 months) CF supplementation on exercise performance in healthy participants (Fraga et al. 2005; Allgrove et al. 2011; Davison et al. 2012; Stellingwerff et al. 2014; Peschek et al. 2014; Patel et al. 2015; Taub et al. 2016). The acute intake of CF (ranging from 246.8-262 mg CF and 89-97 mg epicatechin)

2h pre-exercise did not result in any significant changes in TT performance (15' TT after 2.5 h SS cycling), HR, RPE, VO_{2max} and RER during SS exercise in trained athletes (Davison et al. 2012; Stellingwerff et al. 2014). Only one randomized, single blinded cross-over study examined the effects of acute CF supplementation (350 mg CF/serving; no details on epicatechin) on exercise recovery by examining a 5-km running TT, 48 h after a downhill running protocol which induced muscle soreness, in a rather small population of 8 healthy endurance trained athletes (Peschek et al. 2014). However, these authors could not find any significant improvements in running performance, whole blood CK-concentration, muscle function and self-perceived muscle soreness.

Two-week intake of CF (256 mg CF, 46 mg epicatechin) resulted in an 11% increase of gas exchange threshold compared to the low CF placebo (PL) during a VO_{2max} test in 9 moderate trained healthy men (Patel et al. 2015). Moreover, a 6% increase in 2' cycling TT performance compared to baseline and PL was shown. However, the authors could not find further significant changes in O_2 cost, VO_{2max}, RER and lactate concentrations during the 20' cycling SS preceding the 2' TT (Patel et al. 2015). Allgrove et al. (2011) did not detect any changes in HR, RPE, VO_{2max} during a 1.5 h SS and performance on the subsequent time-to-exhaustion test, following 2-week CF intake (98.7 mg CF, 77.2 mg epicatechin). Fraga et al. (2005) did not find any beneficial effects on the VO_{2max} during a shuttle run test in trained football players after 2 weeks of CF intake (186 mg CF, 39 mg epicachin + catechin) in trained athletes. In contrast, chronic intake (3 months) of CF (26 mg epicatechin) increased VO_{2max} and power output during an incremental cycle test in untrained participants (Taub et al. 2016).

Table 4. The effects of cocoa and exercise on vascular function.

Reference	Study design	Participants	Exercise protocol	Cocoa intervention	Measurements	Outcomes
(Berry et al. 2010)	Randomized, double blind, placebo controlled, cross-over	13 ♂, 8 ♀ overweight or obese Age: 54.9±2.2 yrs BMI: 31.6±0.8 kg/m_2 BP: 134/87 mm Hg	Acute Cycling 10' at 75% age predicted HR_{max}	Acute (2 h pre FMD+ exercise) Liquid <u>HCF</u> *F*: 701 mg *(E: 139 mg; C: 39 mg; PC: 523 mg)* *Cf*: 27.2 mg, *T*: 307.0 mg <u>LCF</u> *F*: 22 mg *(E: 0 mg; C: 9 mg; PC: 13 mg)* *Cf*: 31.1 mg, *T:* 268.1 mg *Wash out: 3-7d*	Pre and post-exercise a) BP b) FMD	a) Pre-exe: HCF=LCF, **Response to exercise** (AUC): DBP: ↑ (HCF)<(68%) ↑ (LCF) (g: 1.63) MBP: ↑ (HCF)<(14%) ↑ (LCF) (g: 1.24) b) **HCF (6.1%) > LCF (3.4%)**
(Davison et al. 2008)	Randomized, double blind, placebo-controlled 4 groups: (1) HCF , (2) HCF + EX, (3) LCF, (4) LCF + EX	18 ♂, 31 ♀ overweight or obese Age: 18-65 BMI=33.5 kg/m2 BP=123/76 mm Hg HOMA2 : 2.4 FMD=4.3%	Chronic: 12 weeks Cycling 3 x 45'/week at 75% age predicted HR_{max}	Chronic (12 weeks) + acute Liquid <u>HCF</u> *F*: 902 mg (E + C + PC unspecified) *Cf:* 36 mg, *T*: 674 mg <u>LCF</u> *F*: 36 mg (E + C + PC unspecified) *Cf*: 42 mg , *T*: 654 mg	Acute: 2h post intake Chronic: after 12 weeks a) BP b) FMD	a) sub-chronic: DBP and MBP: **HCF<LCF (g: 0.19; g: 1.90)** **no effect of exe** b) **acute: HCF > LCF (2.4%)** **sub-chronic: HCF > LCF (1.6%)** **no effect of exe**

Reference	**Study design**	**Participants**	**Exercise protocol**	**Cocoa intervention**	**Measurements**	**Outcomes**
(Fraga et al. 2005)	Randomized, not blind, counterbalanced cross-over	28 ♂ Age : 18±1 yrs BM: 74±1 kg BMI: 24.1±0.2 kg/m_2	Sub-chronic Footbal 2/week training + 1/week match	Sub-chronic (14 days) Solid HCF (105g) F: 186 mg (C + E: 39 mg; PC: 126 mg) Cf: 17.8 mg, T: 178.5 mg LCF (105 white chocolate) F: <5 mg No wash out	Before and after 14 days intake DBP and MBP	**↓ in HCF ⇔=in LCF after 2 weeks (g: 2.0)**
(Patel et al. 2015)	Randomized, single blind, cross-over	9 ♂ (moderately trained) Age: 21±1 BM: 76±9.3 kg Length: 177±9.4 cm VO_{2max}: 41.89±5.4 ml/kg/min	Acute Cycling VO_{2max}-test + 20' 80% gas exchange threshold (SS) + 2' TT	Sub-chronic (14 days) Solid HCF (dark chocolate) 40 g (12887 kJ) *F: 259 mg (E:* 46 mg; *PC:* 213 mg) LCF (white chocolate) (12945 kJ) Wash out: 2 weeks	a) DBP + SBP	a) HCF=control

Reference	Study design	Participants	Exercise protocol	Cocoa intervention	Measurements	Outcomes
(Singh et al. 2006)	Randomized, double blind, cross-over	16 ♂ (8 untrained/ 8 trained) Age: 23±5 yrs BM: 79±11 kg Height: 185±6 cm VO_{2max} trained: 59.5±3.6 mL/kg/min VO_{2max} untrained: 37.5±3.4 mL/kg/min	Acute Cycling 1 h 70% VO_{2max}	Sub-chronic (7 days) – last dose 1 day before exercise "supplement" HCF F: 240 mg No other details LCF: "placebo" Wash out: 1 week	Pre and post-exercise: a) total platelet count b) MPV c) ATP release from platelet granules	a) pre-exe<post-exe HCF=PL b) pre-exe=post- exe HCF=PL c) pre-exe<post-exe HCF=PL
(Soleimani et al. 2013)	Randomized, double blind, placebo-controlled, cross-over	20 ♂ (soccer players) Age: 22±1 yrs H: 178±2.6 cm BM: 72±2.7 kg Fat%: 22.5±1.2% VO_{2max}: 53.7±1.5 ml/kg/min	Acute Walk/run (Bruce test)	Acute (2h pre-exe) HCF F: 360 mg (no details) LCF no details wash out: 1 week	Baseline, Pre-exe, post-exe, 1h post-rec Platelet factors: a) Plt b) MPV c) PDW	a) Post-ex and post-rec > pre-ex **HCF<LCF (g: 8.27 1h post-exe)** b) Post-ex and post-rec > pre-ex **HCF<LCF (g: 2.84 1h post-exe)** c) Post-ex and post-rec > pre-ex **HCF<LCF (g: 2.63 1h post-exe)**

HCF: high cocoa flavanol, LCF: low cocoa flavanol (or placebo or control drink), F: flavanol, E: epicatechin, C: catechin, PC: procyanidins, Cf: caffeine, T: Theobromine, VO_{2max}=maximal oxygen uptake; HR_{max}=maximal heart rate; SS= steady state, TTE=time-to-exhaustion; TT=time trial;%HR_{max}=relative heart rates; exe=exercise; Rec=recovery; BP=Blood Pressure; DBP=diastolic blood pressure, MBP=mean blood pressure; FMD=Flow Mediated Dilation; Body Mass; BMI=Body Mass Index; g=Hedges'g effect size.

Table 5. The effects of cocoa and exercise on oxidative stress and inflammation.

Reference	Study design	Participants	Exercise protocol	Cocoa intervention	Measurements	Outcomes
(Allgrove et al. 2011)	Randomized, single blind, counterbalanced cross-over	20 ♂ Age: 22±4 yrs BM: 74.6±8 kg VO_2 max: 53.1±7.0 ml/kg/min PPO 300±30 W	Acute Cycling 1.5h SS @ 60% VO_{2max} with every 10' min → intensity ↑ until 90% VO_{2max} for 3s + TTE (90% VO_{2max}).	Sub-chronic (2 weeks) + acute DOUBLE DOSE (2h pre-exercise) Solid <u>HCF</u> 80 g dark chocolate intake F: 197.2 mg (C 31.2 mg; E 77.4 mg; Dimer B2: 46.8 mg; Dimer B5: 5.8 mg; Trimer C: 27.8 mg; Tetramer D: 8.2 mg) <u>LCF</u> 56.8 g control chocolate Wash out: 2 weeks	Pre-exe, post-SS, post-TTE + post 1h recovery <u>OS:</u> a) UA b) Vitamin C c) TAC d) free F2-isoprostane e) F2-isoprostanes f) oxidized LDL <u>Inflammation:</u> g) leukocyte count + neutrophil count h) IL-6 i) IL-10 j) IL-1ra	a) - post-SS, post-TTE + post-rec > pre-exe - HCF=LCF b) - post-SS, post-TTE + post-rec > pre-exe - HCF=LCF c) - post-SS, post-TTE > pre-exe - HCF=LCF d) - post-SS, post-TTE > pre-exe **- ↑ HCF< ↑ LCF** (Post-TTE and post-rec) e) **- HCF<LCF** at post-TTE and post-rec f) - post-SS, post-TTE > pre-exe **- HCF< LCF (pre-exe, post-SS, post-TTE)** g) - post-SS, post-TTE + post-rec > pre-exe - HCF=CF h) HCF=LCF i) HCF=LCF j) HCF=LCF

Reference	Study design	Participants	Exercise protocol	Cocoa intervention	Measurements	Outcomes
(Davison et al. 2012)	Randomized, not blind, counterbalanced cross-over	14 ♂ Age: 22±1 yrs BM: 71.6±1.6 kg VO_2 max: 53.1±1.9 ml/kg/min PPO 300±12 W	Acute Cycling 2.5 h @ 60% VO_{2max}	Acute (2 h pre-exercise) Solid HCF (100 g) F: 246.8 mg (C: 39.1 mg, E: 96.8 mg, Dimer B2: 58.4 mg, Dimer B5: 7.3 mg, Trimer C: 34.7 mg, Tetramer D: 10.5 mg) LCF (71 g) F: 0 mg Baseline (BL) no intervention Wash out: 7 days	Pre and Post-exe + after 1h recovery a) Vitamin C b) TAS c) free F2-isoprostane d) Plasma polyphenols e) Cortisol, ACTH f) IL-6 g) Non-esterified fatty acids (NEFA)	a) - post-exe > pre-exe - HCF=LCF b) **- HCF > LCF pre-exe (g: 0.19)** c) - post-exe > pre-exe **- ↑ HCF<↑ LCF post-exe (g: 0.14)** **- post recovery > pre-exe in LCF ⇔ post recovery=pre-exe in HCF (g: 0.14)** d) - E ↑ over time in HCF ⇔ E= over time in LCF - F=over time in HCF and LCF e) - post-exe > pre-exe - HCF= LCF f) - post-exe > pre-exe - HCF= LCF g) - post-exe > pre-exe **- HCF > LCF (trend) (g: 0.11)**
(Fraga et al. 2005)	Randomized, not blind, counterbalanced cross-over	28 ♂ Age : 18±1 yrs BM: 74±1 kg BMI: 24.1±0.2 kg/m2	Sub-chronic Footbal 2/week training + 1/week match	Sub-chronic (14 days) Solid HCF (105g) F: 186 mg (C + E: 39 mg, PC: 126 mg) Cf: 17.8 mg, T: 178.5 mg LCF (105 white chocolate) F:<5 mg No wash out	Before and after 2 week intake: Δ over time a) Urate b) MDA c) Total relative antioxidant potency (TRAP) d) Vit E e) vit E/cholesterol f) vit E/LDL g) Lycopene h) β-carotene i) Coenzyme Q10	**a) ↓ HCF (g: 0,14)⇔=LCF** b) **↓ HCF (12%) (g: 0,24) ⇔ ↑ LCF (10%)** c) = HCF and LCF d) = HCF and LCF **e) ↑ HCF (g: 0,14) ⇔=LCF** **f) ↑ HCF (g: 0,14) ⇔=LCF** g) = HCF and LCF **h) = HCF ⇔ ↓ LCF** i) = HCF and LCF

Reference	Study design	Participants	Exercise protocol	Cocoa intervention	Measurements	Outcomes
(Wiswedel et al. 2004)	Randomized, double blind, cross-over	10 ♂ Nonsmoking untrained	Acute Cycling 29 min: start @ 75W, stepwise ↑ untill 150 W (10')	Acute (2h pre-exe) Liquid (100 ml) <u>HCF</u> F: 187 mg <u>LCF</u> F: 14 mg Wash out: 7 days	BL, pre-exe and 2h + 4h post-exe a) F2-isoprostanes b) relative MDA c) α-tocopherol d) ascorbate e) TAC	a) - post-exe=pre-exe ! - @ pre-exe, 2h + 4h post-exe:=**HCF ⇔ ↑ LCF** b) @ 2h + 4h post-exe: ↑ **HCF<** ↑ **LCF** (trend) c) @ 2h + 4h post-exe: ↓ **HCF<** ↓ **LCF** (trend) d) HCF=LCF e) HCF=LCF
(Singh et al. 2006)	Randomized, double blind, cross-over	16 ♂ (8 untrained/ 8 trained) Age: 23±5 yrs BM: 79±11 kg Height: 185±6 cm VO_{2max} trained: 59.5±3.6 mL/kg/min VO_{2max} untrained: 37.5±3.4 mL/kg/min	Acute Cycling 1h @ 70% VO_{2max}	Sub-chronic (7 days) – last dose 1 day before exercise "supplement" <u>HCF</u> F: 240 mg No other details <u>CONTROL</u> "placebo" Wash out: 7 days	After 7 days, pre-exe (no acute effect of ex) a) TAS	a) HCF=LCF

Reference	Study design	Participants	Exercise protocol	Cocoa intervention	Measurements	Outcomes
(Taub et al. 2016)	Randomized, double blind, placebo-controlled	17 ♂ Sedentary *PL group* (n=8) Age: 49.5±1.6 yrs BMI: 29.5±2.2 (kg/m2) VO_{2max}: 24±1.7 ml/lg/min *HCF group* (n=9) Age: 49.8±2.6 yrs BMI: 27.7±1.8 (kg/m2) VO_{2max}: 22.9±1.9 ml/kg/min	Acute Cycling VO_{2max} test	Chronic (3 months) Solid <u>HCF</u> (20g) F: 175 mg (E: 26 mg; C: 4.6 mg) Cf: 13 m, T: 128 mg <u>LCF</u> F: 1.2 mg (E: 0.3 mg; C: 0.1 mg) Cf: 2 mg, T: 18 mg	Before and after 3 months Muscle biopsy (before exe) a) GSH:GSSG b) Protein carbonylation	Change over 3 months **a) ↑ HCF (g: 2.91) ⇔=LCF** **b) ↓ HCF (g: 1.09) ⇔=LCF**

HCF: high cocoa flavanol, LCF: low cocoa flavanol (or placebo or control drink), F: flavanol, E: epicatechin, C: catechin, PC: procyanidins, Cf: caffeine, T: Theobromine, VO_{2max}=maximal oxygen uptake; HR_{max}=maximal heart rate; SS= steady state; TTE=time-to-exhaustion; TT=time trial;%HR_{max}=relative heart rates; exe=exercise; Rec=recovery; MDA=malondialdehyde; TAC=total antioxidant capacity, TAS=total antioxidant status; IL=Interleukin, UA=Uric Acid, BM= Body Mass; BMI=Body Mass Index; PPO=peak power output; g=Hedges'g effect size.

Table 6. The effects of CF intake and exercise on carbohydrate and fat metabolism.

Reference	Study design	Participants	Exercise protocol	Cocoa intake	Measurements	Outcomes
(Stellingwerff et al. 2014)	Randomized, single blind, placebo-controlled cross-over	16 ♂ Age (yrs): 30.0±6.1 Height (cm): 179.9±7.8 Weight (kg): 72.8±6.0 VO_{2max}: 56.3±5.7 (ml/kg/min)	Acute Cycling 2,5 h SS @ 5% VO_{2max} + 15' TT	Acute (2h pre-exe) Solid HCF (561 kcal) F: 262 mg (E: 89 mg; C: 24 mg; PC B2: 55 mg; PC B5: 37 mg; Trimer C: 37mg Tetramer D: 20mg) Cf: 0.09 g, T: 0.69 g LCF (544 kcal) F:<0.05 mg Cf: 0.01 g, T:<0.01 g 1 week	During pre-exe (t= 0, 15, 30, 45, 60, 90, 120), every 15' during SS, TT and post-rec **a) glucose** **b) insulin** **c) glucagon** d) **triglycerides** (TG) e) FFA f) adrenaline **g) noradrenaline** h) glucose rate of appearance (R_a) **i) glucose rate of disappearance (R_d)** **j) glucose oxidation** **k) muscle glycogen oxidation** l) **Glucose recycling** (gluconeogenesis)	a) **HCF > LCF** during SS, TT and rec b) **HCF > LCF** during rec c) **HCF<LCF** during SS and TT d) **HCF > LCF** during TT and rec e) SS and TT > pre-exe; HCF=LCF f) HCF= LCF g) **HCF<LCF** end of TT h) HCF=LCF i) **HCF<LCF** during SS (16%) (g: 0.11) j) **HCF<LCF** during last 1h SS (18%) (g: 0.18) k) **HCF > LCF** (15%) (g: 0,10) ("compensatory") HCF=LCF during SS

Reference	Study design	Participants	Exercise protocol	Cocoa intake	Measurements	Outcomes
(Allgrove et al. 2011)	Randomized, single blind, counterbalanced cross-over	20 ♂ Age: 22±4 yrs BM: 74.6±8 kg VO_2 max: 53.1±7.0 ml/kg/min PPO 300±30 W	Acute Cycling 1.5h SS @ 60% VO_{2max} with every 10' min 3" @ 90% VO_{2max} + TTE (90% VO_{2max})	Sub-chronic (14 days) + acute DOUBLE DOSE (2h pre-exercise) Solid HCF 80 g dark chocolate intake F: 197.2 mg (C 31.2 mg; E 77.4 mg; Dimer B2: 46.8 mg; Dimer B5: 5.8 mg; Trimer C: 27.8 mg; Tetramer D: 8.2 mg) LCF 56.8 g control chocolate Wash out: 2 weeks	pre-exe, post SS, post-TTE and 1h post recovery a) **FFA** b) glucose c) insulin d) glucagon e) cortisol	a) - Post-SS, post-TTE > pre-exe **-↑ HCF<↑ LCF (21%)** b) - Post-rec<pre-exe - HCF=LCF c) - Post-SS<pre-exe - HCF=LCF d) - Post-SS > pre-exe - HCF=LCF e) - Post-SS=post-TTE=post-rec=pre-exe - HCF=LCF
(Fraga et al. 2005)	Randomized, not blind, counterbalanced cross-over	28 ♂ Age : 18±1 yrs BM: 74±1 kg BMI: 24.1±0.2 kg/m^2	Sub-chronic Footbal 2/week training + 1/week match Acute: Running 20' shutte run	Sub-chronic (14 days) Solid HCF (105g) F: 186 mg (C + E: 39 mg, PC: 126 mg) Cf: 17.8 mg, T: 178.5 mg LCF (105 white chocolate) F:<5 mg No wash out	Fasted state, before and after 14d intake **a) Cholesterol** **b) LDL-cholesterol** c) HDL-cholesterol d) Triglycerides (TG)	Change over 2 weeks **a) ↓ after HCF (g: 0.25) ⇔=after LCF** **b) ↓ after HCF (g: 0.24) ⇔=after LCF** c) = after HCF and LCF d) = after HCF and LCF

Reference	Study design	Participants	Exercise protocol	Cocoa intake	Measurements	Outcomes
(Davison et al. 2008)	Randomized, double blind, placebo-controlled 4 groups: (1) HCF COCOA, (2) HCF COCOA + EX, (3) LCF COCOA, (4) LCF COCOA + EX	18 ♂, 31 ♀ overweighted or obese Age: 18-65 BMI=33.5 kg/m^2 BP=123/76 mm HG HOMA2 : 2.4 FMD=4.3%	Sub-chronic: 12 weeks Cycling 3 x 45'/week @ 75% age predicted HR_{max}	Chronic (12 weeks) + acute (2h pre-exe) Liquid HCF *F*: 902 mg (E + C + PC unspecified) *Cf*: 36 mg, *T*: 674 mg LCF *F*: 36 mg (E + C + PC unspecified) *Cf*: 42 mg , *T*: 654 mg	At week 0, 6 and 12 (fasted state) a) Body composition (dual energy X-ray absorptiometry b) insulin (fasting) c) glucose d) **insulin resistance (HOMA2)** e) fat oxidation during exercise	a) - Abdominal fat%: W 12<W 0 (effect of EXE); HCF=LCF - BMI: W 12=W 0; HCF=LCF - Body fat%: W12=W0; HCF=LCF - Waist circumference: W12= W0; HCF=LCF b) W 12=W 0; HCF=LCF c) W 12=W 0; HCF=LCF **d) HCF<LCF (-0.31%)** e) W 12 > W0 (effect of EXE); HCF= LCF
(Taub et al. 2016)	Randomized, double blind, placebo-controlled	17 ♂ Sedentary *PL group* (n=8) Age: 49.5±1.6 yrs BMI: 29.5±2.2 (kg/m^2) VO_{2max}: 24±1.7 ml/kg/min *HCF group (n=9)* Age: 49.8±2.6 yrs BMI: 27.7±1.8 (kg/m2) VO_{2max}: 22.9±1.9 ml/kg/min	Acute Cycling VO_{2max} test	Chronic (3 months) Solid HCF (20g) F: 175 mg (E: 26 mg; C: 4.6 mg) Cf: 13 mg T: 128 mg LCF F: 1.2 mg (E: 0.3 mg; C: 0.1 mg) Cf: 2 mg T: 18 mg	Before and after 3 months Muscle biopsy (before exe) **a) HDL** **b) TG** c) Glucose d) Cholesterol e) LDL f) CRP g) HbA1c **h) LKB1 and p-LKB1** **i) AMPK and p-AMKB** **j) PGC-1α** k) Porin + mitofilin l) Mitochondrial Volume **m) Citrate synthase (CS)**	Change over 3 months **a) ↑ HCF (g: 0.47) ⇔=LCF** **b) ↓ HCF ⇔=LCF (trend)** c) HCF=LCF (no change) d) HCF=LCF e) HCF=LCF f) HCF=LCF g) HCF=LCF **h) ↑ HCF (g: 1.92)⇔=LCF** **i) ↑ HCF (g: 2.78)⇔=LCF** **j) ↑ HCF (g: 2.28)⇔=LCF** k) No change in HCF and LCF l) No change in HCF and LCF **m) ↑ HCF (g: 5.03) ⇔=LCF**

HCF: high cocoa flavanol, LCF: low cocoa flavanol (or placebo or control drink), F: flavanol, E: epicatechin, C: catechin, PC: procyanidins, Cf: caffeine, T: Theobromine, VO_{2max}=maximal oxygen uptake; HR_{max}=maximal heart rate; SS= steady state; TTE=time-to-exhaustion, TT=time trial;%HR_{max}=relative heart rates; exe=exercise; Rec=recovery; CK=creatine kinase; ACTH=adenocorticotrophic hormone; CRP=C-reactive protein; FFA=Free Fatty Acids, TG=triglyceride; LDL=low density lipoprotein; HDL=high density lipoprotein; IL=Interleukin, BM= Body Mass; BMI=Body Mass Index; PPO=peak power output; g=Hedges'g effect size.

Table 7. The effects of CF intake on exercise performance and recovery.

Reference	Study design	Participants	Exercise protocol	Cocoa intake	Measurements	Outcomes
(Stellingwerff et al. 2014)	Randomized, single blind, placebo-controlled cross-over	16 ♂ Age (yrs): 30.0±6.1 Height (cm): 179.9±7.8 Weight (kg): 72.8±6.0 VO_{2max}: 56.3±5.7 (ml/kg/min)	Acute Cycling 2,5 h SS @ 45% VO_{2max} + 15' TT	Acute (2h pre-exe) Solid HCF (561 kcal) F: 262 mg (E: 89 mg; C: 24 mg; PC B2: 55 mg; PC B5: 37 mg; Trimer C: 37mg, Tetramer D: 20mg) Cf: 0.09 g, T: 0.69 g LCF (544 kcal) F:<0.05 mg Cf: 0.01 g, T:<0.01 g Wash out: 1 week	TT performance	HCF=LCF
(Davison et al. 2012)	Randomized, not blind, counterbalanced cross-over	14 ♂ Age: 22±1 yrs BM: 71.6±1.6 kg VO_2 max: 53.1±1.9 ml/kg/min PPO 300±12 W	Acute Cycling 2.5 h @ 60% VO_{2max}	Acute (2 h pre-exercise) Solid HCF (100 g) F: 246.8 mg (C: 39.1 mg, E: 96.8 mg, Dimer B2: 58.4 mg, Dimer B5: 7.3 mg, Trimer C: 34.7 mg, Tetramer D: 10.5 mg) LCF (71 g) F: 0 mg Baseline (BL) no intervention Wash out: 7 days	During SS a) Mean HR b) Mean RPE c) Mean% VO_{2max} d) Mean RER e) Lactate	a) HCF=LCF b) HCF=LCF c) HCF=LCF d) HCF=LCF e) ↑ during SS; HCF=LCF

Reference	Study design	Participants	Exercise protocol	Cocoa intake	Measurements	Outcomes
(Peschek et al. 2014)	Randomized, single blind, counterbalanced cross-over	8 ♂ Age: 24.6±5.6 yrs Length: 182.1±6.3 cm BM: 73.4±7.0 kg	Acute Downhill running (-10%) 30' @ 70% VO_{2max} + 5 km TT 48h later	Acute **post-exe** (1st hour post-exe + 2h post-exe) Liquid <u>HCF</u> F: 350 mg No details <u>LCF</u> F: 0 mg (= fat, protein, carbohydrate, energy) Wash out: 21 days	Pre-exe, 24h post-ex, 48h post-exe, post-TT a) TT performance b) HR c) RPE d) CK over time e) Muscle soreness f) Muscle function g) Muscle tenderness	a) HCF=LCF b) HCF=LCF c) HCF= LCF d) Post-exe=pre-exe HCF=LCF e) Post-exe > pre-exe (NS!) HCF=LCF f) 24+ 48h post-exe<pre-exe HCF=LCF g) Post-exe=pre-exe HCF=LCF
(Fraga et al. 2005)	Randomized, not blind, counterbalanced cross-over	28 ♂ Age : 18±1 yrs BM: 74±1 kg BMI: 24.1±0.2 kg/m^2	Chronic Footbal 2/week training + 1/week match Acute: Running 20' shutte run	Sub-chronic (14 days) Solid <u>HCF</u> (105g) F: 186 mg (C + E: 39 mg, PC: 126 mg) Cf: 17.8 mg, T: 178.5 mg <u>LCF</u> (105 white chocolate) F:<5 mg No wash out	a) VO_{2max} shuttle run Change after 2 week intake (compared to before) (faster state): b) Lactate c) Lactate dehydrogenase d) Creatinine kinase	a) HCF=LCF **b) = after HCF ⇔ ↓ after LCF** **c) ↓ after HCF (g: 0.80) ⇔=after LCF** d) = after HCF and LCF

Reference	Study design	Participants	Exercise protocol	Cocoa intake	Measurements	Outcomes
(Allgrove et al. 2011)	Randomized, single blind, counterbalanced cross-over	20 ♂ Age: 22±4 yrs BM: 74.6±8 kg VO_2 max: 53.1±7.0 ml/kg/min PPO 300±30 W	Acute Cycling 1.5h SS @ 60% VO_{2max} with every 10' 3" @ 90% VO_{2max} + TTE (90% VO_{2max})	Sub-chronic (2 weeks) + acute DOUBLE DOSE (2h pre-exercise) Solid HCF 80 g dark chocolate intake F: 197.2 mg (C 31.2 mg; E 77.4 mg; Dimer B2: 46.8 mg; Dimer B5: 5.8 mg; Trimer C: 27.8 mg; Tetramer D: 8.2 mg) LCF 56.8 g control chocolate Wash out: 2 weeks	During SS a) HR b) RPE c) % VO_2 max d) RER e) TTE	a) ↑ during SS; HCF=LCF b) ↑ during SS; HCF=LCF c) ↑ during SS; HCF=LCF d) ↓ during SS; HCF=LCF e) TTE: HCF=LCF
(Patel et al. 2015)	Randomized, single blind, cross-over	9 ♂ (moderately trained) Age: 21±1 BM: 76±9.3 kg Length: 177±9.4 cm VO_{2max}: 41.89±5.4 ml/kg/min	Acute Cycling VO_{2max}-test + 20' SS 80% Gas exchange threshold + 2' TT	Sub-chronic (14 days) Solid HCF 40 g (12887 kJ) F: 259 mg *(E:* 46 mg; *PC:* 213 mg) CONTROL (white chocolate) (12945 kJ) + no intervention (baseline) Wash out: 2 weeks	a) VO_{2max} **b) Gas Exchange Threshold SS** c) O_2 cost SS d) RER SS e) Lactate SS **f) TT performance**	a) HCF and LCF > BL, HCF=LCF b) **HCF > LCF** (11%) (g: 0.85) (and > BL (21%)) c) HCF=LCF d) HCF=LCF e) HCF=LCF f) **HCF > LCF** (g: 0.90) (and > BL)

Reference	Study design	Participants	Exercise protocol	Cocoa intake	Measurements	Outcomes
(Taub et al. 2016)	Randomized, double blind, placebo-controlled	17 ♂ Sedentary *PL group* (n=8) Age: 49.5±1.6 yrs BMI: 29.5±2.2 (kg/m2) VO_{2max}: 24±1.7 ml/lg/min *HCF group* (n=9) Age: 49.8±2.6 yrs BMI: 27.7±1.8 (kg/m2) VO_{2max}: 22.9±1.9 ml/kg/min	Acute Cycling VO_{2max} test	Chronic (3 months) Solid <u>HCF</u> (20g) F: 175 mg (E: 26 mg; C: 4.6 mg) Cf: 13 mg, T: 128 mg <u>LCF</u> F: 1.2 mg (E: 0.3 mg; C: 0.1 mg) Cf: 2 mg, T: 18 mg	Before and after 3 months **a) VO_{2max}** **b) Power output**	Change over 3 months a) ↑ HCF ⇔=LCF b) ↑ HCF ⇔=LCF

HCF: high cocoa flavanol, LCF: low cocoa flavanol (or placebo or control drink), F: flavanol, E: epicatechin, C: catechin, PC: procyanidins, Cf: caffeine, T: Theobromine, VO_{2max}=maximal oxygen uptake; HR_{max}=maximal heart rate; RER=respiratory exchange ratio, SS= steady state, TTE=time-to-exhaustion, TT=time trial;%HR_{max}=relative heart rates, T_{VE}=ventilatory threshold; exe=exercise; Rec=recovery; RPE=ratings of perceived exertion; HR=heart rate; BM= Body Mass; BMI=Body Mass Index; PPO=peak power output; g=Hedges'g effect size.

2.5 Discussion

This systematic review examined the effects of CF intake on exercise performance and recovery, and exercise-induced changes in oxidative stress, inflammation, vascular function and carbohydrate and lipoprotein metabolism. Based on 12 studies, we found that acute and sub-chronic CF intake can reduce exercise-induced oxidative stress and positively affect fat and carbohydrate metabolism during exercise. Acute CF intake can improve FMD and reduce BP and temper the exercise-induced increase in BP in obese participants. Despite these positive effects, acute and sub-chronic CF intake do not have ergogenic effects. While the combination of sub-chronic CF intake and exercise training seems a successful strategy to improve cardiovascular risk factors and vascular function, too little evidence exist to answer the question whether exercise training and CF intake have additional effects on energy metabolism, oxidative stress and inflammation.

Quality of included studies

The included studies were of moderate and high quality with a low to very low risk of bias. However, concealment of allocation was only described in 1 out of the 12 studies (Davison et al. 2012) and participants were not blinded to the intervention in 3 studies (Fraga et al. 2005; Davison et al. 2012; Stellingwerff et al. 2014). Researchers were not kept blind to the participants exposure to the intervention and to other important confounding factors in 5 studies (Fraga et al. 2005; Allgrove et al. 2011; Stellingwerff et al. 2014; Peschek et al. 2014; Patel et al. 2015). In order to increase the quality and the level of evidence, future researchers should aim to blind both participants and themselves to the intervention. Moreover, only 2/12 studies performed a priori power analysis, which is recommended to carry out in future studies.

CF, exercise and oxidative stress

All studies except one showed that acute and sub-chronic CF intake enlarged the exercise-induced increase in antioxidant capacity, resulting in less ROS formation and/or lower oxidative damage (Wiswedel et al. 2004; Allgrove et al. 2011; Davison et al. 2012). Within the contracting skeletal muscle, mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase are important sources of ROS (Powers et al. 2016). Moderate levels of ROS serve as signalling molecules that result in increased muscular DNA-repair mechanisms, protein turn-over, mitochondrial biogenesis, glucose uptake and upregulation of redox-sensitive gene expression and antioxidant enzyme levels. Since moderate levels of exercise-induced ROS are required for the beneficial adaptations to exercise training (Powers et al. 2016), chronic CF supplementation during ‘normal’ training periods seems not advisable in healthy persons. Nevertheless, only one study has so far examined the effects of CF intake during a (short) period of exercise training. CF intake did not alter antioxidant capacity, but blunted the increase

in lipid peroxidation seen after 2 weeks of football training (Fraga et al. 2005). Clearly, more research is needed to investigate whether chronic CF intake does indeed blunt beneficial adaptations to exercise training. When (over)production of ROS by exhaustive exercise exceeds the endogenous antioxidant capacity, this deleteriously impacts cell function (Mankowski et al. 2015). The resulting oxidative damage may contribute to muscular fatigue during and after exercise and influence exercise performance and recovery. Allgrove et al. (2011) and Davison et al. (2012) both found that CF reduced exercise-induced oxidative damage, but effects on exercise performance were held off. Based on the calculated effect sizes, all of the reported beneficial outcomes had small effects. Therefore, these results suggest that acute and sub-chronic CF intake might reduce oxidative damage following exhaustive exercise in well-trained athletes, but the consequences for exercise performance and recovery remain elusive.

Two studies included in this systematic review did not observe immune modulatory effects of CF intake in an exercise setting: neither acute nor sub-chronic CF intake prevented the exercise-induced increase in white blood cell count and function and plasma IL-6 and IL-10 in a healthy population (Allgrove et al. 2011; Davison et al. 2012). Ellinger and Stehle (Ellinger and Stehle 2016) suggest that the effects of CF consumption on inflammatory markers might depend on the different stages of vascular inflammation and that especially participants suffering from low basal inflammation or healthy participants exposed to situations of increased inflammatory burden (such as exhaustive exercise) might profit from the beneficial effects of CF. Nevertheless, no anti-inflammatory effects of CF intake were found in response to exercise.

CF, exercise and vascular function

Since CF exert their vasodilatory function through an NO-dependent mechanism, increasing NO bioavailability by CF intake might increase O_2 and nutrient delivery to the working muscles, possibly enhancing performance during exercise. Despite this hypothesis, so far no studies examined the effects of CF on exercise performance together with changes in NO production, NO availability (as measured by nitrite, nitrate or nitrosothiols) or blood flow.

A recent meta-analysis on the effects of chocolate, cocoa and flavanols on cardiovascular health revealed that acute and chronic CF intake, regardless of the dose consumed, improve FMD by 3.19 and 1.34% respectively and reduce mean BP and DBP, with greater effects when the supplement contains > 50 mg epicatechin (Hooper et al. 2012). Studies included in this review indeed showed large beneficial effects of CF intake on mean BP and FMD, evidenced by large effect sizes. This review additionally shows that acute ingestion of CF can attenuate the BP response to exercise in overweight or obese men and women (Berry et al. 2010). The combination of exercise and sub-chronic (186 mg CF) and chronic (902 mg) CF intake can improve FMD and lower BP in football players and overweight men (Fraga et al. 2005; Davison et al. 2008), while exercise alone did not seem to have a positive effect. In contrast, 2-week

intake of CF (259 mg CF) without exercise training did not influence BP in moderately trained men (Patel et al. 2015). Thus, combining sub-chronic CF intake (with high epicatechin concentration) with exercise training may decrease cardiovascular risk and CF may augment exercise-induced benefits on cardiovascular function, especially in participants at-risk.

Mild exercise and CF are both known to suppress platelet activation, which is of interest in the prevention of several pathologies such as coronary artery disease, myocardial infarction and atherosclerosis. However, no studies examined the sub-chronic effects of concomitant exercise training and CF intake on platelet activation. In contrast to mild exercise, strenuous exercise causes intensity-dependent platelet activation, which may rise the risk of heart attacks during exercise (Soleimani et al. 2013). Hypothetically, CF intake might counteract these negative effects of strenuous exercise on platelet activation but results are equivocal. Singh et al. failed to demonstrate that the exercise-induced increase in platelet activity could be normalized by CF supplementation (1 week 236 mg CF/day) (Singh et al. 2006), while Soleimani et al. found that acute CF intake (360 mg CF) reduced the exercise-induced increase in platelet number, MPV and PDW in trained soccer players (Soleimani et al. 2013) and these effects were found to be large. Future research should examine the combined effects of exercise and CF intake on platelet activation, especially in populations with pathologies involving altered platelet activity, and further clarify the inextricable link between exercise and CF and oxidative stress, NO availability, platelet activity and cardiovascular function.

CF, exercise and carbohydrate and lipoprotein metabolism

It is known that NO stimulates glucose transport by glucose transporter 4 (GLUT4) and increases glucose oxidation through activation of the cGMP-AMPK-PGC-1α pathway in the muscle. NO also mediates the stimulatory effect of exercise on glucose transport. Moreover, NO increases fatty acids oxidation by reducing malonyl-CoA availability and decreases triacylglycerol synthesis by inhibiting glycerol-3-phosphate-acyltransferase activity and increases lipolysis (Jobgen et al. 2006). Since CF intake leads to higher NO bioavailability, glucose and fat metabolism during exercise are expected to alter as well.

Based on the studies included in this review, we can state that CF intake can indeed alter carbohydrate and fat metabolism during exercise, but that these effects are small. Acute CF (89 mg epicatechin) intake augmented glucose concentration during exercise (Stellingwerff et al. 2014) and maintenance of blood glucose concentrations appears to enhance prolonged exercise performance (Jeukendrup 2004). It was shown that the increased glucose concentration was caused by a reduced glucose rate of disappearance into the exercising muscle and decreased glucose oxidation. Several *in vitro* studies showed that some polyphenols inhibit the insulin-dependent intracellular GLUT4 translocation to the plasma membrane, which is the primary means of skeletal muscle uptake (Nomura et al. 2008; Strat et al. 2016). Thus, it is

plausible that CF alter glucose metabolism during exercise *in vivo* by the inhibition of GLUT4, but this seems contradictory with the finding that NO upregulates AMPK, which is known to activate GLUT4 translocation and glucose uptake (O'Neill 2013). Thus, we strongly suggest further investigations in the effect of CF intake on glucose metabolism during exercise and the downstream pathways. Besides the effects of CF on carbohydrate metabolism, two-week CF intake (78 mg epicatechin) did increase adipocyte lipolysis and mobilization of free fatty acids during exercise in well-trained athletes (Allgrove et al. 2011). Animal studies and a human study in untrained subjects indeed indicated that upregulation of AMPK, a master metabolic regulator of skeletal muscle energy balance, by CF has a major role in the observed increased fat oxidation (Strat et al. 2016; Taub et al. 2016). For athletes, the observed effects of CF on increased free fatty acids mobilization and fat oxidation are important since they can contribute to the conservation of carbohydrate stores during endurance type exercise and can assist when weight-loss is desired.

While exercise training and chronic CF intake independently improve insulin resistance, LDL and HDL cholesterol, the combined effects are not yet evident. Three-month CF intake improved insulin resistance, but the combination with exercise training did not have an additional effect in overweight participants (Davison et al. 2008). Two-week CF intake in combination with football training lowered cholesterol and LDL-cholesterol levels in football players (Fraga et al. 2005). As the studies of Fraga (Fraga et al. 2005) and Taub (Taub et al. 2016) did not allow a comparison of the effects of exercise training with or without CF intake, we cannot draw conclusions on the additive effects of both interventions. Therefore, this should be the focus of future research.

CF and its ergogenic link

Animal studies reported that epicatechin-rich cocoa enhances exercise capacity in mice (Gutierrez-Salmean et al. 2014). This systematic review showed that CF improves vascular function, reduces exercise-induced oxidative stress and improves fat and glucose metabolism during exercise in humans. Nonetheless, the 4 human studies examining the effects of acute or sub-chronic CF intake on exercise performance in trained athletes, failed to observe an ergogenic effect of either acute or sub-chronic CF intake, independent of the different exercise protocols used. Only 1 out of 5 studies found improved performance (with a large effect size) but it is questionable whether the 2-min TT used in this study, is a reliable outcome measure (Patel et al. 2015). We must emphasize that in a population of trained athletes, NO production, vascular function, energy metabolism and antioxidant capacity are already optimized by regular exercise training. Therefore, it is possible that the additive beneficial effects of CF intake are too small to actually improve exercise performance. Moreover, the reduced exercise-induced oxidative stress might have improved exercise recovery (Bentley et al. 2015; Braakhuis and Hopkins 2015), rather than improving exercise performance, but the included study designs were not optimally

chosen to measure exercise recovery and, moreover, effects of CF on oxidative stress were small. Nevertheless, the only long-term study so far showed an improved VO_{2max} and upregulation of upstream regulators of skeletal muscle metabolic control and mitochondrial efficiency caused by 3-month CF intake (175 mg CF, 26 mg epicatechin) in untrained sedentary men (Taub et al. 2016). This indicates that chronic CF intake may enhance skeletal muscle mitochondrial efficiency and potentially exercise performance, at least in untrained participants.

Practical implications & recommendations

Based on these results, we recommend acute CF supplementation, 430 mg CF or higher, 2 h pre-exercise to reduce exercise-induced oxidative stress and improve vascular function during exercise. There is little evidence supporting beneficial effects of sub-chronic CF intake of lower doses on vascular function and platelet activity during exercise and acute exercise-induced induced oxidative stress. Despite some beneficial effects of CF intake, neither acute nor sub-chronic CF intake seems to have ergogenic effects. Although the ideal dosage and composition of CF intake remains to be established, CF supplementation with higher total flavanol (> 700 mg) and more specifically higher epicatechin (> 80 mg) concentrations show more consistent beneficial effects. It seems advisable to consume CF as a liquid drink, concurrently with carbohydrate intake, in order to optimize epicatechin absorption and bioavailability (Schramm et al. 2003; Badrie et al. 2013).

Unfortunately, not many studies combined long-term CF intake with exercise training in humans, so clearly, more research is needed to determine their synergistic effects. Despite the little evidence, sub-chronic CF intake in conjunction with moderate exercise training seems a good strategy to prevent cardiovascular diseases as the combination has positive effects on blood pressure, antioxidant capacity, glucose and fat metabolism. Moreover, animal studies showed a beneficial effect of chronic consumption of epicatechin, the main CF component, in combination with training (Braakhuis and Hopkins 2015).

Because of its strong antioxidant capacity and effects on NO, CF supplementation could be advisable for well-trained athletes prior to or during periods with increased training loads and decreased recovery, which are perturbing the homeostasis in the body and inducing chronic oxidative stress (Bentley et al. 2015). Furthermore, CF supplementation might be beneficial for people with diseases such as diabetes, obesity, cancer, cardiovascular diseases and in the elderly population in which a pro-oxidant and increased inflammatory shift has been observed, but more research is needed in this field.

It must be noted that commercially available chocolate bars are not only low in CF content, but also show a high batch-to-batch variation in CF content when CF content is not actively controlled (Chin et al. 2013). Moreover, large differences in CF content exist between cocoa plants of different origins and

processing methods of chocolate. Since the beneficial effects of CF in an exercise setting are more evident when high doses of > 400 mg flavanol or > 70 mg epicatechin are consumed, it seems desirable to use CF supplements rather than normal dark chocolate. However, daily consumption of fruits, vegetables and fiber-rich foods is still important to reach an optimal amount of polyphenols to maintain a healthy vascular function and oxidative balance, and CF supplementation is only recommended at moments of (or in patients with) increased need for flavanols.

Limitations of the literature and future recommendations

This systematic review gives an update of the current published research examining the effect of CF intake in an exercise setting. Still, many questions with regards to CF supplementation and exercise remain unanswered. It was extremely difficult to compare the studies because of methodological differences. Studies varied in subject groups (age, BMI, health status, training status), type of CF intervention (duration, dosage and composition of CF supplement intake, as well as the food matrix), type of exercise intervention (intensity and duration) and biomarkers examined. The CF supplementation did not only vary in total CF doses (range: 186–904 mg CF), but also differed in exact composition (e.g. theobromine, caffeine content) and especially the exact amount of epicatechin (range: 31.2–98.7 mg), catechin and the different oligomeric procyanidins. The timing of intake ranged from acute (2 h pre-exercise) to sub-chronic (during 7–14 days) and chronic (during 3 months) and PL were not always matched in color, taste, macro-and micronutrient (e.g. theobromine, caffeine) content. Therefore, we suggest to make clear differences in low (<300 mg), medium (300–699 mg), and high (>700 mg) CF dosages, to clarify the exact composition of the CF supplement (especially for epicatechin, catechin, procyanidins) and to use an appropriate PL, when carrying out further research. Furthermore, the low numbers of participants used in most studies implicate a low statistical power to identify significant differences. Thus, we advise to perform a power calculation in order to arrive at the right sample size.

Future research should focus on whether CF intake could *(i)* prevent exercise-induced effects on oxidative stress and inflammation, *(ii)* influence blood flow and energy metabolism during exhaustive exercise and *(iii)* improve recovery in well-trained athletes, by using a protocol similar to real-life situations. Despite the invasive character of muscle biopsies, this technique allows muscle-specific changes in oxidative stress and energy metabolism during exercise to be studied, and provides insight into underlying biochemical mechanisms. NIRS can offer insight into muscular blood flow and oxygenation during exercise. Moreover, there is a strong need for more studies examining the combined effects of chronic exercise training and CF intake in untrained people and populations at risk of cardiovascular disease, increased levels of oxidative stress, and chronic inflammation.

2.6 Conclusion

This review shows that acute, sub-chronic (2-week) and chronic (3 months) CF intake clearly reduced exercise-induced oxidative stress. Evidence of the effect of CF on exercise-induced inflammation and platelet activation is scarce. CF intake improves vascular function and reduces BP. CF intake can temper the exercise-induced increase in BP in obese participants, but no studies have examined the effect of CF on blood flow during exercise. Acute and sub-chronic CF intake seem to positively affect fat and carbohydrate metabolism during exercise. Despite these beneficial effects, acute and sub-chronic CF intake do not seem to have ergogenic effects. However, the observation that chronic CF intake improves VO_{2max} and muscular mitochondrial efficiency in untrained participants offers promising perspectives. While the combination of sub-chronic CF intake and exercise training seems a successful strategy to improve cardiovascular function, evidence on the synergetic effects of CF and exercise training on oxidative stress, inflammation and fat and glucose metabolism is lacking.

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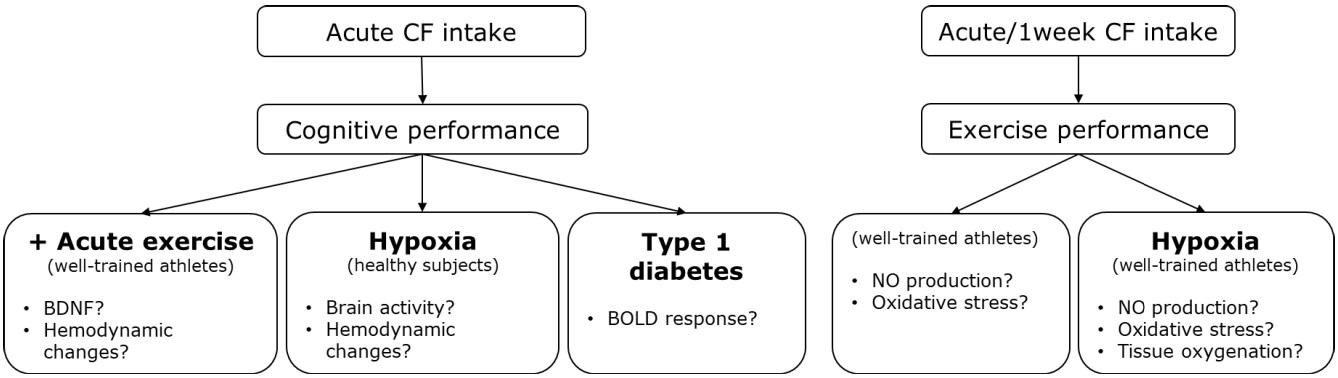
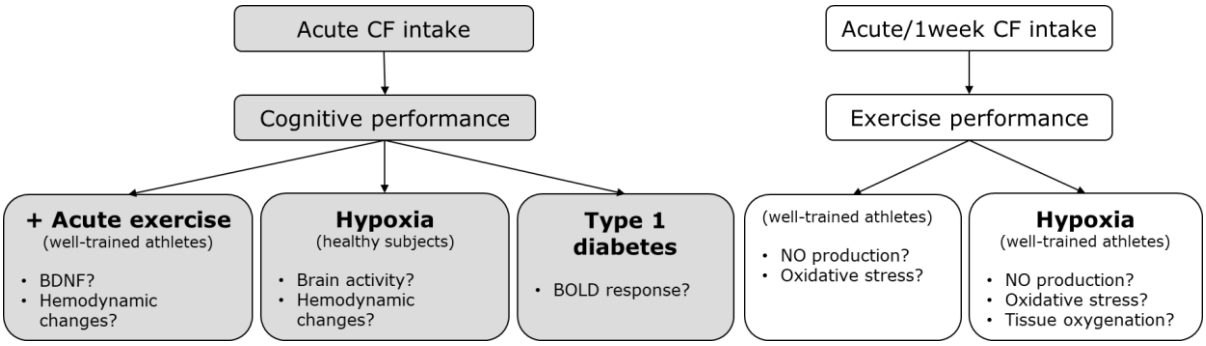
EXPERIMENTAL STUDIES OF THIS PHD



PART 1


Acute CF intake
Cognitive performance
+ Acute exercise
(well-trained athletes)
• BDNF?
• Hemodynamic changes?
Hypoxia
(healthy subjects)
• Brain activity?
• Hemodynamic changes?
Type 1 diabetes
• BOLD response?
Acute/1week CF intake
Exercise performance
(well-trained athletes)
• NO production?
• Oxidative stress?
Hypoxia
(well-trained athletes)
• NO production?
• Oxidative stress?
• Tissue oxygenation?

Chapter 3. Acute cocoa flavanol improves cerebral oxygenation without enhancing executive function at rest or after exercise

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ARTICLE

Acute cocoa flavanol improves cerebral oxygenation without enhancing executive function at rest or after exercise

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3.1 Abstract

Purpose: Acute exercise-induced improvements in cognitive function are accompanied by increased (cerebral) blood flow and increased brain-derived neurotrophic factor (BDNF) levels. Acute cocoa flavanol (CF) intake may improve cognitive function, cerebral blood flow (in humans) and BNDF levels (in animals). This study investigated *(i)* the effect of CF intake in combination with exercise on cognitive function and *(ii)* cerebral hemodynamics and BDNF in response to CF intake and exercise.

Methods: Twelve healthy men participated in this randomized, double-blind, cross-over study. Participants performed a cognitive task (CT) 100 min after acute 903 mg CF or placebo (PL) intake, followed by a 30-min time trial. Immediately after this exercise, the same CT was performed. Prefrontal near-infrared spectroscopy was applied during CT and exercise to measure changes in oxygenated (ΔHbO_2), deoxygenated (ΔHHb) and total haemoglobin (ΔHb_{tot}) and blood samples were drawn and analysed for BDNF.

Results: Reaction time (RT) was faster post-exercise, but was not influenced by CF. ΔHbO_2 during the resting CT was increased by CF, compared to PL. ΔHbO_2, ΔHHb and ΔHb_{tot} increased in response to exercise without any effect of CF. During the post-exercise cognitive task, there were no hemodynamic differences between CF or PL. Serum BDNF was increased by exercise, but was not influenced by CF.

Conclusion: At rest, CF intake increased cerebral oxygenation, but not BDNF concentrations, and no impact on executive function was detected. This beneficial effect of CF on cerebral oxygenation at rest was overruled by the strong exercise-induced increases in cerebral perfusion and oxygenation.

3.2 Introduction

Optimizing cognitive functioning during exercise is of great relevance in sport science, as sport performance depends at least partially on coordination, decision making (executive functions) and motor control (psychomotor speed) (Baker et al. 2014). These cognitive functions are, however, also influenced by acute exercise itself (Chang et al. 2015). The effects of exercise on cognitive function vary depending of the type, intensity and duration of the exercise and the type and duration of the cognitive task (Colcombe and Kramer 2003). In contrast with speculations that exercise intensity affects cognition in a U-shaped fashion, evidence exists that even after acute high intensity exercise, such as a time trial, performance on the Stroop task, a measure of executive function, is improved (Tomporowski 2003). Many factors are associated with exercise-induced increases in executive function. These include increased arousal and increased levels of plasma catecholamines, as well as increased cerebral blood flow and increased levels of brain-derived neutrophic factor (BDNF) (Chang et al. 2015).

For the last decade, nutritional supplements have been growing in popularity in the athletic and non-athletic population (Mankowski et al. 2015). Research is increasingly focussing on dietary constituents that can improve cognitive function, but should also explore possible underlying factors of cognitive enhancements (Meeusen 2014). Yet, studies examining acute effects of cognitive-modulating nutritional supplements in exercise-specific settings are sparse (Baker, Nuccio et al. 2014). Flavonoids, a subgroup of polyphenols, are a class of natural compounds found in the human diet and include subcategories of flavanols, flavonols, iso-flavones, flavones, and anthocyanidins (Del Rio et al. 2013). Intake of flavanols, found in grapes, tea, red wine, apples and especially cocoa (Manach et al. 2004), causes a nitric oxide (NO)-mediated vasodilation (Steffen et al. 2008; Nehlig 2013). As a consequence, cerebral perfusion can be augmented by acute cocoa flavanol (CF) intake (Francis et al. 2006; Lamport et al. 2015). Results of studies investigating the acute CF intake on cognitive function are equivocal (Scholey et al. 2010; Field et al. 2011; Pase et al. 2013; Massee et al. 2015). It seems that the quantity and bioavailability of the consumed CF, as well as the length of the cognitive task, highly impacts its beneficial effects (Scholey et al. 2010; Field et al. 2011; Pase et al. 2013; Massee et al. 2015).

Despite the hypothesis that CF augments cerebral perfusion, consequently leading to improved cognition, only the study of Francis et al. (2006) investigated the effects of CF on cerebral blood flow in combination with a cognitive task. The cognitive task assessed executive control needed for the coordination of multiple tasks. The authors (Francis et al. 2006) found that a medium CF dose (172 mg) taken daily during 5 days increased Blood Oxygenation Level-Dependent contrast (partly reflecting cerebral blood flow and blood volume) in active brain regions during a cognitive task, but without altering cognitive performance. Increased neuronal activation leads to elevated cerebral blood flow which is needed to meet, but also exceeds, the increased oxygen demand. As a consequence, cerebral

oxygenated haemoglobin (HbO_2) is increased while deoxygenated haemoglobin (HHb) is diluted and decreased (Dodd et al. 2015). As CF intake augments cerebral perfusion (Francis et al. 2006; Lamport et al. 2015), hemodynamic changes during a cognitive task and during exercise can possibly also be affected by CF intake. Near-infrared spectroscopy (NIRS) has been extensively used to measure cerebral hemodynamic changes following neuronal activation during cognitive tasks and exercise, even in combination with nutritional interventions (Dodd et al. 2015).

BDNF is a neurotrophic biomarker that is associated with exercise-induced improved cognitive function (Skriver et al. 2014). The effect of CF on BDNF has only been explored in animals so far (Rendeiro et al. 2013; Stringer et al. 2015). Animal studies showed that epicatechin, a main component of CF, can cross the blood-brain barrier (Abd El Mohsen et al. 2002) and that cognitive enhancements after chronic CF supplementation were paralleled by elevated hippocampal BNDF levels (Rendeiro et al. 2013; Stringer et al. 2015). Although the effects of CF on BDNF have never been tested in humans, results from animal studies are promising.

As cerebral blood flow and BDNF are important mediators of exercise-induced cognitive enhancement, we hypothesized that acute CF intake would improve executive function by affecting these same mediators: cerebral hemodynamics and BDNF levels. Consequently, we hypothesized that CF could strengthen the exercise-induced beneficial effects on executive function.

Therefore, the aim of this study was to investigate *(i)* the effect of acute CF intake on executive function in combination with exercise and *(ii)* cerebral hemodynamic changes (as measured by NIRS) and BDNF, two possible underlying mechanisms of cognitive enhancement, in response to acute CF intake and exercise.

3.3 Materials & Methods

3.3.1 Participants

Twelve well-trained men (Performance Level 3 (De Pauw et al. 2013)) with a mean±SD age, height, mass, maximal oxygen uptake (VO_{2max}) of 30±3 years, 177.9±8.8 cm, 72.8±7.8 kg , 63.0±3.5 ml/kg/min, were selected to participate in this randomized, double-blind, cross-over interventional trial. Sample size was calculated based on an estimate of the treatment differences (994 mg CF versus PL) on reaction times in a 10-min lasting reaction test, obtained from previous research from Scholey et al. (2010). Volunteers were excluded from the study if they met any of the following exclusion criteria: (1)<20 years or >35 years, (2) history of severe head injuries, (3) intake of neurological or psychological medication that might alter cognitive function, (4) hypertension, (5) cardiovascular disease, (6) other diseases that can alter cognitive function: diabetes, depression, attention deficit hyperactivity disorder, schizophrenia, (7) smokers. The experimental procedures and potential risks were explained to the participants and a written informed consent was provided and signed prior to inclusion in the study. The study protocol was approved by the ethical research committee of UZ Brussel and the study was conducted according to the Declaration of Helsinki (1964).

3.3.2 Study design

Subjects visited the laboratory 3 times during 3 consecutive weeks, with 7 days wash-out in between. On the first visit, subjects underwent a complete medical screening, performed a maximal incremental cycle test (initial workload of 80W, increased every 3 min by 40 W until volitional exhaustion) on an electromagnetically braked lower extremity cycle ergometer (Lode) to determine VO_{2max} (Metalyzer Cortex (Germany)) and peak power output. Subjects also performed the cognitive task (CT) - that was used in the interventional trials – three times consecutively as a familiarisation to prevent a possible learning effect (Lemay et al. 2004).

Subsequently, subjects underwent 2 interventional trials with a wash-out period of one week, which was largely sufficient since the elimination half-life of cocoa is 2.6 h (Schramm et al. 2003). Subjects reported to the laboratory in a 4 h fasted state at 12:00. Subjects were asked to refrain from intense exercise in the last 48 h and to abstain from caffeine and high-polyphenol foods (e.g. green tea, grapes, olives, dark chocolate, hazel and pecan nuts and berries) for the last 24 h. Both interventions started with a baseline CT lasting 5 min (Figure 11). Then, subjects consumed in a randomised manner either a high CF content chocolate milk (CF - 903.75 mg flavanol, Acticoa®) or a placebo low CF chocolate milk (PL - 15 mg flavanol) which were matched in taste, colour and calories (Table 8). This drink was consumed together with a standardized lunch, composed by a nutritionist to contain a high amount of

carbohydrates to increase flavanol absorption (Schramm et al. 2003) (80% carbohydrate, 15% protein, 5% fat, 10 kcal/kg body weight). As the peak concentration of flavanol in the blood is reached 90-120 min after consumption of the drink (Francis et al. 2006), a blood sample was takens after 90 minutes, followed by a 2nd CT, a 5-min warm up and a time trial, lasting approximately 30 min, on the same cycle ergometer (Lode). Subjects were instructed to cover a fixed amount of work (the equivalent work of 75% of peak power output during 30 min) as fast as possible. The initial workload of the time trial corresponded to 75% of peak power output, but subjects were free to change their power output as desired from the outset, meaning that if the power output was decreased, the duration of the time trial increased (exact time: PL: 29’47”±1’58”; CF: 29’13”±1’19). No feedback regarding time lapse, power output, heart rate or pedal cadence was given, except for total workload that had been completed. Next, a 3rd CT was performed after the time trial, approximately 5 min later. Blood samples were taken upon arrival, before and after the 30-min time trial. Each experimental trial was conducted in a climate room where temperature was controlled and kept constant (20 °C, 60% humidity).

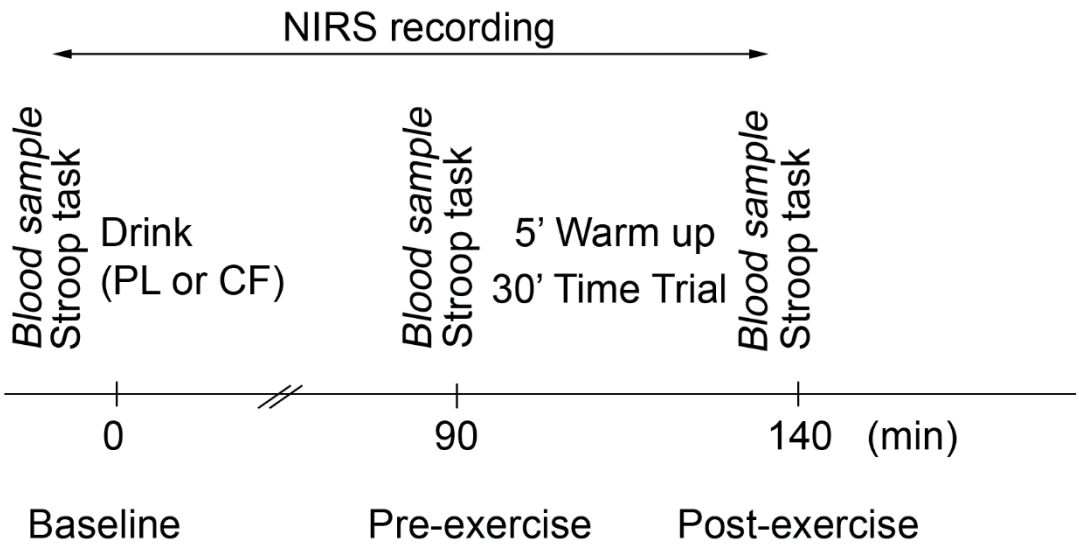

Figure 11. Study protocol. *CF, cocoa flavanol; NIRS, Near-infrared spectroscopy; PL, placebo.*

Table 8. Nutritional profile of cocoa powder (solved in 300 ml skimmed milk).

	Cocoa flavanol (CF)	*Placebo (PL)*
Total Flavanols (mg)	900	15
(−)-Epicatechin	185	0
Catechin	20	0
Cocoa powder (g)		
Acticoa powder	12.0	0.0
Alkalized cocoa powder	3.0	15.7
Potassiumchloride (KCl)	0.7	0.0
Sugar	35.0	35.0
Protein (g)	3.2	2.9
KHO (g)	38.7	38.4
Fat (g)	2.3	2.9
Kcal	193.4	193.4
Caffeine (mg)	30	30
Theobromide (mg)	315	315
Cadmium	<1 ppm	<1 ppm

3.3.3 Measurements

3.3.3.1 Cognitive task

Given that exercise and CF both affect executive functioning (Chang et al. 2012), the Stroop task was used as CT. The Stroop task is a conflict paradigm, which involves inhibition of prepotent response and measures response inhibition (Strauss 2006). The Stroop test was programmed and performed on E-prime 2.0 software (Psychology Software Tools, Inc., Pittsburg, PA). Although Field et al. and Scholey et al. found cognitive enhancements on a longer lasting CT (>45 min), exercise-induced cognitive improvements start to vanish 20 min post-exercise (Chang et al. 2012; Ogoh et al. 2014). Thus, we choose to use a Stroop task with a total test duration of approximately 5 min, consisting of two parts. During the first part, neutral reaction time was measures, as participants were demonstrated with X's coloured in yellow, red, blue and green, and were asked to respond by pushing the corresponding button on a keyboard (AZERTY; F – left middle finger, V – left index finger, B – right index finger, H – right middle finger). In the second part, the words yellow, red, blue and green were shown in matching colours (congruent condition) and non-matching colours (incongruent condition). Participants were asked to push the button corresponding to the word displayed on the screen, disregard the colour of the word. The two parts were separated by a 30-sec rest period. Sixty stimuli were presented in the first part and

60 congruent and 60 incongruent in the second part. The interval response–stimulus onset was set at 500 ms and the stimulus appeared on the screen untill the subject responded. The stimuli were displayed in the middle of the computer screen. Outcome measures were accuracy (%) and reaction time (RT) (ms). To clarify the effect of exercise and CF intake on the Stroop interference, RT for "incongruent-neutral" contrast was calculated.

3.3.3.2 Near-Infrared Spectroscopy

Functional NIRS, a non-invasive optical imaging technique, was used to assess acute changes in local cerebral blood volume (reflecting CBF) and oxygenation (Oxymon continuous-wave NIRS (CW-NIRS) system (ArtinisMedical Systems B.V.). Following introduction of near-infrared light through the skull, HbO_2 and HHb absorb light at slightly different wavelengths (800-940 nm and 600-750 nm, respectively) allowing the measurement of their relative concentrations in the cerebral blood (Perrey 2008). These concentration changes are the result of the interplay between regional cerebral blood flow, blood volume and metabolic rate of oxygen. Whereas HbO_2 and HHb reflect the balance of O_2 delivery and extraction, the sum of both, total haemoglobin (Hb_{tot}) is an index of changes in regional blood volume (Oussaidene et al. 2015).

Intrinsically adjusted for the baseline measurement (at the start of application), this system measures concentration changes across a single recording session. Two nominal wavelengths of light (~765 and 855 nm) were emitted with 4 cm distance between an emitter and receptor and the differential path-length factor was adjusted according to the subjects' age. The modified Beer-Lambert law was used by the software to calculate relative changes in the concentrations of HbO_2, HHb and Hb_{tot} (Oussaidene et al. 2015). Data were collected with a sampling frequency of 5 Hz and were down sampled with factor 5 for analysis.

The emitter/receptor optode pair was positioned over the left prefrontal cortical area between Fp1 and F3, according to the modified international EEG 10-20 system (Rupp and Perrey 2008). The NIRS emitter/receptor optodes pair was set up on the prefrontal cortex at baseline and the position was marked. Ninety min after the CF intake, the optode pair was repositioned and was kept in position until the end of the post-exercise CT. A black cloth was placed over the optode pair to prevent interference of external light and a dark elastic band was wrapped around the head to keep the NIRS-optode pair in place. When the NIRS measurement started, subjects sat still without speaking or moving for 2 min (reference measurement). All NIRS measurements until the end of the time trial, were normalized to reflect changes from this 2-min reference measurement to express the magnitudes of changes. Immediately after exercise, subjects sat still without speaking or moving for 2 min once again before the post-exercise CT started (post-exercise reference measurement). Post-exercise NIRS values were normalized to reflect changes from this post-exercise reference measurement. Mean concentration changes (ΔHbO_2, ΔHHb

and ΔHb_{tot}) were calculated for the two parts of each Stroop task (baseline, pre-exercise and post-exercise CT). ΔHbO_2, ΔHHb and ΔHb_{tot} were calculated during the first ("start) and last ("end") 30 seconds of the TT. The use and limitations of NIRS for monitoring cerebral regional hemodynamics and oxygenation have been extensively reviewed (Rooks et al. 2010).

3.3.3.3 Blood sampling and determination of serum BDNF concentration

A catheter was placed in the forearm upon arrival at the lab. Venous blood samples were collected at baseline, 90 min after consuming the chocolate drink (pre-exercise) and immediately after the time trial. Blood was collected in 8-ml anticoagulant-free tubes and centrifuged (10 min at 3000 RPM, 4 °C) after 30 min at room temperature to allow clotting to obtain serum. Serum was aliquoted and stored at -80 °C until analysis. Serum BDNF was analysed using a commercially available ELISA kit (ChemiKine® BDNF kit, Millipore®, Temecula,CA, USA). The kit has a detection range from 7.8 pg/mL to 500 pg/mL. Intra-assay and inter-assay variations are ± 3.7% (125 pg/ mL) and ± 8.5% (125 pg/mL) respectively. Data were corrected for changes in plasma volume using the determination of haematocrit and the concentration of haemoglobin according to Dill and Costill (Dill and Costill 1974). Pre- and post-exercise values were normalized to the baseline values and expressed as percentage change to baseline.

3.3.4 Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics (version 22; IBM Corp., Armonk, N.Y., USA) and considered significant at $\alpha<0.05$. Normality of the data was tested with the Kolmogorov-Smirnov test and scatter plots. Data was presented as mean±standard deviation, except otherwise indicated.

Since accuracy data of the cognitive tests violated normality, Wilcoxon Signed Ranks test was used to estimate differences between CF drink and PL drink and between post-and pre-exercise. To estimate the effect of CF intake ("intervention") on RT during each part of the Stroop task at the different time points, a two-way *(3 (time) x 2 (intervention))* repeated measure analysis of variance (RM ANOVA) was performed. To estimate the effect of CF and exercise on cognitive control (i.e. the Stroop effect), a three-way (*3 (time) x 2 (intervention) x 2 (congruency))* RM ANOVA was performed. To clarify the effect of exercise and CF intake on the Stroop interference, the "incongruent-neutral" contrast was calculated and subjected to two-way *(3 (time) x 2 (intervention))* RM ANOVA. To estimate the effects of CF intake on serum BDNF concentrations at rest and after exercise ("time" effect), a two-way *(2 (time) x 2 (intervention))* RM ANOVA was performed. Two-way RM ANOVA *(2 (intervention) x 2 (start and end of exercise))* was performed to observe differences in ΔHbO_2, ΔHHb and ΔHb_{tot} during the time trial. Post hoc analyses were performed using the Bonferroni correction. To estimate the effect of CF

intake ("intervention") on ΔHbO_2, ΔHHb and ΔHb_{tot} during the CT before and after exercise, an analysis of Covariance (ANCOVA) was performed to adjust for differences at baseline (day-to-day variability).

3.4 Results

3.4.1 Effect of CF and exercise on cognitive function

RM ANOVA showed a main effect of time on RT on neutral stimuli of Stroop task (F(2)=36.4, p<0.001), but no main effect of intervention and no interaction effects were observed. Independent of the CF or PL drink, subjects reacted significantly faster post-exercise compared to pre-exercise. Subjects reacted faster pre-exercise compared to baseline. Likewise, a main effect of time was detected for RT on the Stroop task for the congruent (F(2)=9.5, p=0.001) and incongruent parts (F(2)=9.9, p=0.001). Independent of the CF or PL drink, subjects performed significantly faster post-exercise compared to baseline and pre-exercise. There was no significant difference between the baseline and pre-exercise CT. Neither a main effect of intervention, nor an interaction effect were detected for both the congruent and incongruent parts (Figure 12). No main effect of time, intervention or interaction effect were found for accuracy on all parts of the Stroop task (no data shown). To assess the effect of CF on executive function in combination with exercise, 3-way RM ANOVA *(3 (time) x 2 (congruency) x 2 (intervention))* was performed. A main effect of time (exercise) (F(2)=10.85, p=0.001) and a main effect of congruency (F(1)=37.62, p<0.001) were evident, but no effect of intervention or interaction effects were significant. These results verified that the Stroop interference were observed in all conditions. To further assess the effect of CF intake and exercise on cognitive control, two-way RM ANOVA *(3 (time) x 2 (intervention))* was performed for Stroop interference (= incongruent – neutral). RM ANOVA exhibited no significant main effects of time, intervention or interaction for Stroop interference.

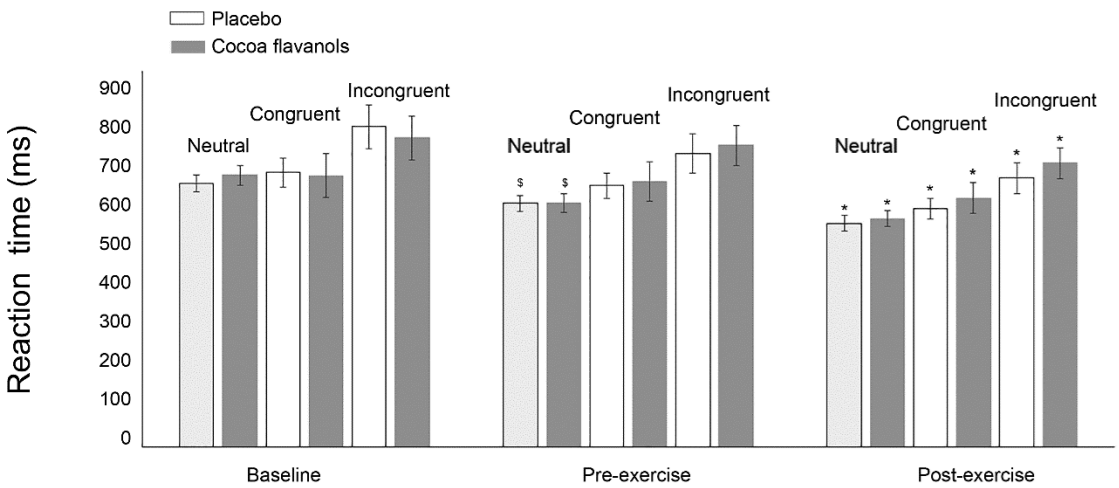

Figure 12. Reaction times (ms) on Stroop task (neutral, congruent and incongruent stimuli) at baseline, pre and post-exercise (30-min time trial), after consumption of a placebo chocolate drink (PL) or cocoa flavanol chocolate drink (CF)*. *: significant change to pre-exercise; $: significant change to baseline (p<0.05). Means±SEM presented.*

3.4.2 Effect of CF and exercise on CBF and oxygenation (NIRS)

Hemodynamic changes during the CT were measured at baseline and before and after exercise (Figure 13, Figure 14). To correct for day-to-day variability in cerebral oxygenation at baseline, ANCOVA with baseline values as covariate was used.

Effect of CF intake on cerebral blood volume and oxygenation during a CT at rest

Compared to PL intake, CF intake induced a significant greater increase in ΔHbO_2 during the first part of the Stroop task ($F(1)=7.1$; $p=0.02$, ES=0.30). A similar increase in ΔHbO_2 during the second part of the Stroop task after CF intake was not significant. No other effects of intervention on cerebral perfusion/oxygenation during the pre-exercise cognitive task were observed.

Effect of CF intake on cerebral blood volume and oxygenation in response to exercise

Two-way RM ANOVA showed a significant increase in ΔHbO_2, ΔHHb and ΔHb_{tot} in response to the time trial (main effect of time; Figure 14,Table 9). No main effect of intervention and no interaction effects were observed.

In the 5-min window between the end of exercise and the start of the post-exercise CT, RM ANOVA showed a significant decrease (main effect of time) in ΔHbO_2, ΔHHb and ΔHb_{tot}, but no interaction effect and no main effect of intervention were observed.

Effect of CF intake on cerebral blood volume and oxygenation during CT after exercise

During the post-exercise Stroop task, ΔHbO_2, ΔHHb and ΔHb_{tot} were not significantly different between CF or PL intake (Figure 13, Figure 14).

3.4.3 Effect of CF and exercise on serum BDNF

Two-way RM ANOVA (intervention (PL or CF) x time (pre-exercise, post-exercise)) on relative changes in serum BDNF concentration showed a significant effect of time ($F(1)=31.8$, $p<0.001$ ES:0.74), but no effect of intervention and no interaction effect. Post-hoc analysis revealed a significant increase in serum BDNF concentration after exercise (+ 67.3±11.9%, $p=0.001$) (Figure 15).

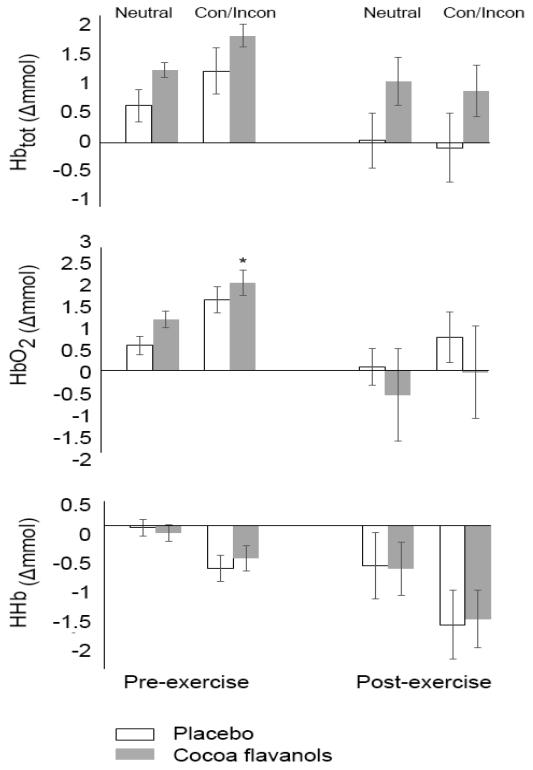

Figure 13. Effect of cocoa flavanol (grey) or placebo intake (white) on hemodynamic changes (oxyhemoglobin (ΔHbO2), deoxyhemoglobin (ΔHHb), and total hemoglobin (ΔHbtot)) during a Stroop task, at pre- and post-exercise (30-min time-trial). *ΔHbO2 during the first part of the Stroop task (neutral stimuli) at pre-exercise was significantly larger after cocoa flavanol intake compared with placebo. Con, congruent; Incon, incongruent. Means±SE are presented. *, Significant change to placebo (p<0.05).*

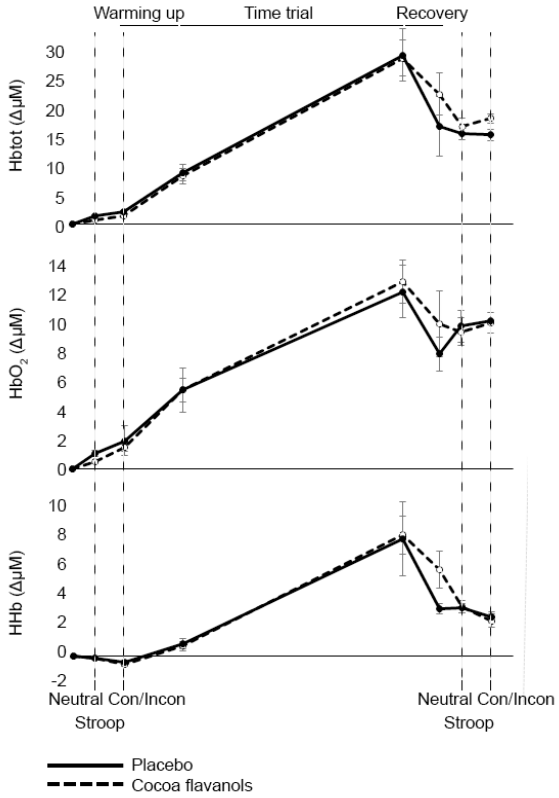

Figure 14. The effect of cocoa flavanol (full lines) or placebo (dashed lines) on hemodynamic changes [oxyhemoglobin (ΔHbO2), deoxyhemoglobin (ΔHHb) and total hemoglobin (ΔHbtot)] during the experimental protocol*. Subjects first performed Stroop task, which was followed by a 5-min warm up and a time trial (TT). Five min after the time trial, the same cognitive task was performed. Means±SEM are presented.*

Table 9. Effects of CF intake on hemodynamic and oxygenation changes in response to an exercise bout (time trial).

	Placebo (n=12)	Cocoa flavanol (n=12)	RM-ANOVA effect
ΔHb_{tot}			
Start exe	6.50±3.31	6.99±3.46	Time: F(1)= 63.4 p<0.001 ES=0.85
End exe	21.85±7.84	22.56±11.10	Intervention: NS Interaction: NS
ΔHbO_2			
Start exe	5.98±2.80	5.98±3.56	Time: F(1)= 17.3 p=0.002 ES=0.66
End exe	13.70±5.19	13.73±5.33	Intervention: NS Interaction: NS
ΔHHb			
Start exe	0.80±1.15	1.01±1.26	Time: F(1)= 30.0 p<0.001 ES=0.73
End exe	8.43±4.94	8.83±6.68	Intervention: NS Interaction: NS

Note: Means±SD are presented. Oxyhemoglobin(ΔHbO_2) , deoxyhemoglobin(ΔHHb) and total hemoglobin (ΔHb_{tot}) significantly increased in response to exercise (exe), independently of the consumed drink (cocoa flavanol or placebo).

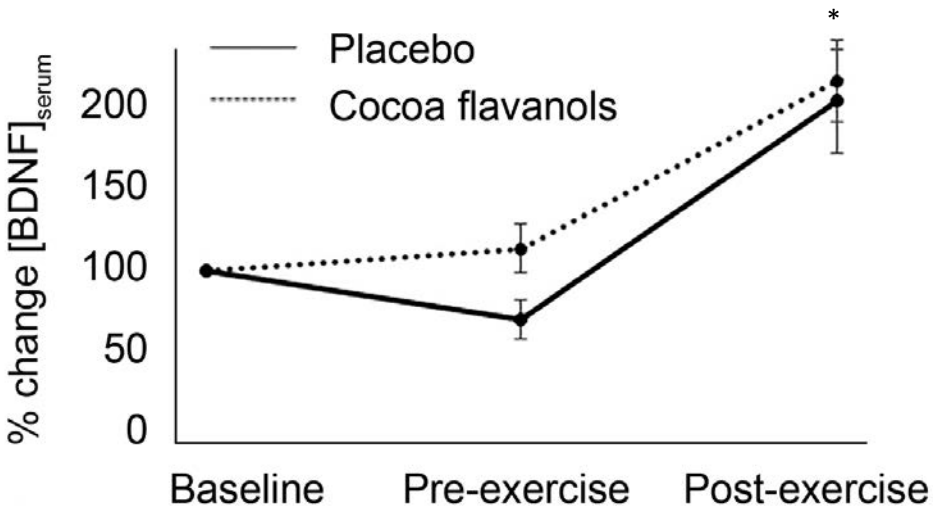

Figure 15. Relative changes in serum BDNF concentrations after the intake of cocoa flavanol (full lines) or placebo (dashed lines), at baseline (BL), before and after exercise (pre and post-exe). *No significant effects of CF intake were found. In both interventions, exercise significantly increased serum BDNF concentrations. Means±SEM are presented. *: p<0.001 vs. pre-exercise*

3.5 Discussion

The aim of this study was to examine the effects of acute (900 mg) CF intake and exercise on cognitive function, taking into account 2 possible underlying factors (cerebral oxygenation/perfusion and BDNF) by which improved cognitive function is paralleled. The main findings of this study were that CF intake increased cerebral oxygenation at rest, but did not increase BNDF and did not improve cognitive performance at rest. Exercise increased cognitive performance, which was paralleled by an increased CBF and increased BDNF. When combining CF and exercise, CF did not have an additive effect on the exercise-induced cognitive enhancement and the associated increased and BDNF.

Cognitive performance

Our study showed that acute exercise improved speed of information processing (RT on the Stroop task). The efficiency of response inhibition, as measured by Stroop interference (incongruent minus neutral stimulus) was not improved significantly in response to exercise. Accuracy was not influenced by exercise. Contrary to our hypothesis, acute intake of CF did not result in improved cognitive performance (RT and accuracy on neutral, congruent and incongruent stimuli and Stroop interference) compared to PL intake, neither at rest nor after exercise. These findings are in line with the studies of Massee et al. (2015) and Pase et al. (2013), who found no effects of a lower (i.e. 250 mg and 500 mg respectively) dose of CF intake on performance on two 30-min cognitive test batteries, including the Stroop task, in healthy subjects. In contrast, Field et al. (2011) and Scholey et al. (2010) found that some parts of cognitive function, in particular spatial memory, visual contrast sensitivity and serial subtraction improved after an acute intake of CF in healthy young adults. Although the amount and timing of CF intake (773 mg; 2 h and 994 mg; 1.5 h) were comparable to those in our study (900 mg; 1.5 h), the cognitive tasks performed, lasting 45 min (Scholey et al. 2010) and 60 min (Field et al. 2011) were substantially longer than the cognitive task lasting 5 min in our study. It could be argued that this short cognitive task was not challenging enough for this young, healthy population and that a ceiling effect of RT had been already reached (Francis et al. 2006). By using a longer cognitive task (45-60 min) in the studies of Field et al.(Field et al. 2011) and Scholey et al. (2010), mental fatigue ratings were increased and cognitive performance was progressively declined even in young subjects, thus eliminating this ceiling effect. Therefore, the beneficial effect of CF might have been easier to detect in this setting. However, in the present study, we aimed to assess the effect of CF on executive function in combination with exercise. Given that acute exercise affects executive functioning, but that these beneficial effects start to vanish 20-min post-exercise (Chang et al. 2012), a shorter cognitive task specifically assessing executive functioning was chosen.

Brain hemodynamic changes

Until now, the acute effect of CF intake on CBF has only been assessed using fMRI, at rest and/or during a CT (Francis et al. 2006; Lamport et al. 2015). In addition to these 2 studies, we wanted to access cerebral perfusion and oxygenation during and after exercise, leading to the choice of NIRS. Compared with functional magnetic resonance imaging (fMRI), NIRS can be applied during exercise and is relatively insensitive to movement artefacts. This technique has already been shown to be sensitive to alterations in CBF during CT in response to other nutritional interventions like caffeine, nitrate and resveratrol (Kennedy et al. 2010; Dodd et al. 2015; Wightman et al. 2015).

Prefrontal ΔHbO_2 and ΔHb_{tot} were increased during the CT (positive values) while ΔHHb was decreased (negative values), which reflects the principle of neurovascular coupling (Perrey 2008). In accordance to the Compensation-Related Utilization of Neural Circuits Hypothesis (CRUNCH) (Paul et al. 2012), which proposes that neural engagement varies with the level of task demand, the increase in ΔHbO_2 and decrease in ΔHHb was significantly larger during the second part of the Stroop task (congruent and incongruent stimuli) compared to the first part (neutral stimuli). The more complex congruent and incongruent stimuli of the Stroop task requires higher activation of the dorsolateral prefrontal cortex, the area which serves as the highest cortical area responsible for executive control and decision making (Leung et al. 2000). In contrast, the "easier" neutral stimuli seemed to require less activation of the prefrontal cortex.

Hemodynamic changes during cognitive task at rest

The current study shows that acute CF intake increases cerebral oxygenation (ΔHbO_2) during the Stroop task. This result is in line with the results of a previous study, showing that 5 days of daily intake of 172 mg CF increased the BOLD response (i.e. change in blood oxygenation, detected with fMRI) in the dorsolateral prefrontal cortex, parietal cortex and anterior cingulate cortex, in response to task switching, without any concomitant effect on cognitive performance (Francis et al. 2006). The fMRI BOLD arises from a complex imbalance of increases in CBF, cerebral blood volume and cerebral metabolic rate of oxygen consumption, whereas NIRS is able to access cerebral blood volume (ΔHb_{tot}) and cerebral deoxygenation rate (ΔHHb) in addition to cerebral oxygenation (ΔHbO_2). Although not statistically significant, the effect of CF intake on ΔHb_{tot} followed the same pattern (increase) as ΔHbO_2 during the CT at rest, suggesting an increased vascular responsiveness and cerebral blood volume after CF consumption (Jackson and Kennedy 2013).

In this study, CF slightly influenced cerebral oxygenation during a CT at rest, but this did not translate into improved executive function. This might partly be explained by the cognitive domains that are activated by the Stroop task. The Stroop task also involves activation of the anterior cingulate and the posterior parietal cortices, but the monitoring of cerebral oxygenation in these brain regions was not

under the scope of this study. However, consumption of resveratrol, another polyphenol with vasodilatory properties, also resulted in increased cerebral blood volume in the prefrontal cortex without improving cognitive performance, during a cognitive task, which was shown to specifically activate the prefrontal cortex (Kennedy et al. 2010).

The CF-induced increases in prefrontal oxygenation (ΔHbO_2) and blood volume ($\Delta Hb_{tot,}$) reflect an increased neurovascular coupling and are most likely related to an increased NO-mediated vasodilation during neuronal activity (Kennedy et al. 2010). However, as oxygen extraction (ΔHHb) and cognitive performance were not altered by CF intake, one can argue whether the CF-induced increase in vascular responsiveness was useful during this short and relatively simple cognitive task. In healthy subjects, the negative feedback control system of neurometabolic regulation assures a balance between oxygen delivery through cerebral blood flow (supply) to its demand (cerebral metabolism). This might explain why additional increases in vascular responsiveness do not result in improved cognitive performance. Therefore, future research should aim to measure the effects of CF intake on hemodynamic changes, concomitantly with neuronal activity by electro-encephalogram (EEG) during longer and more challenging cognitive tasks, or in circumstances where this neurometabolic- neurovascular regulation is under pressure.

Hemodynamic response to exercise and during cognitive task after exercise

This study showed that ΔHb_{tot}, ΔHbO_2 and ΔHHb, significantly increased during acute exercise, which can be attributed to a local vasodilatation likely resulting in a local increased CBF (Oussaidene et al. 2015). However, CF intake did not influence this exercise-induced hemodynamic response. In the 5 min upon cessation of exercise, ΔHbO_2, ΔHHb and ΔHb_{tot} significantly decreased again, with CF intake again not altering this decrease. Noteworthy, we did not detect any influence of CF intake on all NIRS parameters during the post-exercise CT. The large exercise-induced increase in ΔHbO_2 eradicated the small beneficial effect of CF intake on ΔHbO_2, detected during the CT at rest, during the CT post-exercise.

BDNF

Acute exercise-induces cognitive enhancements are linked with increases in BDNF (Skriver et al. 2014). It has been shown in animal studies that CF can cross the blood-brain-barrier (Abd El Mohsen et al. 2002) and that flavanols can interact with the cellular cascade resulting in upregulation of brain BDNF gene or protein expression (Rendeiro et al. 2013). Since human serum BDNF majorly originates from a production by the brain (Rasmussen et al. 2009), we investigated the effect of acute CF intake on serum BDNF. Following exercise, serum BDNF was increased. Contrary to our hypothesis, CF intake did not affect BDNF levels. Chronic intake of CF might be needed to obtain the molecular effect on BDNF,

since it was shown that hippocampal BDNF increased in rat and mice after 6 and 14 weeks of CF consumption (Rendeiro et al. 2013; Stringer et al. 2015).

Limitations and future research

Although a familiarization session with 3 repeats of the CT was implemented 1 week before the study, this could not avoid some learning effect for the first part of the Stroop task (neutral stimuli) (Lemay et al. 2004): independent of CF or PL drink, subjects reacted significantly faster on the pre-exercise Stroop task (neutral stimuli only) compared to the baseline CT. This prevented us from concluding about a positive effect of acute exercise on RT during the first part of the Stroop task (neutral stimuli).

In the present study, cerebral oxygenation was measured by NIRS. A persisting concern with NIRS is the extent to which light is contaminated by the extracranial tissues. However, we used a sufficiently large interoptode distance (4 cm) to avoid the interference of scalp blood flow on cerebral hemodynamic variables quantified by NIRS (Germon et al. 1999). We acknowledge the fact that (left) prefrontal cortex hemodynamic and oxygenation recording is a regional measurement that may not be reflective of global cerebral changes. Lamport et al. (2015) recently reported that CF intake resulted in a regional increase in CBF (as measured with arterial spin labeling MRI) in anterior cingulate cortex and the area from central opercular cortex to the left parietal lobe, but not in the prefrontal cortex. This regional CBF increase might contribute to the observation of a global CBF increase in the gray matter in a pilot study (4 subjects) of Francis et al. (2006). Since we did not measure hemodynamic changes during the absorption period (at rest) and the NIRS system used measured relative and not absolute hemodynamic changes, we cannot draw any conclusions on the effect of CF on CBF at rest. Therefore, the use of a NIRS system measuring absolute hemodynamic changes or measuring hemodynamic changes during the 90-min absorption period would be useful. In future research, it is advised to incorporate multi-channel and multi-site monitoring of cerebral blood volume and oxygenation. Combining the use of NIRS with EEG could reveal the link between neuronal activity and cerebral oxygenation and blood flow after CF intake even further (Muthalib et al. 2013).

In conclusion, acute CF intake increased cerebral oxygenation during a cognitive task assessing executive function, but without any impact on cognitive performance. Serum BDNF was not influenced by CF intake. Exercise improved cognitive function, which was paralleled by increased CBF and oxygenation and increased BDNF levels. When combining CF and exercise, CF had no additive effect on the exercise-induced cognitive enhancement and the associated increased cerebral oxygenation and perfusion and serum BDNF.

3.6 Acknowledgements

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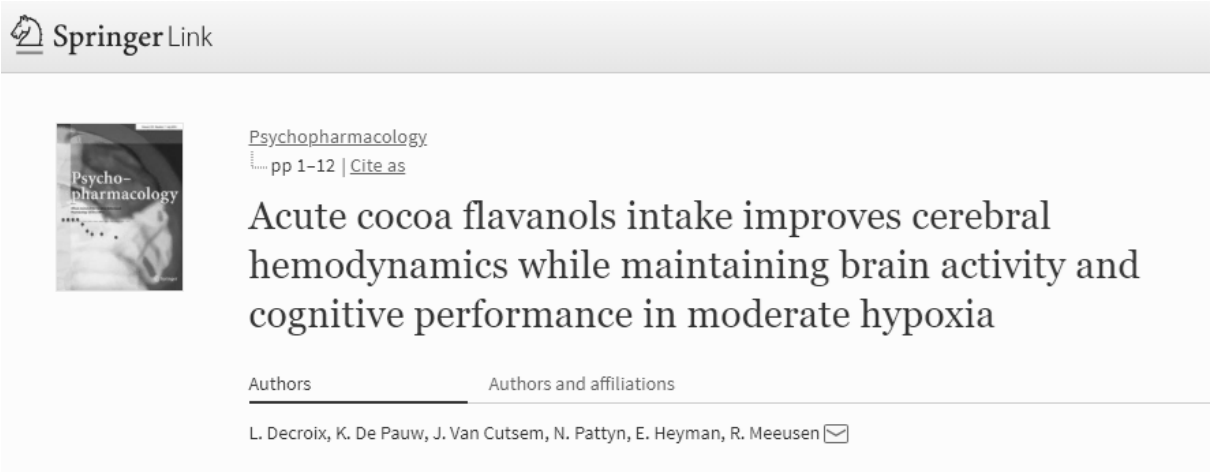
Chapter 4. Acute cocoa flavanol intake improves cerebral hemodynamics while maintaining brain activity and cognitive performance in moderate hypoxia



4.1 Abstract

Introduction: Acute cocoa flavanols (CF) intake has been suggested to modulate cognitive function and neurovascular coupling (NVC). Whether increased NVC is solely driven by improved vascular responsiveness or also by neuronal activity remains unknown. This study investigated the effects of acute CF intake on cognitive performance, NVC and neuronal activity in healthy subjects in normoxia and hypoxia (4000 m simulated altitude; 12.7% O_2).

Methods: Twenty healthy subjects (age: 23.2±4.3 yr) performed 4 trials. Participants performed a Stroop task and "Cognition" battery 2 h after acute CF (530 mg CF, 100 mg epicatechin) or placebo intake, in hypoxia or normoxia. Electroencephalogram and functional Near-Infrared Spectroscopy were used to analyse hemodynamic changes and neuronal activity.

Results: CF enhanced NVC in the right prefrontal cortex during several tasks (risk decision making, visual tracking, complex scanning, spatial orientation), while neuronal activity was not affected. CF improved abstract thinking in normoxia, but not in hypoxia and did not improve other cognitive performances. Hypoxia decreased accuracy on the Stroop task, but performance on other cognitive tasks was preserved. NVC and neuronal activity during cognitive tasks were similar in hypoxia vs. normoxia, with the exception of increased β activity in the primary motor cortex during abstract thinking.

Conclusions: Acute CF intake improved NVC, but did not affect neuronal activity and cognitive performance in both normoxia and hypoxia. Most cognitive functions, as well as NVC and neuronal activity, did not decline by acute exposure to moderate hypoxia in healthy subjects.

Keywords: Electroencephalogram, functional Near-Infrared Spectroscopy, Cocoa flavanol, cognitive performance

4.2 Introduction

Nutritional supplements are popular not only for their potential beneficial effects on general health, but also on brain health (Socci et al. 2017). In this context, there is increasing interest in flavonoids, a subgroup of polyphenols, which are a class of natural compounds found in the human diet and include subcategories of flavanols, flavonols, iso-flavones, flavones, and anthocyanidins (Del Rio et al. 2013). Flavanols are found in grapes, tea, red wine, apples and especially cocoa (Manach et al. 2004). Previous human clinical studies showed that cocoa flavanols (CF) have antioxidant and anti-inflammatory properties and improve vascular function (Andújar et al. 2012). Moreover, evidence exists that CFs have neuroprotective and neuromodulatory effects (Francis et al. 2006; Scholey et al. 2010; Nehlig 2013; Scholey and Owen 2013). It was suggested that long-term CF intake enhances neuronal function by interacting with neuronal intracellular signalling pathways involved in neuronal survival and differentiation, memory and long-term potentiation (Spencer 2008). It seems that cognitive enhancements observed upon acute CF supplementation are associated with its vasodilatory actions (Socci et al. 2017). Previous research has shown improved nitric oxide (NO)-mediated vasodilation in response to acute CF intake, resulting in improved blood flow in the periphery (Grassi et al. 2015) and the brain (Lamport et al. 2015). It has been shown that acute CF intake increased the neurovascular coupling (NVC) during cognitive tasks, assessed by function Near-Infrared Spectroscopy (fNIRS) (Decroix et al. 2016) and functional Magnetic Resonance Imaging (fMRI) blood oxygenation level dependent (BOLD) response (Francis et al. 2006). NVC reflects the local increase in CBF to match oxygen (O_2) supply to neuronal demand during neuronal activation (Ogoh 2017). Several processes underlie NVC, including vasodilation, blood volume, blood oxygenation, neuronal activity and synaptic activity (Steinbrink et al. 2006). An altered hemodynamic response can arise as a result of altered neuronal activity or as a change in vascular responsiveness (or both) (Murta et al. 2015).

While acute CF intake has been shown to improve NVC (Francis et al. 2006; Lamport et al. 2015; Decroix et al. 2016), it remains unclear whether this is solely caused by improved vascular function, or also by altered neuronal activity. Camfield et al. found that chronic CF intake (500 mg CF) altered steady state visually-evoked potential (SSVEP) amplitude at several centro-frontal sites during the spatial working memory test, which was interpreted by the authors as increased neural efficiency (Camfield et al. 2012). Although it may be hypothesized that acute CF intake may enhance neural efficiency (i.e. reducing the neuronal activation required to perform cognitive tasks), it has not been investigated so far. Therefore, we aimed to simultaneously investigate the effects of acute CF intake on neuronal activity and the hemodynamic response (changes in oxyhemoglobin (ΔHbO_2) and deoxyhemoglobin (ΔHHb)) during cognitive tasks. Changes of neuronal activity can be assessed by multichannel electroencephalogram (EEG), and standardised low-resolution brain electromagnetic tomography

(sLORETA) can be utilised to localise regional changes in cerebrocortical activity associated with cognitive performance (Schneider and Strüder 2009). Understanding the relationship between EEG and fNIRS signals should give additional insights into the neuronal substrate of the increased NVC signal, measured by fNIRS (Murta et al. 2015).

Increasing the provision of metabolic substrates by enhancing CBF can result in cognitive benefits (Scholey and Owen 2013). Although there is compelling evidence that acute CF intake increases the cerebrovascular response, the effect of CF on cognitive performance is still ambiguous (Socci et al. 2017). It seems that the quantity and bioavailability of the consumed CF and the subject population used in the studies highly influence the observed beneficial effects (Scholey et al. 2010; Field et al. 2011; Pase et al. 2013; Massee et al. 2015). Pase et al. (2013) and Francis et al. (2006) failed to find beneficial effects of 500 mg and 150 mg CF. The authors stated that the healthy subjects were already performing at a high level of cognitive ability, which would be very difficult to be improve upon. However, several authors (Scholey et al. 2010; Field et al. 2011; Massee et al. 2015) detected improved cognitive performance on working memory tasks and visual function after different dosages of CF in healthy persons (750, 994 and 250 mg). In a recent review, it was noted that in healthy, young subjects, acute CF intake mainly improved cognitive performance in cognitive demanding environments, such as during sustained mental efforts or in fatigued state (Grassi et al. 2016). Acute exposure to hypoxia (12.7% O_2) also forms a cognitive demanding environment. Optimal brain function requires adequate O_2 supply and the decreased arterial pressure of oxygen (PaO_2) and arterial saturation of O_2 (SaO_2) in hypoxia may compromise cerebral O_2 supply and decrease cerebral oxygenation. Previous studies indeed reported cognitive impairments during acute hypoxia, which were related to an insufficient cerebral O_2 supply (Ando et al. 2013). Yet, there appear to be regional differences in the vulnerability/sensitivity to hypoxia, implicating reduced cognitive function in some cognitive domains and not in others (Neubauer and Sunderram 2004).

In the present study, we combined simultaneous recording of hemodynamic changes (fNIRS) with EEG to unmask the physiological origin of NVC, namely vascular responsiveness and/or neuronal activity. We hypothesized that acute CF supplementation would increase NVC (by facilitating NO-mediated vasodilation), without altering neuronal activity, as suggested by Socci et al. (2017). Moreover, we hypothesized that acute CF intake might (partially) counteract hypoxia-induced cognitive impairments as a consequence of increased NVC. Thus, the aim of this study was to investigate the effects of acute CF intake on cognitive function, neuronal activity and NVC during exposure to normobaric hypoxia (4000 m altitude; 12.7% O_2).

4.3 Materials & Methods

A randomized, placebo (PL) controlled, counter-balanced, double-blind, cross-over study design was used.

4.3.1 Participants

Twenty healthy young (male and female) students were selected for participation in this study (age: 23.2±4.3 yr). The number of subjects was chosen after a power calculation based on the results of Scholey et al. (2010), examining the acute effect of CF on cognitive performance in normoxia. Subjects were not acclimatized to a hypoxic environment. Subjects were excluded if they (1) were younger than 18 years or older than 35 years, (2) had severe head injuries in the past, (3) took neuro-modulating medication (psychotropic drugs, beta adrenergic blockers, steroids,…), (4) were hypertensive, (5) had cardiovascular disease, (6) were smokers (7) had other diseases which can alter cognitive function (diabetes, depression…), (8) had a G6PD-deficiency. The experimental procedures and potential risks were explained to the participants and a written informed consent was provided and signed before the start of the study. The study protocol was approved by the Ethical Committee of the Brussels University hospital and was carried out in accordance with the Declaration of Helsinki.

4.3.2 Intervention

During the study, subjects filled out a food frequency questionnaire on 3 separate days, allowing the calculation of daily polyphenol intake.

On the first visit, subjects familiarized with the cognitive task battery by performing the battery three times. Subsequently, subjects performed 4 interventional trials, with a wash-out period of 1 week in between the trials. Subjects were asked not to change their regular sleeping pattern and eating behaviour during the entire study and especially the two nights preceding each interventional trial. They were also asked to abstain from caffeine, alcohol, other psychoactive substances and polyphenol-rich foods (green tea, red wine, dark chocolate (cocoa) and grape (juice)) the last 24 h prior to each intervention trial.

The interventional trials consisted of (1) CF intake before cognitive performance in normobaric hypoxia (4000 m; 12.7% O_2), (2) PL intake before cognitive performance in hypoxia, (3) PL intake before cognitive performance in normoxia (0 m; 21.0% O_2), and (4) CF intake before cognitive performance in normoxia (0 m; 21.0% O_2). All experimental trials were conducted in a normobaric hypoxic chamber, set at 20 °C and with a relative humidity between 30 and 40%.

Subjects reported to the lab in a 3 h-fasted state at the same time of the day for each experimental trial (between 8 am and 4 pm). Subjects then consumed the provided food supplement (4 capsules) (PL or

CF, Naturex, Avignon, France), together with a carbohydrate rich meal, which was carefully selected by a nutritionist to contain 600 kcal, 85% carbohydrates, 10% proteins and 5% fat. The CF supplement was a 1765 mg cocoa extract which contains 530 mg flavanols (of which 100 mg epicatechin, 23 mg catechin), 119 mg theobromine and 17 mg caffeine. Capsules with maltrodextrin, matched in theobromine, caffeine, colour, shape and texture served as PL. The nutritional intervention (i.e. intake of PL versus intake of CF) was double-blind and counter-balanced.

Ninety min after supplement intake, subjects entered the climatic chamber, which was set at the desired altitude. NIRS equipment was applied and a pulse oximeter was positioned on the participants' left index finger (Medlab, Germany) to record heart rate (HR) and indirectly measure arterial O_2 saturation (SpO_2). Subjects were blinded to the hypoxic or normoxic environment. Only one subject experienced symptoms of acute mountain sickness. He was instructed to immediately leave the hypoxic chamber and his results were excluded from data analysis.

4.3.3 Cognitive test

Two hours after CF intake and thirty min upon entrance in the climatic chamber, subjects started the cognitive test battery. During the cognitive testing, subjects were seated, had earplugs inserted and had been instructed to minimize head movement and eye blinking, to avoid frowning and maintain the same posture, in order to minimize movement artefacts in the NIRS data.

First, subjects performed a Stroop task (approximately 9 min) which was immediately followed by the Joggle Cognition test battery.

Stroop test. The Stroop test was programmed and performed on E-prime 2.0 software (Psychology Software Tools, Inc., Pittsburg, PA) and is commonly known as a tool to measure selective attention, cognitive flexibility and response inhibition (MacLeod and MacDonald 2000). This test assesses the ease with which a person can maintain a goal in mind and suppress a habitual response in favour of less familiar ones. The words "green, blue, yellow and red" were shown in matching (congruent) or non-matching (incongruent) colours. Participants had to press the coloured button on the keyboard in which the colour names were printed, disregard their reading content. Outcome measures were accuracy and reaction time (RT) of the decision-making process.

Cognition test battery. The computerized cognitive test battery "*Cognition*" was used because of its sensitivity to multiple cognitive domains at high-level cognitive performance (Basner et al. 2015). *Cognition* consists of 8 neuropsychological tests known to engage specific brain regions evidenced by functional neuroimaging. In particular, the battery consists of the motor praxis test (MPT, measure of sensorimotor speed), visual object learning test (VOLT, measure of spatial learning and memory), abstract matching (AM, measure of abstraction), line orientation test (LOT, measure of spatial

orientation), digit symbol substitution test (DSST, measure of complex scanning and visual tracking), balloon analog risk test (BART, measure of risk decision making), NBACK (measure of working memory) and psychomotor vigilance test (PVT, measure of vigilant attention) and takes approximately 18 min in total.

4.3.4 fNIRS measurements

Functional NIRS, a non-invasive optical imaging technique, was used to assess acute neuronal hemodynamic changes (Octamon continuous-wave NIRS (CW-NIRS) system (Artinis Medical Systems B.V., The Netherlands). By introducing near-infrared light through the skull, oxyhemoglobin (HbO_2) and deoxyhemoglobin (HHb) absorb light at 800–940 nm and 600–750 nm respectively, allowing the measurement of their relative concentrations in the cerebral blood (Perrey 2008). This device uses the modified Beer-Lambert Law to monitor concentration changes in ΔHbO_2 and ΔHHb (in µM.cm) relative to first datum. Concentration changes are the result of the interplay between regional CBF, blood volume and metabolic rate of O_2. NVC is reflected by a increase in ΔHbO_2 and decrease in ΔHHb (Steinbrink et al. 2006).

A six-channel fNIRS system was used to measure ΔHbO_2 and ΔHHb using an age-dependent constant differential path-length factor given by 4.99+0.0067 × (age 0.814) (Duncan et al. 1996). The unit consisted of a headband with 6 light emitters and two light detectors, with an interoptode distance of 3.5 cm. The device placement was replicated between trials by positioning the bottom of the headband 1 cm above the eyebrows, and the middle of the headband in the center of the forehead. Thus, the six fNIRS optodes (six emitters and two detectors) were placed symmetrically over the anterior and medial prefrontal cortex (PFC) (Brodmann areas 46 and 10) (Okamoto et al. 2004). Data were acquired at a sampling frequency of 10 Hz and down sampled by factor 10 for data analysis.

Data were normalized to a 30-sec resting period, during which participants sat still and did not speak, 30 min after entering the climatic chamber and prior to the start of the cognitive test battery. This normalization was done in order to reflect changes from this 30 sec-reference measurement to express the magnitudes of changes. Mean concentration changes (ΔHbO_2 and ΔHHb) during the last 30 seconds of each bout of the cognitive test (durations between 40 sec and 5 min) were calculated for each cognitive test. Data from the channels on the left (T1,T2, T4) and right (T6, T7, T8) PFC were averaged for each cognitive task. The use and limitations of NIRS for monitoring cerebral regional hemodynamic changes and oxygenation have been extensively reviewed (Rooks et al. 2010).

4.3.5 EEG and sLORETA

Before and during the cognitive test battery, brain activity was continuously measured by 32 active Ag/AgCl electrodes attached on the subject's head (Acticap, Brain Products, Munich, Germany),

according to the 10-20 International System. Electrode impedance was kept below 10 kΩ during the entire recording. During a 2-minute baseline measurement, 30 min after entering the climate room, subjects were instructed to sit still, not speak and minimize eye-movement. During the cognitive test battery, events were created in the software to indicate start and endings of each part of the cognitive battery.

Brain Vision Analyzer (version 2.1) was used to preprocess and process the data. First, raw data were down sampled to 256 Hz, filtered (high pass 1 Hz, low pass 45 Hz and Notch 50 Hz, Slope 48 dB/oct) with a Butterworth filter design and re-referenced to an average reference. Then, data were segmented in segments of interest (baseline and each cognitive task) and artefacts were manually removed by raw data inspection. For the segments of the baseline measurement and each part of the cognitive task, artefacts were further removed by using independent component analysis (ICA) and inverse ICA upon manual artefact removal. Subsequently, data were segmented and averaged to a 4 s window (1024 data points, frequency resolution: 0.25 Hz) for analysis in sLORETA. sLORETA is a source localization method which analyses whether brain frequency bands differ between 2 conditions and where these differences take place. This analysis attempts to solve the inverse problem by assuming related orientations and strengths of neighbouring neuronal sources (Pascual-Marqui 2002). EEG files were converted to cross spectra files and the classical frequency bands of interest (i.e. α1 (8.5-10 Hz), α2 (10.5-12 Hz), β1 (12.5–18 HZ), β2 (18.5–21 HZ), β3 (21.5–30 HZ), δ (1.5–6 HZ), θ (6.5–8 HZ)) were selected and the sLORETA program computed the corresponding 3D distribution of the electric neuronal generators. The latter were computed for each subject and dataset, for each aforementioned frequency band.

4.3.6 Statistics

Statistical analysis was carried out by using the Statistical Package for the Social Sciences, version 22 (SPSS Inc., Chicago, IL, USA), with significance set at 0.05 for all analyses. Data are presented as means±standard deviation (SD), unless stated otherwise.

Normality of the data was tested by using one-sample Kolmogorov-Smirnov test, while sphericity was verified by the Mauchly's test. When assumption of sphericity was not met, the significances of F-ratios were adjusted with the Greenhouse-Geisser procedure. When normality was violated, non-parametric testing was used.

For cognitive performance on the Stroop task, a three-way repeated measure ANOVA was used to assess the effects of stimuli (congruent vs. incongruent), supplement (CF vs. PL) and environment (hypoxia vs. normoxia). For cognitive performance on each task of the Joggle Cognition battery, the effects of supplement (CF vs. PL) and environment (hypoxia vs. normoxia) were analysed by two-way repeated

measure (2 x 2) ANOVAs. The effects of environment, supplement as well as time (before/after Stroop and before/after Cognition battery) on physiological measures, HR and spO_2, were examined by three-way repeated measure ANOVAs. For the fNIRS-data, three-way repeated measure ANOVAs were employed to investigate the effects of side (left or right), environment and supplement on ΔHbO_2 and ΔHHb. If significant interaction effects were found in the three-way or two-way repeated measures ANOVAs, two-way repeated measure ANOVAs (per side) or paired t-tests (per environment) were respectively performed in order to interpret the effects of supplement in each environment. If no significant interaction effects were observed in the three-way or two-way repeated measures ANOVAs, main effects of environment and supplement were immediately observed and further interpreted through pairwise comparisons with the Bonferroni correction.

For sLORETA, paired sampled t-tests were computed at each voxel for each cognitive test between the following conditions: PL N vs PL H to assess the effect of hypoxia, PL N vs CF N and PL H vs CF H to assess the influence of the supplement. Non-parametric randomisation tests were performed at all voxels simultaneously, since no 'a priori' hypotheses existed. To correct for these multiple comparisons, the statistical program of sLORETA was based on Fisher's permutation test and relied on a bootstrap method with 5000 randomizations. A nonparametric single-threshold test was assessed, defining a critical threshold (t critical), to correct for multiple comparisons. Voxels with statistic values exceeding this threshold have their null hypothesis, i.e. no difference in EEG power between two conditions, rejected. The omnibus hypothesis (that all the voxel hypotheses are true) was rejected if a voxel value exceeded the critical threshold for $p<0.05$ defined by 5000 randomisations. The statistical non-parametric map method provided voxel information of the results (i.e., Montreal Neurological Institute/Talairach coordinates, Brodmann area (BA), lobe and structure).

4.4 Results

4.4.1 Physiological measures

Acute exposure to hypoxia significantly lowered spO_2 compared to normoxia (hypoxia: 83.8±2.1%; normoxia: 98.0±1.0%; main effect of environment ($F(1,19)=1135.51$, $p<0.001$)). CF supplementation and time did not affect spO_2. HR was significantly elevated during the cognitive test, compared to before the test (main effect of time ($F(1,19)=27.11$, $p<0.001$)) and was 4.3% higher in hypoxia (81.99±2.99) than in normoxia (78.58±2.41) (main effect of environment ($F(2, 34)= 4.47$, $p=0.046$)). HR was not affected by CF supplementation.

4.4.2 Cognitive performance

Stroop task. For accuracy on the Stroop task, main effects of environment ($F(1,15)=4.71$, $p= 0.046$) and stimuli ($F(1,15)=30.71$, $p<0.001$) were found; post-hoc analyses showed that accuracy was significantly lower in hypoxia (90.6±4.5%) compared to normoxia (92.5±4.3%) and that accuracy was significantly lower on the incongruent stimuli (88.9±2.6%) compared to the congruent stimuli (94.2±1.3%) (Table 10). CF intake did not influence accuracy. RT was not slower in hypoxia compared to normoxia, and was not affected by CF intake. The significant main effect of stimuli ($F(1,17)=81.41$, $p<0.001$) indicated that RT was significantly higher on the incongruent stimuli (627.0±70.7 ms) than congruent stimuli (571.1±65.3 ms).

Joggle cognition battery. Performances on the LOT, MPT, NBACK, BART and DSST were neither affected by hypoxia (compared to normoxia) nor by CF intake (compared to PL) (Table 10). The score (expressed as a number/1000) on the PVT tended to be lower in hypoxia (762.3±148.4) compared to normoxia (793.6±160.6) (main effect of environment $F(1,19)=3.19$, $p=0.075$)). RT on the VOLT tended to be slower in hypoxia (1844.6±567.7 ms) compared to normoxia (1753.0±585.6 ms) (main effect of environment ($F(1,19)=3.23$, $p=0.080$)). A 2-way interaction effect *environment x supplement (*$F(1,19)=4.99$, $p=0.038$) was found for RT on the AM. Post-hoc t-tests showed that in normoxia, RT was faster after CF intake (2085.2±171.4 ms) compared to PL intake (2257.2±192.0 ms) ($t=-2.75$, $p=0.013$), while there was no significant difference between CF and PL intake in hypoxia.

Table 10. Cognitive performance in normoxia and hypoxia after acute cocoa flavanol or placebo intake. *MPT: motor praxis task, VOLT: Visual Object Learning Task, AM: abstract matching, LOT: line orientation test, DSST: digit symbol substitution test, BART: balloon analog risk test, PVT: psychomotor vigilance test, RT: reaction time. * main effect of hypoxia in 2-way repeated measures ANOVA, £ main effect of supplement in 2-way repeated measures ANOVA. p<0.05.*

N=20		*Placebo*		*Cocoa flavanol*	
Task	Outcome	Normoxia	Hypoxia	Normoxia	Hypoxia
Stroop-congruent	Accuracy (%)	95.1±2.6	93.8±4.2*	94.8±3.9	93.1±3.9*
	RT (ms)	566.4±66.3	575.2±59.7	567.8±58.9	575.1±76.3
Stroop-incongruent	Accuracy (%)	89.7±5.8	87.2±11.1*	90.3±6.7	88.4±6.4*
	RT	621.8±75.5	631.5±66.8	631.5±68.7	623.2±74.8
LOT	Score (/1000)	815.5±94.7	841.6±62.1	813.0±95.7	820.3±68.3
	RT (ms)	5981.5±1570.5	5491.1±1045.4	5861.2±1925.7	5798.5±1217.6
	Accuracy (%)	61.1±13.7	62.2±12.5	62.7±13.7	60.0±17.6
MPT	Score (/1000)	815.5±94.7	841.6±62.1	813.0±95.7	820.3±68.3
	RT (ms)	5981.5±1570.5	5491.1±1045.5	5861.2±1925.7	5798.5±1217.6
NBACK	Score (/1000)	724.8±142.2	748.0±158.5	687.4±172.3	731.4±179.8
PVT	Score (/1000)	809.0±153.5	765.6±151.9	778.1±158.4	759.0±179.8
	RT (ms)	243.6±24.3	247.4±23.7	248.0±27.2	257.5±43.9
VOLT	Score (/1000)	736.4±114.8	699.5±112.7	728.4±117.3	720.6±112.7
	RT (ms)	1680.2±493.4	1906.5±698.4	1825.7±676.4	1782.7±547.0
AM	Score (/1000)	575.6±85.2	586.4±51.1	557.8±79.4	611.7±51.1
	RT (ms)	2257.2±836.8	1967.6±664.6	2085.2±746.9£	1981.2±679.4
BART	Score (/1000)	920.0±92.2	908.6±110.2	930.0±85.4	914.6±93.4
DSST	Score (/1000)	973.9±21.2	973. 1±13.5	971.7±26.6	960.9±43.5
	RT (ms)	932.0±70.0	918.3±54.0	917.8±67.1	919.9±85.3
	Accuracy (%)	99.1±0.0	99.0±0.0	99.1±0.0	98.5±0.0

4.4.3 fNIRS

The typical hemodynamic response to neural activity is characterized by a concomitant increase in ΔHbO_2 and decrease in ΔHHb. One-sample t-tests compared ΔHbO_2 and ΔHHb with zero for each test in each condition, showing significant changes (and thus hemodynamic coupling) during all cognitive tests (Figure 16).

Stroop task. ΔHbO_2 and ΔHHb during the Stroop task were not affected by hypoxia, nor by CF supplementation. Three-way repeated measures ANOVA showed a main effect of side ($F(1,13)=5.36$, $p=0.046$) for ΔHbO_2 and ΔHHb with a larger increase in ΔHbO_2 and larger decrease in ΔHHb at the right side of the PFC compared to the left side.

Joggle cognition battery. During the PVT, AM, NBACK and VOLT, ΔHbO_2 and ΔHHb were not altered by CF intake nor by hypoxia. During DSST, BART and LOT, CF intake enlarged the decrease in ΔHHb in the right PFC but did not influence ΔHbO_2 (ΔHHb: supplement x side effect: BART ($F(1,9)=7.79$, $p=0.025$), DSST ($F(1,9)=5.20$, $p=0.049$) and LOT ($F(1,9)=5.14$, $p=0.049$)). ΔHbO_2 and ΔHHb were not altered by hypoxia. During MPT, hypoxia increased ΔHbO_2 compared to normoxia (main effect of environment: $F(1,9)=8.03$, $p=0.020$), but ΔHHb was not altered. CF intake did not alter ΔHbO_2 nor ΔHHb during MPT.

4.4.4 sLORETA

sLORETA confirmed that the different cognitive tasks lead to activation of specific brain regions in several frequency bands, compared to the baseline condition. During AM, increased β2 activity was found in hypoxia compared to normoxia, in the frontal lobe precentral gyrus (BA4; $p<0.05$, $t= 5.33$* (t_{crit} for $p<0.05=5.09$)), which was of highest significance at MNI coordinate x,y,z=-35,-20,55 (Figure 17). No further significant changes were obtained for the other frequency bands. During all other cognitive tests, there was no significant difference in brain activity in any of the frequency bands, between hypoxia and normoxia, in any brain region.

Statistical overall analysis by means of the omnibus significance test in sLORETA, revealed no differences in brain activity in none of the frequency bands during all the cognitive tests, between CF and PL intake in normoxia. Similarly, during all the cognitive tests, brain activity in each frequency band was not significantly different in any of the brain regions between CF and PL intake in hypoxia. Table 11 shows a summary of all results.

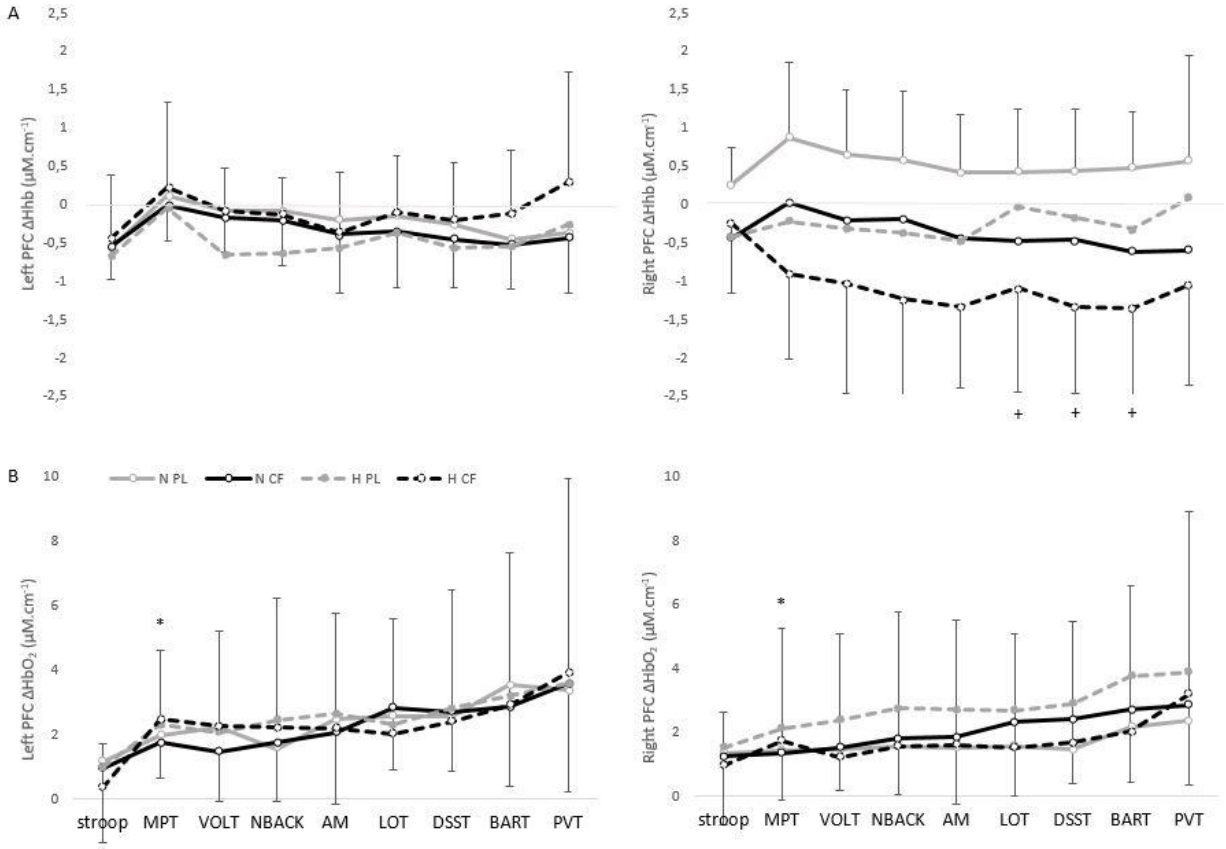


Figure 16. Hemodynamic changes in deoxyhemoglobin (ΔHHb, A) and oxyhemoglobin (ΔHbO₂ , B) in the left and right prefrontal cortex (PFC) during a cognitive test battery at 4000 m simulated altitude (hypoxia (H), dashed lines) and at sea level (normoxia (N), full lines) after acute cocoa flavanol (CF) supplementation (black lines) or placebo (PL, grey lines). **: significant main effect of environment (altitude) (p<0.05); +: significant difference between CF and PL in post-hoc test (significant supplement x side interaction effect (p<0.05)). MPT: motor praxis task, VOLT: Visual Object Learning Task, AM: abstract matching, LOT: line orientation test, DSST: digit symbol substitution test, BART: balloon analog risk test, PVT: psychomotor vigilance test.*

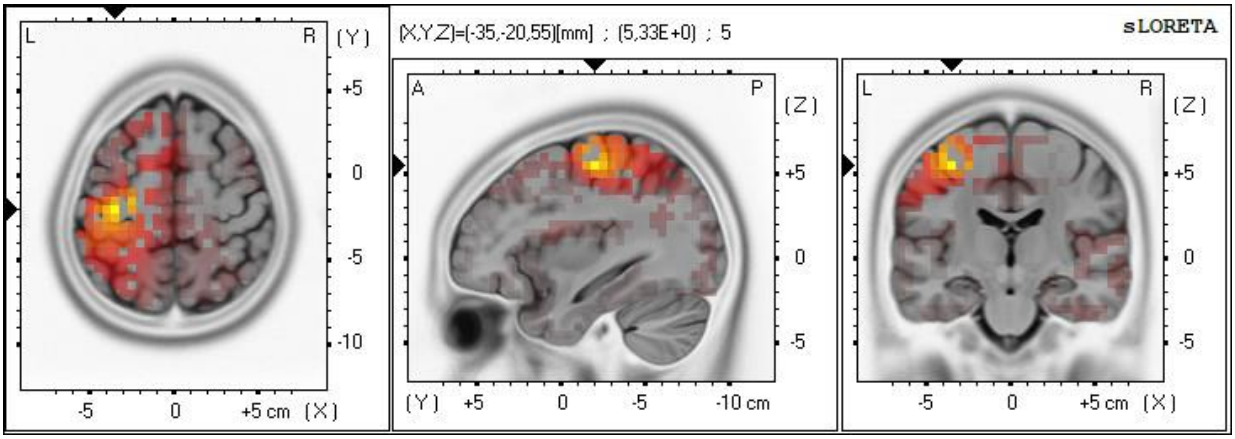


Figure 17. Statistical parametric maps (SPM) of sLORETA during the abstract thinking test after 30 min exposure to hypoxia (4000 m simulated altitude), compared to normoxia*. Red and yellow colours indicate increased activity in the β1-frequency range, which was found to be significant in the frontal lobe precentral gyrus. SPMs are based on voxel-by-voxel t-values of differences. Structural anatomy is shown in grey scale. L: left; R: Right; A: anterior; P: posterior.*

Table 11. Summary of results. *CF: cocoa flavanols, ΔHHb: change in deoxy-hemoglobin, assessed by Near-Infrared Spectroscopy, MPT: motor praxis task, VOLT: Visual Object Learning Task, AM: abstract matching, LOT: line orientation test, DSST: digit symbol substitution test, BART: balloon analog risk test, PVT: psychomotor vigilance test, BA: Brodmann area.*

Test	**Cognitive performance**		**ΔHHb (prefrontal cortex)**		**Neuronal activity**	
	Hypoxia	CF	Hypoxia	CF	Hypoxia	CF
Stroop	↓	=	=	=	=	=
MPT	=	=	↑	=	=	=
VOLT	↓	=	=	=	=	=
NBACK	=	=	=	=	=	=
AM	=	↑	=	=	↑ β2 in BA4	=
LOT	=	=	=	↑	=	=
DSST	=	=	=	↑	=	=
BART	=	=	=	↑	=	=
PVT	↓	=	=	=	=	=

4.5 Discussion

The main findings of this study were as follows: *(i)* Acute CF intake enhanced NVC in the right PFC during tasks known to activate the PFC (risk decision making, visual tracking and complex scanning and spatial orientation). This was not associated by altered neuronal activity. Acute CF intake did not affect any outcome measure of cognitive performance, with the exception of improved RT on abstract thinking in normoxia. *(ii)* Although selective attention and response inhibition were reduced in hypoxia, other cognitive domains were preserved. Exposure to hypoxia did neither change NVC nor brain activity, with the exception of increased β2 activity in the frontal lobe during abstract thinking.

Effects of CF intake

To the best of our knowledge, this was the first study simultaneously investigating the effects of CF on the cerebrovascular response and neuronal activity during cognitive performance, both in normoxia and hypoxia. Acute CF intake increased the hemodynamic response in normoxia and hypoxia: ΔHHb was larger in the right PFC during several cognitive tasks activating the PFC (tests assessing risk decision making, working memory and spatial orientation) after CF intake than PL. A hemispheric lateralization (i.e. greater ΔHHb and ΔHbO_2 in the right compared to left side of PFC) during cognitive tasks was previously also seen in the study of Medvedev et al., pointing to the primary involvement of the right hemisphere in resolving these cognitive tasks (Medvedev et al. 2011). Thus, CF intake induced a larger ΔHHb in the hemisphere which was most involved in solving the cognitive task.

It has been shown that ΔHHb has the highest correlations with the BOLD fMRI response (Steinbrink et al. 2006) and that ΔHHb can be considered as a better indicator of cerebral hemodynamic changes in hypoxic conditions (Davranche et al. 2016). Thus, the lack of a significant change in ΔHbO_2 following CF intake might be explained by interference of hypoxia-induced extracortical changes (e.g. increased HR) with cerebral hemodynamic ΔHbO_2. Alternatively, the effect of CF on ΔHHb, without altering ΔHbO_2 could theoretically also reflect a decreased metabolic rate of O_2 consumption during neuronal activation (Perrey 2008). While global cerebral rate of O_2 consumption can be assessed by magnetic resonance (Scholey 2017), this has not yet been applied to acute CF intake.

The hemodynamic response (decrease in ΔHHb) to neuronal activity originates from an exaggerated regional increase in CBF in response to neuronal activity and O_2 extraction. Evidence exists that acute CF intake stimulates vasodilation and CBF by increasing eNOS-dependent NO production (Schroeter et al. 2006). While we found an increased NVC upon acute CF intake, we could not detect any changes in neuronal activity, as measured by EEG and sloreta. The lack of change in neuronal activity in this study contradicts a “direct” neuromodulatory effect of acute CF intake and points towards a purely

vascular factor as underlying mechanism of the improved NVC (Spencer 2009; Socci et al. 2017). Besides, this shows that acute CF does not improve neuronal efficiency.

While CF intake enhanced NVC, it did not result in better cognitive function. Only during abstract thinking, acute CF intake improved the speed of processing while maintaining the overall accuracy in normoxia. Since this was neither accompanied by a change in hemodynamic response in the PFC nor by changed brain activity in any region, we could not unveil the underlying mechanism. Likewise, Francis et al. also found no behavioural changes despite the increased NVC after 5-day CF (150 mg) intake (Francis et al. 2006). Since impaired NVC is associated with poorer cognitive functions (Sinn and Howe 2008), it was previously hypothesized that improving NVC may enhance cognitive function (Wong et al. 2016). However, our results indicate that in subjects with a healthy circulation, improving NVC by CF intake does not result in better cognitive performance. Alternatively, the question rises whether this cognitive test battery was sensitive enough to detect effects of dietary interventions, such as CF, on human cognitive function to fully assess their efficacy in healthy young subjects (Macready et al. 2009).

Effect of hypoxia

The brain's high energy demand compared to the low energy stores makes the brain critically dependent on adequate glucose and oxygen supply. This renders the brain particularly vulnerable to hypoxic conditions (Ogoh 2017). In contrast to our hypothesis, most aspects of cognitive function were preserved when acutely exposed to normobaric hypoxia (12.7%) in young healthy subjects. During the Stroop task, accuracy was decreased in hypoxia, while speed of information processing, measured as RT, did not decline. RT on VOLT and the score on PVT tended to be deteriorated in hypoxia, suggesting that spatial learning and memory and vigilant attention were slightly decreased. We did not observe any hypoxia-induced changes on sensory-motor speed, abstraction component of executive function, spatial orientation, working memory (complex scanning and visual tracking) and risk taking behaviour. Cognitive function deteriorates with increasing altitude (Taylor et al. 2016) and exposure to more severe hypoxia (simulated hypoxia of 5500 m) showed severely reduced verbal and visual memory, processing speed, executive function, psychomotor speed, cognitive flexibility and complex attention (Turner et al. 2015). Moreover, NVC was similar in hypoxia compared to normoxia during all cognitive tasks, except during the PVC where the increase in ΔHbO_2 was elevated in hypoxia. This most likely reflects the hypoxic cerebral vasodilation (Davranche et al. 2016). Similarly, Davranche et al. (2016), Lefferts et al. (2016) and Shannon et al. (2017) found that acute hypoxia did not change the hemodynamic response during a cognitive task. A larger drop in spO_2 may be necessary to disturb the preservation of cognitive function and hemodynamic response (Taylor et al. 2016). Moreover, neuronal activity was not altered in hypoxia, with the exception of increased β activity, thus increased brain activity (Thompson et al.

2008), in the frontal lobe precentral gyrus during abstract thinking. Schneider et al. (2009) likewise observed increased β activity at rest following hypoxic exposure in the frontal gyrus. Frontal lobe precentral gyrus is involved in planning, control and executive function and is known to be recruited during this abstract matching task (Trans Cranial 2012; Basner et al. 2015). As abstract thinking was not deteriorated, the larger activation of the frontal lobe precentral gyrus was required to preserve cognitive performance in hypoxia and thus reflects a declined neuronal efficiency (Richard and Benjamin 1988).

Limitations and future perspectives

This study was performed in a young, healthy population with a high cognitive level. This study provides insight in how CF intake can affect the healthy brain in normoxia and hypoxia, which is relevant for mountaineers, people moving to high altitude and aircraft pilots. However, we cannot apply these findings to other population with restricted vascular function such as elderly, people with Mild Cognitive Impairment, dementia and Alzheimer's disease (Nehlig 2013). It seems that both age and the "baseline" quality of endothelial function influence the degree in which subjects can benefit from CF consumption; CF induced larger improvements in endothelial function in older adults relative to young adults (Fisher et al. 2006) and CF increased *global* perfusion in younger adults (Francis et al. 2006), while it increased *regional* perfusion (Lamport et al. 2015) in elderly. Therefore, future studies should include subjects with restricted vascular function to examine the beneficial effects of CF on NVC and its influence on cognitive function are larger in such populations.

In this study, we simultaneously used NIRS on the PFC and multichannel EEG, because of its usefulness to correlate hemodynamic responses to neural activity. In interpreting these results, we must keep in mind that despite the excellent temporal resolution to directly measure cortical activity of EEG, its spatial resolution is limited (Thompson et al. 2008). Moreover, another limitation was that we used NIRS only in the PFC and not in the entire cortex. By combining multichannel NIRS with multichannel EEG, more information could be gained between the distinct interplay of neuronal activity and NVC. Both NIRS and EEG are limited to observe changes on the superficial cortex and cannot assess the deeper brain regions, which is only possible with fMRI.

Another limitation of this study is that we did not assess ventilation nor arterial carbon dioxide tension. It is known that CBF changes in hypoxia are not only determined by hypoxic cerebral vasodilation, but also by hyperventilation-induced hypocapnic cerebral vasoconstriction (Ogoh 2017). Furthermore, we used a laboratory setting of acute normobaric hypoxia, which was relatively short. Although this setting provided an excellent framework to study neurophysiological changes in response to hypoxia and the fact that normobaric and hypobaric hypoxia provoke similar physiological responses (Mounier and

Brugniaux 2012), a longer exposure to hypobaric hypoxia would provide more insight into the mechanisms which play a role in real-life exposure to high altitude.

While we focussed on neurophysiological effects of acute CF intake, positive effects on cognition and NVC after chronic CF intake have been shown in aging and clinical populations (Socci et al. 2017). Accordingly, it seems possible that chronic CF intake results in both electrophysiological and neurovascular changes in healthy subjects.

4.6 Conclusion

In healthy subjects, acute CF intake enhanced the hemodynamic response in the PFC during cognitive tasks activating the PFC, but did not alter neuronal activity and did not result in improved cognitive function. Acute hypoxic exposure of 12.7% (simulated altitude of 4000 m) barely disturbed normal functioning of the healthy brain, as cerebrovascular responsiveness, neuronal activity and cognitive performance were maintained, with the exception of increased β2 activity in the frontal lobe during abstract thinking and reduced selective attention and response inhibition in hypoxia.

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Chapter 5. The effect of acute cocoa flavanol intake on the BOLD response and cognitive function in type 1 diabetes: a randomized, placebo-controlled, double-blind cross-over study.

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5.1 Abstract

Objective: Type 1 diabetes is associated with microvascular changes in the brain, which can cause cognitive dysfunction. Cocoa flavanols (CF) can stimulate cerebral blood flow and cognitive function. This study aimed to investigate whether acute CF supplementation can improve cognitive function and hemodynamic responses in type 1 diabetes.

Methods: In this randomized, double-blind, counterbalanced cross-over study, eleven patients with type 1 diabetes and their healthy matched controls consumed CF (900 mg CF) and placebo (PL=15 mg CF) 2 hours before a Flanker test. fMRI was used to measure Blood Oxygen Level Dependent (BOLD) response during the cognitive test.

Results: In patients with type 1 diabetes, cognitive performance was not deteriorated while the BOLD response was larger in the cerebellum, Brodmann area 30 of the ACC and the subgyral temporal lobe, and smaller in the superior temporal gyrus, compared to healthy controls. CF improved cognitive performance in healthy controls and patients with diabetes, compared to PL. CF increased the BOLD response in the supramarginal gyrus parietal lobe and inferior frontal gyrus. After CF intake, the BOLD response in the superior temporal gyrus was larger in patients with diabetes compared to healthy subjects.

Conclusions: Cognitive performance on the Flanker test was not deteriorated in type 1 diabetes, which could be due to a different brain activation pattern as a compensation mechanism. Acute CF intake improved response inhibition and executive function in patients with type 1 diabetes and healthy subjects, and increased the BOLD response in the activated brain areas.

Key words: *type 1 diabetes, cognitive function, MRI neuroimaging, cocoa flavanols, neurovascular coupling*

5.2 Introduction

Type 1 diabetes is a chronic autoimmune disease which is characterized by insulin deficiency and hyperglycemia (Cade 2008). One of the consequences of repetitious hyperglycemia episodes is an impaired vasodilatory response. This can lead to reduced cerebral blood flow (CBF) and vasoreactivity, resulting in deteriorated brain structure and function (Brands et al. 2005). A recent meta-analysis showed that impaired working memory, attention, spatial memory and executive functioning in patients with type 1 diabetes (Tonoli et al. 2014).

Previously, it was stated that adaptive compensation mechanisms could slow-down cognitive impairment, thus explaining why some studies reported no adverse cognitive functioning. By using Blood Oxygenation Level Dependent (BOLD) functional Magnetic Resonance Imaging (fMRI), Guardia-Olmos et al. and Gallardo-Moreno et al. showed increased brain activation in distinct brain regions during cognitive tasks in patients with type 1 diabetes, while maintaining cognitive performance compared to healthy subjects (Gallardo-Moreno et al. 2015; Guàrdia-Olmos et al. 2017).

Recently, there has been growing interest in putative effects of dietary interventions on brain function and CBF. Flavanols, a subgroup of polyphenols, are found in green tea, red wine, fruits such as berries and cocoa (Meeusen 2014). Cocoa contains high amounts of flavanols in its natural form, mainly the monomers epicatechin and catechin (Manach et al. 2004). Cocoa flavanols (CF) can stimulate nitric oxide (NO)-mediated vasodilation, leading to improved blood flow. Moreover, CF improve insulin resistance and glucose tolerance and can reduce oxidative stress (Strat et al. 2016; Araujo et al. 2016). CF are also known to be neuroprotective and to enhance neurocognitive functioning (Nehlig 2013). It has been proposed that the vasodilatory actions of CF account for its acute beneficial effects on cognitive performance (Socci et al. 2017). A recent study using Arterial Spin Labelling (ASL) perfusion fMRI showed that CBF was increased after acute CF intake in older adults (Lamport et al. 2015). Moreover, acute CF intake increased the BOLD signal during a cognitive task in healthy humans (Francis et al. 2006). By using transcranial Doppler, Sorond et al. (2013) observed enhanced neurovascular coupling (NVC) and cognitive function in elderly with impaired vascular function, but not in elderly with intact neurovascular coupling, after 30 days of CF intake. This suggests that populations with vascular impairments can benefit more from CF intake than healthy individuals (Sorond et al. 2013). Whether CF supplementation can improve cerebral vasoreactivity and/or cognitive function in patients with type 1 diabetes has not yet been investigated. Thus, the aim of this study was to analyze the effect of acute CF intake on cognitive performance and the BOLD response in patients with type 1 diabetes and healthy matched controls. We hypothesized that acute CF intake would increase the BOLD response in recruited cortical regions and cognitive performance in patients with type 1 diabetes.

5.3 Materials & Methods

The study protocol was approved by the Ethical Committee of the Brussels University hospital (B.U.N. 143201524680) and was carried out in accordance with the Declaration of Helsinki. The study was registered on www.clinicaltrials.gov (NCT03452605). All subjects provided written informed consent before participation.

5.3.1 Participants

Eleven patients with type 1 diabetes and their healthy matched controls (matched for age, gender, Body Mass Index (BMI) and education level) were recruited through social media and through the Flemish diabetes Liga. Baseline characteristics were collected through questionnaires to allow matching of patients with diabetes with healthy individuals. Inclusion criteria were: (I) non-smoking (II) type 1 diabetes for ≥ 1 year or healthy matched control, (III) ≥ 18 years, (IV) stable medications for ≥ 6 months (no use of cholinesterase inhibitors or prior use of multivitamins), and (V) adequate visual and auditory acuity. Participants were excluded when: (I) enrolled in any investigational drug study within 2 months or longer, depending on the investigational drug half-life, (II) epileptic seizures or any major psychiatric disorder in last 2 years, (III) history or MRI evidence of brain damage (trauma, stroke, hydrocephalus, mental retardation, or neurological disorder), (IV) history of alcoholism or drug abuse, (V) unstable cardiac, renal, lung, liver, or other severe chronic disease, (VI) hypertension or hypotension, (VII) pacemaker or other medical metal devices that precludes performing MRI, (VIII) chronic inflammatory diseases, (including lupus, rheumatoid arthritis, or polymyalgia rheumatic), (IX) macrovascular complications and (X) retinopathy, nephropathy or neuropathy (microvascular complications).

5.3.2 Study design

This study was a randomized double-blind, placebo (PL)-controlled, counterbalanced cross-over study design, investigating the effects of acute CF intake on (I) cognitive functioning and (II) cerebral vasoreactivity (fMRI BOLD response) in patients with type 1 diabetes and their healthy matched controls. The experimental trials took place in the academic hospital (UZ Brussels). Data were collected from October 2015 until March 2017.
This study included 2 conditions: a high CF condition (900 mg CF), and PL condition (15 mg CF) with a 7 day wash-out in between.

5.3.3 Nutrition and CF supplementation

On the morning of each trial, participants were asked to inject their usual dose of short-acting insulin, calculated on the breakfast content, and to consume a standardized breakfast, including the CF or PL drink, 2 hours prior to testing. Breakfast contained a high amount of carbohydrates to increase flavanol

absorption (80% carbohydrate, 15% protein, 5% fat, 10 kcal/kg body weight) (Schramm et al. 2003). During the following 2 hours after breakfast, glycaemia levels were checked regularly in order to reach stable values in subjects with type 1 diabetes (between 5.55 - 8.33 mmol/L).

The order in which CF and PL were consumed was determined by (computerized) randomization. CF and PL drinks were matched in color, flavor, caffeine (30 mg), theobromine (315 mg) and contained similar percentage of macronutrients. The CF drink contained 11.25 g ActiCoA® powder (Barry Callebaut) and 3.75 g alkalized cocoa powder, while the PL drink contained 0 g of ActiCoA powder and 15.37 g of alkalized cocoa powder, both dissolved in 300 ml skimmed milk. The 11.25 g ActiCoA powder (= CF) contained 900 mg CF, including 185 mg (−)-epicatechin, 20 mg (+)-catechin and 691 mg procyanidins. Both researchers and participants were blinded for the nutritional intervention.

Participants had to abstain from caffeine, other psychoactive substances and polyphenol-rich foods (e.g. tea, red wine, dark chocolate (cocoa), berries, fruit juices, soy products and grape (juice)) 24 hours prior to each intervention trial. If a severe hypoglycemic episode within 48h preceding each trial occurred, the trial was postponed.

5.3.4 Procedure during the hospital visit

Ninety-five min after PL or CF intake, participants signed in at the hospital. Next, participants performed the Flanker test twice as familiarization and blood glucose levels were measured. If glycaemia did not reach the requested range of values (5.55 - 8.33 mmol/L), participants were given carbohydrate supplementations until the targeted stable blood glucose value was achieved. Supplements of insulin could not be given, because the peak action of insulin would occur during the fMRI scan, which would influence cognitive functioning. The fMRI scan and cognitive testing (inside the MRI scanner) took place 2 hours post intake of the CF drink, simultaneously with the peak in CBF and plasma epicatechin concentration (Schramm et al. 2003). After fMRI testing, blood glucose levels were assessed again.

5.3.5 Outcome measurements

Cognitive Functioning (Flanker test)

The Flanker test was used to assess executive control and inhibitory control (von der Gablentz et al. 2015). Participants were instructed to respond to which direction the middle (of 5) arrow was pointing by pushing the corresponding button, placed in their left or right hand. The task included congruent (e.g., > > > > >), incongruent (e.g.<< ><<) and neutral stimuli (control condition; e.g. - - > - -) (Nee et al. 2007). Each array of arrows was presented for 200 ms with a variable inter-stimuli interval of 1000-1600 ms. Total duration was approximately 5.5 min, including 120 trials. Participants were instructed to respond as quickly and accurately as possible. Accuracy, response times (RT) and flanker interference effect ($RT_{incongruent\ stimuli}$ minus $RT_{congruent\ stimuli}$) were calculated.

fMRI

The study was carried out on a 3T MRI scanner (GE 750W Discovery) equipped with a 24-channel head coil. We measured 107 consecutive 2D SE-EPI volumes (TR/TE=3000/70 ms, flip angle=90°, 27 slices, slice thickness/gap=4.0/ 1.0 mm, size=64 x 64, FOV=240 x 240 mm) covering the whole brain. During fMRI, stimuli were back-projected onto a flat screen positioned at the subject's feet and viewed via a mirror mounted on the head coil. Before the fMRI experiment, a T1-weighted structural scan (3D BRAVO, TI/TR/TE=450/8.5/3.3 ms, flip angle=12°, matrix=256 x 256, FOV=240 x 240mm, 128 slices, slice thickness 1.2 mm) of the whole head was performed.

Besides the structural T1-weighted scan a FLAIR-sequence (2D T2-flair, FOV=240x240mm2, 27 axial slices, slice thickness=4.0mm, slice gap=1.0mm, TR=2694.8ms, TE=24.0ms, flip angle=111°, TI=826ms, ETL=8, matrix=320x256, NEX=2) was taken. The combination of the different image weightings allows for the detection of cortical or white matter lesions by an experienced radiologist (TV).

Image processing and analysis

fMRI data were processed and analyzed using Statistical Parametric Mapping (SPM8) in Matlab R2016b. Raw data were motion and slice time corrected, normalized to the standard anatomical space (EPI MNI template), smoothed using an 8mm isotropic kernel and high-pass filtered. The anatomical scan was normalized to the standard anatomical space (T1MNI tem-plate) to be used as anatomical underlay for the results. The general linear model (GLM) was used to fit a model containing the offsets of the congruent, incongruent and neutral trials, each convolved with the hemodynamic response function (HRF), the 6 motion parameters and a constant, to the data. Based on the fitting results, for each participant the congruent (congruent > neutral) and incongruent (incongruent > neutral) contrast maps were generated.

5.3.6 Statistical analysis

Statistical analysis of demographical data, glycemic levels and cognitive results were performed using IBM SPSS Statistics (version 23; IBM Corp., Armonk, N.Y., USA). Data were considered significant at α=0.05. Normality of the data was tested using the Kolmogorov-Smirnov test. Baseline differences between the two groups were analyzed using independent student t-tests. Three-way repeated measures (*group x supplement x time (pre/post fMRI)*) analysis of variance (RM-ANOVA) was used for glycemic levels. Three-way repeated measures (*group x supplement x stimulus*) RM-ANOVA was used for RT and two-way (*group x supplement*) RM-ANOVA was performed for the flanker interference effect. The three-way interaction effect, 2-way interaction effects between *supplement x stimulus* and *group x*

supplement, as well as main effects were evaluated to answer the research questions. Further analyses of the significant interaction and main effects were performed using two-way and one-way RM ANOVAs and t-test with post-hoc Bonferroni corrections, respectively.

Differences in the BOLD response were statistically analyzed by performing an ANOVA with *group* as a between subject variable and *supplement* and *stimulus* (congruent > neutral – incongruent > neutral) as within variables based on the subjects' contrast maps. We selected *group x supplement* and *group x supplement x stimulus* interaction effects, as well as the main *group* and main *supplement* effects. The resulting clusters with a voxel height threshold at $\alpha=0.001$ and a minimum cluster size of 20 voxels were considered as significant. To investigate whether significant clusters corresponded to increased or decreased BOLD responses, subsequent post-hoc paired t-tests were performed at $\alpha=0.05$.

5.4 Results

Characteristics of the study population are shown in Table 12. No significant differences were observed between the 2 groups in age, BMI and educational level. Patients with diabetes used an insulin pump (6/11) or insulin pen (5/11).

Table 12. Demographics and clinical characteristics*. No significant differences between groups were detected. Data are presented as mean ±standard deviation (SD=standard deviation, N=number of participants, BMI=body mass index, HbA1c=glycated hemoglobin).*

	Type 1 Diabetes	Controls
Demographic and medical characteristics	Mean±SD	
N	11	11
Age (yrs)	41.3±16.1	41.1±15.4
Gender (M/F)	5/6	6/5
Height (m)	1.7±0.1	1.7±0.1
Body Mass (kg)	78.9±15.1	73.6±13.7
BMI (kg/m^2)	26.1±4.9	24.1±3.1
Education level	3±1	3±1
HbA_{1c} (mmol/mol)	58.1±9.2	/
Diabetes Duration (yrs)	19.8±15.2	N.A
Episodes of severe hypoglycemia (lifetime)	0.8±1.1	NA

5.4.1 Cognitive function

Three-way RM ANOVA showed no significant interaction effects nor main effects for accuracy. For RT, no significant 3-way interaction effect and no significant 2-way interaction effects for *group * supplement,* nor for *supplement * stimulus* were found. RT was faster after CF intake compared to PL (main effect of *supplement (CF vs. PL)* (F(1)=5.23; p=0.03)). There was no difference in RT between healthy controls and patients with diabetes (no main effect of *group*).

A significant interaction effect for *group * stimulus* was found (F(2)=9.60; p<0.001) for RT. Group-wise 2-way RM ANOVA (*supplement * stimulus*) demonstrated a significant main effect of *stimulus* in the healthy matched controls, (F=17.9; p<.001), while no significant main effect of stimulus was found in the diabetes group (F=1.2; p>.05). Healthy subjects had slower RT in incongruent tasks compared to the congruent (p=0.012) and neutral tasks (p<.001), while no differences in RT on the different stimuli were found in the patients with diabetes. Similarly, the Flanker interference effect was smaller in patients with diabetes compared to healthy controls (main *group* effect for Flanker interference (F(1)= 22.26;

p<0.001)). There were no significant differences in RT between patients with diabetes and healthy controls for both congruent and incongruent stimuli. Results are displayed in Table 13.

Table 13. Differences in cognitive function after a CF or PL drink between type 1 diabetes group and their healthy matched controls. *Data are presented as mean±SD. (RT=reaction time, SD=standard deviation, type 1 diabetes= type 1 Diabetic patients, CF=cocoa flavanol, PL=placebo). *=significant main effect of supplement in 3-way RM-ANOVA; $=significant main effect of group in 2-way RM-ANOVA; †=significant difference between congruent and neutral vs. incongruent condition in the healthy matched controls, in the post-hoc test.*

Group	Trial	Condition	Mean±SD
Type 1 diabetes	CF drink *	Congruent (ms)	550.8±81.1
		Incongruent (ms)	560.7±70.5
		Neutral (ms)	559.9±69.3
		Interference effect (ms) $	1.8±41.7
	PL drink	Congruent (ms)	590.1±88.6
		Incongruent (ms)	599.3±96.2
		Neutral (ms)	593.4±81.9
		Interference effect (ms) $	6.4±39.3
Matched controls	CF drink *	Congruent (ms) †	518.0±68.0
		Incongruent (ms)	554.2±50.1
		Neutral (ms) †	509.9±63.3
		Interference effect (ms) $	42.8±51.3
	PL drink	Congruent (ms) †	529.3±72.3
		Incongruent (ms)	572.1±68.3
		Neutral (ms) †	529.7±65.1
		Interference effect (ms) $	36.2±28.0

5.4.2 fMRI

None of the anatomical scans of the recruited participants showed lesions or abnormalities of the brain in patients with diabetes, nor in healthy controls.

Factorial design analysis for the BOLD response during the Flanker test revealed a main effect of *group,* with higher BOLD responses in the subgyral temporal lobe of the right cerebrum (F=32.5; p<.001), the posterior cingulate of the left cerebrum (BA31), BA 30 of the anterior cingulate cortex (ACC) (F=19.5; p<.001) and the posterior lobe of the left cerebellum (F=17.9; p<.001) of patients with diabetes compared to their healthy matched controls (Figure 18A). Voxel wise output of this analysis was further analysed in a second level analysis, consisting of a post-hoc t-contrast analysis to detect the difference in BOLD response between the groups. This showed a smaller BOLD response in the superior temporal gyrus of the right cerebrum (BA22) in patients with diabetes compared to healthy controls (p<.05).

A significant main effect of *supplement* in the BOLD response was present in the supramarginal gyrus of the parietal lobe (BA 40) (F=20.77; p<.001) and the inferior frontal gyrus (BA 47) of the right cerebrum (F=18.84; p<.001) (Figure 18B). Post-hoc analysis showed larger BOLD response after CF intake compared to PL.

Factorial design analysis for the BOLD response during the Flanker test revealed a significant 3-way interaction effect of *group * supplement * stimulus* in the medial frontal gyrus of the right cerebrum (BA46) (F=18.4; p<.001). A significant interaction effect for *supplement * group* was found in the frontal lobe; more specifically in the superior frontal gyrus of the left and right cerebrum (BA10) (F=16.6; p<.001 and F=15.5, p<.001) and the inferior frontal gyrus in the right cerebrum (BA44) (F=16.5, p<.001). A significant interaction effect for *stimulus * group* was found in the posterior cingulate cortex (BA 23) of the left cerebrum (F=22.3; p<.001) and the precentral gyrus of the frontal lobe in the left and right cerebrum (F=16.2; p<.001, F=15.8; p<.001).

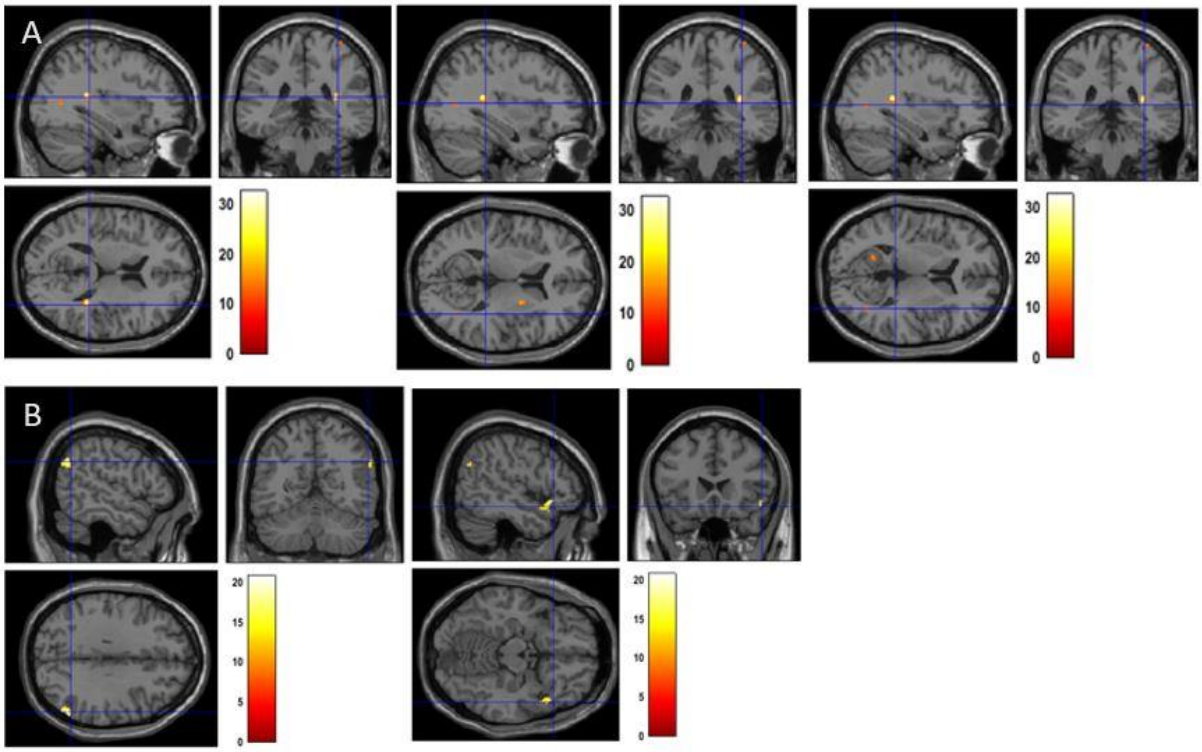

***Figure 18. Flanker-related BOLD response**. **(A) BOLD response in patients with type 1 diabetes compared to healthy matched controls.** Significant higher BOLD response in the sub-gyral temporal lobe of the right cerebrum, posterior cingulate of the left cerebrum and Brodmann Area 30 of the ACC, and in the posterior lobe of the left cerebellum in patients with type 1 diabetes compared to healthy matched controls. **(B) BOLD response after cocoa flavanol intake (CF) compared to placebo (PL).** Significant greater BOLD responses can be seen in the supramarginal gyrus of the parietal lobe of the right cerebrum and in the inferior frontal gyrus of right cerebrum, after CF intake compared to PL. Statistical parametric maps thresholded at p<0.05. Lighter colors correspond to larger BOLD-signal/activation.*

Second level analysis showed that in the PL trial, the BOLD response was lower in patients with diabetes compared to healthy controls in the superior temporal gyrus of the right cerebrum (BA 22) ($p<.05$) (Figure 19A). After CF intake, the BOLD response was lower in patients with diabetes compared to healthy controls in the subgyral frontal lobe in the left cerebrum ($p<.05$). However, the BOLD response was higher in patients with diabetes compared to the healthy controls in the superior temporal gyrus (BA 22) ($p<.05$), the temporal pole (BA 38) ($p<.05$), and the supramarginal gyrus of the parietal lobe (BA 40) of the right cerebrum ($p<.05$) (Figure 19B).

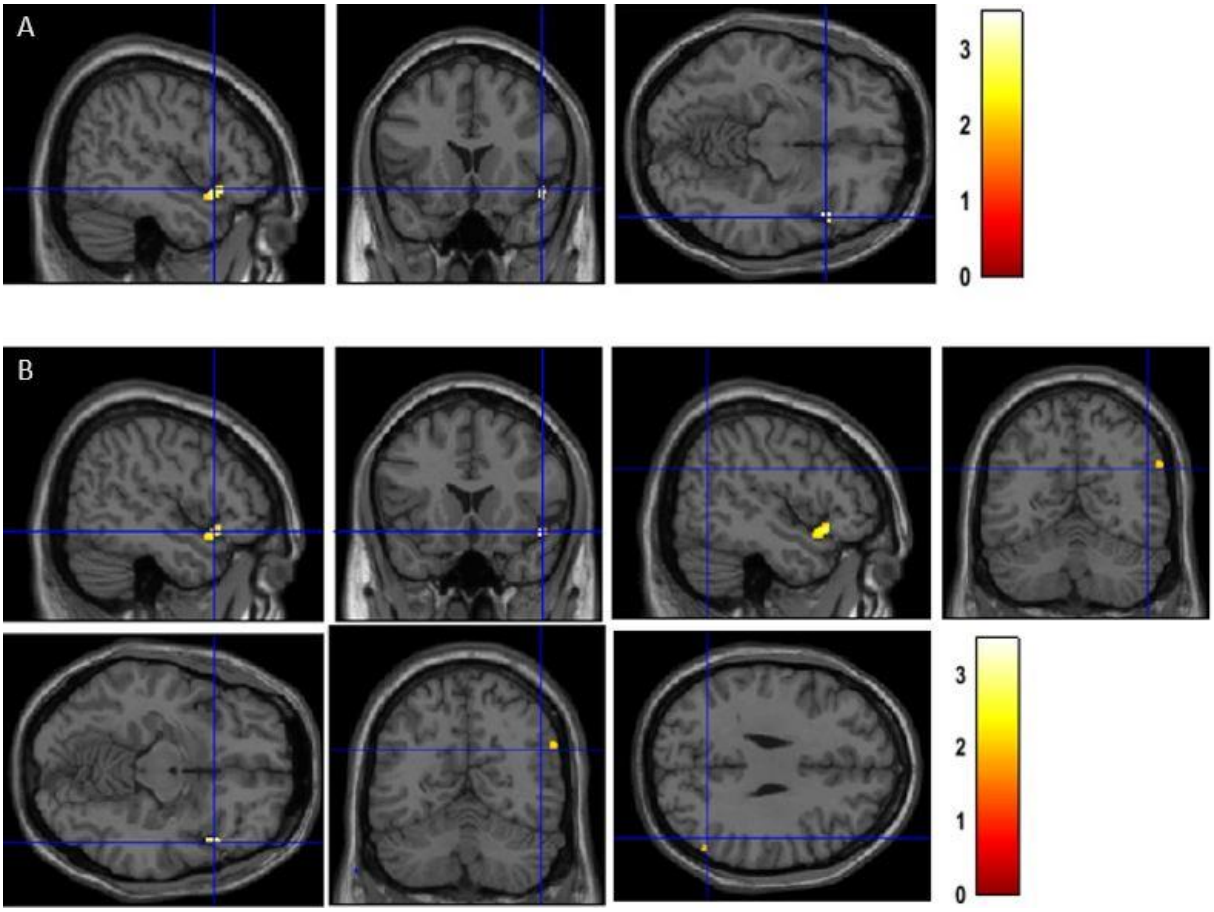

Figure 19. (A) Flanker-related BOLD response after placebo (PL) intake in healthy matched controls vs patients with type 1 diabetes. *After PL intake, BOLD response in the superior temporal gyrus of the right cerebrum of healthy matched controls was significantly higher than in patients with type 1 diabetes.* ***(B) Flanker-related BOLD response after cocoa flavanol (CF) intake in patients with type 1 diabetes vs healthy matched controls****. After CF intake, a significantly higher BOLD response in patients with type 1 diabetes, compared to healthy matched controls was observed in the temporal lobe in the right cerebrum, in the Brodmann area 38 of the right cerebrum, and in the parietal lobe in the right cerebrum. Lighter colors correspond to larger BOLD-signal/activation.*

5.4.3 Glycaemia

Three-way RM ANOVA (*group* * *supplement* * *time*) showed a significant interaction *group* * *time* effect (F(1)=5.92; p=0.024). A significant decrease in post-fMRI glycemic levels (9.77±3.52 in CF, 7.65±2.62 in PL) compared to pre-fMRI (9.77±3.52 in CF, 7.65±2.62 in PL) was found in patients with diabetes, but not in healthy subjects. Patients with diabetes had higher glycemic levels compared to healthy controls (main *group* effect (F(1)=25.19; p<0.001).

5.5 Discussion

The aim of this study was to evaluate whether CF intake could beneficially influence cognitive functioning and cerebral vasoreactivity in patients with type 1 diabetes, compared to healthy controls. CF intake improved cognitive performance and increased the BOLD response in the supramarginal gyrus parietal lobe and inferior frontal gyrus in healthy subjects and patients with type 1 diabetes. CF intake could counteract the diabetes-induced decrease in BOLD response in the superior temporal gyrus.

Type 1 diabetes

Although the interference effect was larger in healthy controls than in patients with diabetes (which was unexpected), there was no significant difference in RT between healthy controls and patients with diabetes on congruent and incongruent stimuli, indicating no deterioration of cognitive function in patients with diabetes. As previously shown, an altered brain activation pattern could have acted as compensation mechanism to maintain cognitive functioning (Gallardo-Moreno et al. 2015). The Flanker test is known to activate several brain regions including the dorsolateral prefrontal cortex, the inferior frontal gyri, the ACC and inferior and superior parietal lobules (Nee et al. 2007). During the Flanker task, a higher BOLD response was observed in the ACC, posterior lobe of the left cerebellum and in the white matter of subgyral temporal lobe of the right cerebrum, in patients with type 1 diabetes compared to healthy controls. The ACC is involved in impulse control and decision making and is activated in inhibitory tasks such as the Flanker test (Nee et al. 2007). The posterior lobe of the cerebellum is involved in motor and executive control (von der Gablentz et al. 2015). In contrast, the BOLD response was smaller in type 1 diabetes compared to healthy controls in the superior temporal gyrus, which is involved in auditory processing and which could be activated by the noise of the fMRI scanner (Trans Cranial 2012). These findings are in line with 2 previous studies showing that in type 1 diabetes, several additional brain regions, that are normally not recruited during the Flanker task, are activated in order to maintain the same level of selective attention and executive functioning (Gallardo-Moreno et al. 2015; Guàrdia-Olmos et al. 2017).

Cocoa flavanols

Acute CF intake improved RT on the Flanker test and increased the BOLD response in the inferior frontal gyrus of the frontal lobe and supramarginal gyrus of the parietal lobe, in healthy subjects and patients with type 1 diabetes. These 2 regions are involved in selective response suppression and hand movement, and are activated during the Flanker test (Forstmann et al. 2008). Thus, the CF-induced increased BOLD response occurred in brain regions known to be activated during the Flanker test. In contrast to these results, Decroix et al. (2016) and Francis et al. (2006) reported beneficial effects of

acute CF intake on the neurovascular response, without altering cognitive performance, in healthy subjects. In contrast to the improved cognitive performance after acute CF intake in both patients with diabetes and healthy controls, Sorond et al. previously found that 30-day CF intake enhanced cognitive function in elderly with impaired vascular function, but not in elderly with intact neurovascular coupling (Sorond et al. 2008).

To the best of our knowledge, the present study was the first to investigate the effect of CF on the BOLD response in patients with type 1 diabetes. CF intake could counteract the diabetes-induced decreased BOLD response in the superior temporal gyrus. Moreover, CF intake also induced a higher BOLD response in patients with type 1 diabetes compared to healthy patients in the temporal pole (BA38) and the parietal lobe of the right cerebrum (BA40). It has been shown that the temporal pole participates in executive function and memory, while the parietal lobe is known to be involved in the executive function network (Trans Cranial 2012).

Mechanisms

In type 1 diabetes, NO inactivation contributes to impaired vasodilatory response and reduced CBF. CF can increase NO levels *in vitro* (Fisher et al. 2006) and acute CF intake can improve NO-mediated vasodilation, vascular function and CBF in humans (Vlachopoulos et al. 2005; Lamport et al. 2015). NO-dependent vasodilation is key to coupling of neuronal activity to the local increase in CBF in the active cerebral tissue (Lieberman 2003), which is measured by the BOLD response. While the CF-induced increase in BOLD signal during the Flanker test observed in this study, can theoretically originate from higher neuronal activity or from an increased vascular responsiveness (higher local CBF) (Steinbrink et al. 2006), it seems most plausible that the latter is responsible for the observed change in BOLD response.

Limitations

Drawbacks of this study were *(i)* the relative small sample size, reducing the power of the statistical measures and *(ii)* heterogeneity in educational level, age and for patients with type 1 diabetes, time of diabetes onset and disease duration.

Future research

We showed that acute CF intake can beneficially influence the BOLD response in the brain of patients with type 1 diabetes. Future research should investigate the effects of *chronic* CF intake on long-term neuro-modulatory effects both in patients with type 1 and type 2 diabetes, as well as in other populations with vascular dysfunctions such as Alzheimer's disease, and elderly with mild cognitive impairments.

5.6 Conclusion

Patients with type 1 diabetes showed a different brain activation pattern during the Flanker test, while maintaining cognitive performance, compared to healthy controls. CF intake improved cognitive performance and increased the BOLD response in the supramarginal gyrus of the parietal lobe and inferior frontal gyrus in both patients with diabetes and healthy controls. CF intake could counteract the diabetes-induced lower BOLD response in the superior temporal gyrus, revealing a beneficial effect of CF intake in patients with diabetes in this brain area. Given the positive effects of CF intake on neurovascular responses and cognitive function in patients with type 1 diabetes, the use of CF as a non-pharmacological aid to stimulate a healthy vascular function and CBF seems promising to prevent or postpone cognitive impairments in type 1 diabetes.

5.7 Acknowledgements

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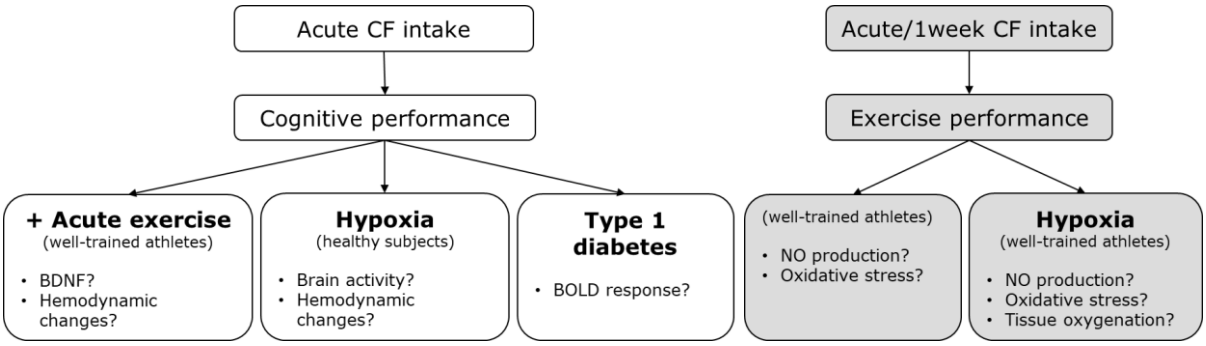
PART 2


Acute CF intake
Cognitive performance
+ Acute exercise
(well-trained athletes)
• BDNF?
• Hemodynamic changes?
Hypoxia
(healthy subjects)
• Brain activity?
• Hemodynamic changes?
Type 1 diabetes
• BOLD response?
Acute/1week CF intake
Exercise performance
(well-trained athletes)
• NO production?
• Oxidative stress?
Hypoxia
(well-trained athletes)
• NO production?
• Oxidative stress?
• Tissue oxygenation?

Chapter 6. Acute cocoa flavanols intake has minimal effects on exercise-induced oxidative stress and nitric oxide production in healthy cyclists: a randomized controlled trial.

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RESEARCH ARTICLE Open Access

Acute cocoa Flavanols intake has minimal effects on exercise-induced oxidative stress and nitric oxide production in healthy cyclists: a randomized controlled trial

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6.1 Abstract

Background: Cocoa flavanols (CF) can stimulate vasodilation by improved nitric oxide (NO) synthesis and have antioxidant and anti-inflammatory capacities. This study aimed to examine whether acute CF intake can affect exercise-induced changes in antioxidant capacity, oxidative stress, inflammation and NO production, as well as exercise performance and recovery in well-trained cyclists.

Methods: Twelve well-trained male cyclists (mean±SD age, VO_{2max}: 30±3 years, 63.0±3.5 ml/kg/min) participated in this randomized, double-blind, cross-over study. On 2 separate occasions, subjects performed two 30-min time trials 1.5 (TT1) and 3 (TT2) hours after CF (900 mg CF) or placebo (PL, 13 mg CF) intake, interposed by passive rest. Lactate, glucose, heartrate, rating of perceived exertion (RPE) and power output were measured during the TTs. Blood was drawn at baseline, before and after each TT and analyzed for epicatechin serum concentrations, trolox equivalent antioxidative capacity (TEAC), uric acid (UA), malonaldehyde (MDA), L-arginine/ADMA, citrulline, interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α plasma concentrations. Relative changes in blood markers and pacing strategy during TT were analysed by repeated measured ANOVA. TT performance was compared between PL and CF by paired t-test.

Results: Epicatechin concentrations were increased by CF intake. Exercise-induced increase in TEAC/UA was improved by CF intake ($F(1)=5.57$; $p=.038$) (post-TT1: PL: 113.34±3.9%, CF: 117.64±3.96%, post-TT2: PL: 108.59±3.95%, CF: 123.72±7.4% to baseline), while exercise-induced increases in MDA, IL-1 and IL-6 were not affected by CF intake. TNF-α was unaltered by exercise and by CF. Exercise-induced decreases in L-arginine/ADMA and increases in citrulline were not affected by CF intake. TT1 and TT2 performance and exercise-induced physiological changes were unaffected by CF intake.

Conclusion: Acute CF intake increased total antioxidant capacity in rest and during exercise, but did not affect exercise-induced lipid peroxidation, inflammation, nor NO production in healthy athletes. Acute CF intake did not improve TT performance and recovery.

Trial registration: ISRCTN32875, 21-11-2016, retrospectively registered

Keywords: cocoa, flavanols, oxidative stress, nitric oxide, exercise

6.2 Introduction

A balanced and well-chosen nutrient intake is not only crucial for a healthy lifestyle, but also for optimal sport performance. There is a widespread use of dietary antioxidants, including vitamin E, resveratrol, beetroot, quercetin and cocoa flavanols (CF) in the athletic field. The main flavanols in cocoa are epicatechin, catechin (monomers) and procyanidins (oligomers). Their antioxidant (and in some situations perhaps pro-oxidant) effect is determined by the tricyclic structure of the flavanols. *In vitro* and *in vivo* studies clearly show that CF have a strong antioxidant capacity (Wiswedel et al. 2004; Katz et al. 2011).

However, the potential role of chronic or acute CF intake to counteract exercise-induced oxidative stress and its effect on exercise performance is still unclear (Allgrove et al. 2011, Davison et al. 2012, Stellingwerff et al. 2014, Peschek et al. 2014, Patel et al. 2015). Oxidative stress refers to the imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and/or molecular damage (Fisher-Wellman and Bloomer 2009). During exhaustive exercise, NADPH oxidase-derived formation of reactive oxygen species (ROS) results in an altered redox state in the muscle (Bentley et al. 2012). In contrast to the initial belief that nutritional antioxidant intake would be beneficial for exercise performance by protecting the body against oxidative stress, it is now hypothesized that exercise-induced oxidative stress promotes the expression of antioxidant defence and the adaptive responses to training (Gomez-Cabrera et al. 2015). However, when exercise-induced ROS formation exceeds the antioxidant capacity, oxidative stress occurs with detrimental effects on proteins, lipids and DNA, possibly leading to contractile muscle dysfunction, accelerated muscle-fatigue and reduced exercise performance (Powers et al. 2011). Thus, at moments when optimal exercise performance is key and training adaptations are of minor importance (e.g. competition), acute intake of antioxidants may help scavenge free radicals and therefore directly prevent a decline in exercise performance and optimize post-exercise recovery (Bentley et al. 2012; Braakhuis and Hopkins 2015).

In the recent years, CF have also been discovered as potent anti-inflammatory agents (Selmi et al. 2008). Exhaustive exercise can cause inflammation (Malaguti et al. 2013), resulting in a potential role for CF as a nutritional intervention to prevent exercise-induced muscle damage. Nevertheless, Allgrove et al. (2011) did not find any beneficial effect of 2 week CF (108 mg) intake on exercise-induced inflammatory markers.

Independent from its antioxidant and anti-inflammatory properties, CF are also known to stimulate vasodilation through increased nitric oxide (NO) availability (Corti et al. 2009). *In vitro* experiments demonstrated that CF lead to the inhibition of arginase, resulting in a greater L-Arginine availability, the substrate of endothelial NO synthase (eNOS) (Schnorr et al. 2008). *In vitro* (Karim et al. 2000) and *in vivo* (Heiss et al. 2005; Balzer et al. 2008) experiments showed that CF intake activates eNOS, which

converts L-arginine, in the presence of molecular oxygen, to NO and citrulline. Besides its role in vasodilation, NO can also react at a diffusion-controlled rate with superoxide to form the highly reactive oxidant peroxynitrite. Consequently, by elevating NO production, CF might hypothetically have a pro-oxidant effect. However, because of its antioxidant capacity, CF can reduce ROS formation (e.g. superoxide) and repress the formation of peroxynitrite, thus elevating NO availability (Schewe et al. 2008). Based on the fact that NO modulates blood flow and mitochondrial respiration during exercise (Bescós et al. 2012; Nosarev et al. 2014), it has been suggested that increased NO production may enhance oxygen and nutrient delivery to active muscles, as well as improve mitochondrial efficiency, hence improving tolerance to physical exercise and recovery mechanisms (Bescós et al. 2012). Although many athletes use supplements of NO because of their potential ergogenic role, the scientific evidence is very scarce (Bescós et al. 2012). eNOS activity and thus NO production are stimulated by exercise-induced shear stress and thus strongly depend on training status (Green et al. 2004). This suggests a minimal effect for CF and other nutritional supplements on eNOS activity and NO production in healthy, well-trained athletes without vascular restrictions. Whether CF supplementation can alter NO production during exercise, and whether it may improve exercise performance and recovery in well-trained athletes, remains elusive (Allgrove et al. 2011, Stellingwerff et al. 2014, Patel et al. 2015).

Therefore, the aims of this study were to examine the effects of CF intake on: *(i)* indirect markers of eNOS dependent NO synthesis, L-arginine and citrulline, during exhaustive exercise *(ii)* exercise-induced changes in antioxidant capacity, oxidative stress and inflammation, and *(iii)* exercise performance and recovery in well-trained athletes. We hypothesized that CF intake *(i)* will have little effect on indirect markers of NO production and exercise performance in these healthy, well-trained, subjects, but *(ii)* will decrease exercise-induced oxidative stress and inflammation, leading to an improved recovery.

6.3 Materials & Methods

6.3.1 Participants

Twelve well-trained male cyclists (Performance level 3 (Depauw et al. 2013)) (mean±SD age 30±3 years, height 177.9±8.8 cm, body mass 72.8±7.8 kg, and VO_{2max} 63.0±3.5 mL/kg/min) participated in this randomized, double-blind, cross-over interventional trial. Volunteers were excluded from the study if they met any of the following exclusion criteria: (1)<20 years or >35 years, (2) hypertension, (3) cardiovascular disease, (4) smokers or history of smoking, (5) habitual antioxidant supplementation. The experimental procedures and potential risks were explained to the participants and a written informed consent was provided and signed prior to inclusion in the study. The study protocol was approved by the research ethical committee of the Vrije Universiteit Brussel and the study was conducted according to the Declaration of Helsinki (1964).

6.3.2 Study design

Subjects visited the lab 3 times during 3 consecutive weeks, with 7 days in between. Training, food intake and lifestyle were kept constant during those 3 weeks. Subjects were asked to complete a 24 h-food recall on 5 random days during the study period (to avoid a potential influence of regular polyphenol intake on the measurements) and training intensity and duration were registered in a training diary. Participants were asked to record their dietary intake of the last 24 h before each lab visit and to replicate this diet in the 24 h preceding each test. On the first visit, subjects underwent a complete medical screening and performed a maximal incremental cycle test (initial workload of 80W, increased every 3 min by 40 W until volitional exhaustion) on an electromagnetically braked cycle ergometer (Lode, Groningen, The Netherlands). Peak power output (PPO) was determined and maximal oxygen uptake (VO_{2max}) was measured using the Metalyzer Cortex (Germany).

Subsequently, subjects underwent 2 interventional (exercise) trials where they consumed a CF or placebo (PL) drink (300 ml), in a randomised order with 1 week in between. Subjects reported to the lab in a 4 h-fasted state at 12 pm. Subjects were asked not to perform heavy exercise in the last 48 h and to abstain from caffeine and foods with a high polyphenol content (green tea, grapes, olives, dark chocolate, hazel and pecan nuts, berries) for the last 24 h. Baseline blood samples were collected, subjects were weighed and blood pressure was taken. Subjects then consumed either a high CF-content chocolate milk (CF - 903.75 mg CF, Acticoa®) or the placebo low CF chocolate milk (PL – 15 mg CF) which were matched in taste, color, calories, macronutrients, caffeine and theobromine. The exact composition of the CF and PL drink, including monomers (epicatechin, catechin) and oligomeric proanthocyanidins are depicted in Table 14. This drink was consumed together with a standardized lunch, carefully selected by a nutritionist to contain a high amount of carbohydrates to increase CF

absorption (Schramm et al. 2003) (80% carbohydrate, 15% protein, 5% fat, 10 kcal/kg body weight). As the peak blood concentration of CF is reached 2 h after consumption of the drink, a 2nd blood sample was taken 100 min later. Subsequently, a 30-min TT was started. Immediately after the TT, a 3rd blood sample was taken. A 4th sample was taken after a 100-minute passive recovery and consequently a 2nd 30-min TT was started. Immediately after the 2nd TT, a 5th blood sample was taken (Figure 20).

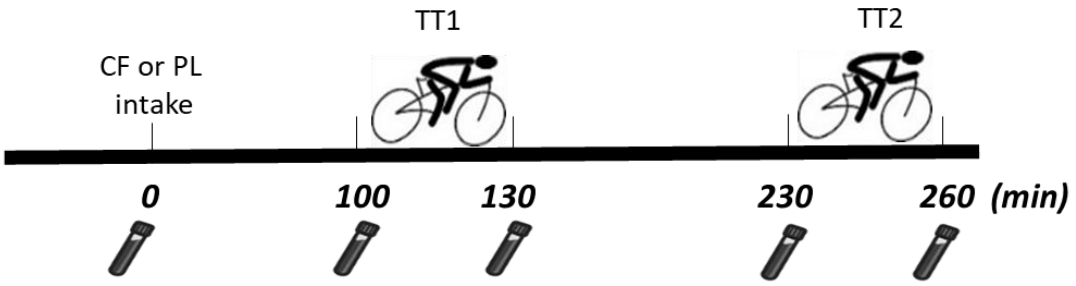

Figure 20. Study protocol *of the 2 interventional trials, performed in randomized order with cocoa flavanol (CF) or placebo (PL), with a washout-out period of 1 week in between. During the time trials (TT), subjects had to cover an amount of work (equal to 75% of peak power output during 30 min) as fast as possible. TT1 was used as a measure of exercise performance, TT2 was used as a measure of exercise recovery.*

Table 14. Nutritional profile of cocoa powder (solved in 300 ml skimmed milk)

	Cocoa flavanol (CF)	***Placebo (PL)***
Total Flavanols (mg)	*900*	*15*
- ***(−)-epicatechin***	*185*	*0*
- ***(+)-catechin***	*20*	*0*
- ***Procyanidins dimer***	*190 (21.9%)*	
- ***Procyanidins trimer***	*117 (13.0%)*	
- ***Procyanidins tetramer***	*113 (12.5%)*	
- *Procyanidins pentamer*	*71 (7.9%)*	
- *Procyanidins hexamer*	*52 (5.8%)*	
- *Procyanidins heptamer*	*51 (5.7%)*	
- *Procyanidins octamer*	*39 (4.3%)*	
- *Procyanidins nonomer*	*33 (3.7%)*	
- *Procyanidins decamer*	*25 (2.8%)*	
Cocoa powder (g)		
- Acticoa powder	*12.0*	*0.0*
- Alkalized cocoa powder	*3.0*	*15.7*
- Potassiumchloride (KCl)	*0.7*	*0.0*
- Sugar	*35.0*	*35.0*
Protein (g)	*3.2*	*2.9*
KHO (g)	*38.7*	*38.4*
Fat (g)	*2.3*	*2.9*
Kcal	*193.4*	*193.4*
Caffeine (mg)	*30*	*30*
Theobromide (mg)	*315*	*315*
Cadmium	*< 1 ppm*	*< 1 ppm*

6.3.3 Measurements

Time trial

After a standardised warm up of 5 min at 120 W, subjects were instructed to cover a fixed amount of work (the equivalent work of 75% of peak power output during 30 min) as fast as possible. The initial workload of the TT corresponded to 75% of the peak power output (280±27.6 W), but subjects were free to change their power output as desired from the outset. If the power output decreased, the duration of the time trial increased. No feedback regarding time lapse, power output, heartrate or pedal cadence was given, except for total workload that was completed. Time to complete the TT and physiological parameters as lactate, heartrate, and rate of perceived exertion (RPE) were used as primary outcome

measures to determine the effect of CF on exercise performance (data from TT1) and recovery (data from TT2). Power output and heartrate were constantly registered during the TT. Relative power output (percent of peak power output), was calculated every 5 min of the TT. Thus, changes in relative power output were analysed at 7 time points during each TT. Lactate, glucose and RPE were measured at the start, after 10 and 20 min, at the end of the TT as well as 5 and 10 min after the TT.

Blood collection and analyses

A catheter was placed in a antecubital vein upon arrival at the lab. Venous blood samples were collected at baseline, 100 min later (pre-TT1), immediately after TT1 (post-TT1), 100 min after finishing TT1 (pre-TT2) and immediately after TT2 (post-TT2). Blood was collected in two 5 ml EDTA tubes, one 5 ml heparinized tube and one 8 ml anticoagulant-free tube and centrifuged immediately to obtain plasma or after 30 min at room temperature to allow clotting to obtain serum (10 min at 3000 rpm, 4 °C). Plasma and serum were aliquoted and stored at -80 °C until further analyses. All biochemical data were corrected for changes in plasma volume using the determination of haematocrit and the concentration of haemoglobin according to Dill and Costill (Dill and Costill 1974). Pre-and post-TT1 and pre-and post-TT2 values were normalized to the baseline values and expressed as% change from baseline. Blood lactate and glucose were measured in a capillary earlobe sample. Lactate was analysed by a Biosen 5030 (EKF, Magdeburg, Germany) and glucose by a photometrical method (using the hexokinase Roche).

1. Determination of serum flavanols concentration

Serum samples were analysed for epicatechin and catechin concentrations as described by Warden et al. (2001). In detail, 0.5 ml of serum was mixed with 1.0 ml phosphate buffer (100 mM, pH 5, containing ascorbic acid (20 mg/ml), EDTA (1.5 mg/ml)) and 20 µl glucuronidase/sulfatase(100 000 and 7500 units/ml, respectively) and incubated at 37 °C for 30 min to hydrolyse glucuronate and sulfate conjugates of epicatechin and catechin. Then, 5 ml tert-butylmethylether was added and vortexed for 1 min. For phase separation, the mixture was centrifuged at 10 °C for 5 min at 5000 rpm.

The organic phase was transferred to a new tube and after a second extraction with 5 ml tert-butylmethylether, the combined extracts were dried under a stream of nitrogen and stored at -80 °C. For HPLC analyses, the dry residues were reconstituted in 200 µl methanol, vortex-mixed and centrifuged at 5 °C at 14.000 rpm for 5 min. 20 µl of the supernatant were injected onto the HPLC-column. For HPLC analysis, a reversed-phase RP18 end-capped column (Lichrospher 100, 5 µm, 250 x 4 mm; Merck) coupled with a guard column (Lichrospher 100, 5 µm, 4 x 4 mm; Merck) was used. Detection was accomplished at an excitation wavelength of 280 nm and an emission wavelength of 310 nm. Data were recorded by HPLC-System Manager software (Merck/Hitachi; Darmstadt, Germany).Samples were eluted from the column at 20 °C using a step-gradient as follows: from 0 to 18 min 53%

acetonitrile/H_2O/acetic acid (150:846:4) / 47% H_2O/acetic acid (1000:5), from 18 to 27 min 80% acetonitrile/H_2O/acetic acid (150:846:4) / 20% H_2O/acetic acid (1000:5). To elute retained compounds the column was flushed from 27 to 60 min with 100% acetonitrile/acetic acid (1000:4) and subsequent equilibration from 60 to 80 min with starting conditions. Flow rate was 1.5 ml/min. Under these conditions the analytes elute with retention times of 15.4 min for catechin and 24.9 min for epicatechin. Peak areas of catechin and epicatechin were used to calculate the concentrations applying the external standard method. Standard curve linearity was observed in the range from 0.125 to 20 µM for both compounds.

2. *Quantification of total antioxidant capacity (TEAC), uric acid (UA) and lipid peroxidation (MDA) in plasma*

Plasma antioxidant capacity was quantified as trolox equivalent antioxidant capacity (TEAC) according to Fischer et al. (Fischer et al. 2005) and corrected for plasma uric acid (UA) as a major antioxidant in the blood (Weseler et al. 2011). In this procedure, the decolorization of the preformed green-blue 2,2`-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$) by the sample within a fixed amount of time reflects the antioxidant capacity of the blood sample. MDA in EDTA-plasma samples were quantified after derivatization with thiobarbituric acid by using HPLC with fluorometric detection as described by Lepage et al. (1991).

3. *Quantification of inflammatory markers in plasma*

The inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 were quantified by means of commercially available ELISA kits (PeliKine Compact human ELISA kits, CLB/Sanquin). The limits of sensitivity were 4 pg/ml for TNF-α, 2.5 pg/ml for IL-1 and 0.5 pg/ml for IL-6.

4. *Determination of mediators of NO-pathway (citrulline, L-arginine, ADMA)*

An internal standard (150 µL, 1 µM ADMA and 50 µM arginine, methanol mixture) was added to 10 µL of plasma and centrifuged (13000 rpm, 10 min, 4 °C) to remove the precipitated proteins. Supernatant was collected and dried under a stream of nitrogen at 70 °C. The dried extract was dissolved in 100 µL of a butanol solution containing 3N HCl and kept at 70 °C for 40 min. The solvent was removed by evaporation under nitrogen flow at 70 °C. The sample was then dissolved in 2.5 mL of water-methanol (90:10, v:v) containing 0.1% formic acid and 5 µL was injected into an analytical column (Kinetex C18 (5µm, 2.1x100mm)). Mass spectrometric analysis was performed using an UFLC-XR Shimadzu coupled with an QTRAP® 5500 hybrid system, equipped with a Turbo VTM ion197 source (AB Sciex, Foster City, CA, USA). Multiple reaction monitoring (MRM) measurement was performed using optimal cone and collision energy values. The run was performed at a flow rate of 500µL*min^{-1} at 30 °C, lasting 9 min in total. A gradient profile consisted of solution A (water with 0.1% (v/v) formic

acid) and solution B (methanol with 0.1% (v/v) formic acid). The percentage of organic solution B was changed linearly as follows: 0 min, 2%; 4 min, 7%; 6 min, 50%; 7 min, 2%; 9 min, 2%. Data were acquired with Analyst Software version 1.5.2. Calibration curves, performed in water, were obtained adding increasing concentrations of ADMA and SDMA from 0.1 to 4 µM and L-arginine and citrulline from 12.5 to 125 µM.

6.3.4 Sample size calculation and statistical analysis

The minimal sample size was calculated using G*Power using results from previous studies examining effects of CF intake on markers of oxidative stress and exercise performance (Davison et al. 2012; Stellingwerff et al. 2014; Patel et al. 2015). To detect a difference at a power of 0.8 with 95% confidence, a minimal sample size of 12 individuals with each intervention was necessary. Statistical analysis was performed with IBM SPSS Statistics (version 22; IBM Corp., Armonk, N.Y., USA) and considered significant at α=0.05. Normality of the data was tested with the Kolmogorov-Smirnov test. Data are presented as mean±standard deviation. A paired samples t-test was used to determine differences in TT performance. Since relative power output during the time trial was not normally distributed, non-parametric Friedman and Wilcoxon signed rank tests were used to determine differences in pacing strategy between CF and PL intake. To correct for the large inter-individual variation in some plasma markers, relative changes (% change to baseline) were calculated and used for data analysis. To examine the effects of CF and exercise on physiological markers, plasma flavanol metabolites and markers of oxidative stress and inflammation, two-way repeated measures *(trial x time)* analysis of variance (RM-ANOVA) was performed. To evaluate the effect of CF intake on markers of the NO-pathway before and after exercise, RM-ANOVA (*2 (trial; PL and CF) x 3 (time; baseline, before and after TT1))* was used.

6.4 Results

6.4.1 Epicatechin/catechin serum concentrations

CF intake significantly increased serum epicatechin concentrations before and after TT1 (100 min and 130 min after intake), as well as before and after TT2 (230 and 260 min after intake), compared to baseline (main effect of time: F=13.23, p< 0.001). The peak concentration was reached before and after TT1. After PL intake, epicatechin levels remained unchanged (Figure 21). Serum catechin concentrations did not significantly differ between CF and PL intake and between the different time points.

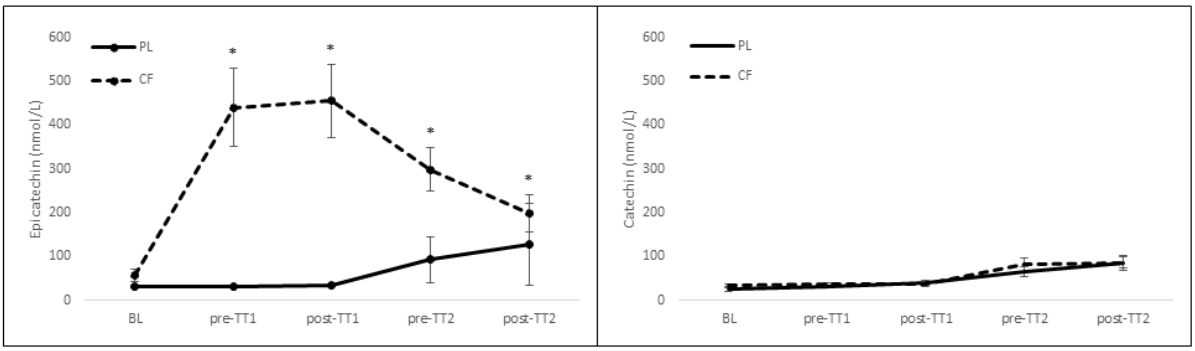

Figure 21. Serum epicatechin and catechin concentrations (expressed in nmol/L) after cocoa flavanol (CF) or placebo (PL) intake, before and after the two time trials (TT). **: p<0.05 compared to baseline (BL).*

6.4.2 Plasma concentrations of mediators of the NO-pathway

A main effect of time was found for L-arginine/AMDA and citrulline plasma concentrations (RM-ANOVA (5*2), L-arginine/ADMA: F(4)=3.69; p=.01; citrulline: F(4)=11.88; p<.001)). However, no effect of trial nor an interaction effect were observed. Post-hoc analysis showed that exercise (both TTs) significantly decreased L-arginine/ADMA and increased citrulline concentrations in plasma (Figure 22).

6.4.3 Markers of oxidative stress

Plasma concentration of UA was significantly increased by CF, compared to PL, as indicated by a significant main effect of trial (F(1)=7.21; p=0.02). UA was increased after TT1 and TT2 compared to before TT1 and TT2 and was significantly lower before TT2, compared to after TT1 (main effect of time (F(4)=17.7; p<0.001)) (no interaction effect).

Exercise and CF intake elevated total plasma antioxidant capacity reflected by the TEAC values corrected for UA concentrations (TEAC/UA) (main effect of time (F(4)=11.84; p<0.001), main effect of trial (F(1)=5.57; p=0.038) (no interaction effect). Post-hoc analysis showed that TEAC/UA was

significantly higher in the CF trial than in the PL trial. After each TT, TEAC/UA was significantly increased compared to the concentrations before the TT. Before TT2, TEAC/UA was significantly lower compared to after TT1.

MDA plasma concentration significantly changed over time, but was not affected by CF intake (effect of time: (F(4)=2.98; p=0.029), no effect of trial/interaction effect). Post-hoc analysis showed that MDA concentrations were significantly higher after TT1 compared to before TT1 and compared to before TT2 (Figure 23).

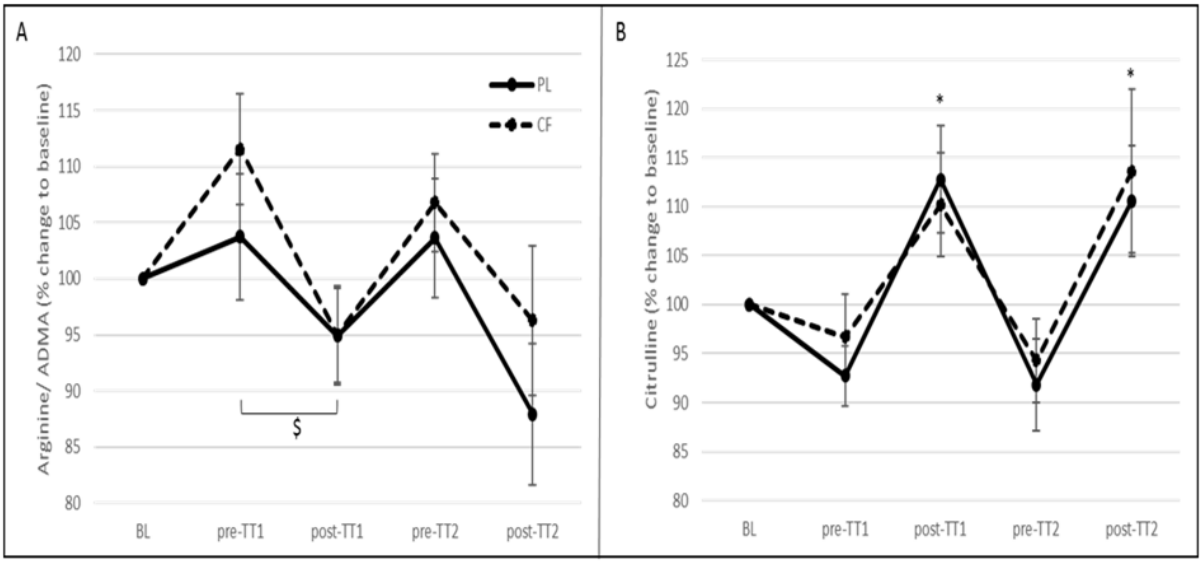

Figure 22. Influence of cocoa flavanols (CF) and exercise on plasma concentrations of arginine/ADMA and citrulline, mediators of the NO-pathway. *(A) Both time trials (TT1 and TT2) decreased arginine/ADMA concentrations after CF and placebo (PL) intake.*

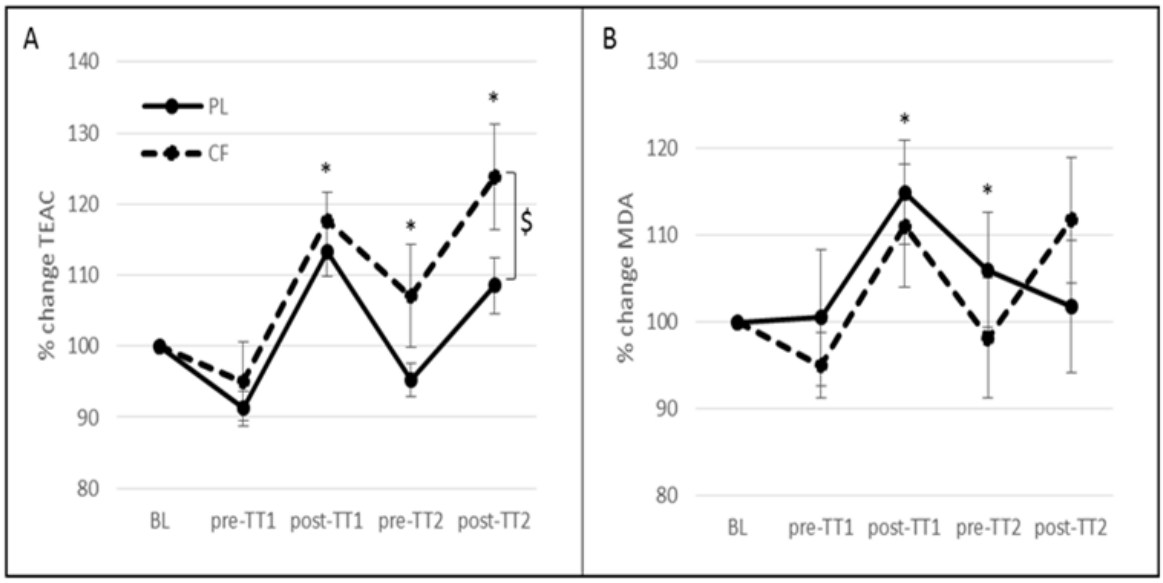

Figure 23. Relative changes in total antioxidant capacity of plasma (TEAC/UA) (A) and plasma malondialdehyde concentrations (MDA) (B) in response to cocoa flavanols (CF) or placebo (PL) and 2 time trials (TT). *$: p<0.05 CF vs PL. *: p<0.05 compared to the previous time point. Mean±SEM presented.*

6.4.4 Plasma markers of inflammation

Neither CF intake, nor exercise changed TNF-α (data not shown). For IL-1 and IL-6, a main effect of time was observed (IL-1: F(4)=2.96; p=0.03; IL-6: F(4)=18.88; p<0.001). Post-hoc analysis revealed that IL-1 and IL-6 were both increased after TT1 (IL-1, PL:+ 12.2±16.0%; CF: + 12.8±12.6%) (IL-6, PL:+ 64.9±10.5%; CF: + 70.2±14.2%). However, TT2 did not induce any significant increases in IL-1 or IL-6 (data not shown). CF intake did not influence IL-1 or IL-6 (no main effect of trial, no interaction effect).

6.4.5 Impact of CF intake on exercise performance: TT1 performance and pacing strategy

Time to complete TT1 tended to be faster after CF intake compared to PL intake, although not significant (PL: 29’47”±1’58”; CF: 29’13”±1’19”; p=0.09). The difference in mean power output during TT1 was + 3±8 Watt (+ 1.22±3.03%) after CF intake compared to PL. This increase is smaller than the typical error of measurement for a TT and thus not relevant to improve performance in real-life. Friedman test showed no significant changes of pacing strategy at 7 different time points in the PL trial and in the CF trial. Wilcoxon signed rank test showed no significant differences in relative power outputs between CF and PL at the start, 5, 10, 15, 20 min and the end of the TT. A significant higher power output in the CF trial, compared to the PL trial, was found after 25 min in TT1 (PL: 73.09±5.3%;CF: 76.75±4.9%; p=0.03; absolute difference: + 14±0.3 W after CF intake (280±29 W) compared to PL (266±29 W) (Figure 24).

During TT1, RM ANOVA *(time (5) x trial (2))* for blood lactate, glucose and RPE showed a main effect of time ($p<0.001$), but no significant main effect of trial and no significant interaction effect. RM ANOVA (time (4)*trial (2)) for heartrate showed a main effect of time ($F=841.38$; $p<0.001$), but neither significant main effect of trial nor significant interaction effect (Figure 25).

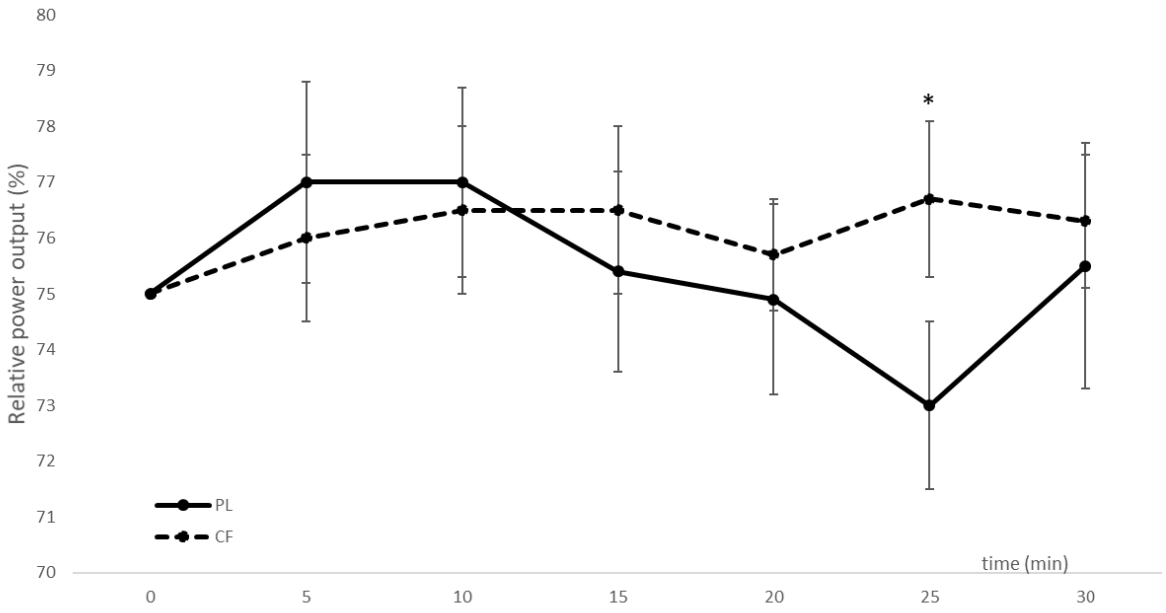

Figure 24. Time trial performance. Pacing strategy of TT1 after cocoa flavanol (CF) or placebo (PL) intake. *The relative power output after 25 min was significantly higher after CF intake compared to PL intake. *: p<0.05 compared to PL.*

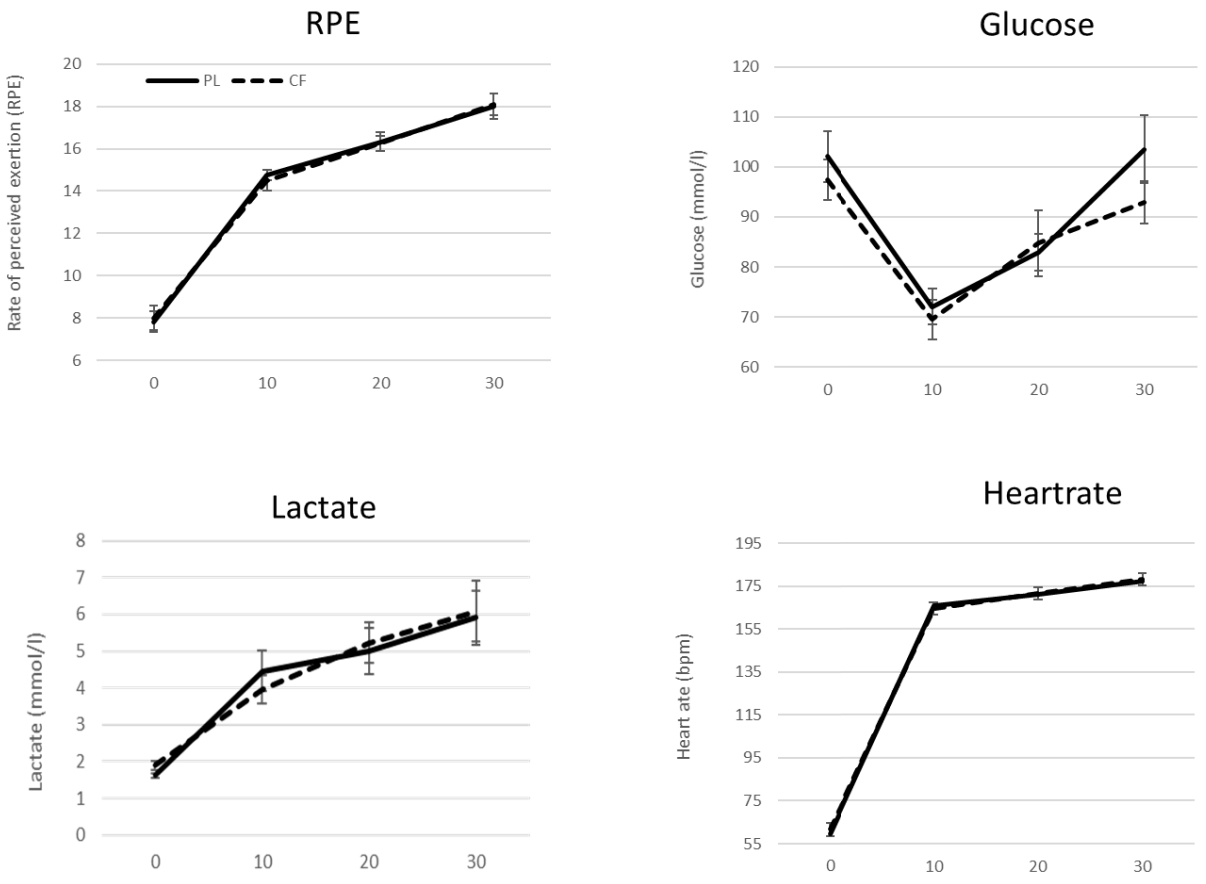

Figure 25. Physiological parameters during time trial 1 (TT1). *No significant differences in rate of perceived exertion (RPE), lactate, glucose or heartrate during TT1 were seen after cocoa flavanol (CF) compared to placebo (PL) intake.*

6.4.6 Impact of CF intake on exercise recovery: performance on TT2

Time to complete TT2 was not different between CF intake and PL intake (PL: 30’±1’35”; CF: 29’34”±49”; p=0.41). In both the PL and CF trial, relative power output changed significantly during TT2 (PL: p=0.02; CF: p<0.01), with a significant increase in power output during the last 5 min of TT2. Relative power output was not significantly different between CF and PL at any of the time points. During TT2, glucose, lactate, heartrate and RPE increased significantly (main effect of time), but were unaffected by CF intake (no main effect of trial, no interaction effect) (data not shown).

6.5 Discussion

This study aimed to examine the effect of CF intake on: *(i)* indirect markers of eNOS-dependent NO production during exercise, *(ii)* exercise-induced changes in antioxidant capacity, oxidative stress and inflammation, and *(iii)* exercise performance and recovery.

Epicatechin, one of the main components of CFs, is at least partially associated with the observed improvements in NO synthesis and endothelial function (Schroeter et al. 2006). CF intake increased serum epicatechin concentrations reaching its peak concentration (approximately 450 nM) between 100 and 130 min after intake, exactly during the first exercise bout, i.e. TT1. We hypothesized that CF intake, through the subsequent increase in epicatechin, would increase NO production, which could influence TT performance. In this study, NOS-dependent NO production was indirectly estimated by measuring its substrate L-arginine/ADMA and its by-product citrulline (Willoughby et al. 2011), because of our inability to directly assess NOS activity in our experimental model. L-arginine, a rate limiting factor for NO production, is converted into NO and citrulline by eNOS and arginine supplementation has been shown to affect the release of NO (Koppo et al. 2009). As intracellular ADMA and arginine compete for NOS binding, ADMA and arginine levels regulate NO production (Sibal et al. 2010). In addition, extracellular ADMA and arginine also compete for cell transport (CAT-2) (Brinkmann et al. 2015). Thus, the higher the arginine/ADMA-ratio, the more arginine may be expected to be available as a substrate for eNOS to produce NO. Although it was shown *in vitro* that CF inhibits arginase activity, L-arginine/ADMA was not increased by acute CF intake in our study.

Exercise-induced shear stress is the principal physiological trigger for eNOS activation and NO production, promoting vasodilation and blood flow (Newsholme et al. 2010). Thus, in this population of well-trained cyclists, NO production is already optimized by their regular training (Green et al. 2004). We examined whether acute CF intake could improve their exercise-induced NO production even more. Citrulline increased and L-arginine/ADMA decreased after both TTs, suggesting an exercise-induced increase in eNOS activity and NO production, but we did not observe an improved eNOS activation by CF as there were no differences in L-arginine/ADMA (eNOS substrate) and citrulline (by-product) after CF intake, neither in rest, nor in response to exercise. However, recent *in vitro* (Karim, et al. 2000) and *in vivo* (Heiss et al. 2005; Balzer et al. 2008) experiments showed that CF intake activates eNOS and improved NO availability. Thus, untrained subjects or a clinical population suffering from reduced NO bioavailability, would probably be more likely to benefit from the CF-induced increase in NO synthesis (Nosarev et al. 2014). Moreover, a very recent pilot study of Taub et al. (2016) showed that chronic (3 months) intake of epicatechin-rich chocolate increased VO_{2max} and enhanced mitochondrial efficiency in healthy, but untrained people. Further research measuring NO production (e.g. nitrate, nitrite, nitrosospecies, peroxynitrite, eNOS activity), tissue hemodynamics (e.g. Flow Mediated Dilation,

Magnetic Resonance Imaging, Doppler) and mitochondrial efficiency is needed to elucidate the exact role of CF on eNOS activation and NO production in combination with exercise.

In this study, time to complete TT1 was not influenced by CF intake, despite the small significant increase in relative power output at the end of TT1. Increases in RPE, lactate, HR and glucose during TT1 were not affected by CF intake. This suggests that acute CF intake has very limited ergogenic effects in well-trained cyclists. Comparing these results directly with similar studies is difficult because of different exercise protocols, as well as different doses, timing, exact composition of the CF extract and the food matrices of CF intake and placebo used. Davison et al. (2012) investigated the effects of the acute intake of dark chocolate containing 247 mg CF (of which 97 mg epicatechin) on physiological parameters during 2.5 h cycling at 60% of their VO_{2max}. Similar to our study, heartrate, RPE and respiratory exchange ratio (RER) during the exercise protocol were not affected by CF intake. Exercise performance, however, was not measured or reported in this study. Stellingwerff et al. (2014) similarly examined the effects of the acute intake of dark chocolate containing 240 mg CF (of which 89 mg epicatechin) on exercise performance, but used a different exercise protocol. Two hours after CF intake, subjects performed 2.5 h of SS exercise at 45% of VO_{2max}, followed by a 15-min TT. The authors did not find any improvements in the 15-min TT performance. This might be explained by the timing of CF intake and the consequent peak of epicatechin in relation to the exercise protocol: the sum of all epicatechin metabolites reached its maximal concentration (approximately 700 nM) during the SS exercise (after 190 min) and was already decreased by 65% at the start of the subsequent TT.

The second goal of this study was to examine the effects of acute CF intake on exercise-induced changes in antioxidant capacity, oxidative stress and inflammation and its possible implications for exercise recovery. Post-exercise recovery can be optimized by acute intake of antioxidants by scavenging free radicals (Bentley et al. 2012; Braakhuis and Hopkins 2015). Antioxidant capacity refers to the cumulative action of all antioxidants present in the plasma and is modulated by either radical overload or by exogenous antioxidant intake (Ghiselli et al. 2000). UA, a strong scavenger of free radicals, contributes to more than 60% of the total plasma antioxidant capacity. However, UA is also upregulated by exercise as a result of an elevated energy-rich purine phosphate catabolism (Jówko et al. 2014). Therefore, next to assessing the total antioxidant capacity of plasma as TEAC values, UA plasma concentrations were determined and TEAC/UA was assessed (Morillas-Ruiz et al. 2006). Consistent with previous research which showed that acute exercise induces oxidative stress and antioxidant capacity (Fisher-Wellman and Bloomer 2009), TT1 and TT2 increased both TEAC/UA and UA plasma concentrations. In line with our hypothesis that CF intake enhances the antioxidant capacity, TEAC/UA and UA were significantly increased by CF intake, before and after exercise. In the study of Davison (Davison et al. 2012), resting antioxidant capacity was also slightly increased by acute dark chocolate intake. Their exercise protocol (2.5 h cycling at 60% VO_{2max}) was longer and less intense than ours, but

also increased TEAC values. However, in contrast to our results, they found that dark chocolate intake blunted the exercise-induced rise in antioxidant capacity. In the study of Allgrove (2011), a 2-week dark chocolate supplementation tended to increase TEAC at each time point, independently of exercise, which is consistent with our results. MDA, a by-product of lipid peroxidation, is the most frequently studied marker of oxidative stress during exercise (Morillas-Ruiz et al. 2006). Although TT1 increased MDA plasma concentrations, this temporal change was not affected by CF intake. Davison et al. (2012) and Allgrove et al. (2011) used F2-isoprostanes as a marker of lipid peroxidation and found that acute and chronic chocolate intake lowered post-exercise plasma free F2-isoprostane levels. In the study of Wiswedel et al. (2004), plasma F2-isoprostane concentrations increased after acute CF (187 mg) intake in rest, whereas plasma TEAC and MDA remained unaffected by CF intake. Thus, we cannot rule out that other biomarkers (such as F2-isoprostane) of oxidative stress might have been affected by CF intake in the present study. Although acute CFs intake upregulated the exercise-induced antioxidant capacity, the resultant oxidative stress (lipid peroxidation) was not decreased, suggesting that the small CF-induced increase in antioxidant capacity is not sufficient to counteract the increase in ROS formation during exhaustive exercise.

The TTs induced an increase in inflammatory cytokines IL-1 and IL-6, but not in TNF-α. Although anti-inflammatory properties of CF are well documented by *in vitro* and *ex vivo* studies (Corti et al. 2009), acute intake of CF did not modify the exercise-induced inflammatory response. Similarly, Davison et al. (2012) and Allgrove et al. (2011) could not find any beneficial effects of acute and chronic dark chocolate intake on exercise-induced inflammatory markers. IL-6, next to its role as pro-inflammatory cytokine, also serves as a myokine produced by skeletal muscle in response to acute exercise to regulate muscle metabolism (Ertek and Cicero 2012). Therefore, the inability of acute CF to blunt exercise-induced IL-6 elevations is not detrimental in this context. However, future research should focus on the effect of chronic CF intake on chronic diseases involving inflammation. Gastrointestinal, nervous, and cardiovascular diseases might benefit from chronic anti-inflammatory properties of CF (Becker et al. 2013), as evidenced by a lowered TNF-α and IL-6 in type 2 diabetes patients following 6 weeks of CF supplementation (Parsaeyan et al. 2014).

Given that CF intake did not reduce time to complete a second TT, exercise-induced inflammation and lipid peroxidation, a biomarker of oxidative stress, we failed to support the hypothesis that CF intake could enhance post-exercise recovery. However, the finding that acute CF intake increased total antioxidant capacity in response to exercise is promising. Future research should focus on its implications on a broader range of biomarkers of oxidative stress, metabolic and vascular parameters in healthy and diseased populations.

6.6 Conclusion

In a population of well-trained cyclists, acute CF intake upregulated exercise-induced total antioxidant capacity, but did not affect lipid peroxidation and exercise-induced inflammation. Acute CF intake did not alter exercise-induced decreases in L-arginine and increases in citrulline (respectively substrate and by-product of eNOS-dependent NO production). Acute CF intake did not improve exercise performance and exercise recovery in a population of well-trained cyclists. Further research on the effects of CF on NO bioavailability, oxidative stress and its consequences in an exercise setting is needed to confirm these data.

6.7 Declarations

The results of the current study do not constitute endorsement of the product by the authors or the journal. This investigation is performed thanks to Lotto Sport Science Chair. L.D. has a PhD grant sponsored by "Nationale Loterij" Belgium. The authors declare no conflict of interest.

Ethics approval and consent to participate

The experimental procedures and potential risks were explained to the participants and a written informed consent was provided and signed prior to inclusion in the study. The study protocol was approved by the research ethical committee of the Vrije Universiteit Brussel and the study was conducted according to the Declaration of Helsinki (1964). The approval was given on 28/01/2015 with reference number 143201523265.

Availability of data and material

The datasets used and/or analysed during the current study is available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest, financial or otherwise. The authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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Authors' contribution

Experimental design:	DL, TC, MR
Data acquisition:	DL, TC, SDD
Biochemical analyses:	DA, DRM, SW
Data interpretation:	DL, WA, SW, HE, MR
Manuscript writing:	DL, TC, SDD, WA, BA, HE, MR

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Chapter 7. One week CF intake increases prefrontal cortex oxygenation at rest and during moderate-intensity exercise in normoxia and hypoxia

7.1 Abstract

Introduction*:* During exercise in hypoxia, O_2 delivery to brain and muscle is compromised and oxidative stress is elicited. Cocoa flavanols (CF) have antioxidant capacities and can increase blood flow by stimulating endothelial function. We aimed to examine the effects of 7-day CF intake on oxidative stress, nitric oxide production and tissue oxygenation in response to exercise in normobaric hypoxia (14.3% O_2).

Methods*:* In a randomized, double-blind, cross-over study, 14 well-trained male cyclists completed 4 trials: exercise in normoxia or hypoxia, after 7-day CF or placebo intake. Flow-mediated dilation (FMD) was measured before intake of the last dose CF or placebo. One hundred min later, 20-minute steady-state (SS, 45% VO_{2max}) and 20-minute time trial (TT) (cycling) were performed. Blood samples were taken. Prefrontal and muscular oxygenation were assessed by near-infrared spectroscopy.

Results*:* At baseline, FMD was increased by CF. Hypoxia increased exercise-induced elevations in lipid peroxidation and antioxidant capacity. CF suppressed exercise-induced lipid peroxidation, but did not influence antioxidant capacity. At rest and during SS, prefrontal and muscular oxygenation were decreased by hypoxia. CF elevated prefrontal oxygenation, but did not impact muscular oxygenation. During TT, hypoxia accelerated the exercise-induced decrease in prefrontal oxygenation, but not in muscular oxygenation. During TT, CF didn't alter prefrontal and muscular oxygenation. CF did not change plasma nitrite, nitrate and arginine:citrulline.

Conclusion*:* During high-intensity exercise, CF did neither improve tissue oxygenation, nor performance in well-trained athletes. At rest and during moderate-intensity exercise, CF reduced exercise-induced lipid peroxidation and partially restored the hypoxia-induced decline in prefrontal oxygenation.

New and noteworthy:

For the first time, we showed that CF had beneficial effects on endothelial function at rest, as well as on prefrontal oxygenation at rest and during moderate-intensity exercise, both in normoxia and hypoxia. Moreover, we showed that CF intake inhibited oxidative stress during exhaustive exercise in hypoxia.

7.2 Introduction

Several sports such as skiing, mountaineering, and sometimes cycling and running involve exercise at altitude. The lower barometric pressure at altitude reduces the partial pressure of inspired oxygen, which results in reductions of O_2 delivery to the active muscles and the brain (Subudhi et al. 2007) and elicits the formation of reactive oxygen species (ROS) (Mcginnis et al. 2014). This leads to a faster development of peripheral and central fatigue, resulting in decreased exercise performance (Verges et al. 2012). Thus, enhancing O_2 delivery by improving blood flow at altitude could improve tolerance to physical exercise and recovery thereafter.

One of the key molecules regulating blood flow is nitric oxide (NO). NO is endogenously produced by the conversion of arginine into citrulline by endothelial NO synthase (eNOS), in the presence of O_2. NO exerts its vasodilatory function via stimulating guanylate cyclase and relaxing smooth muscle cells. eNOS-dependent NO production can be limited in conditions of low O_2 availability and high levels of oxidative stress (Ostergaard et al. 2007; Rochette et al. 2013). Besides, oxidative stress decreases NO availability by increased NO degradation through the reaction of NO with superoxide, the precursor of most other ROS, to form peroxynitrite (Beckman et al. 1990). Both exercise (Powers et al. 2016) and hypoxia (Mcginnis et al. 2014) independently elicit the formation of ROS. While ROS have important roles in cell signalling, apoptosis, gene expression and ion transport, excessive ROS leads to oxidative modification and damage of DNA, RNA, proteins and lipids. In the context of exercise, especially at altitude, the excessive ROS formation can lead to impaired muscle contractile and mitochondrial function, resulting in faster development of exercise-induced muscle fatigue and decreased NO availability (Powers et al. 2016).

It has been reasoned that modulating NO metabolism by nutritional interventions may influence physiological responses to exercise and thus exercise performance in both normoxia and hypoxia (Domínguez et al. 2017). Dietary nitrate supplementation, for example via beetroot juice, has beneficial effects on NO availability, muscle oxygenation and exercise performance (Domínguez et al. 2017), but other supplements hold promise to increase NO availability too. The intake of cocoa flavanols (CF), a subgroup of polyphenols with antioxidant capacities causes NO-mediated vasodilatation, clinically measured by flow-mediated dilation (FMD) (Hooper et al. 2012; Grassi et al. 2015). *In vitro* and *in vivo* data showed that (−)-epicatechin, the main bioactive constituent of cocoa, increased nitrite concentration, an indirect marker of eNOS dependent NO production (Loke et al. 2008a; Brossette et al. 2011). Furthermore, CF and/or their metabolites are strong antioxidants, by directly scavenging superoxide, inhibiting NADPH oxidase (Steffen et al. 2008) and/or by modulating the endogenous antioxidant defence (Ruijters et al. 2013).

Despite the existing evidence of the beneficial effects of CF on endothelial function and oxidative stress, few studies investigated the possibilities of CF to modulate exercise-induced oxidative stress and/or exercise performance (Wiswedel et al. 2004; Fraga et al. 2005; Allgrove et al. 2011; Davison et al. 2012; Stellingwerff et al. 2014; Patel et al. 2015; González-Garrido et al. 2015; Taub et al. 2016). Because of the large variety of study designs, subject samples, type of exercise, timing and dosages of CF intake used in these studies, results concerning the CF-induced decrease in oxidative stress after exercise are inconsistent. These studies were all performed at sea level. However, it may be conceivable that this nutritional strategy is more efficient in hypoxia, where O_2 delivery is reduced and where ROS formation is exaggerated.

Consequently, the objectives of this study were to investigate the effects of a 7-day CF intake on *(i)* selected plasma markers of NO availability and oxidative stress, *(ii)* muscle and cerebral oxygenation in response to an acute exercise bout in normoxia (sea level) and normobaric hypoxia (simulated altitude of 3000 m, 14.3% O_2) and *(iii)* the implications for exercise performance. We hypothesized that, compared to placebo (PL), CF intake would increase NO availability, decrease oxidative stress and increase cerebral and muscular oxygenation during exercise in normoxia and hypoxia and enhance exercise performance.

7.3 Methods

7.3.1 Participants

A sample size calculation, based on the results of Allgrove et al.(2011), Patel et al. (2015) and Wiswedel et al. (2004), indicated that 14 subjects were required to detect differences at P value $p<0.05$ with 90% power. The recruitment started in January 2016. Subjects were excluded when *(i)* younger than 18 years or older than 36 years, *(ii)* smoking or smoking in the past, *(iii)* took antioxidant supplementation, *(iv)* trained less than 10 h per week, *(v)* had stayed at high altitude (> 2000m) for more than 3 weeks during the last 6 months, or *(vi)* if the medical examination prior to the experiment revealed they were hypertensive or had cardiovascular disease. Fifteen healthy well-trained male cyclists were selected for participation in this study. One subjects dropped out because of an injury (knee injury). The study was approved by the UZ Brussel Ethics Committee and was in accordance with the declaration of Helsinki. The experimental procedures and potential risks were explained to the participants and a written informed consent was provided and signed before the start of the study. This trial was registered at clinicaltrials.gov as NCT03135314 and this manuscript is compliant with CONSORT (Consolidated Standards for Reporting Trials).

7.3.2 Study design

A randomized, placebo controlled, counter-balanced, cross-over study design was used. The study was conducted at the Department human physiology of the Vrije Universiteit Brussel (VUB, Brussels, Belgium) from March 2016 until July 2016. On the first lab visit, subjects underwent a complete medical screening (including skinfolds measures) and performed a maximal incremental cycle test on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). During this test, initial work rate was set at 80W and work rate was then increased every 3 min by 40 W until volitional exhaustion. Maximal oxygen uptake (VO_{2max}) was determined using the Metalyzer cortex (Biophysik GmbH, Germany) and peak power output (PPO) was determined.

Subsequently, subjects visited the lab once every 2 weeks for 8 weeks (4 visits): each visit was preceded by a 1-week wash-out (except for the first visit) and a 1-week nutritional intervention (PL or CF). The sequence of the 4 nutritional interventions was randomly assigned for each participant by using a computer-based randomly permutated block method. The allocation list was generated by CT (co-author), recruitment of participants was conducted by LD (first author) and allocation of participants was conducted by a third author (EL). Participants and researchers involved in data collection, outcome assessment and statistical analysis were blinded to the nutritional intervention. Participants and all researchers, except for LD, were blinded for the fraction of inspired O_2 (F_IO_2).

Subjects performed 4 interventional trials in randomised order: (1) exercise in (normobaric) hypoxia (H) (3000 m; 14.3% O_2) preceded by 7 days of CF intake [H-CF], (2) exercise in H (3000 m; 14.3% O_2) preceded by 7 days of PL intake [H-PL] (3) exercise in normoxia (N) (0 m; 21.0% O_2) preceded by 7 days of CF intake and [N-CF] (4) exercise in N (0 m; 21% O_2) preceded by 7 days of PL intake [N-PL]. All experimental trials were conducted in 20 °C and relative humidity was kept between 30 and 40%.

7.3.3 Supplementation

Subjects were asked to consume the provided supplements (PL or CF, Naturex, Avignon, France) every morning at breakfast during the 6 days prior to the testing day. On the testing day, subjects consumed the last dose of supplements upon arrival in the lab. The daily dose of CF consisted of 4 capsules, containing a total of 1765 mg cocoa extract of which 100 mg epicatechin, 23 mg catechin, 119 mg theobromine and 17 mg caffeine (Table 15). The dose and duration of the supplementation were based on the finding that 1-week of CF intake enhances vascular function in a dose-dependent manner, with an optimal effect of 100 mg epicatechin (Grassi et al. 2015) and on the pooled results from a recent meta-analysis where 1 week of polyphenol intake increases performance (Somerville et al. 2017). The PL capsule contained 1765 mg maltodextrine and was matched with the CF capsule in colour and shape, theobromine and caffeine content. From the blinding check, it was clear that subjects were unable to distinguish the 2 interventions. The nutritional intervention was double-blind and counter-balanced. Subjects were provided with a list of foods rich in polyphenols which they should avoid throughout the 8-week study. They were asked to abstain from caffeine during the last 24 h prior to each intervention trial and to repeat the same nutritional regimen during the last 24 h prior to each intervention trial. Subjects completed a 24 h-food recall on 3 random days during the study, to check for a potential influence of polyphenol intake on the measurements.

Table 15. Composition of cocoa flavanol (CF) and placebo (PL) supplementation (Naturex) (daily dose).

Content in 4 pills	PL	CF
Cocoa extract (mg)	0	1764
Maltodextrine (mg)	1764	136
Total flavanols (mg)	0	530
Total monomers (mg)	0	121
(−)-Epicatechine (mg)	0	100
(+)-Catechine (mg)	0	21
Theobromine (mg)	119	119
Caffeine (mg)	17	17

7.3.4 The four interventional trials

Subjects were asked to keep a training diary for the entire duration of the study and to repeat the same weekly training regimen (volume and intensity) for the duration of the study. They were instructed to abstain from intensive training the last 24 h prior to each intervention trial. On each visit, subjects arrived at the lab at the same time of the day in a 3 h fasted state. The entire protocol is depicted in Figure 26.

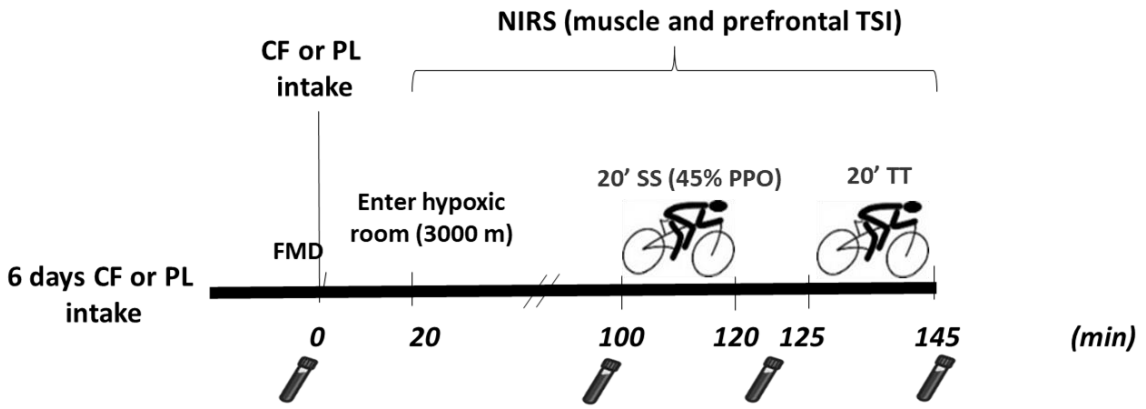

Figure 26. Interventional exercise protocol; *twice executed in hypoxia and twice in normoxia, following 7 days of cocoa flavanol (CF) or placebo (PL) intake. FMD: Flow mediated dilation. NIRS: Near infrared spectroscopy at M. vastus lateralis and prefrontal cerebral cortex. SS: steady state, TT: time trial.*

First, a baseline FMD measurement took place. Subsequently, a catheter was placed in a forearm vein and a first venous blood sample was collected. Subjects then consumed the last dose of their supplementation, together with a carbohydrate rich meal, which was carefully selected by a nutritionist to contain 600 kcal, 85% carbohydrates, 10% proteins and 5% fat. After the meal, subjects entered the isobaric hypoxic chamber, which was pre-set at the desired% O_2. Subjects were asked to sit down and relax. It was shown that the maximal concentration of plasma flavanols is reached 100 min after acute CF intake and the plasma concentration of flavanols remains at a maximum for 50 min (Schramm et al. 2003). Therefore, ninety-five min after the last supplement intake, a second blood sample was taken. Subsequently, the participants started a 20-minute steady state (SS) cycling exercise, one hundred min after the last dose. During the SS, power output was fixed at 45% of their PPO. SS was followed by five min passive rest in seated position and a blood sample was taken. The 20-minute TT then started at 75% of PPO, but subjects were free to increase or decrease their power output as desired from the outset. The goal was 'to perform as much as possible during 20 min'. Subjects received information on the time lapsed, but did not receive any feedback regarding power output or heart rate (HR). HR and saturation (SaO_2) were recorded continuously during the experimental trial using a chest belt and Polar HR monitor and a pulse oximeter which was positioned on the participants' left index finger (Medlab, Germany). Rating of perceived exertion (RPE) was measured at the start and after 5, 10, 15 and 20 min of the SS and the TT. Blood lactate was enzymatically determined in a capillary blood sample from the ear lobe

(Ekf, Biosen 5030, Magdeburg, Germany), at the start, after 10 and 20 min of the SS, and the TT. During the 20-minute TT, the completed work (kJ) was used as the main outcome parameter of exercise performance. The occurrence of acute mountain sickness was assessed using the Lake Louise Questionnaire at the end of each trial, but none of the subjects experienced any symptom of AMS.

7.3.5 Measurements

Primary outcome measures were muscle and cerebral oxygenation, markers of oxidative stress and exercise tolerance and performance. Secondary outcome measures included flavanol metabolites, markers of NO availability, FMD and mean arterial pressure.

7.3.5.1 Flow-mediated dilation (FMD) and mean arterial pressure (MAP)

Upon arrival at the lab, subjects were instructed to relax in supine position for 10 min, during which MAP was measured automatically (Medisana BU510, Kerkrade, Netherlands). Then, arterial endothelial function was assessed by FMD. The operator, blinded to PL or CF condition, took images of the vessel upon arrival of the participants (baseline measure) with a 5-10 Hz linear array probe. The scan of the right brachial artery, approximately 2-7 cm above the antecubital fosse (marked on the first visit, ensuring that measurement occurred at the same place for each scan), was evaluated in the longitudinal image. The sphygmomanometer placed around the forearm (distal) was inflated 50 mmHg above the systolic blood pressure for 5 min. A continuous scan of the brachial artery was then performed during 90 s after the rapid deflation of sphygmomanometer, which induced shear-stress and endothelium-dependant dilation. Each brachial artery diameter was manually measured three times at end of diastole, 60 s after the cuff deflation. The FMD (%) was calculated as ((hyperaemic diameter – pre-inflation diameter)/pre-inflation diameter) x 100.

7.3.5.2 Muscle and cerebral oxygenation during exercise

Near-infrared spectroscopy (NIRS) (Portalite continuous-wave NIRS system (Artinis, Elst, Netherlands), a non-invasive optical imaging technique, was used to assess changes in oxygenation status of the *prefrontal cerebral cortex* and the *M. vastus lateralis* during exercise. The use of NIRS to assess tissue oxygenation, including its limitations, has been extensively described (Ferrari et al. 2004).

Upon entrance in the isobaric hypoxic chamber, one emitters/receptor optode pair was positioned over the left prefrontal cortical area between Fp1 and F3, according to the modified international EEG 10-20 system. One emitters/receptor optode pair was attached to the (shaved) skin on the lower third of the belly of the right *M. vastus lateralis* (middle between the lateral epicondyle and trochanter). Skinfold measures during the medical screening assured that the adipose tissue thickness was well below 1.5 cm to allow the NIRS photons to penetrate into the muscle (Ferrari et al. 2004). The inter-optode distance

for both probes was 4 cm and the probes were covered with a black cloth to minimize intrusion of extraneous light. A dark elastic band was wrapped around the head and the leg to keep the NIRS-optode pairs in place.

NIRS data collection was started 20 min prior to the start of exercise. To collect a baseline NIRS value, the mean of a 2-minute period during which subjects sat still without speaking or moving, was calculated. The tissue saturation index (TSI) was determined by spatially resolved spectroscopy and offers a surrogate measure of the fraction of O_2 saturated haemoglobin and myoglobin, reflecting a tissue oxygenation status in percentage (%) (Ferrari et al. 2004). Data were collected at a sampling frequency of 5 Hz and were down sampled with factor 5 for analysis. During exercise, mean TSI was calculated per 30 second window. NIRS values of the following 30-sec epochs were used for data analysis: 0, 5, 10, 15 and 20 min of the SS and TT.

7.3.5.3 Blood analyses

Venous blood samples were collected at baseline, and at the start and end of the SS and the TT. Blood was collected into 5 ml EDTA tubes, 5 ml heparinized tubes and 8 ml anticoagulant-free tubes and were centrifuged immediately to obtain plasma or after 30 min at room temperature to allow clotting to obtain serum (10 min at 704 *g*, 4 °C). Plasma and serum were aliquoted and stored at -80 °C until further analyses. Values were corrected for changes in plasma volume using the haematocrit and haemoglobin concentration according to Dill and Costill (Dill and Costill 1974). Hemoglobin concentration was measured in duplibcate using an azidemethemoglobin double wavelength photometer method (Hemocue Hb201+, Angelholm, Sweden), while haematocrit was determined by microcentrifugation in triplicate (Heraeus Pico 17, Germany).

Serum flavanols

Serum samples were analysed for epicatechin and catechin concentrations as described by Neukam et al (Neukam et al. 2007). In detail, 0.5 ml of serum was mixed with 1.0 ml phosphate buffer (100 mM, pH 5, containing ascorbic acid (20 mg/ml), EDTA (1.5 mg/ml)) and 20 µl glucuronidase/sulfatase (100,000 and 7500 units/ml, respectively) and incubated at 37 °C for 30 min to hydrolyse glucuronate and sulfate conjugates of epicatechin and catechin. Then, 5 ml tertbutylmethylether was added and vortexed for 1 min. For phase separation, the mixture was centrifuged at 10 °C for 5 min at 1957 *g*. The organic phase was transferred to a new tube and after a second extraction with 5 ml tertbutylmethylether, the combined extracts were dried under a stream of nitrogen and stored at −80 °C. For HPLC analyses, the dry residues were reconstituted in 200 µl methanol, vortex-mixed and centrifuged at 5 °C at 15 339 *g* for 5 min. 20 µl of the supernatant were injected onto the HPLC-column. For HPLC analysis, a reversed-phase RP18 end-capped column (Lichrospher 100, 5 µm, 250 × 4 mm; Merck) coupled with a guard column (Lichrospher 100, 5 µm, 4 × 4 mm; Merck) was used. Detection was accomplished at an

excitation wavelength of 280 nm and an emission wavelength of 310 nm. Data were recorded by HPLC-System Manager software (Merck/Hitachi; Darmstadt, Germany). Samples were eluted from the column at 20 °C using a step-gradient as follows: from 0 to 18 min 53% acetonitrile/H_2O/acetic acid (150:846:4) / 47% H_2O/acetic acid (1000:5), from 18 to 27 min 80% acetonitrile/H_2O/ acetic acid (150:846:4) / 20% H_2O/acetic acid (1000:5). To elute retained compounds the column was flushed from 27 to 60 min with 100% acetonitrile/acetic acid (1000:4) and subsequent equilibration from 60 to 80 min with starting conditions. Flow rate was 1.5 ml/min. Under these conditions the analytes elute with retention times of 15.4 min for catechin and 24.9 min for epicatechin. Peak areas of catechin and epicatechin were used to calculate the concentrations applying the external standard method. Standard curve linearity was observed in the range from 0.125 to 20 µM for both compounds.

Plasma nitrite and nitrate

Plasma nitrite (NO_2^-) and nitrate (NO_3^-) were analysed by gas phase chemiluminescence analysis. Plasma was deproteinised with ice-cold ethanol. For NO_2^- analysis, samples were injected into a glass purge vessel containing 5ml glacial acetic acid and 1ml NaI solution, which reduces NO_2^- to nitric oxide (NO) gas, that is carried into the NO detector in inert nitrogen. For NO_3^- analysis, samples were reduced in a solution of vanadium (III) chloride in 1 M hydrochloric acid (0.8% w/v). Quantification of NO was enabled by the detection of light emitted during the production of nitrogen dioxide formed upon reaction of NO with ozone. Luminescence was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence nitric oxide analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). The concentrations of NO_2^- and NO_3^- were determined by plotting signal area (mV) against a calibration plot of 25nM to 1µM sodium nitrite and 100nM to 10µM sodium nitrate respectively. The NO_3^- concentration was corrected by deduction of the NO_2^- value, since the vanadium chloride solution also reduces nitrite.

Plasma arginine and citrulline

150 µL of internal standard (50 µM arginine, methanol mixture) was added to 10 µL of heparinized plasma and centrifuged (13000 rpm, 10 min, 4 °C) to remove the precipitated proteins. Supernatant was collected and dried under a stream of nitrogen at 70 °C. The dried extract was dissolved in 100 µL of a butanol solution containing 3N HCl and kept at 70 °C for 40 min. The solvent was removed by evaporation under nitrogen flow at 70 °C. The sample was then dissolved in 2.5 mL of water-methanol (90:10, v/v) containing 0.1% formic acid and 5 µL was injected into an analytical column (Kinetex C18 (5µm, 2.1x100mm)). Mass spectrometric analysis was performed using an UFLC-XR Shimadzu coupled with an QTRAP® 5500 hybrid system, equipped with a Turbo VTM ion197 source (AB Sciex, Foster City, CA, USA). Multiple reaction monitoring (MRM) measurement was performed using optimal cone and collision energy values. Each run was performed at a flow rate of 500 µL/min at 30°C,

lasting 9 min in total. A gradient profile consisted of solution A (water with 0.1% (v/v) formic acid) and solution B (methanol with 0.1% (v/v) formic acid). The percentage of organic solution B was changed gradually as follows: 0 min, 2%; 4 min, 7%; 6 min, 50%; 7 min, 2%; 9 min, 2%. Data were acquired with Analyst Software version 1.5.2. Calibration curves, performed in water, were obtained adding increasing concentrations of arginine and citrulline from 12.5 to 125 µM.

Total antioxidant capacity (TEAC), uric acid (UA), malondialdehyde (MDA) in plasma

Plasma antioxidant capacity was quantified as trolox equivalent antioxidant capacity (TEAC) according to Fischer et al. (2005). In this procedure, the decolorization of the preformed green-blue 2,2`-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical by the heparinized plasma within a fixed time reflects the antioxidant capacity of the sample. To correct plasma TEAC values for individual differences in uric acid (UA) concentrations, the most abundant antioxidant in blood, UA plasma concentrations were quantified by HPLC (Ruijters et al. 2013). MDA, a marker of lipid peroxidation as a result of oxidative stress, was quantified in EDTA-plasma samples after derivatization with thiobarbituric acid by using HPLC with fluorometric detection as described by Lepage et al. (1991).

7.3.6 Statistical analyses

A power calculation to determine the minimal sample size required to assess whether CF intake would affect exercise-induced markers of oxidative stress and exercise performance (TT) (n=15, p=0.05, power= 0.8) was based on the results of Allgrove, Patel and Wiswedel (Wiswedel et al. 2004; Allgrove et al. 2011a; Patel et al. 2015).

Statistical analyses were performed with IBM SPSS Statistics (version 22; IBM Corp, Armonk, USA) and considered significant at $p<0.05$. Data are presented as mean±standard deviation (SD) for n=13, except when otherwise indicated. Normality and sphericity of the data were assessed by the Kolmogorov-Smirnov test and Mauchly's test. To follow the absorption and metabolism of epicatechin and catechin after 6-day CF intake and after intake of the final dose of CF, a three-way repeated measures ANOVA *(F_IO_2 x supplement x time (baseline, start SS, start TT, end TT))* was used. Two-way repeated measures ANOVA *(fraction of inspired O_2 (F_IO_2) x supplement)* at *baseline* was employed to assess the baseline differences in nitrite, nitrate, arginine:citrulline ratio (arg:citr), TEAC, UA, MDA, MAP and FMD between 6-day CF and PL intake, with interpretation of the main effect of supplement. Two-way repeated measures ANOVAs *(F_IO x supplement)* were used to assess differences in nitrite, nitrate, arg:cit, TEAC, UA, MDA and muscular and prefrontal TSI between CF and PL in H and N at start of SS and start of TT. For significant interactions between F_IO_2 and supplement, pairwise comparisons were performed using the post hoc Bonferroni correction. Two three-way repeated measures ANOVAs (*F_IO_2 x supplement x time)* were used to assess differences between CF and PL in H and N during

exercise (1 for SS, 1 for TT) for the following outcome parameters: nitrite, nitrate, arg:citr ratio, TEAC, UA, MDA, work performance during TT, muscular and prefrontal TSI. The effects of H and CF intake on TT performance (work performed after 20 min) was assessed by a two-way repeated measures ANOVA *(F_IO_2 x supplement).* Significant interactions in the three-way repeated measures ANOVA were further analysed by two-way repeated measures ANOVA with subsequent paired t-tests to interpret the effect of the supplement over time at each F_IO_2 (N and H) and the effect of F_IO_2 over time after CF or PL intake. If no significant interaction effects were observed, main effects were immediately interpreted through pairwise comparisons with the Bonferroni correction. Significant interactions in the two-way repeated measures ANOVA *(supplement x F_IO_2)* were further analysed by paired t-tests to interpret the effect of the supplement at each F_IO_2 (N and H) and the effect of F_IO_2 after each supplementation. RPE was not normally distributed and was therefore analysed by Friedman tests and Wilcoxon signed rank tests. A Pearson correlation was used to assess correlations between baseline concentrations of nitrite and nitrate and relative increases in nitrite and nitrate concentration after 6 days of CF intake.

7.4 Results

7.4.1 Subject characteristics

The 14 well-trained athletes included in this study were 30.7±3.1 years old, had a height of 1.80±0.05 m, weight of 73.4±7.4 kg and BMI of 22.5±1.5. They had a VO_{2max} of 62.9±5.8 mL/min*kg and PPO of 366±45 W.

7.4.2 Effects of CF intake on (−)-epicatechin and (+)-catechin

At baseline, there was no significant difference between 6-day CF and PL intake on serum epicatechin concentration. Three-way repeated measures ANOVA showed a significant *supplement x time* interaction (F=14.70, p<0.001). Post-hoc analysis showed that 100 min after acute CF intake, serum epicatechin was elevated compared to baseline (+ 238±43%, H and N pooled, p<0.05). After CF intake, serum epicatechin further increased during SS exercise (+ 359±32%, H and N pooled, p<0.001 compared to baseline), while a plateau phase in serum epicatechin was reached during the TT (no further increase post-TT compared to pre-TT) (Figure 27). After PL intake, epicatechin did not change over time. Serum catechin was not affected by CF intake, compared to PL intake, at any time points and in both N and H (Table 16) for baseline values, other data not shown).

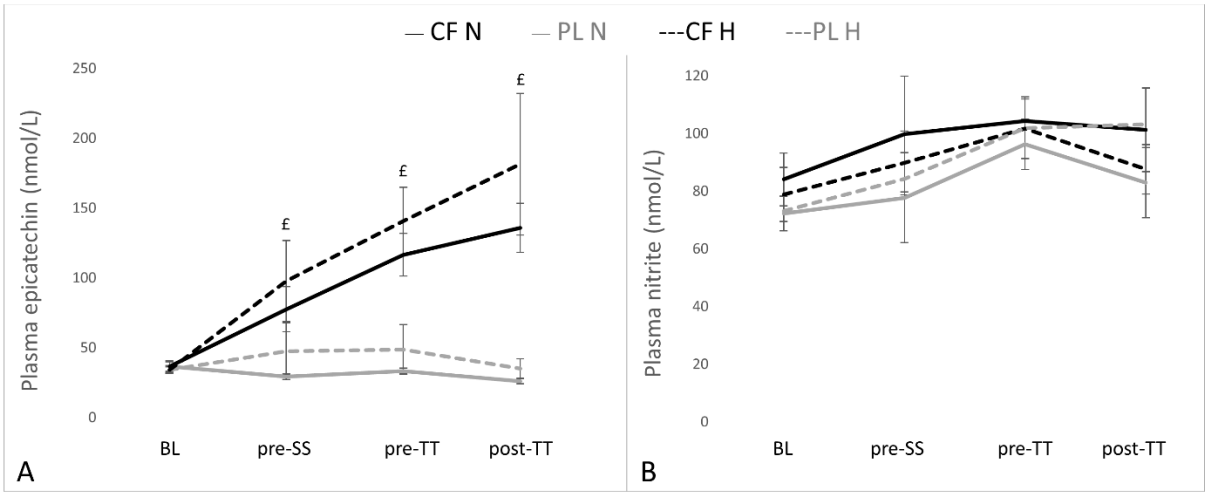

Figure 27. Effect of 7-day cocoa flavanol (CF, black lines) or placebo (PL, grey lines) supplementation in hypoxia (H, dashed lines) and normoxia (N, full lines) on plasma epicatechin (A) and plasma nitrite (B) concentrations. *BL: baseline, before intake of the last dose, Pre-SS: at the start of the 20-minute steady state exercise (45% of peak power output), pre-TT: at the start of the 20-min time trial, post-TT: at the end of the 20-min time trial. Mean±SE presented. £: p<0.05: main effect of supplement.*

Table 16. Baseline measures following 6-day (before intake of last dose) cocoa flavanol (CF) or placebo (PL) intake (n=14).

	PL	***CF***
(−)-Epicatechin (nM)	35.4±10.8	35.3±9.2
(+)-Catechin (nM)	19.7±10.8	21.7±14.7
Nitrite (nM)	69.9±17.5	81.4±19.9
Nitrate (µM)	43.8±22.9	47.5±21.5
MDA (µmol/L)	1.17±0.25	1.19±0.21
arg:citr ratio	1.93±0.56	1.92±0.27
TEAC	442.8±28.7	447.4±49.3
UA (µmol/L)	302.0±39.3	303.5±40.4
MAP (mm Hg)	95.5±7.1	94.0±7.1
FMD (%)	0.56±2.26	2.15±2.19*

MAP: mean arterial pressure, FMD: flow mediated dilation. p<0.05 between CF and PL. Epicatechin and catechin,were measured in serum, nitrite, nitrate, malonaldehyde (MDA), Arginine (Arg), Citrullin (Citr), Uric acid (UA) and Trolox Equivalent Antioxidant Capacity (TEAC) were measured in plasma.*

7.4.3 Effect of CF intake on NO availability during exercise in H

eNOS dependent NO production

eNOS dependent NO synthesis was reflected by plasma nitrite and nitrate concentrations and by the plasma ratio of arg:citr (Sureda and Pons 2012). Plasma nitrite and nitrate did not change during exercise and were not affected by H (Figure 27). Arg:citr ratio significantly decreased at the end of exercise compared to pre-exercise (main effect of time: F=14.1, p=0.003) and was significantly higher in H compared to N (main effect of F_IO_2: F=10.0, p=0.008) (Figure 28).

CF intake did not significantly change plasma nitrite, nitrate and arg:citr ratio, neither before (thus at "baseline"), nor after the final dose. CF intake did not change plasma nitrite, nitrate and arg:citr ratio after exercise. However, a significant negative correlation was found between baseline nitrite (after 6 days of PL intake) and the relative increase in nitrite concentration after 6 days of CF intake (R^2=0.67, p<0.001, Pearson correlation).

Oxidative stress and antioxidant capacity

Two-way repeated measures ANOVAs at baseline and pre-exercise showed that MDA was neither affected by 6-day CF intake (Table 16) nor by the final dose of CF, in rest. Three-way repeated measures ANOVA during exercise revealed a significant interaction effect of *time x supplementation* (F=7.95 p=0.018): the significant exercise-induced increases in plasma MDA concentrations after PL intake

(+12.2±5.5%, p=0.047 in N and +19.0±6.8%, p=0.016 in H), were suppressed by CF intake in both N (+ 2.9±4.4%, NS) and H (+ 2.0±4.4%, NS) (Figure 28).

Two-way repeated measures ANOVAs at baseline and pre-exercise showed that total plasma antioxidant capacity, measured as TEAC, was neither affected by the intake of 6 day CF intake, nor by the last dose of CF in N and H in rest. Three-way repeated measures ANOVA revealed that the exercise-induced increase in TEAC was larger in H than in N (time x F_IO_2: F=4.83, p=0.05), but was not affected by CF intake. To correct for individual differences in UA, the most abundant plasma antioxidant that contributes to plasma TEAC, UA concentrations were quantified in every sample. Two-way repeated measures ANOVAs showed that UA was neither affected by 6-day CF intake, nor by intake of the final dose of CF at rest. Three-way repeated measures ANOVA showed that UA concentrations were elevated after exercise, and in H compared to N (main effect of time: F=25.26, p<0.001; main effect of F_IO_2: F=6.48, p=0.026), while CF did not influence this response (Figure 28).

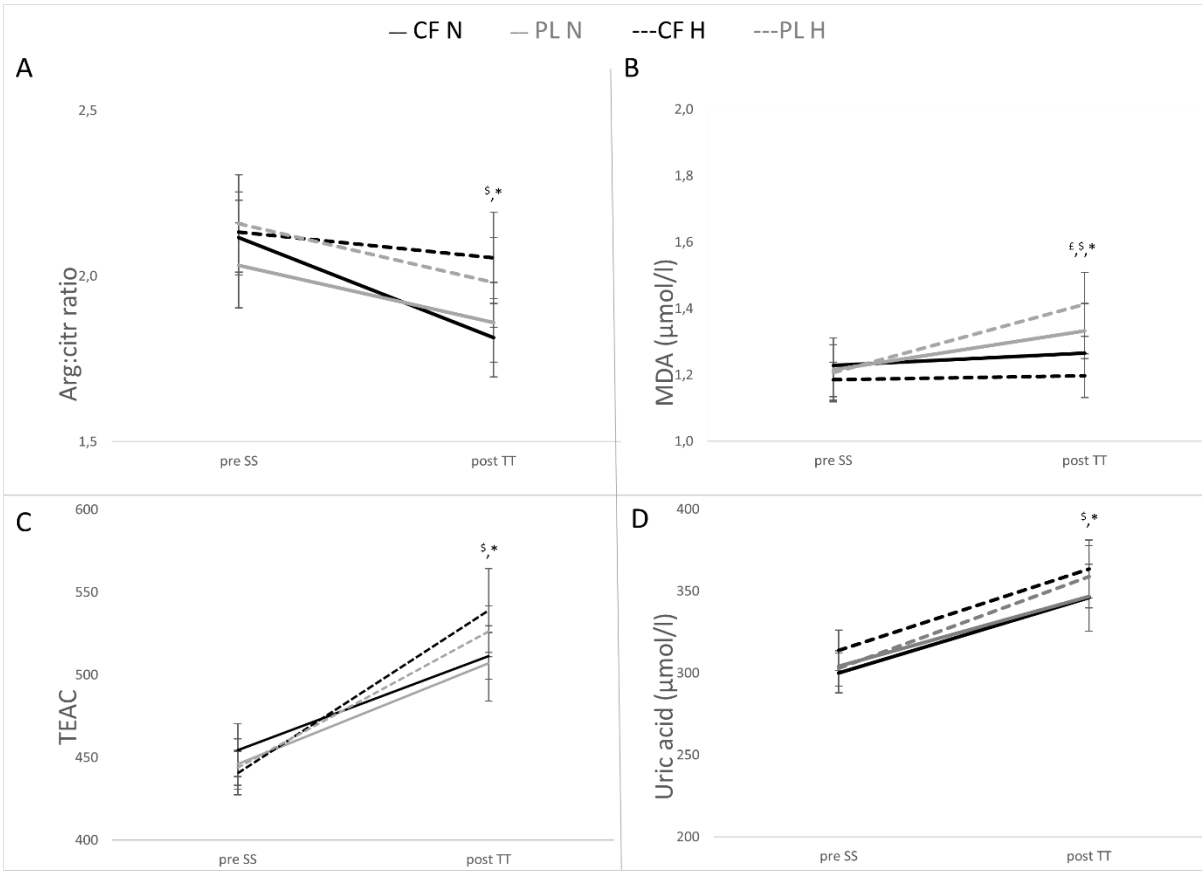

Figure 28. Effect of 7-day cocoa flavanol (CF, black lines) or placebo (PL, grey lines) supplementation in hypoxia (H, dashed lines) and normoxia (N, full lines) on exercise-induced changes in plasma arginine:citrulline ratio (A), plasma malondialdehyde concentration (MDA) (µmol/L) (B), plasma trolox equivalent antioxidant capacity (TEAC) (C) and plasma uric acid concentration (UA) (µmol/L) (D). *Pre-SS: at the start of the 20-minute steady state exercise (45% of peak power output), Post-TT: at the end of the 20-min time trial. Mean±SE presented. *: p<0.05: main effect of F_IO2; £: p<0.05: main effect of supplement; $: p<0.05: main effect of exercise.*

7.4.4 Vasoreactivity

At baseline, MAP was not different between 6-day CF and PL intake. FMD was significantly increased after 6 days of CF intake compared to PL (main effect of supplement: F=5.59, p=0.042) (Table 16). The change in FMD after 6 days of CF intake compared to 6 days of PL was not correlated with the relative increases in nitrite and (−)-epicatechin concentration after 6 days of CF intake compared to PL.

Muscle oxygenation during exercise

At the start of SS exercise, TSI in the M. vastus lateralis was not affected by the supplement and H. During SS, a significant interaction *time x* F_IO_2 effect was found for TSI (F=11.95, p<0.001) (Figure 29). Post-hoc Bonferroni corrections showed that TSI decreased during the first 5 min and then stabilized. This exercise-induced decrease was aggravated in H, compared to N, while CF intake had no effect.

At the start of the TT, TSI was significantly lower in H than in N (main effect of F_IO_2: F=6.96, p=0.02). Three-way repeated measures ANOVAs showed a main effect of time during the TT for TSI (F=71.65, p<0.001): TSI significantly decreased during the first 5 min and stabilized during the last 15 min. H and CF intake did not influence TSI during the TT.

Prefrontal cortex oxygenation during exercise

At the start of SS exercise, both H and CF influenced TSI (main effect of F_IO_2: F=7.05, p=0.02, main effect of supplement: F=7.66, p=0.017) (Figure 29). TSI was significantly lower in H compared to N. TSI was significantly higher after CF intake compared to PL (Figure 29). During SS, 3-way repeated measures ANOVA showed a significant main effect of supplement (F=12.28, p=0.004) and a significant FIO_2 *x time* interaction for TSI (F=24.10 p<0.0001). CF intake significantly increased prefrontal TSI during SS exercise. TSI significantly decreased in H, but not in N.

At the start of the TT, TSI was significantly lower in H than in N (*effect of* F_IO_2: F=6.43, p=0.026), while CF intake had no significant effect. Three-way repeated measures ANOVA showed a significant F_IO_2 *x supplement x time* interaction effect for TSI during the TT (F=4.11, p=0.016). In N, TSI decreased significantly for the entire duration of the TT after both PL and CF intake. However, a larger decrease was observed after CF intake, compared to PL (significant *supplement x time* interaction effect (F=6.38, p<0.001)). In H, TSI enormously decreased during the first 5 min and did not change significantly during the remaining 15 min after both PL and CF intake and no interaction effect of *supplement x time* was found.

7.4.5 Exercise tolerance and performance

Steady state

Two-way repeated measures ANOVA showed that at the start of SS, SaO_2 was significantly lower in H than in N (Table 17). At rest, CF intake did not alter SaO_2. HR and lactate were similar in H and N and were not different after CF intake, compared to PL. Three-way repeated measures ANOVAs showed a significant F_IO_2 *x time* interaction effect for SaO_2, HR and lactate during SS. An exercise-induced decrease in SaO_2 occurred in H, but not in N. The exercise-induced increase in HR was larger in H than N. In N, lactate decreased during SS, but in H, lactate increased during SS. During SS, RPE was significantly higher in H than in N. The (significant) difference in SaO_2 between CF and PL intake during SS exercise in H (-1.21±.48% in CF vs. PL) was smaller than the accuracy range (2 – 3%) claimed by the distributor of the pulse oximeter used and might thus not be reliable. CF did not influence HR, lactate and RPE during SS exercise.

Time trial

TT performance (work performed during the 20 minute-TT) decreased in H compared to N, but CF intake did not influence TT performance (Table 17). Two-way repeated measures ANOVAs at the start of the TT showed that SaO_2 and lactate were significantly lower (-8±1%, $p<0.001$) and higher (+0.9±0.2 $mmol.L^{-1}$, $p<0.001$) in H compared to N. No significant difference between N and H was observed for HR. CF did not influence SaO_2, lactate or HR. Three-way repeated measures ANOVAs showed a significant F_IO_2 *x time* interaction effect for SaO_2, HR and lactate during the TT. Post hoc analysis showed a larger drop in SaO_2 and a larger increase in lactate during the TT in H compared to N. Post hoc analysis showed a faster elevation of HR, but lower HR_{max} at the end of the TT in H than in N. RPE was significantly higher in H than in N during the first half of the TT, but there was no difference during the second half. CF intake did not influence any of these physiological changes.

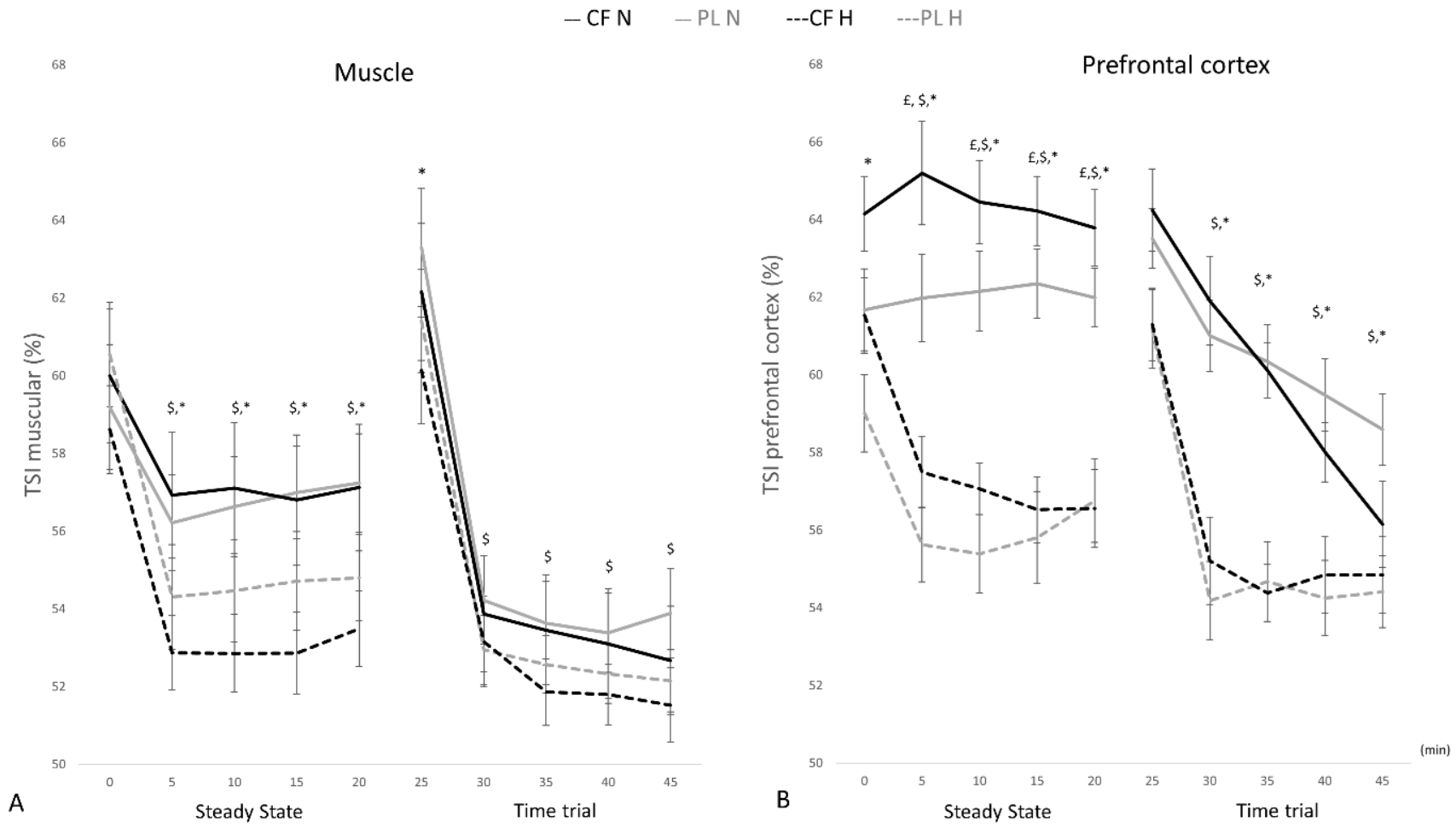

Figure 29. Effect of 7-day cocoa flavanol (CF, black lines) or placebo (PL, grey lines) supplementation in hypoxia (H, dashed lines) and normoxia (N, full lines) on tissue oxygenation (TSI,%) in the M. vastus lateralis (A) and prefrontal cerebral cortex (B). *0-20 min: steady state exercise (45% of peak power output), 20-25 min: passive rest in seated position on the bike, 25-45 min: time trial. Mean±SE presented. *: p<0.05: main effect of FIO2; £: p<0.05: main effect of supplement; $: p<0.05: main effect of exercise.*

Table 17. Effect of 7 day cocoa flavanols intake on physiological changes during moderate and high intensity exercise in hypoxia and normoxia (n=14).

	PL- N	CF- N	PL- H	CF-H	*2-way RM anova (start)*	*3-way RM anova*
SaO_2 - SS						
- **Start**	97±4	98±1	89±3*	88±6*£	*S: NS*	*O_2 x S: F=5.11 p=0.05*
- **5'**	97±2	95±4	79±5*	78±5*£	*O_2: F= 122.00*	*O_2 x T: F=26.90 p<0.0001*
- **10'**	97±2	97±1	79±4*	76±4*£	*p<0.001*	
- **15'**	95±2	96±1	79±4*	76±3*£		*S: F= 6.48 p=0.027*
- **End**	96±2	96±2	80±3*	78±3*£		*O_2: F=624.55 p<0.001*
						T: F=25.20 p<0.0001
SaO_2 - TT						
- **Start**	97±1	97±2	88±1*	89±4*	*S: NS*	*T x O_2 : F=34.92 p<0.001*
- **5'**	93±2	94±2	79±3*,$	78±2*	*O_2: F=100.3*	
- **10'**	93±2	93±2	79±3*	79±3*	*p<0.001*	*S: NS*
- **15'**	93±2	93±2	80±3*	79±3*		*O_2: F=102.61 p<0.0001*
- **End**	93±2	94±2	80±2*	80±3*		*T: F=113.13 p<0.0001*
HR - SS						
- **Start**	72±5	69±3	72±3	68±3	*S: NS*	*T x O_2: F=14.54 p<0.001*
- **5'**	122±3	123±3	136±3	136±3	*O_2: NS*	
- **10'**	127±3	129±3	141±3	143±3		*S: NS*
- **15'**	130±3	132±3	142±2	146±4		*O_2: F=44.19 p<0.0001*
- **End**	131±3	134±2	146±3	148±3		*T: F=530.72 p<0.0001*
HR - TT						
- **Start**	91±3	95±4	93±2	94±3	*S: NS*	*T x O_2: F=8.06 p<0.001*
- **5'**	162±2	165±2	168±3	168±2	*O_2: NS*	
- **10'**	170±2	173±2	172±2	171±2		*S: NS*
- **15'**	175±2	176±2	173±2	171±2		*O_2: NS*
- **End**	180±2	181±2	177±1	176±1		*T: F=140.60 p<0.0001*
Lactate - SS						
- **Start**	1.4±.2	1.4±.2	1.5±.1	1.5±.1	*S: NS*	*T x O_2: F=34.34 p<0.001*
- **10'**	1.1±.1	1.0±.1	1.9±.2	1.8±.2	*O_2: NS*	
- **End**	.9±.1	.8±.1	1.9±.2	2.0±.3		*S: NS*
						O_2: F=30.57 p<0.0001
						T: NS
Lactate - TT						
- **Start**	.9±.1	.8±.1	1.7±.2	1.7±.2	*S: NS*	*T x O_2: F=4.24 p=0.026*
- **10'**	4.2±.6	4.5±.4	6.9±.8	7.2±.7	*O_2: F= 53.02*	
- **End**	6.5±.8	7.2±.8	8.9±.6	7.8±.7	*p<0.001*	*S: NS*
						O_2: F=22.90 p<0.0001
						T: F=109.44 p<0.0001
RPE- SS					*Wilcoxon*	*Wilcoxon*
- **5'**	10±1	11±2	11±2	11±2		
- **10'**	10±1	11±2	12±2*	12±2*		

- **15'**	11±1	11±2	12±2*	12±2*		
- **End**	11±1	11±2	12±2*	12±2*		
RPE- TT					*Wilcoxon*	*Wilcoxon*
- **5'**	14±1	14±1	16±1*	16±1*		
- **10'**	15±1	16±1	17±1*	17±1*		
- **15'**	17±1	17±1	18±1	18±1		
- **End**	18±1	19±1	19±1	19±1		
Work (kJ) performed						
- **5'**	79.1±9.2	79.5±10.2	75.2±10.0	74.7±9.1	*S: NS*	*T x O_2: F=46.74 p<0.001*
- **10'**	160.9±20.9	162.8±22.8	147.7±18.7*	147.7±19.1*	*O_2:F=46.67*	
- **15'**	244.0±34.0	246.2±36.0	216.9±27.5*	217.2±28.4*	*p<0.0001*	*S: NS*
- **End**	327.5±46.7	330.9±49.9	287.2±37.7*	288.3±37.4*		*O_2: F=35.98 p<0.0001*
						T: F=651.3 p<0.0001

*PL: placebo, CF: cocoa flavanol, H: hypoxia, N: normoxia, SS: steady-state, TT: time trial, SaO_2: peripheral oxygen saturation, HR: heart rate, RPE: rate of perceived exertion. *: p<0.05: main effect of F_IO_2 (O_2) (H-PL compared to N-PL and H-CF compared to N-CF); £: p<0.05: main effect of supplement (S) CF compared to PL); $: p<0.05: main effect of time (T) (compared to previous timepoint)*

7.5 Discussion

The important novel findings of this study were that in well-trained cyclists, 1-week CF intake can *(i)* increase prefrontal oxygenation at rest and during moderate-intensity exercise and thus can partially restore the hypoxia-induced decline in oxygenation during exercise at altitude and *(ii)* reduce exercise-induced oxidative stress, which is substantially higher in hypoxia than in normoxia. CF does not improve exercise performance in normoxia and hypoxia.

It is well documented that CF intake leads to improved endothelial function, as reflected by FMD, in individuals with and without cardiovascular risks (Heiss et al. 2007; Grassi et al. 2015). We found that this beneficial effect also occurs in well-trained athletes who already have an enhanced endothelial function by regular exercise training. Studies using either NO-synthase inhibitor (L-NMMA) (Heiss et al. 2005) or parallel measure of circulating nitrites (Heiss et al. 2007) suggested that the CF-induced improvement in FMD can originate from an effect of CF on NO metabolism. However, in the current study, nitrite concentration and arg:citr ratio, two indirect markers of eNOS dependent NO production and NO availability (Fekkes et al. 2007; Brossette et al. 2011), were not altered by CF intake and no correlation between the change in FMD and change in nitrite concentration was found. One hypothesis to explain this result could be that CF could also act on other molecules than NO, which would play a role in smooth muscles relaxation. As for example, Grassi et al. (2015) showed that 7-day CF supplementation improved FMD and decreased concentrations of endothelin 1, a substance known to act directly on smooth muscles by inducing vasoconstriction in healthy volunteers. Despite a greater CF-induced increase of plasma nitrites in subjects with lower initial levels of nitrite, we did not find greater FMD improvements in those subjects. This result might be an additional argument for a putative role of other dilator substances, besides NO, in CF effects.

CF intake did not affect nitrite, nitrate and arg:citr ratio in response to exercise and hypoxia. Moreover, nitrite and nitrate were neither altered by acute hypoxia, nor by exercise. Similarly, nitrite was similar to pre-exercise levels after a 3 h cycling race in the study of Sureda et al. (2006) and Kelly et al. (2014) found no effect of exercise in normoxia and hypoxia on nitrite after PL intake. However, the interpretation of these data is not straightforward, since plasma nitrite is likely to reflect the dynamic balance between NOS-derived NO production and the reduction from nitrate to nitrite and further NO, which is expected to be facilitated in hypoxia (Kelly et al. 2014). Furthermore, in the current study, the arg:citr ratio was lowered after exercise and the magnitude of this decrease was smaller in hypoxia compared to normoxia. This seems consistent with the notion that enzymatic production of NO depends on the availability of O_2 (Ostergaard et al. 2007) and that hypoxia triggers superoxide anion generation, causing depletion of tetrahydrobiopterin, the essential eNOS cofactor, which results in eNOS uncoupling and decreased eNOS-dependent NO production (De Pascali et al. 2014).

Previously, it has been shown that CF intake leads to inhibition of NADPH oxidase (Katz et al. 2011). The generation of the superoxide anion radicals by NADPH oxidase results in scavenging of NO, eNOS uncoupling and reduced NO availability, but also triggers oxidative stress. CF intake may decrease oxidative stress after different types and durations of exercise in humans at sea level (Wiswedel et al. 2004; Fraga et al. 2005; Allgrove et al. 2011a; Davison et al. 2012). At altitude, the magnitude of exercise-induced oxidative stress is elevated compared to at sea level (Mcginnis et al. 2014). For the first time, we demonstrated that 7-day CF intake can inhibit the exercise-induced increase in lipid peroxidation in N, but also in H. Lipid peroxidation, which is the result of a multistep chain reaction where ROS attack lipids in cell membranes (Ayala et al. 2014), was affected by CF intake. However, CF did neither affect plasma UA concentrations, nor the total plasma antioxidant capacity measured as TEAC, in response to exercise and hypoxia. The plasma antioxidant capacity does not necessarily correlate with changes in lipid peroxidation since hydrophilic antioxidants are not efficient against lipid peroxidation (Niki 2010). Previous *in vitro* studies showed that CF can directly scavenge free radicals, act as a chain-breaking antioxidant in lipid peroxidation and/or regulate ROS-related enzymes (Lü et al. 2010; Katz et al. 2011; Andújar et al. 2012). Our results propose that during exercise in hypoxia, CF mainly reduces oxidative stress in the environment of membranes and lipoproteins. The diminished oxidative stress raises the possibility for CF to prevent muscle damage and thus have a beneficial effect on exercise recovery.

Consistent with previous research, the exercise-induced drops in tissue oxygenation were larger in hypoxia than in normoxia during moderate-intensity exercise (Masschelein et al. 2012; Gatterer et al. 2013). The decreased muscular oxygenation in hypoxia was paralleled by elevated blood lactate concentration, indicating a higher reliance on anaerobic glycolysis, but was not affected by CF intake. Thus, the effects of hypoxia to inhibit oxidative energy production during moderate-intensity exercise were not suppressed by CF. In contrast to the muscle, CF intake beneficially impacted cerebral oxygenation at rest and during moderate-intensity exercise in hypoxia. Although no other studies have examined muscular nor prefrontal oxygenation changes in response to CF intake, we might speculate that there is a tissue-specific reaction to CF supplementation. Using another supplement (beetroot) during moderate-intensity exercise in hypoxia, Masschelein et al. (2012) found a tissue-specific reaction, but in the opposite way with improved muscular oxygenation, but no difference in prefrontal oxygenation. However, beetroot is known to influence the NO metabolism, while we found no differences in nitrate and nitrite concentrations after CF intake. Thus, the specific tissue responsiveness to CF supplementation merits further investigation.

The beneficial effects of CF on prefrontal oxygenation vanished during high-intensity exercise, indicating that the physiological alterations in response to exhaustive exercise largely overruled any beneficial effects of CF. CF intake could not increase muscular oxygenation and could not prevent

greater reliance on anaerobic glycolysis during the TT in hypoxia, as evidenced by the higher blood lactate concentration. Moreover, CF intake did not have ergogenic effects in hypoxia and normoxia.

Future research may address some of the potential limitations of the current study. The measured markers of NO availability and oxidative stress in plasma might not exactly reflect changes in the endothelium, brain and muscle. While NIRS is currently the only method allowing the measurement of muscular and cerebral blood flow and oxygenation continuously during whole-body exercise, it only provides indirect information.

For the first time, we showed that CF intake inhibited oxidative stress during exhaustive exercise in hypoxia. CF had beneficial effects on endothelial function at rest, as well as on prefrontal oxygenation at rest and during moderate-intensity exercise. This is not only relevant for athletes exposed to altitude, but also for hypoxemic patients suffering from a reduced blood oxygenation, as well as for patients suffering from chronic diseases involving increased levels of oxidative stress.

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Chapter 8. General discussion

In this dissertation, we aimed to investigate the effects of acute and 1-week CF intake on cognitive and exercise performance and the underlying mechanisms.

It was the purpose of this PhD to answer the following research questions:

- Can acute CF intake improve executive function in combination with exercise in well-trained athletes? Are cerebral hemodynamic changes and/or changes in BDNF altered in response to acute CF intake and exercise? (Chapter 3)
- Can acute CF intake improve cognitive performance at (simulated) altitude in young, healthy subjects? Are cerebral hemodynamic changes and/or brain activity during cognitive performance in moderate hypoxia altered by CF intake? (Chapter 4)
- What is the effect of acute CF intake on cognitive performance and the BOLD response (cerebral hemodynamic change) in patients with type 1-diabetes (and healthy controls)? (Chapter 5)

- Can acute CF intake increase exercise-induced NO production and/or reduce oxidative stress and inflammation? What are the implications of acute CF intake for exercise performance and recovery in well-trained athletes? (Chapter 6)
- Are NO production, oxidative stress and tissue oxygenation during exercise in hypoxia altered in response to 1-week CF intake in well-trained athletes? Are there implications of 1-week CF intake for exercise performance in hypoxia? (Chapter 7)

8.1 Cocoa flavanols, the brain and cognitive performance

Our systematic review showed that, until 2017, no studies were conducted which examined the combined effects of CF and exercise on cognitive performance. We performed a randomized, double-blind, cross-over study with 12 healthy well-trained men to investigate the effect of acute CF intake in combination with exercise on cognitive function, while focusing on the role of prefrontal oxygenation, NVC and BDNF. We found that 900 mg CF intake (196 mg epicatechin) increased prefrontal oxygenation during a Stroop task performed at rest in healthy, young athletes. However, cognitive performance was not affected during this relatively short and easy Stroop task, assessing executive function. Immediately after exercise, cognitive function and prefrontal oxygenation were improved, but CF supplementation did not increase the exercise-induced effects. Thus, it was clear that the effects of acute high-intensity exercise largely overruled the effects of CF intake on prefrontal cortex oxygenation.

Very recently, Tsukamoto et al. performed a similar study, where they determined whether CF intake (563 mg CF) could enhance the exercise-induced improvement in cognitive function. Subjects performed 30 min cycling at 60% of their peak O_2 intake and performed a Stroop task and face-name matching task pre-exercise (30 and 60 min after CF intake) and post-exercise (100, 130 and 160 min after CF intake=immediately, 30 min and 60 min after exercise). They found that executive function was improved by CF intake before and after exercise and that the exercise-induced improvements were larger after CF intake compared to PL. In contrast, CF consumption and moderate-intensity exercise did not improve memory. The authors advised that combining CF intake and aerobic exercise may be beneficial for improving executive function (Tsukamoto et al. 2018). Interestingly, executive function was already improved 30 min after CF intake, while the peak of systemic CF levels is known to occur much later (2 h post intake) (Holt et al. 2002). It was suggested that CF intake could have an early effect on cognitive function and thus, that the increased systemic CF levels and cerebral neural activation in response to CF intake are not related to the improvement in cognitive function. We did not measure cognitive function and prefrontal oxygenation at those early time points, as our first (pre-exercise) measurement was 90 min after CF intake. Thus, we cannot confirm or contradict this hypothesis. Unfortunately, the authors did neither measure epicatechin levels, nor cerebral oxygenation, and did not describe the exact composition of the CF drink. This prevents us from a further in depth comparison between our study and this recently published study. However, the CF and PL drinks did not seem to be matched in caffeine content, which could also explain the early beneficial effect on cognitive function. Caffeine intake and even mouth rinsing improve cognitive function immediately (De Pauw et al. 2015). While the authors proposed that circulating NO, BDNF and lactate levels play a role in the CF induced improvements in cognitive function, none of the above were measured. In our interventional study, we

did not find any effect of CF on BDNF levels, indicating that BDNF does not seem to be involved in the acute effects of CF on cognitive function.

Acute hypoxic exposure is known to place an extra burden on cognitive performance. It was previously suggested that acute CF intake would mainly improve cognitive performance in cognitive demanding environments (Socci et al. 2017). Thus, in a subsequent study, the effect of acute CF intake (100 mg epicatechin) on performance on a demanding cognitive test battery was assessed in normoxia and hypoxia (simulated altitude 4000 m). EEG and fNIRS were used to analyse neuronal activity and hemodynamic changes in order to unravel whether CF-induced hemodynamic changes were solely driven by changes in vascular responsiveness or also by altered neuronal activity. Acute CF intake increased the hemodynamic response, both in normoxia and hypoxia, without any effect on neuronal activity and cognitive performance. Thus, this study added on to the existing literature by showing that *(i)* the increased fNIRS response observed after CF intake must be driven by improved vascular responsiveness, rather than by altered neuronal activity and *(ii)* the effects of CF intake were similar during acute exposure to hypoxia and normoxia in healthy, young subjects.

In contrast to the hypothesis, most cognitive functions, as well as NVC and neuronal activity, did not decline by acute exposure to moderate hypoxia in healthy subjects. These findings showed that young, healthy persons have a very efficient NVC, which governs adequate CBF in response to neuronal activity even during hypoxic exposure. Additional increases in vascular responsiveness by CF supplementation do not lead to better cognitive function in persons with a healthy (neuro)vascular function.

Acute CF intake did not influence several domains of cognitive function in healthy subjects in our 2 first interventional studies. However, response inhibition and executive function (measured during the Flanker test) were improved after acute CF intake (196 mg epicatechin) in patients with type 1 diabetes, and also in their healthy matched controls. CF increased the BOLD response in the supramarginal gyrus parietal lobe and inferior frontal gyrus, two brain areas activated during this specific cognitive task. However, we did not perform correlations between BOLD fMRI and behavioural alterations after CF intake and thus cannot clearly demonstrate the link between improved cognive performance and altered BOLD response.

While cognitive performance was not deteriorated in the patients with type 1 diabetes, they showed a different brain activation pattern compared to healthy controls and this brain activation pattern was altered after CF intake. It is important to notice that the healthy matched controls in this study were older (41±15 years) and less fit/trained (<3 h sport/week) compared to the young, healthy well-trained men (30±3 years, >10 h training/week) who participated in the first study. The fact that exercise training improves cognitive function and that cognitive function deteriorates with age, could explain why acute CF intake (900 mg CF, 196 mg epicatechin) did not improve executive function (measured during Stroop

task) in the young, well-trained (healthy) men in the first study, but had a beneficial effect on executive function (measuring during Flanker test) in the “healthy matched controls” in the last study.

To conclude, acute CF intake clearly improved prefrontal oxygenation and cerebrovascular responsiveness during cognitive tasks at rest. While CF intake improved cognitive function in patients with type 1 diabetes and healthy, untrained subjects, it did not alter executive function in healthy, well-trained, young subjects at sea level and in hypoxia. Compared to exercise, the magnitude of the CF-induced neurovascular and cognitive changes is small.

8.2 Cocoa flavanols, oxidative stress, NO availability and exercise performance

Our systematic review showed that acute (247 mg and 186 mg CF), 2 week (197 mg, 186 mg) and 3 month (240 mg) CF intake reduced exercise-induced lipid peroxidation (positive effect in 5/6 studies), while plasma antioxidant capacity did not seem to be affected by acute CF intake (positive effect in 1/5 studies). Although evidence is scarce so far, it seems that reducing exercise-induced oxidative damage by CF intake does not influence exercise performance and thus, consequences for exercise performance and recovery remain elusive.

Theoretically, a reduction in oxidative stress (as seen after CF intake) can be due to decreased ROS production and/or increased antioxidant capacity. Based on the results of the systematic review, a decrease in ROS production seems the cause of lowered oxidative damage of CF intake, as the antioxidant capacity was barely affected after CF intake. This would be in line with *in vitro* studies showing that CF inhibits NADPH oxidase, and thus lower $O_2^{\cdot -}$ production and peroxynitrite formation (Steffen et al. 2008). However, we must be cautious in interpreting these results as the plasma antioxidant capacity (TEAC), which was mostly used in the studies included in the systematic review, is not suitable to assess the efficiency of lipid peroxidation inhibition (Niki 2010). Moreover, several factors contribute to plasma TEAC, with albumin and UA accounting for 2/3 of TEAC. In the study of Allgrove et al., UA was not affected by CF intake even though lipid peroxidation was attenuated (Allgrove et al. 2011b). Other small antioxidant molecules and proteins account for the remaining 1/3. Thus, upregulation of endogenous antioxidants such as SOD and catalase by CF might not necessarily result in a significant increase in plasma TEAC (Ramirez-Sanchez et al. 2013).

Even though there is a strong interplay between NO production and oxidative stress, no studies have investigated the effects of CF intake on the interaction between NO production and oxidative stress during exhaustive exercise so far. Thus, our randomized, placebo-controlled, cross-over study examined the effects of acute CF intake on markers of NO metabolism and oxidative stress during exhaustive exercise, in well-trained cyclists. A single dose of 900 mg CF (with 196 mg epicatechin) was ingested, which was a much higher dose than used in previously published studies. We found that a 30-minute TT increased the plasma total antioxidant capacity, which was further upregulated by CF supplementation. However, the exercise-induced increase in lipid peroxidation was not reduced. It has been suggested that in contrast to *in vitro* experiments where CF clearly reduced oxidative stress, it seems more difficult to observe beneficial effects of antioxidants on biomarkers of oxidative stress *in vivo*. This is especially the case in healthy athletes where the endogenous antioxidant defence is already promoted to a very large extent by their regular exercise training (Niki 2010; Powers et al. 2016). We propose that the additional antioxidant boost of CF was not sufficiently large to prevent oxidative stress and could not lead to improved exercise performance nor recovery. Indirect markers of NO production,

which were increased during exhaustive exercise, were not influenced by acute CF intake. This could indicate that any potential effect of CF intake on NO production, through activation of eNOS or inhibited eNOS uncoupling by reduced ROS production, is minimal or inexistent compared to the trigger of exercise-induced shear stress to stimulate eNOS-dependent NO production. Alternatively, the indirect markers of NO metabolism used in this study may not be sufficiently sensitive to reflect the *in vivo* situation of NO production and availability. Therefore, in a following study, we used the clinical method to assess NO-dependent endothelial function, FMD and chemoluminescence to analyse nitrite and nitrate to assess NO availability.

In a dose-response study, Grassi et al. showed that 1 week intake of 500 mg CF (with 100 mg epicatechin) elicited maximal improvements in vascular function (Grassi et al. 2015). Therefore, we used this dose in the following study. We examined whether 1-week CF intake (100 mg epicatechin) could influence markers of oxidative stress and NO production and availability, as well as vascular responsiveness and tissue oxygenation during low-and high-intensity exercise in normoxia and hypoxia. The eNOS dependent NO production, as reflected by arg:citr ratio, was increased during exercise in a larger extent in normoxia than in hypoxia, but was not influenced by 1-week CF intake. NO availability, as assessed by nitrite and nitrate, was also not affected by CF intake. Nevertheless, clinical outcome measures of vascular responsiveness, FMD and prefrontal oxygenation, were beneficially influenced by 1-week CF intake. While CF did not influence muscular oxygenation, prefrontal oxygenation was improved at rest and during low-intensity exercise, both in normoxia and hypoxia. Thereby, CF intake could partially counteract the hypoxia-induced decline in prefrontal oxygenation at rest and during high-intensity exercise. The beneficial effects of CF on prefrontal oxygenation vanished during high-intensity exercise, indicating that the physiological alterations in response to exhaustive exercise largely overruled any beneficial effects of CF. The exercise-induced increase in plasma antioxidant capacity was larger during hypoxic exposure compared to normoxia. CF intake did not influence the antioxidant capacity, but significantly blunted exercise-induced lipid peroxidation in normoxia and in hypoxia, which was in line with the results of our systematic review. CF intake did not influence exercise performance during a 30-min TT and not alter heart rate, lactate nor RPE during the TT in normoxia and hypoxia.

In our 2 interventional studies, we did not observe any changes in indirect markers of NO production and/or NO availability at rest and during exercise, despite some clinical changes in vascular responsiveness after 1 week CF intake. However, previous studies using a NO-synthase inhibitor (L-NG-monomethyl Arginine acetate (L-NMMA)) (Heiss et al. 2005) or parallel measure of circulating nitrites (Heiss et al. 2007) suggested that the beneficial effect of CF on vascular function was mediated through NO-dependent vasodilation. Our results might be an argument for a putative role of other dilator substances besides NO, in the effects of CF. Indeed, other mechanisms that do not involve NO have

been suggested to underlie the vasodilatory effects of CF as well (Ludovici et al. 2017). In humans, CF inhibit endothelin-1 production, a strong vasoconstrictor (Loke et al. 2008b; Calderón-Garcidueñas et al. 2013) and inhibit angiotensin-converting enzyme (ACE), leading to decreased angiotensin 1, which also has vasoconstrictive properties (Persson et al. 2011). Other putative mechanisms of CF to stimulate vasodilation include inhibition of thromboxane A2 (vasoconstrictor), stimulation of endothelium-derived hyperpolarizing factor (leading to more relaxation) and stimulation of prostacyclin (Ludovici et al. 2017) (Figure 30). However, future research should further investigate these putative underlying mechanisms during exercise as well, because exercise-induced vasodilation is orchestrated by the interaction of several muscle-related vasodilators (e.g. lactate, CO_2, potassium, adenosine) with endothelium-derived vasodilators (Sarelius and Pohl 2010).

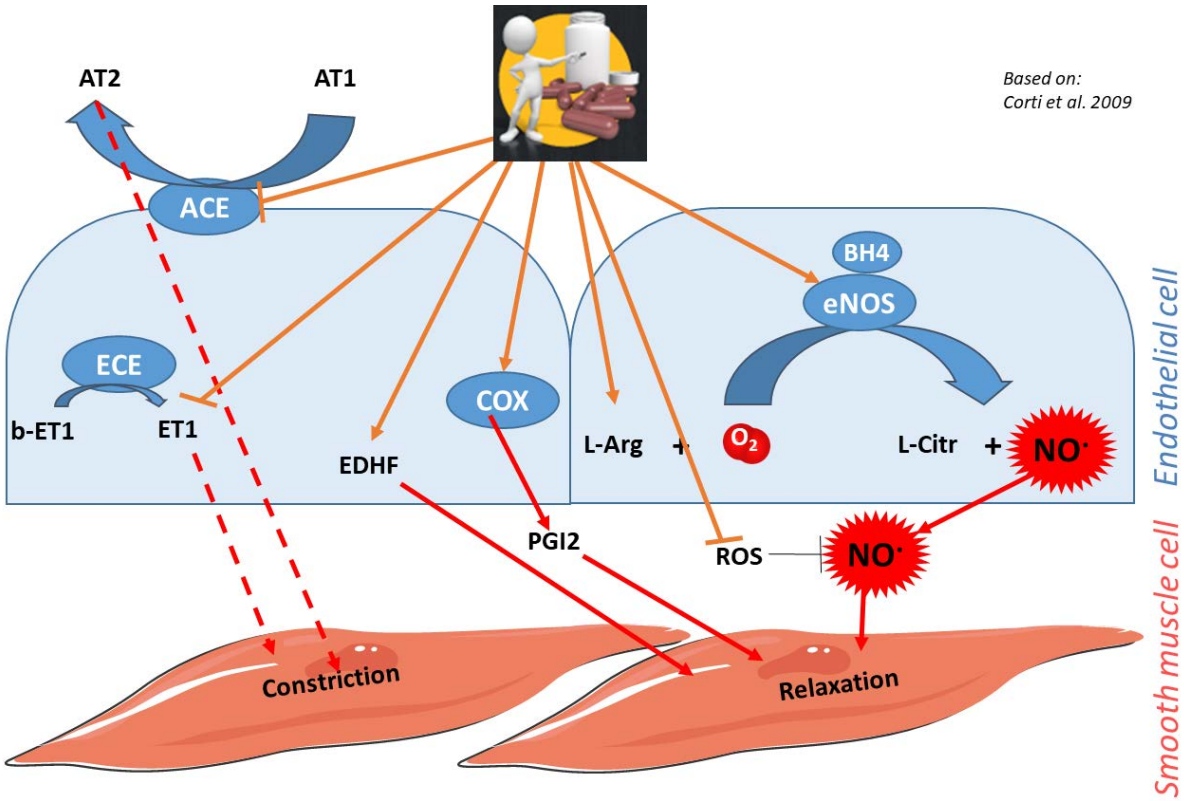

Figure 30. Putative mechanisms for the vasodilatory effects of cocoa flavanols (CF), as proposed by Ludovici et al. 2017*. CF increases the availability of vasodilator nitric oxide (NO) through i) the stimulation of endothelial NO synthase (eNOS), which converts L-arginine (L-arg) into L-citrulline (L-citr) and NO in the presence of oxygen (O_2) and its cofactor tetrahydrobiopterine (BH_4), ii) inhibiting arginase, thus augmenting L-arg levels, and iii) reducing the production of reactive oxygen species (ROS) which react with NO and decrease NO availability. Other putative mechanisms include activation of the vasodilators endothelium-derived hyperpolarizing factor (EDHF) and endothelial prostacyclin release (PGI2) by stimulating cyclooxygenase (COX). CF may also inhibit Angiotensin Converting Enzyme (ACE), which converts Angiotensin 1 (AT1) into Angiotensin 2 (AT2) or inhibit the synthesis of endothelin-1 (ET1), thereby reducing vasoconstriction. Figure based on Corti et al. 2009.*

During our 2 interventional studies, high-intensity exercise increased the plasma antioxidant capacity and lipid peroxidation. These studies, together with the results from the systematic review clearly show a beneficial effect of CF in upregulating antioxidant capacity and/or decreasing lipid peroxidation during exhaustive exercise. In Figure 31, we present an overview of the effects of CF and acute exercise on oxidative stress and NO metabolism, measured during this dissertation. Interestingly, acute CF intake (196 mg epicatechin) was found to increase TEAC without reducing exercise-induced lipid peroxidation during a TT, while 1 week CF intake (100 mg epicatechin) reduced exercise-induced lipid peroxidation during a TT, but did not affect TEAC. These differences may be explained by the different dose and timing of CF supplementation, as well as by the different subjects participating in the 2 studies. While the beneficial effect of acute CF intake on TEAC likely reflects an upregulation of endogeneous antioxidants, the effect of 1-week CF intake on reducing lipid peroxidation suggests direct scavenging of CF. Clearly, more research is needed to clarify the molecular mechanisms of CF *in vivo*. Based on these results, it seems possible that 1 week, rather than 1 day of CF intake is needed to reduce oxidative stress elicited during exhaustive exercise. Further research should investigate the effects of acute vs. 1- week CF intake on antioxidant enzyme levels and other markers of oxidative stress in both trained and untrained subjects to unravel the observed effects of the 2 studies in this dissertation.

The molecular mechanisms behind the CF-induced changes in oxidative stress and antioxidant capacity during exercise were not investigated in this dissertation. A recent meta-analysis examined the efficacy of procyanidins (PC), polymers of epicatechin and catechin, against *in vivo* cellular oxidative damage in animals. The authors showed that PC improve the total antioxidant capacity, driven by elevated SOD, GSH, GPX and catalase levels, which results in reduced lipid peroxidation, measured as MDA in tissue and serum (Li et al. 2015). Ramirez-Sanchez et al. were the only ones so far examining the effects of 3-month CF intake on oxidative stress specifically in skeletal muscle, in a human interventional study. They found that in patients with heart failure and type 2 diabetes, CF supplementation led to increases in SOD and catalase protein expression and activity levels (Ramirez-Sanchez et al. 2013). Thus, it seems that CF, at least upon chronic intake, not only *(i)* inhibit NADPH oxidase, thereby reducing ROS production (Steffen et al. 2008) and *(ii)* act as a direct scavenger to neutralize ROS (Ruijters et al. 2014), but *(iii)* also stimulate the expression and activity of endogenous antioxidants, such as SOD, GSH, catalase and GPX. However, more human interventional trials, assessing biomarkers of oxidative stress not only in blood but also in muscle tissue, are needed to confirm these results.

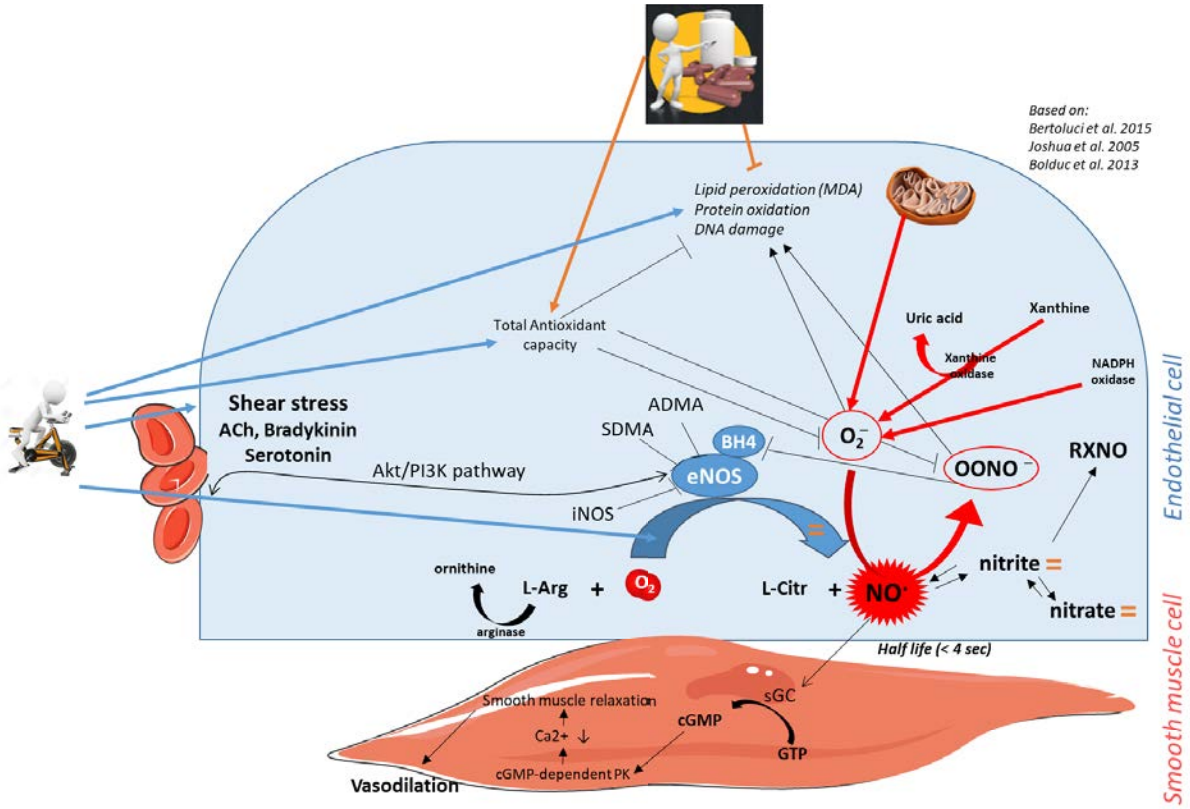

Figure 31. The effect of high-intensity exercise (blue lines) and cocoa flavanol (CF) intake (orange lines) on nitric oxide (NO) production and oxidative stress, measured in vivo in the interventional studies of this PhD. *Pointed arrow: increase; blocked arrow: inhibition,=: no effect. NO production was augmented during exercise, but CF intake had no effect. Nitrite and nitrate, 2 indirect markers of NO availability, were not altered during exercise and were also not affected by 1- week CF intake (100 mg epicatechin). Exercise upregulated the total antioxidant capacity of plasma, but also increased lipid peroxidation (malondialdehyde: MDA), a marker of oxidative stress. Acute CF intake (196 mg epicatechin) increased the total antioxidant capacity, but did not inhibit lipid peroxidation. One week CF intake (100 mg epicatechin) reduced the exercise-induced lipid peroxidation, but did not increase the total antioxidant capacity, both in normoxia and during acute exposure to hypoxia. Note: nitrite, nitrate, MDA and TEAC were measured in plasma, and not directly in the endothelial cell as indicated in the figure. O_2: oxygen, L-Citr: L-citrulline, L-Arg: L-arginine, sGC: guanyl cyclase, GTP: guanosine triphosphate, cGMP: cyclic guanosine monophosphate, PK: protein kinase, Ca2+: calcium, iNOS: inducible NO synthase, SDMA: symmetric dimethylarginine, ADMA: asymmetric dimethylarginine, eNOS: endothelial NO synthase, $O_2^{\cdot-}$: superoxide, BH4: tetrahydrobiopterin, $OONO^-$: peroxynitrite, Ach: acetylcholine.*

8.3 Strengths and limitations of this dissertation

In this dissertation, we used fMRI and fNIRS to measure the hemodynamic response to neuronal activity. As mentioned before, this hemodynamic response depends on the physiological cascade of cerebral metabolism and blood flow. Changes in baseline physiological parameters, pharmacological interventions, or disease-related vascular changes may disturb the NVC. Therefore, several experimental measures have been proposed to improve the interpretability of hemodynamic responses. These include the assessment of baseline brain perfusion, vascular reactivity, simultaneous EEG-fMRI or EEG-fNIRS measurements and the measurements of CBV and rate of metabolic O_2 consumption (Lindauer 2010). While we assessed the effects of CF intake on the fNIRS response or BOLD response during cognitive tasks in 2 studies, we also combined fNIRS and EEG in an attempt to look further into the underlying physiology of the measured hemodynamic response in a 3rd study. However, we did not assess baseline brain perfusion, which can be done by ASL or transcranial Doppler. Combining BOLD response, which depends on changes in CBF and cerebral metabolic rate of O_2 ($CMRO_2$), with ASL, which only depends on CBF changes, allows for the calculation of changes in $CMRO_2$ by using a mathematical model proposed by Davis et al. (1998). By performing a simple cognitive test in a condition of mild hypercapnia (5% CO_2), which affects CBF but not $CMRO_2$, the BOLD signal can be "calibrated" to non-invasively determine the fractional changes in $CMRO_2$ during neuronal activity (Ances et al. 2009). This calibrated-BOLD approach could be used in future research to further unravel the relationship between O_2 metabolism, CBF and blood oxygenation in response to CF intake. While we combined EEG and prefrontal fNIRS in one study, future research could also combine EEG and BOLD-fMRI over the entire cortex. This will help us to better understand functional brain imaging based on hemodynamic changes.

In several studies of this dissertation, we measured NVC or oxygenation in the prefrontal cortex. Although this brain region is involved in executive function and motor control, it is a limitation that we did not measured hemodynamic changes over the entire brain. Moreover, fNIRS cannot measure activation of deeper brain structures, and does not enable exact localization of brain region because of its limited spatial resolution. Regarding the data analysis of fNIRS, no standardized methods have been established yet. We did not use any pre-processing techniques and chose to 'traditionally' analyse the data by using the mean activation response during the last 30 seconds of each cognitive task, compared to a baseline period before cognitive performance. Recently, pre-processing steps including the use of different filters and independent component analysis have been suggested to reduce artefacts and optimize the signals (Perrey 2014). Mandrick et al. (2013) proposed an alternative method to analyse fNIRS-derived cerebral hemodynamic responses by analysing the slope in ΔHbO_2 and ΔHHb from onset of stimulus to the peak. This proposed slope method allowed a better discrimination in terms of cortical activation among all levels of mental workload than the traditional method. Therefore, future research

could use this more sensitive technique to analyse the hemodynamic changes by fNIRS in response to nutritional interventions.

In 2 interventional trials, several indirect markers of NO production and oxidative stress were measured in venous blood samples, in response to exercise and CF intake. While increased radical production in the active skeletal muscle certainly contribute to these oxidized biomarkers in the blood, other tissues and (white) blood cells may also generate ROS in some situations and thus affect biomarkers measured in the blood (Powers and Smuder 2010). Therefore, researchers should use caution when extrapolating findings from blood samples to events occurring in other tissues. Measuring oxidative damage in skeletal muscle by using an invasive muscle biopsy may be considered in future research.

We measured the total antioxidant capacity (TEAC) because it represents the end-result of the interactions between different antioxidants. Moreover, it is less expensive and less time consuming than measuring each individual antioxidant component. However, this method is based on a one-electron transfer and so cannot measure chain-breaking antioxidant activity, which is involved in the prevention of lipid peroxidation (Powers and Smuder 2010). Also, measuring antioxidant compound level does not give any information on the molecular mechanisms of action of CF. Thus, measurement of antioxidant enzyme levels (e.g. including SOD, catalase, GPX, TRX), oxidation products of antioxidants (e.g. biopyrrin) and the ratio of oxidized to reduced forms of e.g. GSSG/GSH *in vivo*, is worth considering for future research. Understanding mechanisms of antioxidant action of CF is important for designing appropriate experimental methods in the future (Niki 2010). We measured only 1 biomarker of oxidative damage, being lipid peroxidation. When feasible, we would also asses other markers of oxidative damage on DNA (e.g. comet assay, thymine glycol) and proteins (e.g. protein carbonyl, disulphide –SS-) in blood and/or skeletal muscle in future research. In a first study examining the effect of CF on NO metabolism (Chapter 6), we measured indirect markers of NO production. Recent recommendations from the American Heart Association call for the use of electron paramagnetic resonance (EPR) with NO spin trap $Fe[DETC]_2$ to detect NO, or ozone-based chemiluminescence to detect NO, nitrite, nitrate and nitrosothiols with suitable accuracy (Griendling et al. 2016). Therefore, we used the ozone-based chemiluminescence method to detect nitrite and nitrate in the last study (Chapter 7).

8.4 Guidelines for further research

During this PhD, we examined the effects of acute and 1-week CF intake on several parameters. However, our systematic review showed that evidence is still lacking on the synergistic effects of long-term CF intake and exercise training on cognitive function, oxidative stress, inflammation and fat and glucose metabolism.

Six week ad libitum epicatechin intake improved spatial memory in mice. These effects were enhanced by exercise and changes were associated with increased angiogenesis and neuronal spine density in the hippocampus, upregulation of genes associated with learning and downregulation of markers of neurodegeneration in the hippocampus (van Praag et al. 2007). These promising results from animal studies merit further investigations in humans. Thus, future research should address the following research questions:

- Can chronic CF intake (> 3 month), combined with aerobic exercise improve cognitive function, to a larger extent than each intervention alone?
- Can chronic CF intake (> 3 month), combined with aerobic exercise result in structural and/or neurovascular changes in the brain, measured by MRI, in healthy young subjects and/or healthy elderly?
- Can chronic CF intake (> 6 months), combined with aerobic exercise prevent the age-related shrinkage of the hippocampus, to a larger extent than exercise alone (in healthy elderly with or without mild cognitive impairment)?

Until today, the role of ROS in training adaptation, exercise performance and exercise recovery remains unclear. Moderate exercise training causes improved endogenous antioxidant defence, leading to better protection against ROS during subsequent exercise sessions. When exhaustive exercise causes high amounts of ROS that overwhelm the endogenous antioxidant system, it results in oxidative stress and cell damage (Steinbacher and Eckl 2015). Acute antioxidant intake may then reduce oxidative stress. Still, the use of antioxidants remains controversial, because chronic intake of antioxidants in high doses (e.g. vitamin C and vitamin E) can blunt training adaptations because they block molecular mediators of endogenous ROS defence and attenuate mitochondrial biogenesis following exercise (Gomez-Cabrera et al. 2012). However, for CF, it was shown that 3 month CF intake (100 mg epicatechin) upregulated SIRT1, SIRT3 and PGC1-α (Ramirez-Sanchez et al. 2013) and improved mitochondrial structure and mitochondrial biogenesis (Taub et al. 2012) in skeletal muscle of patients with heart failure and diabetes type 2. Furthermore, in a proof-of-concept study with 6 subjects, hand grip strength and the ratio of plasma follistatin/myostatin were increased by 1 week CF intake (25 mg epicatechin/day), showing the potential of CF to stimulate muscle growth and differentiation (Gutierrez-Salmean et al. 2014). Also in untrained subjects, 3 month CF intake (26 mg epicatechin/day) increased AMP-activated

protein kinase (AMPK) and PGC1-α levels, upstream regulators of metabolic control of skeletal muscle, and mitochondrial efficiency. These changes were associated with improved VO_2 max (Taub et al. 2016). While these beneficial effects were observed in untrained subjects and patient populations, it remains the question whether they also take place in well-trained athletes. Thus, more research is needed to verify that chronic CF intake increases or attenuates mitochondrial biogenesis and endogenous ROS defence following exercise training, in well-trained athletes. Also, it was suggested that periods of overload training can compromise the antioxidant defence system, but whether it is helpful to use antioxidant supplementation under these conditions remains to be investigated (Steinbacher and Eckl 2015). Furthermore, future research should focus on the role of exercise and CF intake on a broader range of biomarkers of oxidative stress, metabolic and vascular parameters in diseased or at-risk populations.

Future research should examine the following research questions:

- Can chronic CF intake (> 3 months), combined with aerobic exercise improve cardiovascular health and reduce oxidative stress in patients at-risk for CVD or patients with restricted cardiovascular function?
- Does chronic CF (3-month) intake alter some training adaptive responses (mitochondrial efficiency and biogenesis, endogenous antioxidants) in well-trained and untrained subjects?
- Can 1- or 2-week CF intake improve exercise recovery during a period of intensified training in well-trained athletes? (muscle function and oxidative stress and inflammation after muscle damaging exercise?)
- What are the molecular mechanisms behind CF-induced improvements in vascular function and reduced oxidative stress *in vivo* in humans?

Several authors suggest that a combination of antioxidants may induce larger effects in combating ROS than one single antioxidant alone, because the antioxidant defence system works as a complex interaction of several endogenous antioxidants, with each one being effective at quenching a specific type of ROS/RNS (Bentley et al. 2015). Moreover, CF and nitrate both increase NO availability and when consumed together in low doses, CF and nitrate have additional effects on vascular function. However, CF does not further improve FMD after high nitrate intake (Rodriguez-mateos et al. 2014). Thus, future research could also focus on combining CF with other antioxidants and/or NO-related supplements to study exercise-related oxidative stress and exercise performance.

In this PhD, we assessed the effect of CF on the brain during exercise. The importance of the gut-brain axis in exercise-induced stress has long been recognized, but the role of microbiota in influencing this axis has emerged the last years. Recent evidence showed a correlation between physical and emotional stress during exercise and changes in the microbiota composition (Clark and Mach 2016). Recent

literature has shown a mutual relationship between gut microbiota and polyphenols including CF. The bioavailability of CF metabolites depends on the biotransformation by gut microbiota, but CF can also alter the gut microbiota community (Ozdal et al. 2016). Thus, future research may investigate *(i)* the effects of chronic CF intake on the microbiome, the brain-gut axis and exercise-induced stress and *(ii)* the role of the composition of microbiota in modulating the effects of CF on exercise and cognitive performance.

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Chapter 9. General conclusions

The purpose of this PhD was to identify the putative effects of CF on physical and cognitive performance, which both influence sports performance. The following results were obtained:

- ✓ Acute CF (196 mg epicatechin) intake improved prefrontal oxygenation during cognitive tests, but did not increase serum BDNF and did not improve cognitive performance in well-trained athletes. The beneficial impact of exercise on cognitive performance and CBF was much larger than the small effects of CF, which could not enlarge these exercise-induced cognitive benefits. (Chapter 3)
- ✓ Acute CF (100 mg epicatechin) intake improved the hemodynamic response during cognitive tasks in normoxia and hypoxia, but without altering cognitive performance and brain activity in young, healthy subjects. (Chapter 4)
- ✓ Acute CF (196 mg epicatechin) intake improved cognitive performance in patients with type 1 diabetes and increased the BOLD response in the supramarginal gyrus parietal lobe and inferior frontal gyrus, two brain areas activated during this specific task. (Chapter 5)
- ✓ Acute CF intake (196 mg epicatechin) increased the exercise-induced total antioxidant capacity, but did not reduce exercise-induced oxidative damage (lipid peroxidation) and did not increase NO production during exercise in well-trained athletes. (Chapter 6)
- ✓ Acute CF (196 mg epicatechin) intake did neither improve exercise performance, nor exercise recovery in well-trained athletes. (Chapter 6)
- ✓ One week CF (100 mg epicatechin) intake reduced oxidative stress (lipid peroxidation) elicited during exercise in hypoxia (and normoxia) in well-trained athletes. NO production and NO availability were not altered by one-week CF intake. (Chapter 7)
- ✓ One week CF (100 mg epicatechin) intake improved flow-mediated dilation in well-trained athletes. (Chapter 7)
- ✓ One week CF (100 mg epicatechin) intake improved prefrontal oxygenation at rest and during moderate-intensity exercise (in normoxia and hypoxia), but these effects vanished during exhaustive exercise. (Chapter 7)
- ✓ One week CF (100 mg epicatechin) intake did not improve exercise performance in (normoxia and) hypoxia. (Chapter 7)

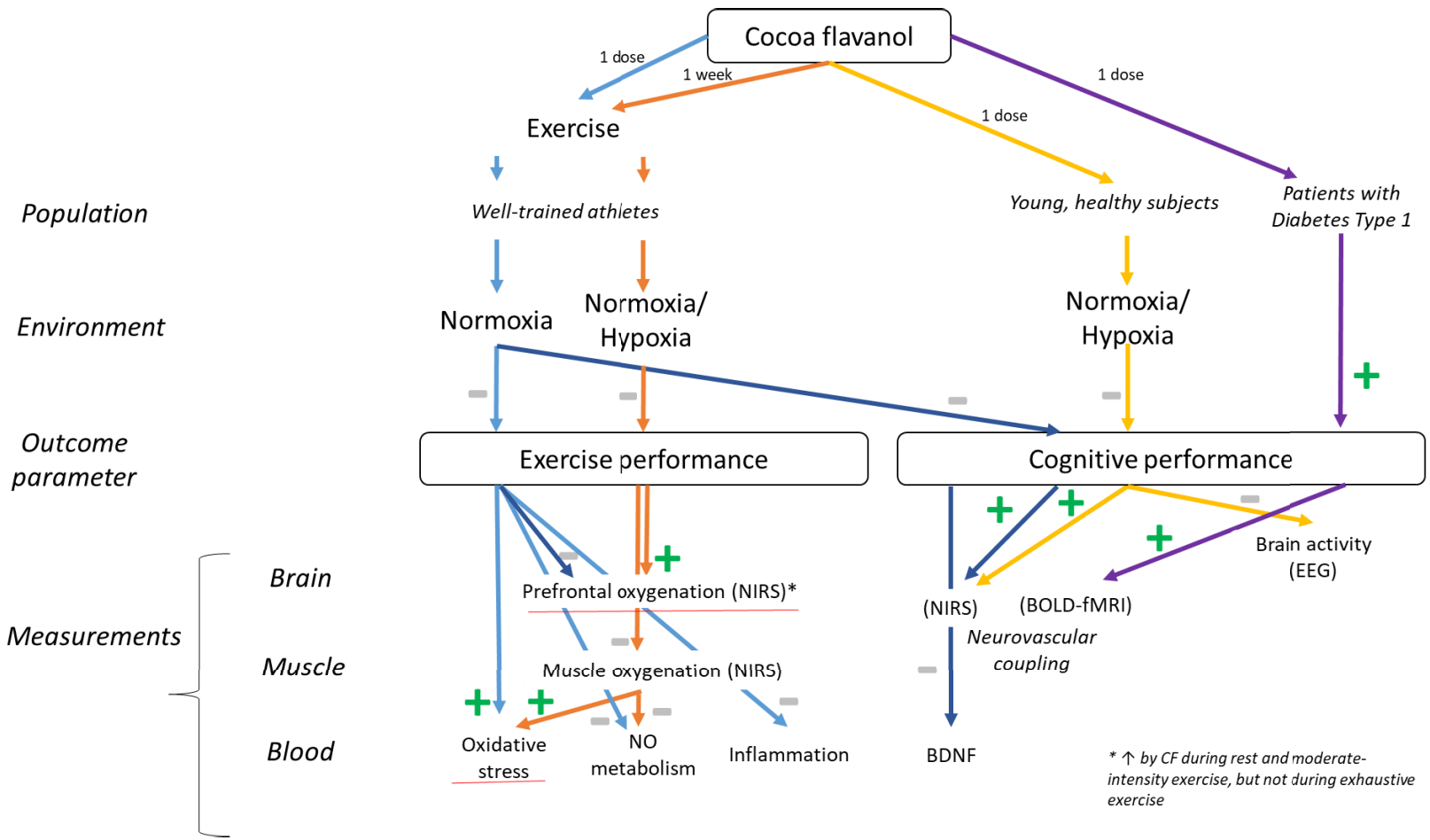

Figure 32.Overview of results obtained in the interventional studies of this PhD*.Light blue: study described in chapter 6, darnk blue: study described in chapter 3, orange: study described in chapter 7, yellow: study described in chapter 4, purple: study described in chapter 5. + : positive effect of cocoa flavanol, - : no effect of cocoa flavanol, red line: negative effect of hypoxia. NIRS: near infrared spectroscopy, BOLD: blood oxygenation level dependet, fMRI: function magnetic resonance imaging, EEG: electroencephalography, NO: nitric oxide, BDNF: brain derived neutrophic factor.*

Figure 32 shows a summary of the results of the interventional studies of this PhD. To conclude, we showed that both acute (196 mg epicatechin) and 1-week (100 mg epicatechin/day) CF intake neither improve cognitive performance nor physical performance in healthy, young athletes. Therefore, CF should not be consumed as performance-enhancing aid. However, CF intake can be considered as a nutritional aid to stimulate cerebrovascular responsiveness and to maintain cerebral oxygenation in hypoxia, both at rest and during low-intensity exercise.

We showed that CF intake can assist in the antioxidant defence system battling ROS production and preventing oxidative stress, induced by exhaustive exercise and by hypoxic exposure. Further research is warranted to investigate whether and when these antioxidant capacities of CF intake are beneficial for athletes, keeping in mind that small levels of ROS are needed in the process of training adaptation. For now, CF intake seems not advisable for athletes during the season preparation, when they aim to increase their adaptive response to training, but may be advantageous during training camps at altitude or stage races, when recovery is vital.

List of publications- scientific CV

Publications in international peer-reviewed journals as first author (*: included in PhD dissertation)

1. Decroix L, Van Muylder V, Desender L, Sampaolesi M, Thorrez L. Tissue clearing for confocal imaging of native and bio-artificial skeletal muscle. Biotechnic & Histochemistry. 2015;90(6):424-431.

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1. Decroix L. Bio-artificial muscles: beyond myofibers. Oral presentation at: 2ndBelgian symposium on tissue engineering; Micro to macro: translating between scales; 2013 October 24-25; Leuven, Belgium.

2. Decroix L. Guidelines to classify female subject groups in sport science research. Oral presentation at: European Congress of Sport Science; 2015 June 24-27; Malmö, Sweden.

3. Decroix L. Acute effect of cocoa flavanol intake on cognitive performance and cerebral blood flow in rest and following exercise in well trained athletes. Oral presentation at: 2nd International congress on chocolate and cocoa in medicine; 2015 September 25-26; Barcelona, Spain.

4. Decroix L. Acute cocoa flavanol intake affects exercise performance, oxidative stress and the NO-pathway in well trained athletes. Oral presentation at: Cycling and Science; 2016 June 29-30; Caen, France.

5. Decroix L. Acute cocoa flavanol intake, exercise and cerebral hemodynamics in healthy atheles. Oral presentation at: European Congress of Sport Science; 2016 July 6-9; Vienna, Austria.

6. Decroix L. Can the Lamberts Submaximal Cycle Test reflect overreaching in professional female cyclists? Oral presentation at: Vereniging voor Kinesiology Symposium; 2016 December 2; Gent, Belgium.

7. Decroix L. Can cocoa flavanols partially restore hypoxia-induced decline in cerebral oxygenation during exercise in healthy athletes? Oral presentation at: VasCog Symposium; 2016 December 15; Lille, France.

8. Decroix L. Brein en voedingssupplementen in de sport. Oral presentation at: Dag van de wetenschap, breinwijzer “I-brain sport: brein en bewegen”; 2017 November 26; Gent, Belgium.

9. Decroix L. Overtraining syndrome in women. Oral presentation at: International Congress of Physiotherapy: Women and sports; 2018 January 27; Brussels, Belgium.

10. Decroix L. Het effect van bewegen en voeding (en supplementen) op het brein. Oral presentation at: Ons brein in beweging (Brain awareness week, Center for Neuroscience); 2018 March 12; Brussels, Belgium.

Poster presentations

1. Decroix L, Lespagnol E, Meeusen R, Heyman E. Can cocoa flavanols partially restore hypoxia-induced decline in cerebral oxygenation during exercise in healthy athletes? VasCog Symposium, 2016 December 15th, Lille, France.

2. Decroix L, Lamberts R, Meeusen R. Can the Lamberts Submaximal Cycle Test reflect overreaching in professional cyclists? American College of Sports Medicine, ACSM Annual Meeting, 2017 May 30-June 3, Denver, USA.

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Summary

Amongst athletes, the use of nutritional supplements is widespread because of the belief that they can enhance sports performance. While sports performance largely depends on physical factors, cognitive functioning also plays a great role, as performance in many sports depends on decision making and skill accuracy. Thus, nutritional supplements as potential ergogenic aids cannot only impact muscle, but also the brain. Cocoa flavanols (CF) have antioxidant and anti-inflammatory capacities and can improve vascular function by stimulating NO-dependent vasodilation. CF intake might thus improve exercise performance and recovery through increasing antioxidant capacity, reducing oxidative stress and increasing NO availability. Besides, it has been shown that CF intake can improve executive function (decision making) and motor control, two important factors influencing sports performance. Thus, it can be speculated that CF intake can also enhance sport performance by boosting cognitive function. It is the purpose of this PhD to identify the putative effects of CF on physical and cognitive performance.

Elite athletes often train and perform at high altitude, where the decreased partial pressure of O_2 in the air results in reduced tissue O_2 delivery, which places an extra burden on physical and cognitive performance. Therefore, we aim to investigate whether CF intake can reduce the negative effect of acute hypoxia on cognitive and physical performance.

First, we performed a systematic review on the existing literature regarding the effect of CF intake on *(i)* exercise performance and recovery and *(ii)* the acute and chronic exercise-induced changes in vascular function, cognitive function, oxidative stress, inflammation and carbohydrate and lipoprotein metabolism. Thirteen studies were included. A first finding was that acute and sub-chronic CF intake can reduce exercise-induced oxidative stress and alter carbohydrate and fat metabolism during exercise in trained participants, but without improving exercise performance. Secondly, combining sub-chronic CF intake and exercise training improves cardiovascular risk factors and vascular function in both healthy and overweight participants, but evidence on the synergistic effects of CF and exercise training on oxidative stress, inflammation and fat and glucose metabolism was lacking.

Then, the effect of acute CF intake on cognitive performance was examined, while focusing on the role of brain oxygenation and BDNF. We found that 900 mg CF intake could increase prefrontal oxygenation in healthy, young athletes, but without affecting cognitive performance on a relatively short and easy Stroop task, measuring executive function. BDNF was not affected by CF intake. It was clear that the effects of acute high-intensity exercise largely overruled the effects of CF intake: large beneficial effects of exercise on both prefrontal oxygenation and cognitive function were observed and CF supplementation did not enlarge these effects.

Acute hypoxic exposure is known to place an extra burden on cognitive performance and it was previously suggested that acute CF intake would mainly improve cognitive performance in cognitive demanding environments. Thus, in a next study, the effect of acute CF intake (530 mg CF) on

performance on a difficult and demanding cognitive test battery in normoxia and hypoxia (simulated altitude 4000 m) was assessed. Electroencephalogram and fNIRS were used to analyse neuronal activity and hemodynamic changes in order to unravel whether changes in neurovascular coupling (NVC) were solely driven by changes in vascular responsiveness or also by neuronal activity. NVC is the process which adapts regional cerebral blood flow to neuronal activity. Acute CF intake improved NVC, but did not affect neuronal activity and cognitive performance in both normoxia and hypoxia. Thus, this study added on to the existing literature by showing that the increased NVC observed after CF intake must be driven by improved vascular responsiveness, rather than by altered neuronal activity. In contrast to the hypothesis, most cognitive functions, as well as NVC and neuronal activity, were not altered by acute exposure to moderate hypoxia in healthy subjects. These findings showed that young, healthy persons have a very efficient neurovascular coupling, which governs adequate cerebral blood flow even during hypoxic exposure. Additional increases in vascular responsiveness by CF supplementation do not lead to better cognitive function in persons with a healthy (neuro)vascular function.

However, acute intake of 900 mg CF resulted in enhanced cognitive performance on the Flanker test (response inhibition and executive function) in patients with type 1 diabetes, and also in their healthy matched controls. CF increased the BOLD response in the supramarginal gyrus parietal lobe and inferior frontal gyrus, two brain areas activated during this specific task. While cognitive performance was not deteriorated in patients with type 1 diabetes, a different brain activation pattern during the cognitive task was observed, compared to healthy controls. Moreover, this brain activation pattern was altered after CF intake. To conclude, acute CF intake clearly improves prefrontal oxygenation and cerebrovascular responsiveness. This can be associated with better cognitive function in patients with type 1 diabetes, but does not result in improved executive function in healthy persons and thus will not improve exercise performance through better cognitive functioning. Compared to exercise, the magnitude of the CF-induced neurovascular changes is small.

Two studies were conducted examining the effects of CF intake on exercise-induced oxidative stress, NO availability and its implications for exercise performance, in well-trained cyclists. First, we examined whether acute CF intake (900 mg CF) could influence oxidative stress, NO production and inflammation during high-intensity exercise (time trial). While acute CF improved the exercise-induced increase in total antioxidant capacity, the exercise-induced increase in lipid peroxidation was not reduced. NO production was increased during exercise, but was not influenced by acute CF intake. IL-1 and IL-6 increased during exercise as well, but were neither influenced by CF intake. Exercise performance and recovery were not improved by acute CF intake in these well-trained cyclists. In a next study, we then examined how 1-week CF intake (530 mg CF) could influence oxidative stress, NO production and tissue oxygenation during low-intensity and high-intensity exercise in normoxia and hypoxia. One week CF intake did neither change NO production, nor NO availability at rest and during

exercise. Vascular function, measured at rest, was improved by CF intake. At rest and during low-intensity exercise, CF intake improved prefrontal oxygenation, but did not influence muscular oxygenation, both in normoxia and hypoxia. Thus, CF partially restored the hypoxia-induced decline in prefrontal oxygenation during rest and low-intensity exercise, but not during high-intensity exercise. During high-intensity exercise, CF did neither improve tissue oxygenation, nor performance in well-trained athletes. Interestingly, CF intake reduced exercise-induced lipid peroxidation, but did not alter total antioxidant capacity.

Thus, we showed that both acute (900 mg CF, 196 mg epicatechin) and 1-week (530 mg CF, 100 mg epicatechin/day) CF intake cannot improve cognitive performance, nor physical performance in healthy, young athletes and should therefore not be consumed as a performance-enhancing aid. However, CF intake can be considered as a nutritional aid to stimulate cerebrovascular responsiveness and to maintain cerebral oxygenation in normoxia and hypoxia, both at rest and during low-intensity exercise. Moreover, we showed that CF intake can increase the antioxidant defence system battling ROS production and prevent oxidative stress, induced during exhaustive exercise. However, further research is warranted to investigate whether and when these antioxidant capacities of CF intake are beneficial for athletes, keeping in mind that small amounts of oxidative stress are needed in the process of training adaptation.

Samenvatting

De laatste decennia hebben voedingssupplementen aan populariteit gewonnen. Een voedingssupplement dient als aanvulling op de normale voeding en bevat een geconcentreerde bron van één of meerdere nutriënten of andere stoffen met een nutritioneel of fysiologisch effect. Onder topsporters komt het gebruik van supplementen vaak voor, in de hoop dat ze prestatie bevorderend zouden zijn. Echter, slechts voor een zeer beperkt aantal is er voldoende wetenschappelijk bewijs dat het supplement ook nuttig is. Echter, van veel supplementen is er nog geen bewijs dat ze effectief werken en sommige kunnen zelfs schadelijk zijn. Daarom is het van cruciaal belang dat wetenschappelijk onderzoek gebeurt naar het effect van bepaalde supplementen op de sportprestatie.

Een sportprestatie is uiteraard afhankelijk van fysieke capaciteiten, maar ook van cognitieve capaciteiten. Tijdens een sportwedstrijd moet men namelijk vaak beslissingen nemen, bewegingen correct uitvoeren, tijdig en accuraat reageren op wat gebeurt tijdens de wedstrijd, etc. Sporters kunnen dus trachten om hun sportprestatie te verbeteren door de inname van voedingssupplementen die zowel de fysieke als cognitieve prestatie kunnen beïnvloeden.

De cacaoboon, maar ook bepaalde "verrijkte" cacaoproducten bevatten een hoog gehalte aan cacao flavanolen (CF). Deze CF werken als antioxidant, maar daarnaast bevorderen ze ook de vasculaire functie via het verhogen van de beschikbaarheid van stikstofmonoxide (NO). NO is een vasodilatator die bovendien ook een rol speelt in mitochondriale efficiëntie en biogenesis. Dankzij het verbeteren van de vasculaire functie, wordt ook de hersendoorbloeding bevorderd en kan cognitieve prestatie verbeteren na inname van CF. Het zou dus kunnen dat CF de sportprestatie bevorderen, via het reduceren van oxidatieve stress tijdens inspanning, via het verbeteren van NO beschikbaarheid, via het verhogen van de bloedtoevoer en aanvoer van zuurstof en nutriënten naar de spieren en hersenen en/of via het verbeteren van de cognitieve prestatie.

Het doel van dit doctoraat is om het effect van CF inname op cognitieve en fysieke prestaties bij zeer goed getrainde atleten na te gaan, alsook om de onderliggende mechanismen te onderzoeken. Topsporters trainen en presteren vaak op grote hoogte, waar de fysieke en cognitieve prestatie vermindert door de gedaalde partiële druk van zuurstof in de lucht (hypoxie). Daarom is een bijkomend doel van deze thesis om te onderzoeken of CF inname de negatieve effecten van hypoxie op weefseloxygenatie zou kunnen reduceren. Terwijl CF positieve effecten kunnen hebben op vasculaire functie bij mensen met een verminderde gezondheid (in preventie van bv. cardiovasculaire aandoeningen), is het mogelijk dat er bij topsporters geen effect is omdat hun dagelijkse training reeds heel veel bijdraagt tot de optimale werking/elasticiteit van de bloedvaten. Daarom willen we ook het effect van CF op cognitieve prestatie nagaan in een populatie met een verminderde (cerebro)vasculaire functie, nl. patiënten met type 1 diabetes.

Aan de hand van een systematische review werd de bestaande literatuur rond CF en sportprestatie in kaart gebracht. Dertien studies werden geïncludeerd. De studies toonden aan dat de inname van CF, eenmalig en gedurende 2 weken, oxidatieve stress tijdens zware fysieke inspanning verlaagt, alsook het koolhydraat-en vetmetabolisme tijdens inspanning verandert. Echter, CF bevordert de fysieke prestatie niet. Daarnaast blijkt uit deze systematisch review dat de combinatie van fysieke activiteit en CF inname het risico op een cardiovasculaire aandoening verlaagt en de vasculaire functie verbetert, bij gezonde mensen en bij mensen met overgewicht. Er is wel nood aan studies die het gezamenlijke effect van inspanning en chronische CF inname onderzoeken op oxidatieve stress en inflammatie.

Vervolgens werden enkele experimenten uitgevoerd die het effect van CF op cognitieve en fysieke prestatie nagingen. De eerste vraag die werd gesteld, was of acute CF inname het positief effect van inspanning op cognitie kon versterken in jonge, gezonde proefpersonen. Onze studie toonde aan dat de inname van 900 mg CF de vasculaire respons/oxygenatie in de prefrontale cortex tijdens cognitieve taken verbetert, maar de prestatie op een korte cognitieve taak niet wordt beïnvloed. Bovendien was het effect van de inspanning op zowel oxygenatie, als op cognitieve prestatie veel groter dan het effect van CF. CF inname kon het positief effect van inspanning niet versterken. De vraag of een neurogene factor, nl. BDNF, beïnvloed wordt door CF inname, werd negatief beantwoord.

In een vroegere studie werd reeds gesuggereerd dat CF inname voornamelijk een positief effect zou hebben wanneer het cognitief vermogen tegen zijn limieten aanleunt, bv. bij een slaaptekort of zeer belastende en cognitieve taken. Tijdens een volgende studie werd nagegaan wat het effect van eenmalige CF (530 mg CF) inname was op de cognitieve prestatie tijdens uitdagende cognitieve testen die uitgevoerd werden op een gesimuleerde hoogte van 4000 meter of op zeeniveau. Elektro-encefalografie en NIRS (Near infrared spectroscopie) werden gebruikt om respectievelijk de hersenactiviteit en neuro-vasculaire respons tijdens de cognitieve testen te meten. Het resultaat van deze studie was dat de neuro-vasculaire respons weliswaar groter wordt door inname van CF, maar dat de hersenactiviteit niet verandert. Ook de cognitieve prestatie verbeterde niet door CF inname. Bovendien was er nauwelijks verschil merkbaar tussen de situatie op zeeniveau en 4000 m, wat aantoont dat de neuro-vasculaire respons (en hersenactiviteit) van een jonge, gezonde persoon zeer efficiënt werkt en aldus een optimale cognitieve functie gehandhaafd kan worden, zelfs wanneer men onderhevig is aan hypoxie. De bijkomende verhoging in cerebrovasculaire responsiviteit als gevolg van CF inname resulteert niet in een verbeterde cognitieve prestatie bij gezonde personen met een goede (neuro)vasculaire functie.

Veel patiënten met type 1 diabetes hebben echter een verminderde neuro-vasculaire functie. Uit een volgend onderzoek bleek dat bij deze patiënten acute inname van 900 mg CF wel resulteert in een verbeterde cognitieve prestatie. Ook in de controle groep, die dezelfde karakteristieken vertoonde als de patiënten met type 1 diabetes en in vergelijking met de populaties uit de voorbije studies, een stuk ouder

en minder getraind was, verbeterde de hersenfunctie na CF inname. Bovendien resulteerde acute CF inname in een verhoogde neuro-vasculaire respons, gemeten via fMRI, in een aantal hersengebieden die geactiveerd worden tijdens de cognitieve taak. Er was geen verschil merkbaar in cognitieve prestatie tussen de gezonde personen en de patiënten met type 1, maar het patroon van hersenactivatie tijdens deze test was wel degelijk verschillend. Dit zou kunnen suggereren dat bij patiënten met type 1 diabetes bijkomende hersenregio's worden geactiveerd ter compensatie van "aftakelende" hersenregio's, en dit zo de cognitieve achteruitgang kan remmen.

We kunnen dus besluiten dat acute CF inname prefrontale oxygenatie en cerebrovasculaire responsiviteit verbetert. Terwijl dit resulteert in een betere executieve functie bij patiënten met type 1 diabetes, heeft dit geen effect op de cognitieve prestatie van gezonde, jonge personen. In vergelijking met het effect van fysieke inspanning, is het positief effect van CF echter zeer klein.

Verder werd ook onderzocht wat het effect is van CF inname op oxidatieve stress en NO productie tijdens inspanning, en of CF inname de fysieke prestatie en/of herstel bevordert. Deze studie toonde aan dat eenmalige inname van 900 mg CF de anti-oxidatieve capaciteit, die wordt getriggerd door fysieke inspanning, verhoogt. De oxidatieve stress die ontstond tijdens een tijdrit werd niet afgeremd door CF inname. Tijdens de tijdrit verhoogde de NO productie, maar dit werd niet beïnvloed door CF inname. CF inname had eveneens geen effect op de productie van IL-1 en IL-6, die toenam tijdens de tijdrit. Noch de fietsprestatie, noch het herstel, verbeterde door CF inname bij deze zeer getrainde wielrenners.

Tenslotte gingen we na hoe de inname van 530 mg CF gedurende 1 week de oxidatieve stress, NO productie en weefseloxygenatie tijdens een matig intensieve en maximale fysieke inspanning beïnvloedt. Dit zowel op zeeniveau als op een gesimuleerde hoogte van 3000 m. In hypoxie was de weefseloxygenatie en tijdritprestatie duidelijk verlaagd in vergelijking met de situatie op zeeniveau. Na 6 dagen CF inname verbeterde de vasculaire functie. Zowel in rust, als tijdens matig intensieve inspanning, verbeterde de oxygenatie van de prefrontale cortex door CF inname. Hierdoor kon CF inname het negatief effect van hypoxie op breinoxygenatie afremmen. Tijdens de maximale inspanning verdween het positieve effect van CF op breinoxygenatie. CF inname had geen invloed op spieroxygenatie en verbeterde de prestatie op de tijdrit niet. NO productie en NO beschikbaarheid werden niet beïnvloed door CF inname, terwijl CF inname de oxidatieve stress tijdens fysieke inspanning wel reduceerde.

We kunnen dus besluiten dat zowel eenmalige inname van 900 mg CF, als de inname van 530 mg CF gedurende 1 week, noch de fysieke, noch de cognitieve prestatie bevordert bij jonge, gezonde atleten. We raden dan ook niet aan om CF in te nemen als prestatie bevorderend voedingssupplement. CF inname lijkt wel veelbelovend om een goede neurovascularisatie te stimuleren en oxygenatie van het brein tijdens blootstelling aan hypoxie op pijl te houden. Bovendien werd tijdens dit

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Résumé

1. Introduction

Les suppléments nutritionnels sont nombreux et populaires pour leurs vertus santé. En particulier, les athlètes les utilisent de plus en plus, avec pour objectif d'améliorer leur performance sportive, bien que cet effet ne soit pas toujours validé scientifiquement (Maughan et al. 2018). La performance sportive peut dépendre à la fois de facteurs physiques, mais également de facteurs cognitifs impliqués dans la prise de décision et l'ajustement des mouvements par exemple (Baker et al. 2014). Par conséquent, l'étude des effets de certains suppléments nutritionnels sur la performance physique et cognitive et leurs mécanismes d'action est d'un intérêt majeur pour le monde scientifique.

La famille des antioxydants est l'une des plus populaires des suppléments nutritionnels. Dans la famille des antioxydants, les polyphénols composent un grand groupe qu'on trouve principalement dans les fruits, les légumes, le vin rouge et le cacao. Ces polyphénols sont divisés en flavonoïdes et non-flavonoïdes. Les flavonoïdes eux-mêmes peuvent être divisés en flavones, flavanones, isoflavones, flavonols, anthocyanidins et flavanols. Ce dernier groupe, les flavanols, et en particulier le monomère épicatéchine, sont présents en grande quantité dans les fèves de cacao (Meeusen and Decroix 2018). Pendant la préparation du chocolat, la plupart des flavanols sont perdus, mais il est possible d'optimiser ce processus et d'obtenir des produits et suppléments de cacao riches en flavanols (CF) (Katz et al. 2011).

Les CF ont des effets bénéfiques sur le système vasculaire, qui sont attribués à une action directe sur la fonction endothéliale, ainsi que des effets anti-athérosclérotiques, anti-hypertenseurs et anti-inflammatoires. Les CF stimulent la production du vasodilatateur monoxyde d'azote (NO) par la NO synthase endotheliale (eNOS) et préviennent la dégradation du NO par les radicaux libres dérivés de l'oxygène (reactive oxygen species ; ROS) (Heiss 2014). En améliorant la vasoréactivité, l'apport en oxygène (O_2) aux tissus peut augmenter. En plus d'être vasodilatateur, le NO est aussi impliqué dans la biogenèse et la fonction mitochondriale musculaire, ce qui peut également favoriser la performance sportive (Jones 2014).

Il a été également démontré *in vitro* que la formation et l'accumulation de ROS est diminué par les CF (Nabavi et al. 2015). Ainsi, une supplémentation en CF peut réduire le stress oxydant, qui est associé avec certaines maladies chroniques et qui est aussi induit pendant l'exercice exhaustif (Powers and Ji 2011). Le stress oxydant est causé par une augmentation des systèmes pro-oxydants (comme la NADPH oxydase) et/ou une réduction de l'activité des systèmes antioxydants (Powers et al. 2016). Le stress oxydant induit pendant l'exercice exhaustif serait impliqué dans la fatigue musculaire et il a été suggéré que la réduction du stress oxydant pendant l'exercice pourraitt améliorer la récupération après (Powers and Jackson 2010). Néanmoins, les preuves montrant que les CF peuvent améliorer la performance

sportive en réduisant le stress oxydant et/ou en augmentant la biodisponibilité de NO restent à ce jour limitées.

D'autre part, chez l'homme, la consommation de CF pourrait améliorer les fonctions cognitives via une action sur le débit sanguin cérébral (Socci et al. 2017). Ainsi, nous faisons l'hypothèse que la consommation de CF pourrait aussi améliorer la performance sportive en améliorant les fonctions cognitives (Meeusen and Decroix 2018).

L'objectif de cette thèse est donc d'analyser les effets d'une consommation aigue, ou pendant une semaine, de CF, sur la performance physique et cognitive en prenant en compte les mécanismes sous-jacents à ces effets dans diverses populations: chez des athlètes de haut niveau, chez des sujets actifs, et chez des personnes ayant un diabète de type 1 (DT1). Ces derniers présentent la particularité de pouvoir atteindre un très haut niveau dans le sport de compétition, mais également d'être sujets à des dysfonctions endothéliales précoces (même avant l'apparition clinique de signes de complications vasculaires), visibles par exemple en réponse à des situations physiologiques comme l'exercice. De plus, nous nous attacherons à investiguer les effets des CF en hypoxie, puisque les compétitions et les entraînements de certains sportifs de haut niveau ont lieu en altitude, où la pression partielle en oxygène, la saturation artérielle en oxygène et l'oxygénation des tissus sont diminués. Ainsi, l'hypoxie limite la performance cognitive et physique (Petrassi et al. 2012; Davranche et al. 2016). Les questions de recherche sont les suivantes :

- La consommation aigue de 900 mg de CF peut-elle améliorer la fonction cognitive en combinaison avec l'exercice, chez des athlètes de haut niveau ? L'hémodynamique cérébrale et le le facteur neurotrophique derivé du cerveau (BDNF) sont-ils influencés par cette consommation aigue de CF et l'exercice ?
- La consommation aigue de 900 mg de CF peut-elle améliorer la fonction cognitive en altitude, via un effet sur l'hémodynamique cérébrale et/ou l'activité neuronale, chez des sujets jeunes et en bonne santé ?
- Quel est l'effet de la consommation aigue de 900 mg de CF sur la fonction cognitive et le signal BOLD (dépendant du flux sanguin cérébral et de la consommation d'oxygène cérébrale) chez des personnes avec un DT1 et des témoins sains ?

- La consommation aigue de 900 mg de CF peut-elle augmenter la production de NO et réduire le stress oxydant, induit par l'exercice intense exhaustif ? Quelles en sont les implications pour la performance physique et la récuperation chez des sportifs de très haut niveau?
- La consommation de 530 mg de CF/jour pendant 1 semaine peut-elle améliorer l'oxygénation cérébrale et musculaire, la production de NO et réduire le stress oxydant pendant un exercice intense

et exhaustif en hypoxie? Quelles en sont les implications pour la performance physique chez des sportifs de très haut niveau?

Dans le premier chapitre, nous nous intéresserons à la littérature existante sur les effets des CF sur les adaptations à l'exercice telles que la fonction vasculaire, le stress oxydant, le métabolisme des glucides et des protéines et la fonction cognitive (revue systématique). Dans les chapitres suivants, nous présenterons les différentes études expérimentales de ce projet doctoral qui ont examiné les effets de la consommation de CF sur la performance cognitive et physique. Les dosages des supplémentations, les populations, et les méthodes utilisées dans les différentes études sont synthétisées dans la figure 33.

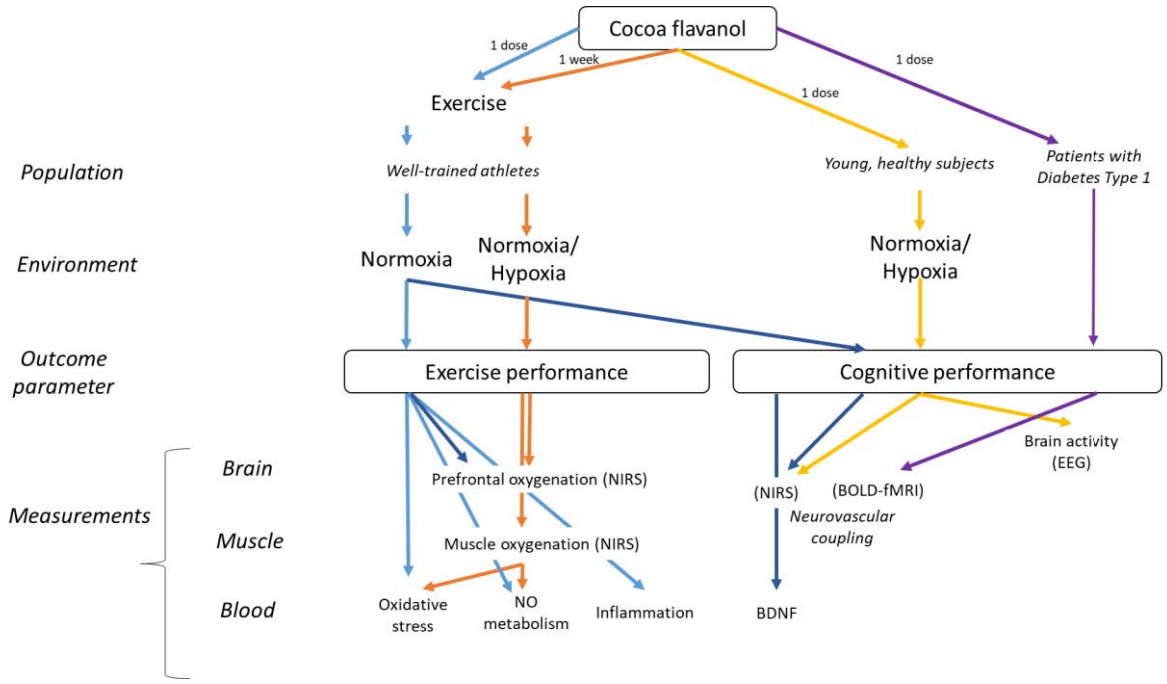

Figure 33. Résumé des dosages de supplémentation, les populations, les méthodes utilisés et paramètres de résultats, des études de ce projet doctoral. Bleu clair : étude décrite au chapitre 6, blue foncé : étude décrite au chapitre 3, orange : étude décrite au chapitre 7, jaune : étude décrite au chapitre 4, violet : étude décrite au chapitre 5. *NIRS: spectroscopie dans le proche infrarouge; BOLD-fMRI : le signal dépendant du niveau d'oxygène sanguin- l'imagerie par résonance magnétique; BDNF : facteur neurotrophique dérivé du cerveau ; NO : monoxyde d'azote.*

2. Revue systématique

Référence

Decroix L, Soares DD, Meeusen R, Heyman E and Tonoli C. (2018) Cocoa Flavanol Supplementation and Exercise: A Systematic Review. Sport Med 2018;48(4):867-8921–26.

Objectif

Les CF sont connus pour leurs capacités antioxydantes et anti-inflammatoires et leur capacité à augmenter la fonction vasculaire. Notre hypothèse est que la consommation de CF pourrait améliorer la performance sportive. Cette revue systématique visait à étudier la littérature sur les effets des CF sur les réponses à l'exercice notamment vasculaires, sur le stress oxydant, sur le métabolisme des glucides et lipides, et sur les fonctions cognitives.

Méthodologie

Deux bases de données électroniques ont été consultées jusqu'à Mars 2017 : Pubmed et Web of Science. Nous avons seulement inclus des articles rapportant les dosages exacts de CF. Treize études, avec un total de 240 sujets (70 sujets obèses, 35 sujets non-entrainés et 135 sujets très entrainés) ont été inclus dans cette revue systématique. Nous avons examiné la qualité de la méthodologie et le degrés de biais.

Résultats et discussion

Une consommation aigue, ainsi qu'un consommations de 2 semaines ou de 3 mois de CF peuvent réduire le stress oxydant induit par l'exercice. Par contre, il n'existe pas de preuves scientifiques de l'effet des CF sur l'inflammation et l'activation plaquettaire induites par l'exercice. La consommation aigue de CF réduit l'hypertension au repos et atténue l'élévation de tension artérielle à l'exercice chez les personnes obèses. La consommation aigue, ou sur 2 semaines, de CF peut modifier le métabolisme des glucides et des lipides pendant l'exercice. La consommation de CF n'a pas d'effet ergogène dans une population d'athlètes. Par contre, 3 mois de supplémentation en CF augmente l'efficacité mitochondriale musculaire chez des sujets non-entrainés.

Ainsi, la combinaison de CF à l'exercice peut améliorer les facteurs de risque cardiovasculaire et la fonction vasculaire. Par contre, aucun travail n'a montré des effets synergiques des CF et de l'exercice sur le stress oxydant, l'inflammation et le métabolisme des lipides et des glucides.

En conclusion, dans les études réalisées jusqu'à présent, les CF peuvent améliorer la fonction vasculaire, réduire le stress oxydant pendant l'exercice et modifier le métabolisme des lipides et des glucides pendant l'exercice, mais les répercussions sur la performance sportive restent minimes. Néanmoins,

d'autres études doivent compléter ces données dans différentes populations et conditions (exemple : hypoxie) auxquelles les sportifs peuvent être soumis.

3. L'effet des CF sur la performance cognitive et les mécanismes sous-jacentes

3.1. La prise aigue de cocoa flavanols améliore l'oxygénation cérébrale sans affecter les fonctions exécutives au repos et après l'exercice

Référence

Decroix L, Tonoli C, Soares DD, Tagougui S, Heyman E and Meeusen R (2016) Acute cocoa flavanol improves cerebral oxygenation without enhancing executive function at rest or after exercise. Appl Physiol Nutr Metab 41:1225–1232

Objectif

L'amélioration de la fonction cognitive suscitée par un exercice ponctuel est accompagnée d'une augmentation du débit sanguin cérébral et d'une augmentation de la concentration du facteur neurotrophique dérivé du cerveau (BDNF). La consommation de CF pourrait améliorer la fonction cognitive, le débit sanguin cérébral (chez l'homme) et la concentration de BDNF (chez les animaux). Cette étude analyse les effets de la consommation de CF combinée à l'exercice physique sur *(i)* la fonction cognitive et *(ii)* l'hémodynamique cérébrale et le BDNF sérique.

Méthodologie

Douze hommes en bonne santé participent à cette étude randomisée contrôlée en double aveugle. Les participants effectuent une tâche cognitive 100 min après avoir consommé 903 mg de CF (196 mg epicatechin) ou de placebo (PL) suivie d'un exercice de contre la montre de 30 min. La tâche cognitive consiste en un test de Stroop, qui évalue les fonctions exécutives et la prise de décision, et qui prend environ 5 minutes. Immédiatement après l'exercice, les participants effectuent à nouveau la même tâche cognitive. Les variations de volume sanguin local (ΔHb_{tot}) et d'oxygénation (ΔHbO_2) sont évalués au niveau du cortex préfrontal par spectroscopie dans le proche infrarouge (NIRS). Le BDNF est analysé sur sérum dans les échantillons de sang prélevés.

Résultats et discussion

Le temps de réaction est plus court après l'exercice, mais n'est pas influencé par les CF. ΔHbO_2 est plus élevée dans la condition CF comparativement à la condition PL durant la tâche cognitive au repos, avant l'exercice. ΔHbO_2, ΔHHb et ΔHb_{tot} augmentent en réponse à l'exercice, et ce, sans effet des CF. Durant la tâche cognitive post-exercice, on n'observe pas de différence de l'hémodynamique entre CF et PL

(Figure 34). Le BDNF sérique augmente à l'exercice, mais n'est pas influencé par la supplémentation en CF.

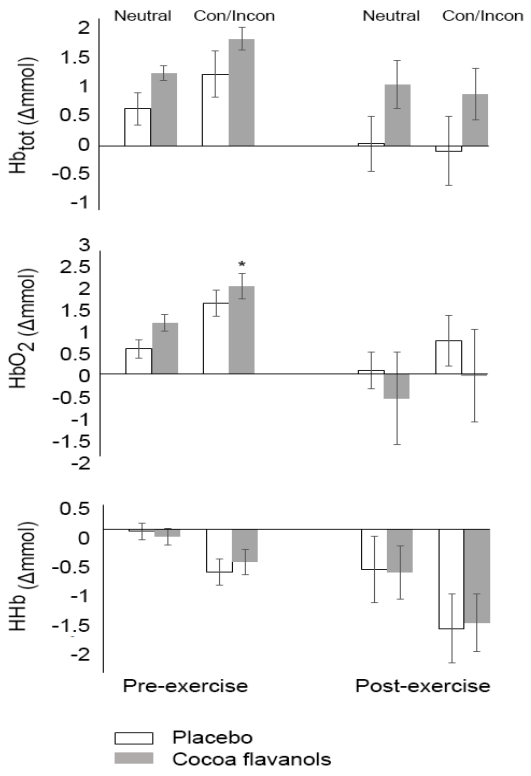

Figure 34. L'effet des cocoa flavanols (CF) et placebo (PL) sur l'hémodynamique cérébrale (variations de la concentration d'hémoglobine oxygénée (ΔHbO2), désoxygénée (ΔHHb) et totale (ΔHbtot)) pendant le test cognitif avant et après l'exercice de 30 minutes (contre-le-montre). *Pendant la première partie du test de Stroop (stimules neutres) avant l'exercice, ΔHbO2 est plus élevée après consommation de CF, comparé au PL. Légende: Con : stimulis congruents, Incon : stimulis incongruents.*

En conclusion, au repos, la consommation de CF suscite une augmentation de l'oxygénation cérébrale, mais pas de la concentration sérique du BDNF et n'a pas d'effet notable sur la fonction cognitive, évaluée par un test court et relativement facile, chez des sportifs sains. L'effet bénéfique des CF sur l'oxygénation cérébrale au repos est dépassé par l'ampleur de l'augmentation de la perfusion et de l'oxygénation cérébrale à l'exercice physique et n'est donc plus visible en post-exercice.

3.2. La consommation aigue de cocoa flavanols améliore l'hémodynamique cérébrale sans affecter l'activité neuronale et les fonctions cognitives en hypoxie modérée

Référence

Decroix L, De Pauw K, Van Cutsem J, Pattyn N, Heyman E and Meeusen R (2018) Acute cocoa flavanol intake improves cerebral hemodynamics while maintaining brain activity and cognitive performance in moderate hypoxia. Submitted to: Psychopharmacology.

Introduction

Le couplage neurovasculaire est le mécanisme régulant le débit sanguin cérébral (local) en fonction de l'activité neuronale et est donc dirigé par l'activité neuronale et la vasoreactivité. Il a été montré qu'une consommation de CF pouvait augmenter la perfusion et la vasoreactivité cérébrales et il est suggéré que les CF peuvent améliorer la performance cognitive surtout dans des conditions extrêmes, comme en hypoxie. En altitude, la pression partielle d'oxygène et donc l'oxygénation tissulaire sont diminués, ce qui peut limiter la performance cognitive (Petrassi et al. 2012; Davranche et al. 2016). Par conséquent, notre objectif est examiner si une consommation aigue de CF pourrait limiter les effets négatifs de l'hypoxie (12.7% oxygène) sur la performance cognitive pendant un test cognitif assez exigeant et difficile. De plus, nous nous intéresserons aux mécanismes sous jacents possibles tels que l'hémodynamique cérébrale et l'activité cérébrale, déterminant le couplage neurovasculaire.

Méthodologie

Vingt sujets jeunes et en bonne santé (âge : 23.2±4.3 ans) participent à cette étude randomisée contrôlée en double aveugle, consistant en 4 visites, espacées d'une semaine. Les participants entrent la chambre climatique qui se trouve en hypoxie normobare, ou en normoxie, 1 h 30 min après avoir consommé 530 mg de CF (100 mg épicatéchine) ou de PL. Les participants effectuent une tâche cognitive, consistant en un test de Stroop et une batterie de tests (la « Cognition battery ») évaluant plusieurs domaines cognitives, 2 h après la consommation de CF ou PL. L'oxygénation et le volume sanguin local sont évalués par spectroscopie proche infrarouge au niveau du cortex préfrontal et l'activité neuronale des aires cérébrales est évaluée au moyen de l'électroencéphalographie (EEG).

Résultats et discussion

Les CF augmentent la réponse hémodynamique préfrontale durant plusieurs parties de la tâche cognitive, examinant la prise de risques, le suivi visuel, l'analyse complexe, l'orientation spatiale (Figure 3), mais n'affecte pas l'activité neuronale. Les CF améliorent la pensée abstraite en normoxie, mais n'améliorent aucun autre domaine des fonctions cognitives. Seule la précision lors du test de Stroop est diminuée par

l'hypoxie. De plus, la réponse hémodynamique préfrontale et l'activité neuronale ne diffèrent pas en hypoxie vs. en normoxie.

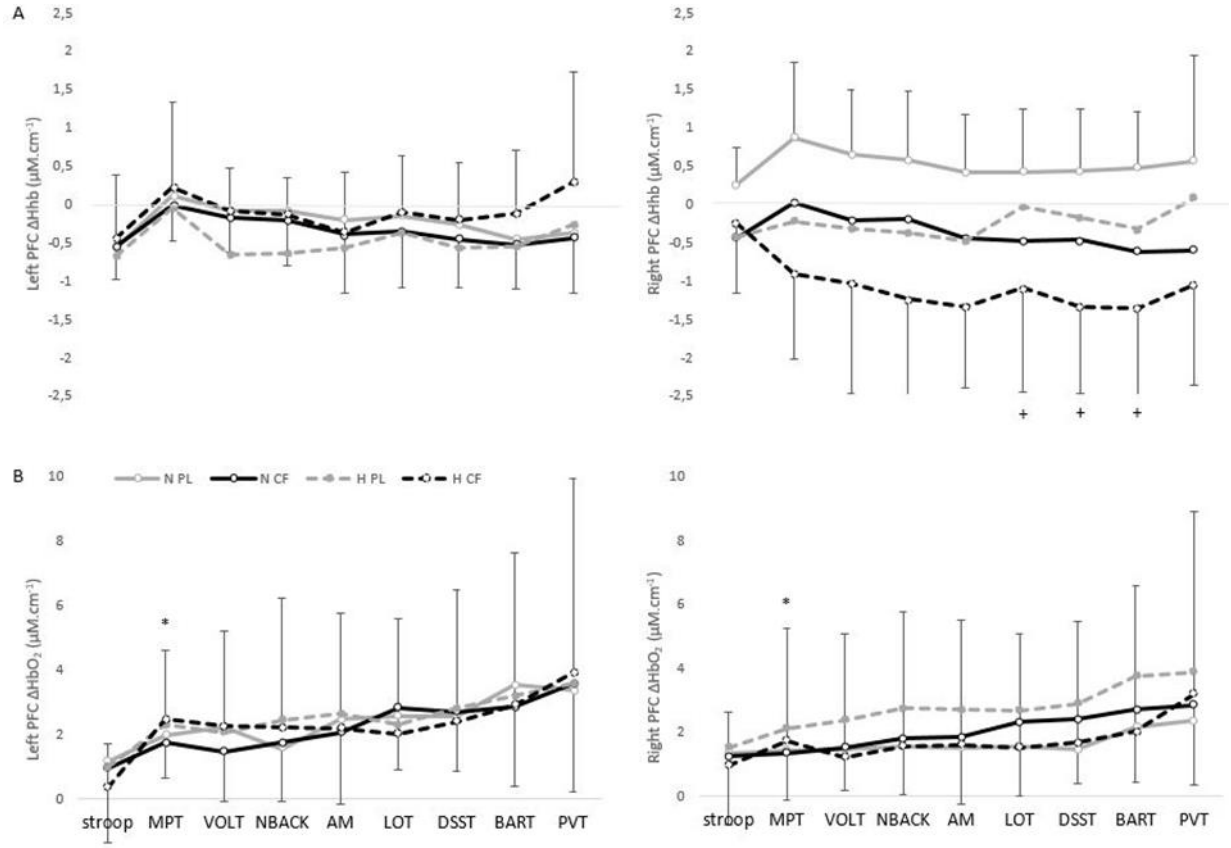

Figure 35. L'effet des cocoa flavanol (CF) et placebo (PL) en hypoxie (H) et normoxie (N) sur l'hémodynamique cérébrale au cortex préfrontal (PFC) gauche (left) et droit (right) (variations de la concentration d'oxyhémoglobine (ΔHbO₂) et de déoxyhémoglobine (ΔHHb)) en réponses aux tests cognitifs. **: effet significatif de l'hypoxie (p<0,05); +: effet significatif des CF (p<0,05). MPT: motor praxis task, VOLT: Visual Object Learning Task, AM: abstract matching, LOT: line orientation test, DSST: digit symbol substitution test, BART: balloon analog risk test, PVT: psychomotor vigilance test.*

Ces résultats montrent que la fonction cognitive, et le couplage neurovasculaire, qui régule l'augmentation de flux sanguin local en fonction de l'activité neuronale, sont bien préservés en hypoxie modérée (altitude simulée de 4000m) chez des sujets jeunes et en bonne santé. Même si la consommation aigue de CF stimule ce couplage neurovasculaire, ceci ne résulte pas en une meilleure performance cognitive.

3.3 La consommation aigue de cocoa flavanols améliore le signal BOLD et la fonction cognitive dans le diabète de type 1

Référence

Decroix L, Van Schuerbeek P, Tonoli C, Soares DD, Heyman E, Vanderhasselt T, Raeymaekers H, De Mey J and Meeusen R (2018) The effect of acute cocoa flavanol intake on the bold response and cognitive function in type 1 diabetes: a randomized, placebo-controlled, double-blind cross-over study. Submitted to: Plos One

Introduction

Le diabète de type 1 (DT1) est associé à des complications microvasculaires, qui sont causées par des épisodes d'hyperglycémie. Le signal BOLD, mesuré par l'imagerie par résonance magnétique (fIRM), reflète les variations locales et transitoires de la quantité d'oxygène transportée par l'hémoglobine, en fonction de l'activité neuronale du cerveau. Chez les patients DT1, une modification du signal BOLD lors de tâches cognitives, a déjà été observée en comparaison de sujets sains (Gallardo-Moreno et al. 2015; Guàrdia-Olmos et al. 2017). Ces derniers présentent la particularité d'être à risque d'une dysfonction endothéliale précoce (même avant l'apparition clinique de signes de complications vasculaires). Cette dysfonction endothéliale constitue un des facteurs de déclin cognitif lié au diabète (Tonoli et al. 2014). Nous avons observé une augmentation de flux sanguin cérébral et des réponses hémodynamiques augmentés après une consommation aigue de CF chez des sujets sains (Lamport et al. 2015; Decroix et al. 2016). Cette étude a pour objectif d'investiguer l'effet d'une consommation aigue de CF sur les fonctions cognitives et le signal BOLD chez des patients DT1.

Méthodologie

Dans cette étude randomisée contrôlée en double aveugle, 11 patients DT1 (41.3 ±16.1 ans ; 5 hommes et 6 femmes; HbA_{1c} 7.5± 3%) et 11 sujets sains, appariés sur l'âge, le sexe et l'indice de masse corporelle ont été inclus. Les participants effectuent le Flanker test, qui estime le contrôle exécutif et inhibitoire, 2 h après avoir consommé 903 mg de CF (196 mg d'épicatéchine) ou de PL. Le signal BOLD est mesuré par fIRM pendant les tâches cognitives.

Résultats et discussion

Les performances cognitives ne sont pas altérées chez les patients DT1 en comparaison des sujets sains. Chez les diabétiques, en comparaison des témoins sains, le signal BOLD est plus élevé dans le cervelet, dans l'aire de Brodmann 30 du cortex agranulaire retro-limbique et dans le lobe temporal subgyral et plus faible dans le gyrus temporal supérieur. La consommation aigue de CF améliore les fonctions

cognitives chez les patients DT1 et les sujets sains, en comparaison de PL. CF augmente le signal BOLD au niveau du gyrus supramarginal, du lobe pariétal et du gyrus frontal inférieur. Le signal BOLD est augmenté au niveau du gyrus temporal supérieur chez les patients DT1, en comparaison des sujets sains, après consommation de CF.

En conclusion, les performances cognitives ne sont pas altérées chez les patients DT1 de cette étude, en comparaison des sujets sains. Il se pourrait que l'activation cérébrale différente que nous avons observée joue un rôle compensatoire. La consommation aigue de CF peut améliorer les capacités d'inhibition et les fonctions exécutives chez des patients DT1 et des sujets sains, et peut augmenter le signal BOLD dans les régions du cerveau activées par les tâches cognitives.

3.4 Conclusions

Les résultats des 3 études montrent que la consommation aigue de CF augmente l'oxygénation et la vasoréactivité cérébrale. Tandis que ces changements sont associés avec une meilleure fonction cognitive chez des patients DT1, ce n'est pas le cas des sujets très entraînés, ni au niveau de la mer, ni en altitude simulée. Malgrè les effets bénéfiques des CF sur la vasoreactivité cérébrale lors de tâches cognitives, l'exercice physique reste un moyen beaucoup plus efficace pour stimuler la vasoreactivité cérébrale et les fonctions cognitives.

4. L'effet des CF sur la performance physique, le stress oxydante et la production du NO

4.1. Une consommation aigue de CF a des effets minimes sur le stress oxydant induit par l'exercice et la production du NO chez des cyclistes de haut niveau : une étude randomisée contrôlée en double aveugle.

Référence

Decroix L, Tonoli C, Soares DD, Descat A, Drittij-Reijnders MJ, Weseler AR, Bast A, Stahl W, Heyman E and Meeusen R (2017) Acute cocoa Flavanols intake has minimal effects on exercise-induced oxidative stress and nitric oxide production in healthy cyclists: a randomized controlled trial. J Int Soc Sports Nutr 14:28

Introduction

L'exercice intense induit la production de radicaux libres. Ces derniers peuvent causer du stress oxydant et donc diminuer la force musculaire. Ils peuvent également dégrader le NO et donc réduire la biodisponibilité en NO. Les CF stimulent la vasodilatation via une augmentation de la biodisponibilité

du NO, en augmentant la production de NO dépendante de la eNOS, et/ou en diminuant sa dégradation via une réduction du stress oxydant. L'objectif de cette étude est de chercher les effets d'une consommation aigue de CF sur la capacité antioxydante, le stress oxydant, la production de NO et l'inflammation pendant l'exercice intense chez des sportifs très entraînés. Nous nous intéresserons aussi aux implications pour la performance physique et la récupération.

Méthodologie

Douze cyclistes masculins de très haut niveau et en bonne santé (30±3 ans, VO_{2max} : 63.0±3.5 mL/kg/min) participent à cette étude randomisée contrôlée en double aveugle. Les participants viennent à 2 reprises au laboratoire, séparées par une semaine. Ils effectuent 2 sessions d'exercice intense exhaustif (soit un contre-le-montre (TT) de 30 minutes) 1.5 h (TT1) et 3 h (TT2) après consommation de 903 mg CF (196 mg d'épicatéchine) ou PL, séparés par une récupération passive. Pendant les TT, nous mesurons le lactate, le glucose, la fréquence cardiaque (FC), la perception de l'intensité de l'effort (RPE) et la puissance développée. Des échantillons sanguins veineux sont récoltés à l'arrivée au laboratoire (valeur basale, avant prise des CF ou PL) puis avant et après chaque TT afin de mesurer les concentrations d'épicatéchine, la capacité antioxydante en équivalent trolox (TEAC), l'acide urique (UA), le malonaldehyde (MDA), le rapport L-arginine/ADMA, la citrulline, l'interleukine (IL)-1, l'IL-6 et le facteur de nécrose tumorale (TNF-α).

Résultats et discussion

La consommation aigue de CF augmente les concentrations d'épicatéchine. L'exercice exhaustif augmente le rapport TEAC/UA, et les CF potentialisent cet effet. Toutefois, les augmentations induites par l'exercice de MDA , IL-1 et IL-6 ne sont pas influencées par la consommation de CF. Nous n'observons aucun changement du TNF- α, ni par l'exercice, ni par les CF. L'exercice induit des variations des biomarqueurs indirects de production de NO, comme le rapport L-arginine/ADMA (baisse) et la citrulline (élévation), ce qui indique que la production du NO est stimulée pendant l'exercice. Par contre, cette production de NO n'est pas influencée par la consommation CF ou PL. La consommation aigue de CF n'a pas d'effet bénéfique sur la performance physique (TT1), ni sur la récupération (performance lors du TT2). De plus, les paramètres physiologiques, comme la fréquence cardiaque, ne sont pas affectés par les CF.

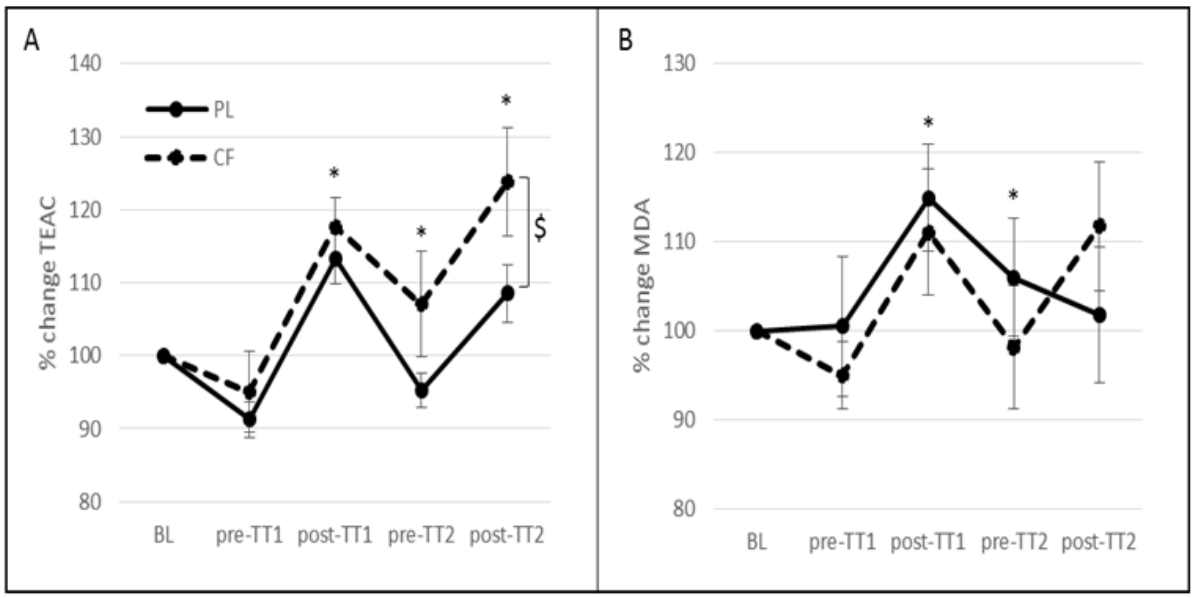

Figure 36. Changements relatifs en capacité antioxydante (TEAC) et peroxydation lipidique (malondialdehyde (MDA)) pendant les 2 sessions d'exercice (contre la montre (TT)), après consommation aigue de cocoa flavanols (CF) ou de placebo (PL). *$: p<0.05 CF vs PL. *: p<0.05 en comparaison du moment précèdent.*

En conclusion, chez les athlètes, la consommation aigue de 903 mg CF augmente la capacité antioxydante au repos et pendant l'exercice, mais sans réduire la peroxydation lipidique (marqueur de stress oxydant), l'inflammation et la production de NO. La consommation aigue de CF n'augmente pas la performance physique, ni la récupération.

4.2 La prise de CF pendant une semaine augmente l'oxygénation préfrontale au repos et pendant l'exercice d'intensité modéré en normoxie et en hypoxie

Référence

Decroix L, Tonoli C, Lespagnol E, Balestra C, Descat A, Drittij-Reijnders MJ, Blackwell JR, Stahl W, Jones A, Weseler AR, Bast A, Meeusen R and Heyman E (2018) One week CF intake increases prefrontal cortex oxygenation at rest and during moderate-intensity exercise in normoxia and hypoxia. J Appl Physiol March 15

Introduction

En hypoxie, l'oxygénation du cerveau et des muscles est diminuée, ce qui peut affecter la performance physique. L'exercice en hypoxie induit un stress oxydant plus élevé qu'en normoxie. Les CF ont des capacités antioxydantes et stimulent la fonction vasculaire et le flux sanguin cérébral. Dans cette étude,

il s'agit d'étudier les effets d'une consommation de 530 mg CF (dont 100 mg d'épicatéchine) sur le stress oxydant, la biodisponibilité du NO et l'oxygénation préfrontale et musculaire pendant un exercice en normoxie et en hypoxie (14.3% oxygène normobare).

Méthodologie

Quatorze cyclistes masculins de très haut niveau (30.7±3.1 ans, VO_2max: 62.9±5.8mL.kg^{-1}.min^{-1}) participent à cette étude randomisée contrôlée en double aveugle. Les participants viennent au laboratoire à 4 occasions, après avoir consommé 6 jours de CF ou PL. Le 7ième jour, la dernière dose de CF ou PL est donnée au laboratoire, après les mesures de base, qui consistent en une mesure de la dilatation médiée par le flux sanguin (FMD) et en un prélèvement sanguin. Cent minutes après la dernière consommation de CF ou PL, les participants effectuent un premier exercice d'intensité modérée, à savoir 20 minutes à 45% du VO_2max,, suivi d'un exercice intense exhaustif (contre-la-montre de 20 minutes). L'oxygénation préfrontale et musculaire est mesurée en continu par NIRS. Nous mesurons également des biomarqueurs indirects de la production de NO et de sa biodisponibilité, le stress oxydant et la capacité antioxydante, dans les échantillons de sang prélevés.

Résultats

Le FMD est augmentée par la consommation de CF pendant 6 jours. L'exercice induit une augmentation de la peroxydation lipidique et de la capacité antioxydante, et ce d'autant plus pendant la situation d'hypoxie en comparaison à la normoxie. La prise de CF atténue la peroxydation lipidique induite par l'exercice, mais n'influence pas la capacité antioxydante. Au repos et pendant l'exercice modéré, l'oxygénation préfrontale et musculaire sont réduites par l'hypoxie. La consommation de CF améliore l'oxygénation au niveau préfrontal, mais pas au niveau musculaire lors de l'exercice modéré. Pendant le TT, i.e. l'exercice intense, l'hypoxie aggrave la baisse d'oxygénation préfrontale induite par l'exercice, sans moduler l'oxygénation musculaire. Par contre, la consommation de CF n'améliore plus l'oxygénation préfrontale et musculaire pendant cet exercice intense. La consommation de CF ne modifie pas les marqueurs indirects de production de NO (rapport arginine:citrulline) ou de biodisponibilité du NO (nitrite et nitrate).

Ainsi, la consommation de CF pendant une semaine peut augmenter la fonction endothéliale de repos, même chez des sportifs de très haut niveau, qui ont déjà une excellente fonction vasculaire en raison de leur entrainement physique quotidien. La consommation de CF pendant une semaine peut réduire les effets nuisibles de l'hypoxie sur l'oxygénation préfrontale au repos et pendant l'exercice modéré. Par contre, cet effet disparait pendant l'exercice intensif. La performance physique n'est pas augmentée par la consommation de CF.

4.3 Conclusions

Figure 37 présente le résumé des effets de l'exercice et de la consommation de CF sur production de NO et stress oxydant, mesuré *in vivo* dans les études de cette thèse doctorale.

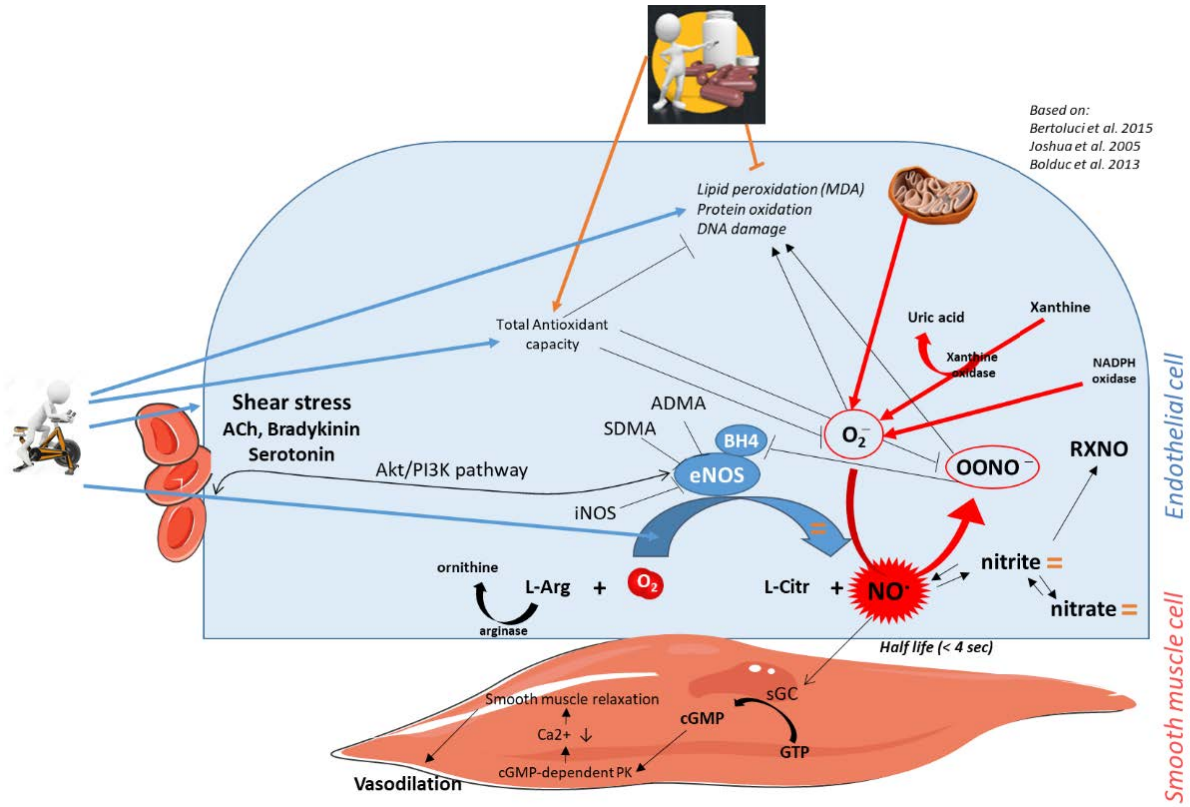

Figure 37. Effets de l'exercice (lignes bleues) et la consommation de cocoa flavanols (CF) (lignes oranges) sur la production de monoxyde d'azote (NO) et le stress oxydant, mesuré chez des sportifs très entraînés pendant les études de cette thèse doctorale. *Flèches pointues: augmentation ; flèches bloquées: inhibition; =: pas d'effet. La production de NO est augmentée par l'exercice, mais il n'y pas d'effet de la prise de CF. Les nitrites et les nitrates, 2 marqueurs indirects de la disponibilité en NO, ne changent pas pendant l'exercice et ne sont pas affectés par la consommation de 903 mg CF pendant 1 semaine. Pendant l'exercice intense, la capacité antioxydante plasmatique (TEAC) augmente, mais la peroxydation lipidique (malondialdehyde : MDA) s'amplifie aussi. La consommation aigue de 903 mg CF (196 mg d'épicatéchine) améliore la capacité antioxydante, mais n'atténue pas la peroxydation lipidique, donc le stress oxydant. La consommation de 530 mg CF (100 mg d'épicatéchine) pendant une semaine réduit la peroxydation lipidique induite par l'exercice, sans augmenter la capacité antioxydante plasmatique, en hypoxie et en normoxie. Remarque : les nitrite, nitrate, MDA et TEAC sont mesurés dans le plasma et non directement dans les cellules endothéliales, comme indiqué dans la figure. O2: anions superoxyde, L-Citr: L-citrulline, L-Arg: L-arginine, sGC: guanyl cyclase, GTP: guanosine triphosphate, cGMP: cyclic guanosine monophosphate, PK: protein kinase, Ca2+: calcium, iNOS: inducible NO synthase, SDMA: symmetric dimethylarginine, ADMA: asymmetric dimethylarginine, eNOS: endothelial NO synthase, O2·-: superoxide, BH_4: tetrahydrobiopterin, OONO-: peroxynitrite, Ach: acetylcholine.*

Nous avons montré que la consommation de CF, soit aigue, soit pendant 1 semaine, peut réduire le stress oxydant et/ou augmenter la capacité antioxydante pendant l'exercice exhaustif chez des sportifs très entraînés. Par contre, les CF n'ont pas d'effet ergogène chez des athlètes de ce niveau.

5. Conclusion générale

Nous pouvons conclure que la prise aigue de CF (196 mg d'épicatéchine) et la prise de CF sur une semaine (100 mg d'épicatéchin/jour) n'augmentent ni la performance cognitive, ni la performance physique chez des sportifs sains très entraînés. Ainsi, il ne semble pas recommandé de consommer des CF pour une aide ergogène. Par contre, la consommation de CF pourrait être considérée comme aide nutritionnelle pour ses effets stimulateurs de la fonction endothéliale et de la vasoreactivité cérébrale et pour réduire partiellement les effets nuisibles de l'hypoxie sur l'oxygénation cérébrale au repos et pendant un exercice modéré. De plus, nous avons montré que la consommation de CF peut aider le système antioxydant en limitant la production de ROS et en prévenant le stress oxydant induit par l'exercice exhaustif. Toutefois, des recherches complémentaires sont nécessaires pour examiner si, et dans quelles conditions, les capacités antioxydants des CF sont intéressants pour les athlètes de haut niveau. En effet, des quantités de ROS et de stress oxydant minimaux sont nécessaires dans le processus d'adaptation à l'entraînement. Ainsi, avant de s'engager dans une supplémentation en CF, il faut bien peser les avantages sur la fonction vasculaire et la vasoreactivité cérébrale contre les inconvénients éventuels des effets antioxydants. La figure 5 résume les résultats des études de cette thèse doctorale.

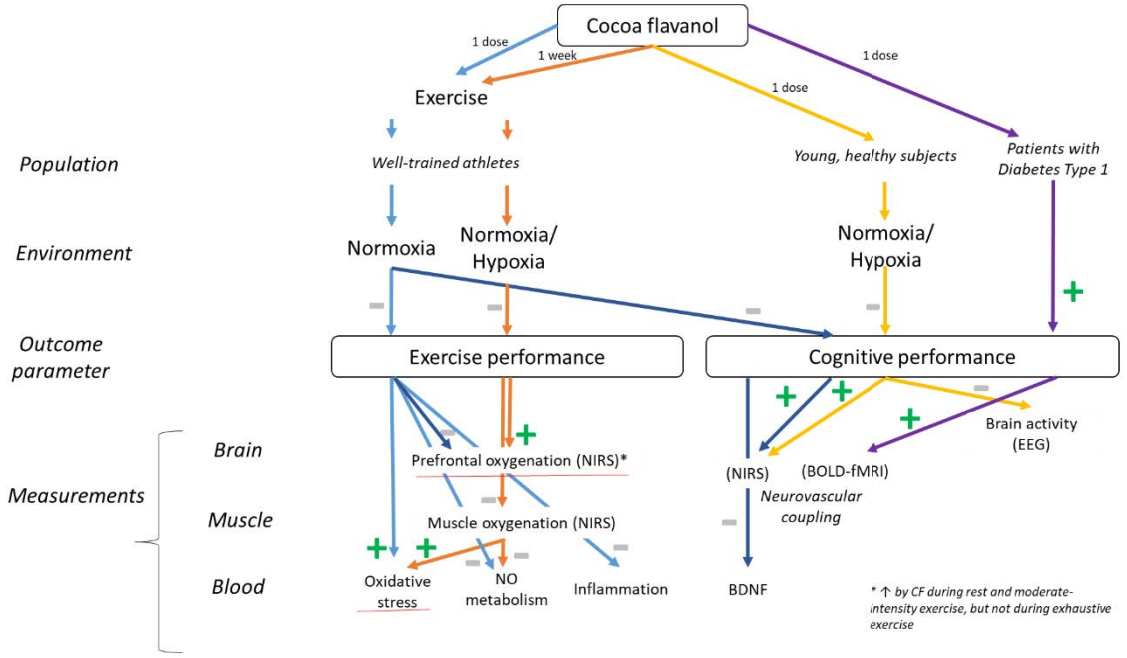

***Figure 38. Résumé des résultats de cette thèse doctorale**. Bleu clair : étude décrite au chapitre 6, blue foncé : étude décrite au chapitre 3, orange : étude décrite au chapitre 7, jaune : étude décrite au chapitre 4, violet : étude décrite au chapitre 5. +: effet positif des cocoa flavanol (CF); -: pas d'effet des CF ; ligne rouge : effet négatif d'hypoxie.. NIRS: spectroscopie dans le proche infrarouge; BOLD-fMRI : le signal dépendant du niveau d'oxygène sanguin- l'imagerie par résonance magnétique; BDNF : facteur neurotrophique dérivé du cerveau ; NO : monoxyde d'azote.*

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