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Effets d'une prise unique de flavanols du cacao sur la réactivité vasculaire périphérique chez des patients atteints de diabète de type 2

Anouk Tanghe

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Université de Lille

GHENT UNIVERSITY

Acute effects of cocoa flavanols on peripheral vascular reactivity in patients with type 2 diabetes mellitus

Anouk Tanghe

“ A chocolate a day, keeps the doctor away? ”

Acute effects of cocoa flavanols on peripheral vascular reactivity in patients with type 2 diabetes mellitus

Anouk Tanghe

THESE DE DOCTORAT

Co-tutelle de thèse Université de Gand – Université de Lille

Spécialité

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Anouk TANGHE

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Effets d'une prise unique de flavanols du cacao sur la réactivité vasculaire périphérique chez des patients atteints de diabète de type 2.

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Abbreviations

ACEi	Angiotensin converting enzyme inhibitor
ADA	American Diabetes Association
AGE	Advanced glycation end products
AHD	Antihypertensive drugs
ARB	Angiotensin receptor blocker
ATP	Adenosine triphosphate
BMI	Body mass index
BP	Blood pressure
C	(+)-Catechin
CF	Cocoa flavanols (flavanols extracted from the cocoa bean)
CVD	Cardiovascular disease
DM	Diabetes mellitus
EC	(-)-Epicatechin
eNOS	Endothelial nitric oxide synthase
$FADH_2$	Flavin adenine dinucleotide
FFA	Free fatty acids
FMD	Flow-Mediated dilation
GLcNAc	N-acetlyglucosamine
GLUT	Glucose transporter
HbA1c	Hemoglobin A1c
HDL	High density lipoprotein
LDL	Low density lipoprotein
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NF-kB	Nuclear factor kappa-light-chain-enhancer
NO	Nitric oxide
PKC	Protein Kinase C
NIRS	Near-Infrared spectroscopy
ROS	Reactive oxygen species
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
(*)	Is indicated when further explanations are described below

Chapter 1: Introduction

1. Diabetes mellitus

Diabetes mellitus (DM), represents a group of complex metabolic disorders with hyperglycemia as main feature. This hyperglycemia is caused by lack of insulin production, insufficient relative insulin action and/or resistance of target tissues to insulin [1].

Generally, DM is roughly divided into Type 1 (auto-immune loss of insulin production capacity, T1DM), and Type 2 DM (T2DM). The latter is identified by insulin resistance with mostly relative insulin deficiency and accounts for 90 – 95 % of worldwide DM cases [1].

Prevalence and burden

The international Diabetes Federation reported that the prevalence of DM in adults has more than tripled over the past 20 years making DM one of the fastest growing health challenges of the 21st century. In 2019, the estimated worldwide prevalence of adults with DM was 463 million, which is expected to increase to 579 million adults in 2030 and 700 million adults in 2045 [2]. In 2020, the Belgian prevalence of adults with DM was 6.8 % resulting in 561 200 cases [3]. Although there is a global increase in prevalence, more than 80 % of patients with T2DM live in low-to-middle-income countries [4]. As DM demands high health care costs (10% of global health expenditure), it places substantial socioeconomic pressure [5]. The annual global health expenditure on DM (direct costs) is estimated at USD 760 billion in 2019 and is expected to increase to USD 825 billion by 2030 [2]. In Belgium, in 2018, the direct and indirect costs of DM to the Belgian social security system accounted for 5.82 billion euros of which only 6 % is related to drug costs and 94 % is related to treatment of complications [6].

DM is also a very important risk factor for cardiovascular diseases (CVD). Based on a meta-analysis with 698 782 participants, an increased risk was demonstrated for ischemic stroke (hazard ratio 2.27; 95% CI: 1.95–2.65), coronary heart disease (hazard ratio 2.00; 95% CI: 1.83–2.19), and other deaths related to vascular disease (hazard ratio 1,73; 95% CI: 1,51–1,98) in patients with DM [7]. The Global Burden of Disease Study 2013 indicated a global increase in age-standardized mortality rates of 9 % from 1990 to 2013 due to DM. Moreover, DM is globally classified as the seventh major cause of diminished life expectancy within non-communicable diseases [8]. Furthermore, in 2015, DM was recognized as the sixth leading cause of disability [9]. In Europe, the mean age for T2DM diagnosis was around 62 years old; however, partly due to increasing prevalence of obesity, particularly in younger people, the mean age of diagnosis is decreasing, which exerts higher risks for cardiovascular and non-cardiovascular diseases and mortality [10-14].

Hence, DM is a complex rapidly increasing disease with high burden that poses a global challenge.

Pathophysiology of Type 2 diabetes

The name 'Diabetes Mellitus' originates from '*diabainen* (go through) *mellitus* (sweet)' illustrating the initial diagnostic characteristic, namely polyuria. Polyuria means excessive passing of urine, here sweet urine because of high sugar levels, through the kidneys [15]. It was only in around 1910 - 1920 that a relation between pancreas, insulin, and DM was discovered [16-18].

The pancreas is localized between the stomach and the small intestine and consists of 2 different types of glandular tissue: the endocrine cells excreting hormones into the bloodstream and exocrine cells excreting enzymes into the intestine, the digestive tract. β-cells (majority of cells in the islets of the pancreas) releasing insulin and amylin, α-cells producing glucagon, δ-cells secreting somatostatin, and F-cells generating pancreatic polypeptides are its major types of endocrine cells [19].

When serum glucose levels rise, insulin is released by the β-cells and causes transport of energy sources (carbohydrates, lipids, proteins) in the bloodstream towards organs for uptake, metabolism, and storage and so have anti-catabolic, systemic effects. Figure 1 illustrates direct and indirect insulin actions in white adipose tissue (anti-lipolysis, lipogenesis), liver (glycogen synthesis, anti-gluconeogenesis), and muscles [20]. Furthermore, insulin and amino acids as such inhibit protein breakdown and stimulate protein synthesis during whole-body and skeletal muscle protein metabolism [21-23].

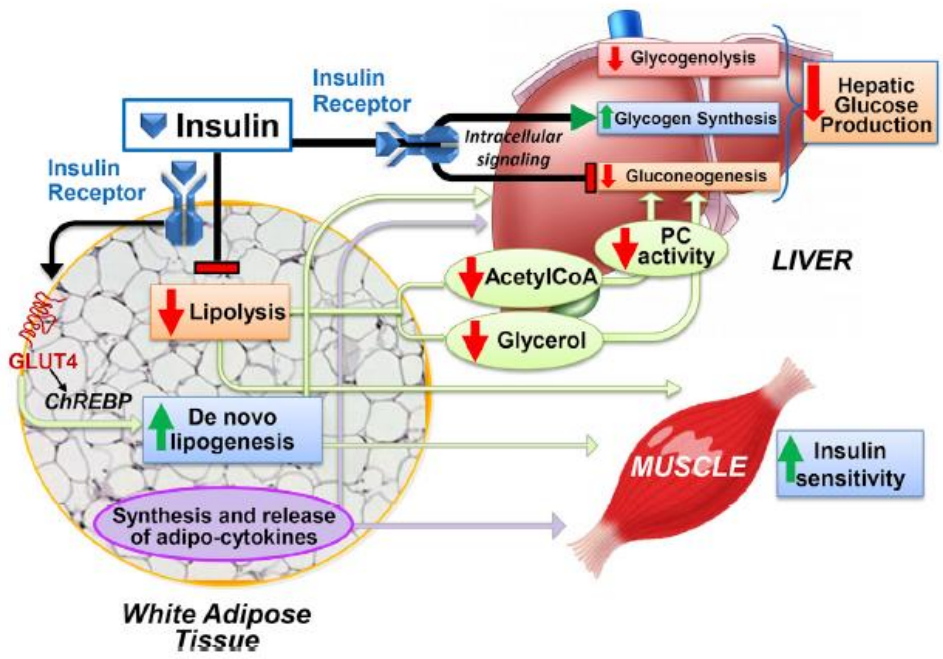

Figure 1: Systemic effects of insulin.
Black arrows indicate insulin direct effect. green arrows indicate insulin indirect effects; acetyl-CoA, acetyl coenzyme A; ChREBP= carbohydrate-responsive element binding protein; GLUT4= glucose transporter type 4; PC= pyruvate carboxylase [20].

Insulin has also vasoactive properties like production of nitric oxide (NO) causing vasodilation and production of endothelin-1 causing vasoconstriction with NO being the dominant insulin-stimulated vasomodulator [24]. Moreover, it has a great influence on the microcirculation, predominantly in the pre-capillary arterioles, by exerting vasodilation through relaxation of the vascular smooth muscle cells increasing microvascular perfusion. Hence, the capillaries have a high recruitment of insulin and so play the key role in the availability of insulin and glucose in body tissues [25, 26].

Intracellular, in the adipocytes and predominantly in the myocytes, insulin activates translocation of glucose transporter (GLUT)-4 towards the non-permeable lipid bilayer membranes leading to greater glucose transport, metabolism, and storage. Insulin-induced GLUT-4 translocation occurs via signaling pathways, especially the phosphatidylinositol 3-kinase. In short, as presented in Figure 2, when insulin is produced, it binds to its receptors on the surface of target cells and thus activates its intracellular tyrosine-kinase domain.

Upon this activation, the receptor phosphorylates various substrates, of which insulin receptor substrates 1 and 2 are the main substrates in fat and muscles cells. Tyrosine-phosphorylated insulin receptor substrate proteins attract more effector molecules like phosphatidylinositol 3-kinase, who target protein kinase B, also known as Akt (a serine/threonine-specific protein kinase), and the atypical protein kinase C isoform (the latter is not shown). These targets then translocate GLUT-4 towards the membrane [27, 28]. In addition, insulin leads to movement of GLUT-2 towards the membrane in hepatocytes, pancreatic β-cells, renal tubular cells, and enterocytes and accordingly increases glucose uptake in these cells (Figure 2) [28, 29]. Furthermore, insulin limits hepatic glucose formation (gluconeogenesis) and glucagon secretion, and decline serum free-fatty acid (FFA) levels by both restricted lipolysis and increased storage of FFA as triglycerides [28, 30].

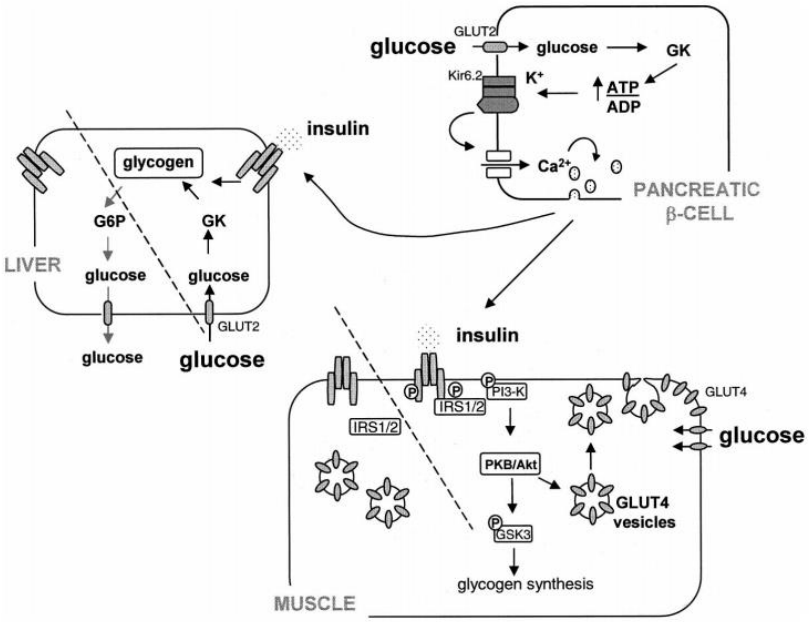

Figure 2: Glucose-induced Insulin signaling pathways regulating glucose uptake.
ADP= Adenosine diphosphate; Akt= serine/threonine kinase; ATP= Adenosine triphosphate; Ca^{2+}= calcium; G6P= glucose-6-phosphate; GK= glucose kinase; GLUT= glucose transporter; GSK3= glycogen synthase kinase 3; IRS= insulin receptor substrate; K^{+}= potassium; PI3-K= phosphatidylinositol 3-kinase; PKB= protein kinase B [29].

Hence, insulin plays a key role in whole body energy transport, metabolism, and storage. When one develops insulin resistance, a diminished sensitivity of the effector cells for insulin (*), higher insulin levels are needed to attain normal blood glucose values. If β-cells succeed in sufficient insulin production, a state of euglycemic hyperinsulinemia will occur; however, as long as insulin resistance remains present (e.g. due to obesity), the β-cells will be chronically stressed and might fail to compensate leading to chronic hyperglycemia, a constant excessive blood glucose level, hence T2DM [31-35]. Although both insulin resistance and β-cell dysfunction lead to the progression from normal glucose to impaired glucose to T2DM [33], their relative contribution can vary. Up to 13 years before diagnosis, abnormal insulin sensitivity can be detected [36].

Risk factors for the development of insulin resistance and β-cell dysfunction and so T2DM, are predominantly environmental factors like the Western life style including an increased unhealthy food consumption and a sedentary life [37-39]. However, also increasing age, sex, ethnicity, and genetic predisposition would be important contributors [40-44].

**Development of insulin resistance*

Insulin resistance is a very complex pathological condition containing varied components of which most are unresolved yet. Here, we only focus on 2 main aspects, namely (1) obesity and greater FFA levels and (2) impaired GLUT-4 expression and/or activation.

(1) Chronic overfeeding and especially high fat intake and food with a high glycemic index (a measure of the glycemic response to carbohydrate-containing foods) and a high glycemic load (a measure of both carbohydrate quality and quantity) [45], will overwhelm one's ability to safely store these nutrients (fat and carbohydrates) in the subcutaneous fat locations, hence evoking a 'fat spill over' and visceral and ectopic fat storage. Because of limited ability of fat storage in these visceral and ectopic tissues, metabolic stress and lipotoxicity arise causing organ-specific damage, like insulin resistance [44]. This metabolic stress is mainly initiated and resided in adipose tissue leading to abnormal adipocyte hypertrophy and insufficient proliferation and differentiation. Furthermore, this metabolic stress evokes a protective unfolded protein response in the endoplasmic reticulum activating inflammatory signaling pathways that can lead to inflammatory and modified metabolic responses. The stress-induced activation of c-Jun N-terminal kinase, for example, will activate activator protein-1 causing inflammation, but will also phosphorylate insulin receptor substrate 1 with serine and so impair the activation of the downstream phosphatidylinositol 3-kinase-dependent pathways [30, 46-48].

Since adipocytes of obese people are progressively less sensitive for the antilipolytic and storing effect of insulin, they release more FFA into the bloodstream. Increased FFA levels demands greater FFA oxidation, thereby increasing ROS production and subsequently creating even more inflammation, activation of c-Jun N-terminal kinas, and damage. Besides muscles, adipocytes, and liver, β-cells are mainly vulnerable to an excess flux of FFA. As β-cells have very few storage capacities for triacylglycerol there is a rather rapid accumulation of toxic lipid

metabolites, such as diacylglycerol and ceramide, which are shunted into damaging metabolic pathways (glucolipotoxicity) [30, 47-50].

Next to its metabolic and immunological functions, adipose tissue acts as an endocrine organ producing hormones, adipokines, that are positively (such as leptin, visfatin, tumor necrosis-α) or negatively (such as adiponectin) correlated with the amount of adipose tissue. The more adipose tissue, the more/ less adipokines will be present in plasma causing detrimental reactions like insulin resistance, inflammation, ROS, and atherosclerosis [47, 51].

In summary, obesity causes insulin resistance in adipose tissue, muscle, and liver (with stimulation of both gluconeogenesis [52] and glycogenolysis [53]), provokes β-cell failure, and increases oxidative stress, starting a vicious circle towards the development of T2DM [30]. (Figure 3)

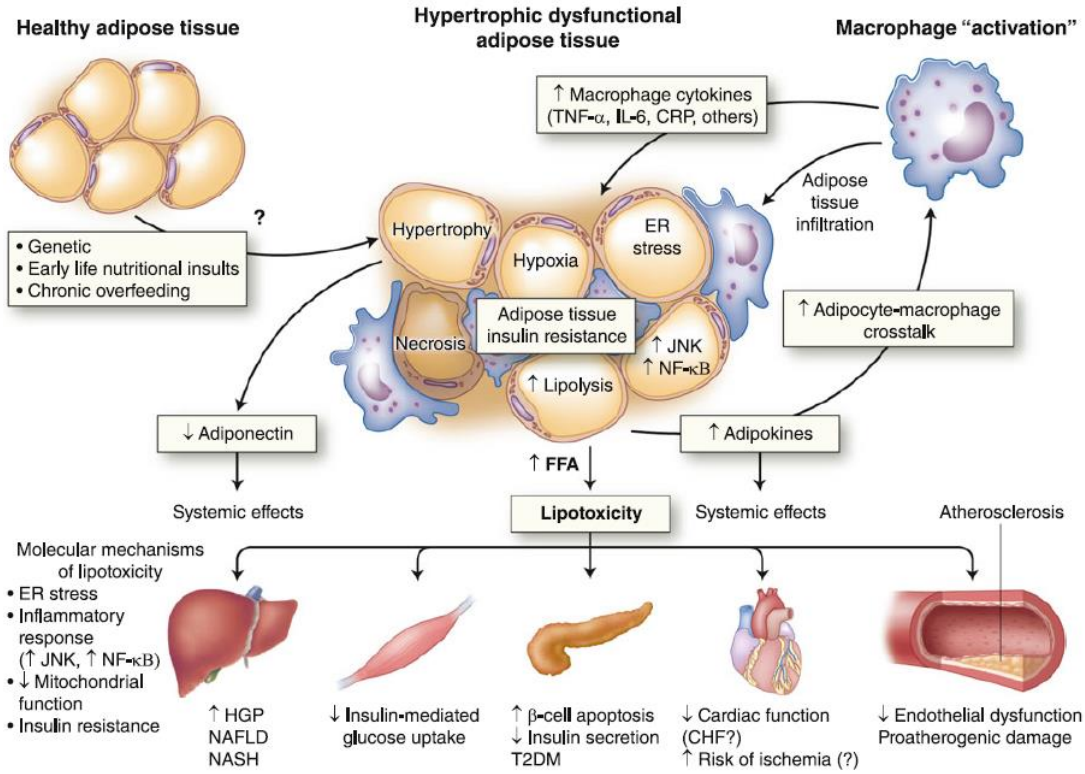

Figure 3: Lipotoxicity-induced metabolic dysfunction.
CHF= congestive heart failure; CRP= C-reactive protein; ER= endoplasmic reticulum; FFA= free fatty acids; HGP= hepatic glucose production; IL-6= interleukin-6; JNK= c-Jun N-terminal kinase; NAFLD= nonalcoholic fatty liver disease; NASH= nonalcoholic steatohepatitis; NF-κB= nuclear factor-κB; T2DM= type 2 diabetes mellitus; TNF-α= tumor necrosis factor-α [30].

(2) Furthermore, as GLUT-4 translocation is essential for glucose metabolism, a reduced expression of GLUT-4 or a lower expression of the insulin receptor substrate, an impaired insulin-signaling pathway regulating GLUT-4 translocation or a deficient translocation of GLUT-4 as such, are several causes for developing insulin resistance [28, 54]. As depicted in Figure 4, both insulin and muscle contractions induce translocation of GLUT-4 towards the membrane facilitating glucose uptake [55]. Muscle contractions provoke activation of protein

kinase C isoforms, AMP-activated protein kinases (because of increased adenosine monophosphate: adenosine triphosphate ratio), and several calcium dependent mechanisms to activate the GLUT-4 translocation [56].

Besides, physical activity also induces weight loss and could thereby prevent limited GLUT-4 expression in adipose tissue and impeded GLUT-4 translocation in skeletal muscles due to obesity [28].

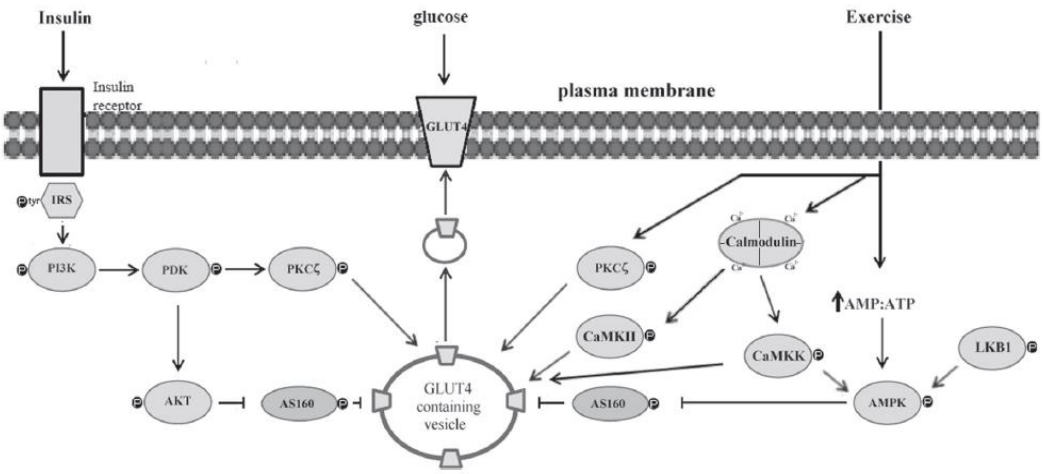

Figure 4: Glucose uptake in skeletal muscles through insulin signaling pathways and through muscle contractions.
Akt= protein kinase B; AMPK= AMP-activated protein kinase; AS160= 160 kDa protein; CaMKII= Calcium/calmodulin-dependent kinase II; CaMKK= calcium/calmodulin-dependent protein kinase kinase; GLUT4= glucose transporter – 4; IRS= insulin receptor substrate; LKB1= serine threonine kinase; PDK= phosphoinositide-dependent kinase; PI3K= phosphatidylinositol 3-kinase; PKCζ= protein kinase C isoform [56].

Diagnosis of type 2 diabetes

Based on the criteria of the American Diabetes Association (ADA), a person is diagnosed with DM if one of the following criteria is fulfilled [1]:

(i) HbA1c (glycated hemoglobin) ≥ 6.5%

(ii) Glucose ≥ 126 mg/dL (7,0 mmol/L) in fasting condition (minimal 8 hours)

(iii) Glucose ≥ 200mg/dL (11,1 mmol/L) during an oral glucose tolerance test

(iv) A random glucose ≥ 200 mg/dL (11,1 mmol/L) with classic symptoms of hyperglycemia or hyperglycemic crisis (polyuria, polydipsia, weight loss, polyphagia, and blurred vision)

Although the ADA formulated clear thresholds for diagnosis (as listed above), since both insulin and glucose levels increase with increasing insulin resistance, the development of DM is a continuous process making the formulated thresholds (cut-offs) relatively arbitrary [32]. An alternative classification system based on 6 variables [i.e. age at diagnosis, body mass index

(BMI), auto-antibodies (i.e. glutamate decarboxylase antibodies), glycemic control, and homeostasis model assessment of β-cell function and surrogates of insulin resistance] was recently set up as a start towards a more precise, clinically valuable stratification. This alternative classification underlines the heterogeneity within DM, especially T2DM, nevertheless, is not (yet) in use nor approved in the DM medicine [57, 58].

Diabetic vascular complications

Patients with DM are at high risk for atherosclerotic damage impeding the most important functions of the arterial tree: (1) delivery of blood to the organs and (2) ensuring a continuous flow of this blood through a dampening/ cushioning effect and thus reducing exposure of the arterial tree to the cardiac pulsatile energy [59].
Two phenotypes of the atherosclerotic damage are atherosclerosis and arteriosclerosis. Atherosclerosis is a regional abnormality causing arterial stenosis/ obstruction and so organ ischemia. It starts in the intima, the inner layer of the vessels, containing the endothelial cells (Figure 5). The endothelium is the major regulator of vascular homeostasis, which induce several vasoprotective effects (e.g. vasodilation, inhibition of inflammatory responses, and suppression of smooth muscle cell growth) mainly mediated by NO production. Hence, a deficit of NO production or activity leads to endothelial dysfunction, an early marker of atherosclerosis [60].
Arteriosclerosis is rather systemic and is caused by arterial stiffening, which develops with reduction in the elastic fibers, increase in the collagen content, and proliferation and hypertrophy of the vascular smooth muscles cells (Figure 5). Arterial stiffening increases the cardiac afterload, decreases the diastolic blood supply causing an impaired coronary blood supply, and exerts microvascular damage because of high pulsatile flow in the small vessels [59].

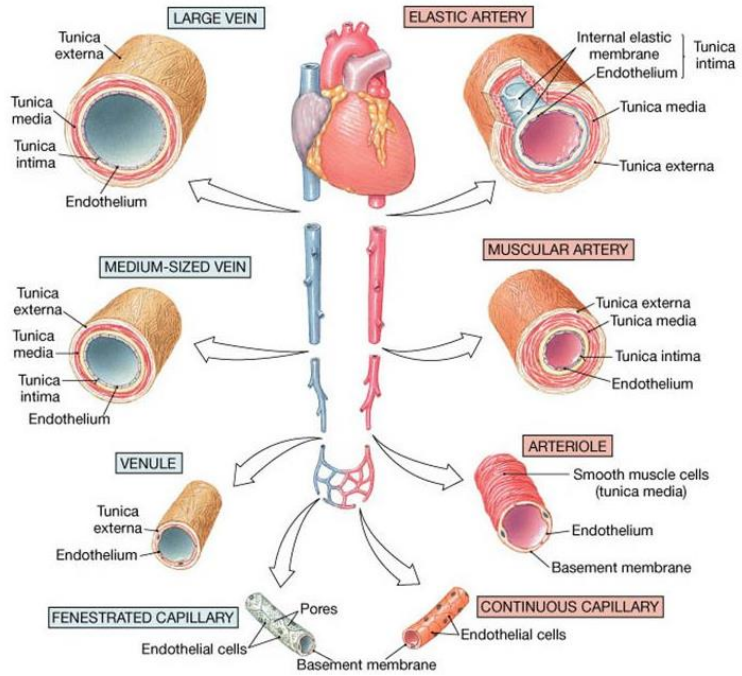

Figure 5: Presentation of the vascular tree and vascular layers [61]

In the following paragraphs, I will explain how DM may cause/ accelerate the development of atherosclerotic damage:

Chronic hyperglycemia provokes long-term dysfunction, failure, and even damage of varied organs like blood vessels, heart, kidneys, eyes, and nerves [1, 38]. Diabetic vascular complications are frequent in patients with T2DM with 50 % having microvascular (retinopathy, nephropathy, and neuropathy) and 30 % having macrovascular (cardiovascular, cerebrovascular, and peripheral artery disease) complications [39, 62, 63]. Endothelial dysfunction would be a major contributor for these complications. However, also dysfunction of the vascular smooth muscle cells, predominantly in the pre-capillary arterioles, should be regarded as this would impair microvascular perfusion, hence glucose and insulin delivery in body tissues [64]. The onset of diabetic vascular complications forms an important limitation of quality of life and increases the global burden of T2DM in terms of health care costs (more than 50 % of direct global health care costs accounts for management of complications [2]), morbidity, and even mortality [65-67]. An adequate prevention or treatment for T2DM as well as for these vascular complications are thus essential.

The 'unified hypothesis' as introduced by Brownlee [68] proposes that overproduction of ROS by the mitochondrial electron transport chain (*) plays the key role in the development of these vascular complications. The mitochondrial ROS overproduction in patients with DM is triggered by intracellular hyperglycemia for microvascular complications and triggered by increased FFA oxidation, induced by insulin resistance, for macrovascular complications [68]. Increased levels of ROS lead to the activation of poly-ADP-ribose polymerase to counter hyperglycemia-induced harm of DNA. Subsequently, poly-ADP-ribose polymerase could inhibit glyceraldehyde 3-phosphate dehydrogenase, which in turn activates the upstream accumulation of glycolytic metabolites that are diverted into 5 pathways, also called the non-oxidative glucose pathways [68-70] (Figure 6):

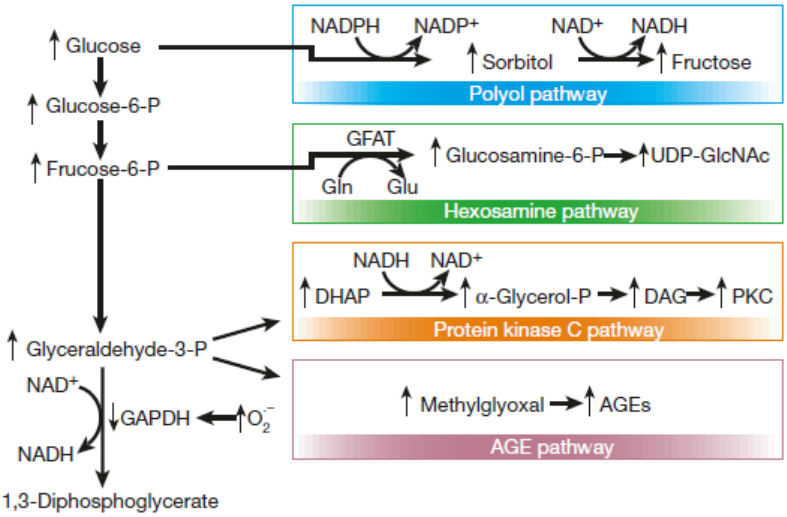

Figure 6: Non-oxidative glucose pathways.

AGE= advanced glycation end product; DAG= diaglycerol; DHAP= dihydroxyacetone phosphate; GAPDH= glyceraldehyde-3-phosphate dehydrogenase; GFAT= glutamine:fructose-6-phosphate amidotransferase; NAD(P)H= Nicotinamide adenine dinucleotide (phosphate); $O_2^{\bullet-}$= superoxide; PKC= protein kinase C; UDP-GlcNac= uridine diphosphate N-acetylglucosamine [71].

(1) Greater flux through the polyol pathway (Figure 7). The increased flux elevates the consumption of NADPH and so lowers concentrations of glutathione, an important intracellular antioxidant, and reduces the synthesis of NO [72]. Furthermore, stimulation of this pathway has been regarded as the main source of NADH/NAD^+ redox imbalance increasing oxidative stress [73, 74], decreasing sirtuins activity, and possibly also upregulating poly-ADP-ribosylase activity. As sorbitol and fructose are main products of the polyol pathway, their production will also accumulate which can evoke osmotic stress and might lead to increased AGE formation respectively [75-77].

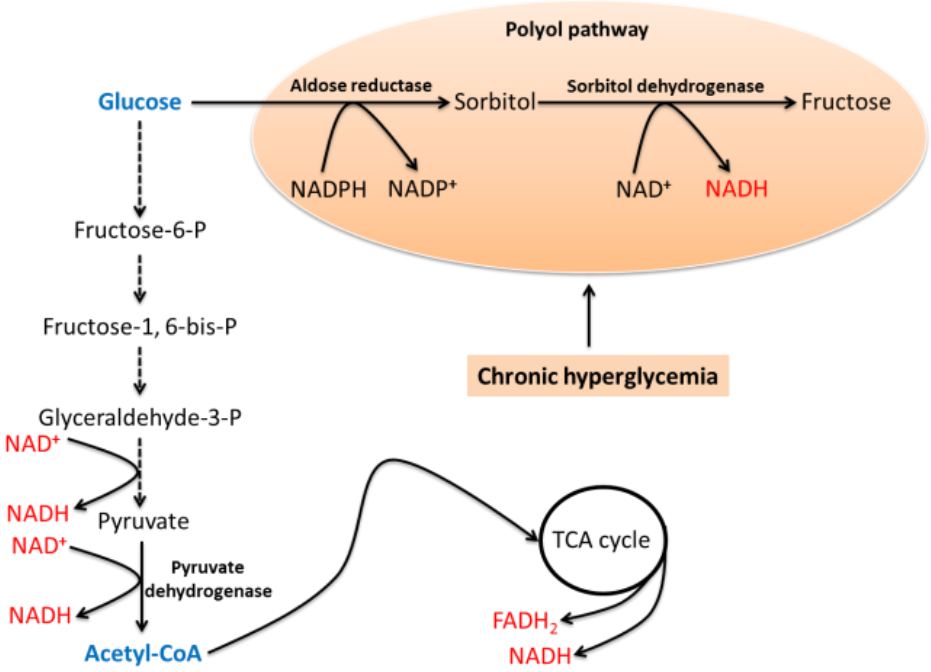

Figure 7: The polyol pathway

$FADH_2$= flavin adenine dinucleotide; GFAT= glutamine:fructose-6-phosphate amidotransferase; NAD(P)H= Nicotinamide adenine dinucleotide (phosphate); TCA= tricarboxylic acid [78].

(2) Boosted activity of the hexosamine pathway provoking *O*-N-acetylglucosamine (*O*-GlcNAc)-modification on serine/threonine residues of certain nuclear and cytoplasmatic proteins (Figure 8). Higher flux through this hexosamine pathway results in higher levels of Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), which is a substrate for the glycosylation of essential intracellular modulators like transcription factors, and thus may affects expression of certain genes that control cardiovascular function. More specifically, *O*-GlcNAcylation of specificity protein 1 can trigger activation of transforming growth factor-β1 in arterial endothelial cells and plasminogen activator inhibitor-1 in both arterial endothelial and vascular smooth muscle cells [79, 80]. *O*-GlcNAcylation of eNOS proteins constrains eNOS activity in arterial endothelial cells and accelerate atherosclerosis formation through lipid accumulation, endoplasmic reticulum stress, and higher expression of inflammatory genes [81-85]. Furthermore, nuclear *O*-GlcNAcylation could diminish sarcoplasmic/endoplasmic Ca^{2+}-ATPase expression impairing myocardial contractility and thus impacting cardiac myocyte

function [86]. It is also proposed that this pathway may induce oxidative stress itself and inhibit fatty acid β-oxidation via acetyl-CoA carboxylase-β induction [87-90].

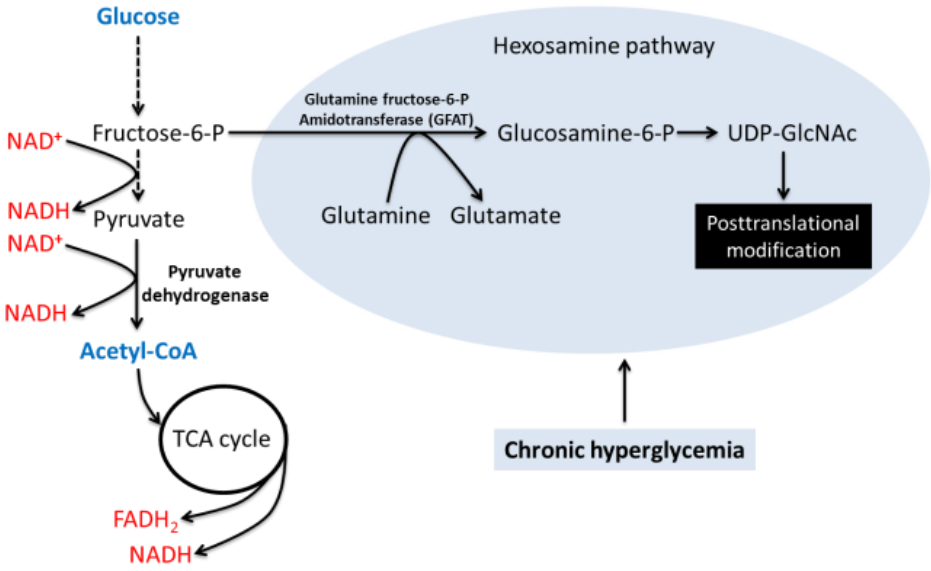

Figure 8: The hexosamine pathway

$FADH_2$= flavin adenine dinucleotide; NAD(P)H= Nicotinamide adenine dinucleotide (phosphate); TCA= tricarboxylic acid; UDP-GlcNac= uridine diphosphate N-acetylglucosamine [78].

(3) Increased formation of diacylglycerol, hence activation of protein kinase C (PKC) (Figure 9). Activation of PKC isoforms, especially β1/2 and δ, may induce several vascular abnormalities: A decrease in endothelial NO synthase (eNOS) activity and an increase in endothelin-1 expression as well as a raise in cytosolic phospholipase A2 inhibiting Na^+-K^+-ATPase, all leading to increased contractility and decreased vascular flow. Furthermore, an elevation of vascular endothelial growth factor creating an increased permeability, angiogenesis, and cell turnover. Higher levels of transforming growth factor-β1 causing thickening of the basement membrane, extracellular matrix expansion, and cardiomyopathy. Also, greater plasminogen activator inhibitor-1 reducing fibrinolysis and greater nuclear factor kappa-light-chain-enhancer (NF-kB) provoking a pro-inflammatory gene expression. Moreover, PKC could increase levels of nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidases eliciting even more ROS production. Besides, it is demonstrated that PKC induced vascular pathologies could also be provoked by changing insulin's actions on blood vessels [91-93].

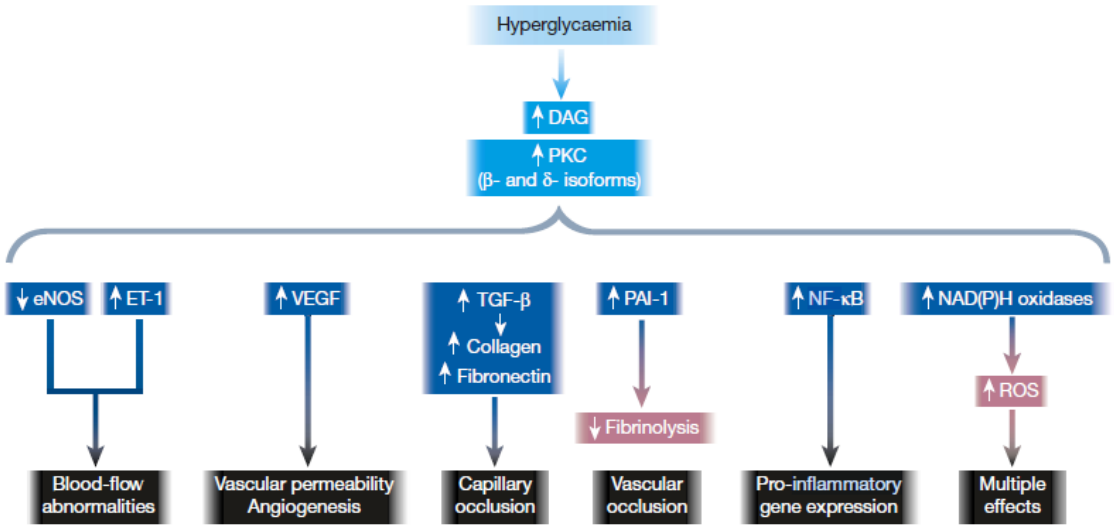

Figure 9: Consequences of hyperglycemia-induced activation of protein kinase C (PKC)
DAG= diacyglycerol; eNOS= endothelial nitric oxide synthase; ET-1= endothelin-1; NAD(P)H= Nicotinamide adenine dinucleotide (phosphate); PAI-1= plasminogen activator inhibitor-1; PKC= protein kinase C; ROS= reactive oxygen species; TGF-β = transforming growth factor-β; VEGF= vascular endothelial growth factor [71].

(4) Accelerated formation of intracellular advanced glycation end products (AGEs) as well as (5) higher expression of their receptors and the activation ligands (Figure 10). AGEs may modify structure and function of target proteins, like high density lipoproteins (HDL), low density lipoproteins (LDL), collagen, serum albumin, and several intracellular proteins such as proteins regulating gene transcription. AGEs on the extracellular vascular matrix may elicit endothelial dysfunction through decreasing vascular elasticity and NO bioavailability [94]. As already mentioned, NO is important for a general vascular health. It controls vascular tone as it elevates intracellular cyclic guanosine monophosphate levels in vascular smooth muscles cells hence induce vasorelaxation, but also regulates myocardial contractility, leukocyte adhesion and migration, platelet adhesions and aggregation, and smooth muscle cell proliferation [95-98]. Furthermore, AGEs could also trigger vasoconstriction, e.g. by enhanced expression of endothelin-1, a potent vasoconstrictor [99]. Binding of AGEs and its receptors could generate oxidative stress evoking platelet and macrophage activation, vascular inflammations, thrombosis, and accelerated atherosclerosis [100-104].

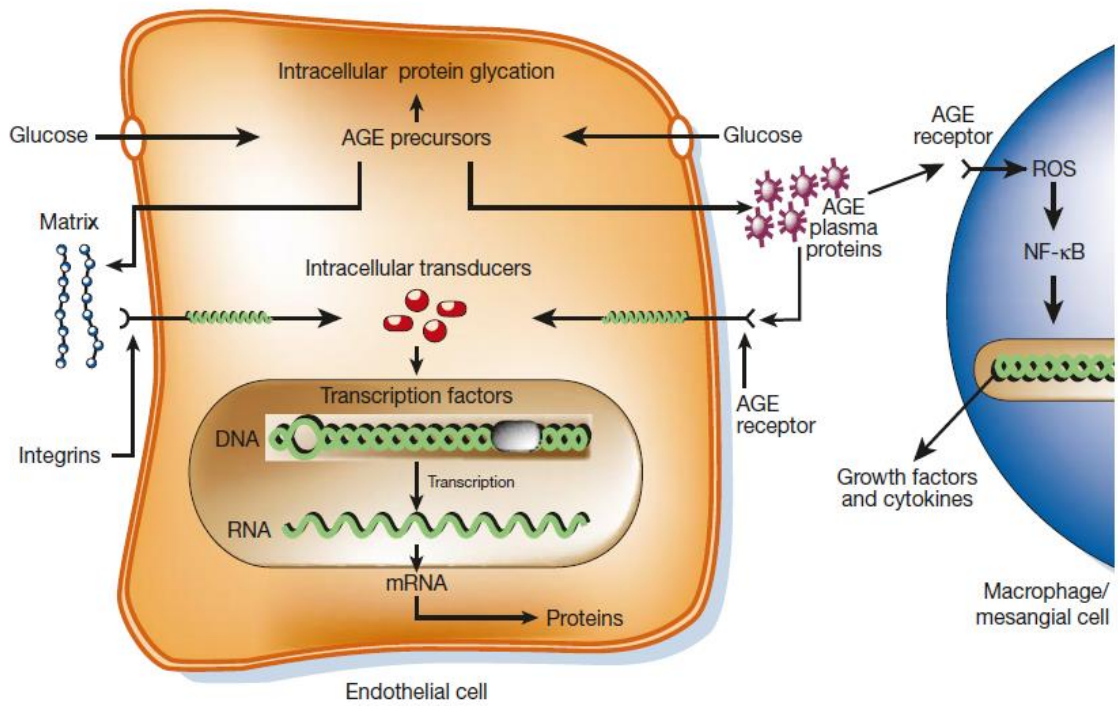

Figure 10: Mechanisms by which intracellular production of advanced glycation end-product (AGE) precursors damages vascular cells

AGE= advanced glycation end-product; DNA= Deoxyribonucleic acid; mRNA= messenger ribonucleic acid; NFκB= nuclear factor- κB; ROS= reactive oxygen species [71].

We did not describe all proposed detrimental effects of the non-oxidative glucose pathways into little detail and insights in these pathways are still increasing through additional research on animals and humans. However, it is clear that oxidative stress and the resulting deficit of NO, which decreases endothelial-mediated vascular relaxation and increases vascular stiffness [105-107], play a key role in the onset of diabetic vascular complications.

Besides, hyperglycemia also increases arginase activity and so decreases availability of L-arginine and causes eNOS uncoupling increasing formation of $O_2^{\bullet -}$, which will rapidly bind with NO to form peroxynitrite, a strong oxidant. These mechanisms also contribute to endothelial dysfunction in T2DM [108, 109]. (Figure 11)

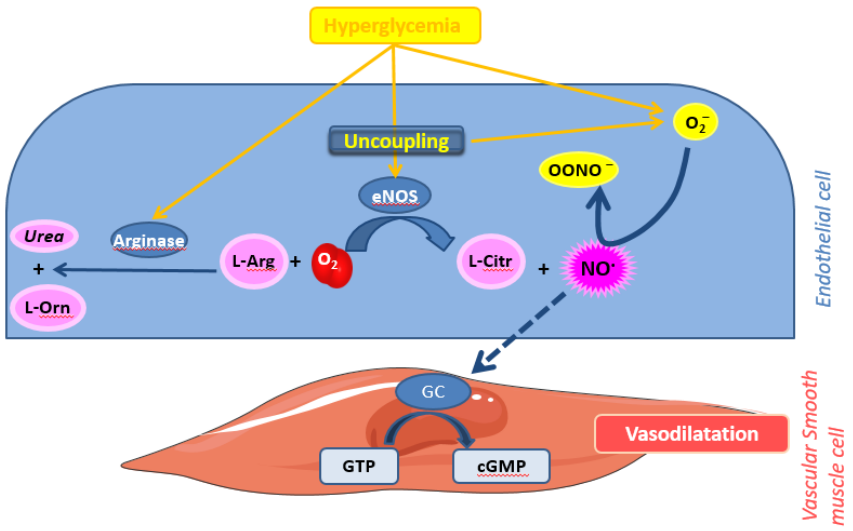

Figure 11: Impact of hyperglycemia on vasodilation.

cGMP= cyclic guanosine monophosphate; eNOS= endothelial nitric oxide synthase; GC= guanylate cyclase; GTP= guanosine triphosphate; H_2O_2 = hydrogen peroxide; L-arg= L-arginine; L-citr= L-citrulline; L-orn= L-ornithine; O_2= oxygen; $O_2^{\bullet-}$= superoxide: $ONOO^-$= peroxynitrite.

Furthermore, impaired autonomic nervous system activity have been identified in patients with T2DM. Sympathetic overactivity, related to plasma insulin and glucose levels, triggers the renin-angiotensin-aldosterone system, stimulates sodium reabsorption, evokes platelet activation, elicits insulin resistance, increases heart rate and stroke volume, and induces vasoconstriction and peripheral vascular resistance, which all contribute to hypertension (present in more than 60 % of all patients with T2DM [110]) and atherosclerosis, hence greater cardiovascular risk [111-114].

Since hyperglycemia activates the 5 interacting non-oxidative glucose pathways that further fuel its own activation through generating of even more oxidative stress, since hyperglycemia impairs antioxidant defense mechanisms via multiple interacting pathways, since hyperglycemia increases arginase activity and provokes eNOS uncoupling, and since hyperglycemia induces sympathetic overactivity, it is suggested that hyperglycemia creates a vicious metabolic cycle inducing diabetic vascular complications [69, 112].

**Mitochondrial overproduction of reactive oxygen species*

The central role of glucose in cellular homeostasis, namely providing enough energy through adenosine triphosphate (ATP) formation, requires a continuous availability of glucose and is maintained via glucose production by liver (gluconeogenesis) in fasting state and via peripheral glucose uptake after oral glucose ingestion (meal/ drinks) [28]. The metabolism of glucose starts with the transformation of glucose into pyruvate generating nicotinamide adenine dinucleotide (NADH) (Glycolysis in the cytoplasm). Pyruvate can be transformed into lactate, a substrate for gluconeogenesis in liver, or into CO_2 and H_2O, hereby generating 4 molecules of NADH and 1 molecule of reduced flavin adenine dinucleotide ($FADH_2$) (Tricarboxylic acid cycle, also known as Krebs cycle, in mitochondria). NADH and $FADH_2$

provide electrons that can be transported through the inner membrane-associated enzyme complexes I, II, III, and IV of the electron transport chain (Figure 12). This electron transport system ensures a precise regulation of ATP levels: Electrons are transported from left to right. Part of the energy of these electrons is used for pumping protons through complexes I, III, and IV, creating a voltage across the mitochondrial membrane. The energy of this generated voltage gradient drives ATP formation via ATP synthase. (Oxidative Phosphorylation in mitochondria). To ensure adequate ATP generation, uncoupling proteins can bleed down the created voltage gradient through heat generation [68].

In postprandial state, higher levels of glucose are metabolized generating higher levels of NADH and $FADH_2$, and thus higher levels of electrons that passes through the electron transport chain. Hence, the voltage gradient increases. When a critical threshold is reached, complex III impedes electron transport so that electrons accumulate in coenzyme Q. Coenzyme Q then donates electrons to molecular oxygen (O_2) resulting in superoxide ($O_2^{\bullet -}$, ROS) formation.

Although a certain amount of ROS is required for metabolic purposes, too many ROS formation causes harm. Intrinsic protective mechanisms include manganese superoxide dismutase, which is the mitochondrial form of superoxide dismutase and scavenges generated free radicals via the following chemical reaction [71, 115, 116]:

$$Mn^{3+} + O_2^{\bullet -} \leftrightarrow Mn^{2+} + O_2$$
$$Mn^{2+} + O_2^{\bullet -} + 2\ H^+ \leftrightarrow Mn^{3+} + H_2O_2$$

In addition, in normal conditions, a feedback loop based on crosstalk between β-cells in pancreas and insulin-sensitive tissues keeps glucose levels within a narrow range [37]. Besides the explained enzymatic antioxidant system, vitamin C, vitamin E, and α- and β-carotene are examples of important non-enzymatic dietary antioxidants that counter increasing ROS levels [117].

In diabetic cells with chronic hyperglycemic states, there is a chronic increased flux through the Krebs cycle generating a lot of NADH and $FADH_2$ and consequently pushing continuously more electrons into the electron transport chain. Here, the capacities of uncoupling proteins and manganese superoxide dismutase will be exceeded increasing oxidative stress.

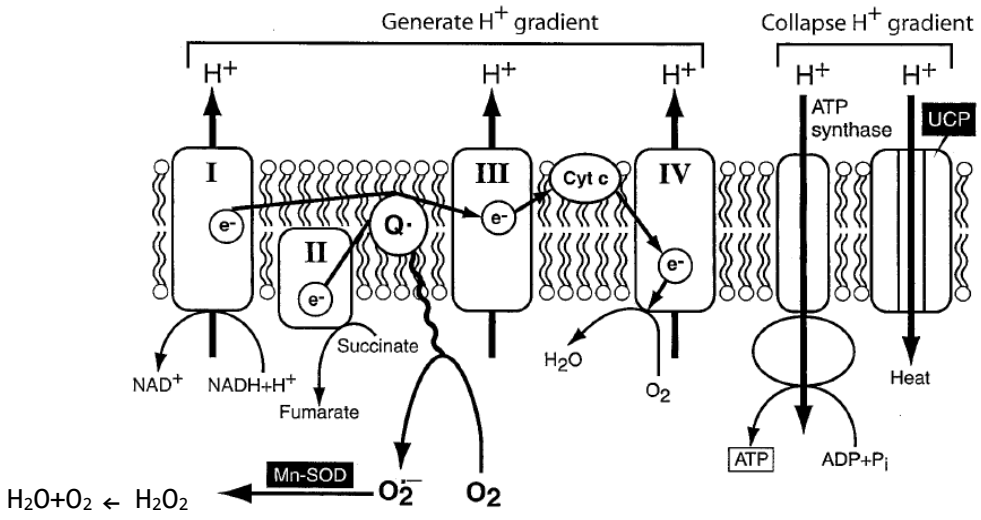

Figure 12: Schematic presentation of electron transport chain.
ATP= Adenosine triphosphate; e^- = electron; H^+= proton; Mn-SOD= manganese superoxide dismutase; NADH= nicotinamide adenine dinucleotide; O_2= molecular oxygen; $O_2^{\bullet-}$= superoxide, reactive oxygen species; Q^-= coenzyme Q; UCP= uncoupling proteins, which may control the voltage gradient to generate heat as a way of keeping the rate of ATP generation constant [68].

Management of type 2 diabetes

The management of T2DM involves a pharmacological and a non-pharmacological approach.

The non-pharmacological approach, the only causal therapy, entails lifestyle modifications and is the first-line strategy for both prevention and treatment of T2DM [39, 118]. Physical inactivity, unhealthy food consumption, and overweight/ obesity are major modifiable risk factors for developing and treating T2DM.

In general, previous research indicated that physical activity, defined as any bodily movement generated by skeletal muscles leading to energy expenditure [119], would lower genetic susceptibility to higher central pressure augmentation [120]. In addition, it has been reported that exercise, defined as planned, structured, and repetitive physical activities to improve or maintain the physical fitness [119], potentially upregulates eNOS, enhances arterial compliance, improves cardiac autonomic function, and mitigates age-related reductions in central arterial compliance [121-123]. As explained, next to insulin, muscle contractions cause translocation of GLUT-4, which is mandatory for an adequate glucose metabolism [54]. Therefore, physical activity is once more important in obese people as they are progressively less sensitive to insulin actions. In patients with DM, aerobic exercise, resistance exercise, combination of both, and also interval training have beneficial impact on insulin sensitivity, endothelial function, and vascular compliance [124, 125]. The World Health Organization guidelines on physical activity (2020) stated that physical activity improves mental and cognitive health, sleep, amount of adipose tissue, but also decreases incident of hypertension, T2DM, cancer, and cardiovascular disease, and lowers all cause-mortality. They indicated that

these beneficial effects are obtained if adults perform regular physical activity and do at least 150 – 300 minutes of moderate-intensity aerobic physical activity or at least 75 – 150 minutes of vigorous-intensity aerobic physical activity or an equivalent combination of moderate- and vigorous-intensity activity throughout the week. Also muscle-strengthen activities of major muscles groups at moderate or higher intensity on at least 2 days a week are recommended for additional health benefits [126]. ADA recommends patients with T2DM to perform physical activity for at least 150 minutes/ week, preferably both aerobic and resistance exercise training [125]. However, these quantifications of regular physical activity are arbitrary. Several studies demonstrate beneficial health effects with only small bouts of physical activity [127-130]. Hence, the main message, some physical activity is always better than no physical activity. (Figure 13)

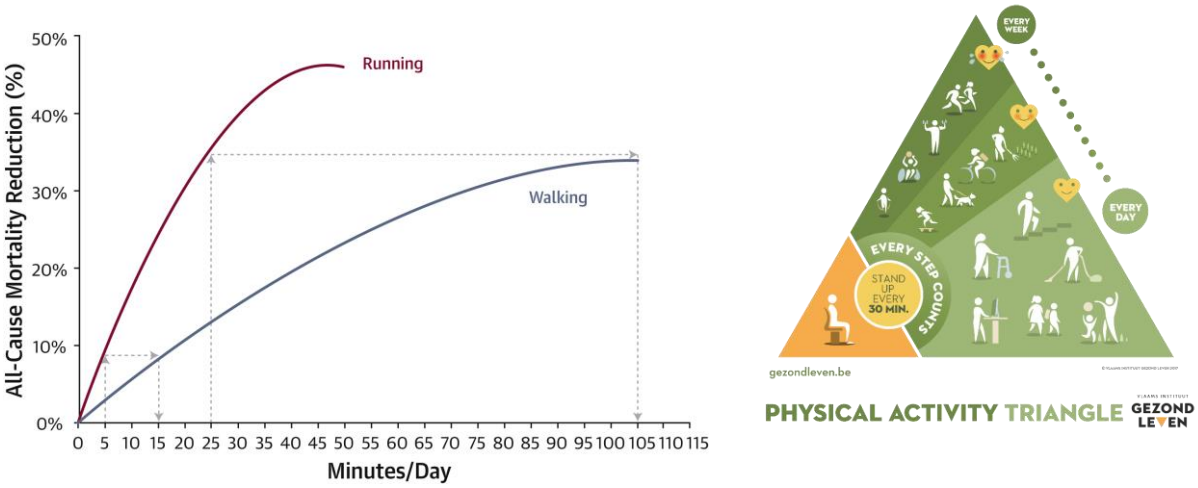

Figure 13: Left: Relationship all-cause mortality and daily physical activity (walking compared to running) [130]. - Right: The World Health Organization guidelines were translated into a Flemish physical activity triangle [131] .

Physical activity is also associated with weight loss and more importantly an altered body composition, which in turn provokes further health benefits. Weight loss is proposed to lower arterial stiffness and hypertension and to protect against insulin resistance [132-136]. Not only weight loss as such, but also a healthy food intake are mandatory for protection against DM and its complications. Although in the past DM-specific food recommendations to the treatment and prevention of DM were setup [137, 138], the composition of one's 'ideal' diet varies. As recently reported by the ADA, there is no single 'magic' diet for patients with DM since everyone's body reacts differently to varies types of foods and diets [139]. The ADA formulated a Nutrition Consensus Report [140], in which over 600 publications about diets or eating patterns in patients with DM were reviewed. The main conclusion here was to encourage the patients to discover oneself (together with their care provider) what fits and supports them best to manage their blood sugar. Some highlights from this consensus report indicated that independent of chosen type of diet or eating pattern, ensure to include many non-starchy vegetables, limit refined grains and added sugars, and choose whole, minimally processed foods. Furthermore, they advised 'food swaps' like changing foods high in saturated fats to foods predominantly containing unsaturated fat, e.g. olive oil instead of butter. The

percentage of calories from carbs, fat, and proteins as well as the amount of carbs as such have to be individualized.

Each individual food pattern should be discussed with their care provider to avoid hypo- or hyperglycemia, to reduce weight and maintain this reduction, and to improve cardiovascular health [140]. A well balanced diet with enough fruits and vegetables should be recommended. Previous studies that focused on the antioxidant properties of fruits and vegetables examined β-carotene, vitamin C, and vitamin E, but, it is suggested that main antioxidant sources are provoked by other compounds, like flavonoids, previously designated as vitamin P [141-143], part of polyphenols [144]. Polyphenols have antioxidant properties and the ability to influence insulin sensitivity, endothelial function, inflammatory mediators, and fat and carbohydrate metabolism and so have promising health benefits for patients with T2DM [145, 146]. Recommendations for herbal and micronutrient supplementations are not yet provided because of their high variability that complicates unambiguous research [140].

If the lifestyle approach is not enough to reach target values, which are mostly below 7.5 % for HbA1c, however patient-dependent, medical treatment is required. The pharmacological approach focuses on reducing and maintaining glucose levels as close to normal values and can be divided in oral drugs, like biguanides (e.g. metformin), sulfonylureas (e.g. gliclazide), dipeptidyl peptidase 4 inhibitors (e.g. alogliptin), and sodium-glucose co-transporter 2 inhibitors (e.g. canagliflozin) and injectable drugs, like rapid-, intermediate- or long-acting insulin (e.g. insulin aspart, isophane insulin, insulin zinc-crystalline respectively) and incretins, more specifically glucagon-like-peptide-1 receptor agonists (e.g. exenatide) [37] (Table 1).

Table 1: Pharmacological treatment for T2DM [37, 147].

Mechanism of action	Medication
Increasing insulin sensitivity	Biguanides, Thiazolidinediones
Increasing insulin secretion	Sulfonylureas, Glinides
Reducing glucose reuptake	Sodium-glucose co-transporter 2 inhibitors
Increasing insulin production and decrease glucagon secretion	Dipeptidyl peptidase 4 inhibitors, Incretins
Exogenous insulin	Rapid-, intermediate- or long-acting insulin

However, ‘glycemic memory’, also called ‘legacy effect’, expresses that treatment solely concentrating on glycemic values does not prevent the onset of diabetic vascular complications. ‘Glycemic memory’, introduced via the Diabetes Control and Complications Trials in patients with T1DM [148] and later also reported in patients with T2DM via the UK prospective Diabetes Study [149], indicates that hyperglycemia causes long-term epigenetic changes in the promotor of the NF-kB p65 subunit, which is an important determinant in atherosclerotic processes [150], leading to higher expression of p65 gene and p65-dependent proinflammatory genes. Hence, it underlines the detrimental long-term effects of even short-term, HbA1c-independent, hyperglycemic events. As normalization in mitochondrial ROS production could counter these epigenetic changes, there is high need for treatment focusing on antioxidative mechanisms [70, 151].

Furthermore, the pharmacological approach involves therapy that fights detrimental effects of one of the 5 activated non-oxidative glucose pathways. For example, research showed a decrease in AGE formation through alagebrium chloride, an AGE crosslink breaker, and suppression of AGE receptors via simvastatin [152-155]. Furthermore, aldose reductase inhibitors, like carboxylic acids (epalrestat, zopolrestat) and hydantoins (sorbinil), might be used to fight abnormal polyol pathway activation [156-158]. Another example is ruboxistaurin, a selective inhibitor of PKC-β2, which might add benefits to the already determined treatments for retinopathy and nephropathy [69, 159, 160].

Also, antihypertensive therapy that targets both arterial stiffness and blood pressure (BP) are of special interest and show greatest survival. It has been reported that more than 60 % of all T2DM patients have hypertension [110]. The presence of DM in combination with hypertension substantially increases the risk of cardiovascular disease. Therefore, treatment should focus on both reducing hyperglycemia and cardiovascular events [161]. As explained, oxidative stress and NO depletion impair endothelial function and cause arterial stiffness. Arterial stiffness is a marker of cardiovascular risk, but may also directly trigger formation of atherosclerotic plaques. Therefore, prevention or treatment that reduce arterial stiffness will impede excess cardiovascular morbidity and even mortality. Both endothelial dysfunction and arterial stiffness are related to hypertension, which is associated with high risk for vascular mortality [107, 162, 163]. Specific beta-blockers, angiotensin-converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARB), and calcium channel blockers reduce sympathetic activity and lower BP, but also release NO improving endothelial function and arterial stiffness [111, 112, 164-169].

2. Cocoa flavanols

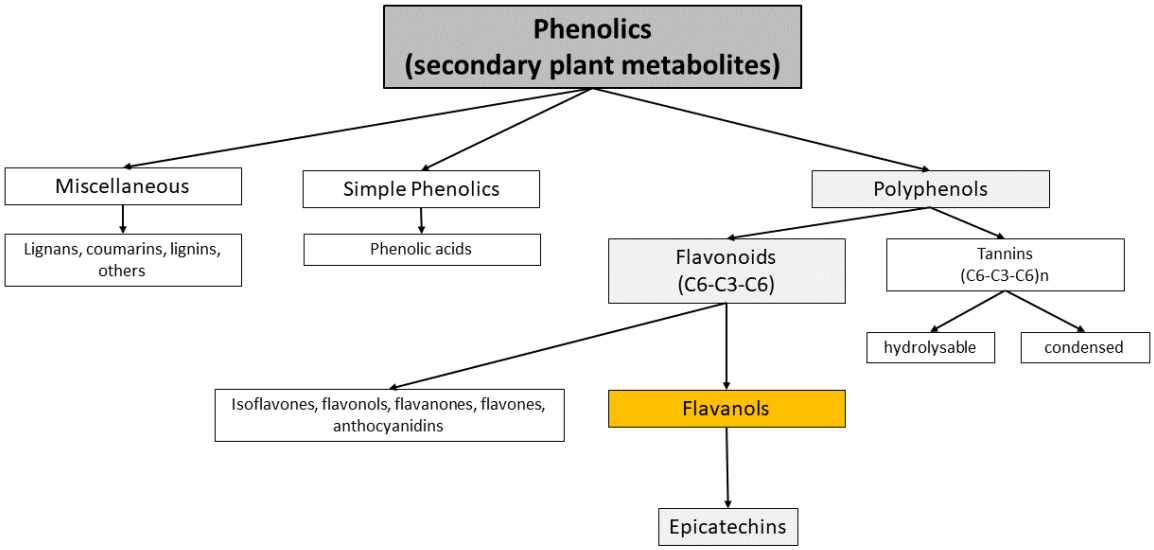

Figure 14: Flowchart presenting classification of phenolics (classification according to the number of phenol subunits).
(Figure based on Robbins et al. (2006) [144])

Polyphenols – Flavonoids – Flavanols

Polyphenols are secondary plant metabolites produced by higher plants, and many of them have been found in plant-based foods [170]. They are abundant micronutrients in our diet [171]. Since it is demonstrated that polyphenols are associated with disease prevention, have antioxidant properties, and are the most abundant antioxidants in our foods, research on the structure and effects of polyphenols has increased. Research with interest on the antioxidant activity of polyphenols mainly focus on flavonoids and phenolic acids (e.g. chlorogenic acid, caffeic acid) as they comprise a catechol group, a double bond with an oxo group, and/or several hydroxyl groups (Figures 14 and 15 A) [145, 172].

Chemically, flavonoids have a C6-C3-C6 structure consisting of 2 benzene rings (A and B) coupled by an oxygenated heterocycle (C) (Figure 15A). They are formed via the phenylpropanoid pathway, which converts phenylalanine into 4-coumaroyl-CoA, followed by the flavonoid biosynthesis pathway [173]. Depending on the differences of the basic chemical structure, more specifically the degree of oxidation and the pattern of substitution of the oxygenated heterocycle (C), flavonoids can be split up in (at least) 9 subclasses, namely flavones, flavonols, dihydroflavonols, chalcones, dihydrochalcones, flavanones, isoflavones, anthocyanidins, and flavanols, also named flavan-3-ols (Figures 14 and 15). In nature, flavonoids occur as glycosides, aglycones (mainly flavanols), and methylated derivates [143, 174].

Previous research indicated the potential cardiovascular health benefits of flavonoids, especially flavanols [175-179]. Flavanols are the most complex subfamily of flavonoids as they

include monomers, (-)-epicatechin (EC) and (+)-catechin (C), and oligomeric and polymeric forms of these monomers, also called procyanidins. Monomers can be present in the aglycone form or esterified with gallic acid to form the gallate derivatives. As presented in Figure 15C and 15D, flavanols are characterized by a hydroxyl group at position 3 of the oxygenated heterocycle (C) and monomers differ in pattern of substitution of the 2 benzene rings (A and B) [174, 180].

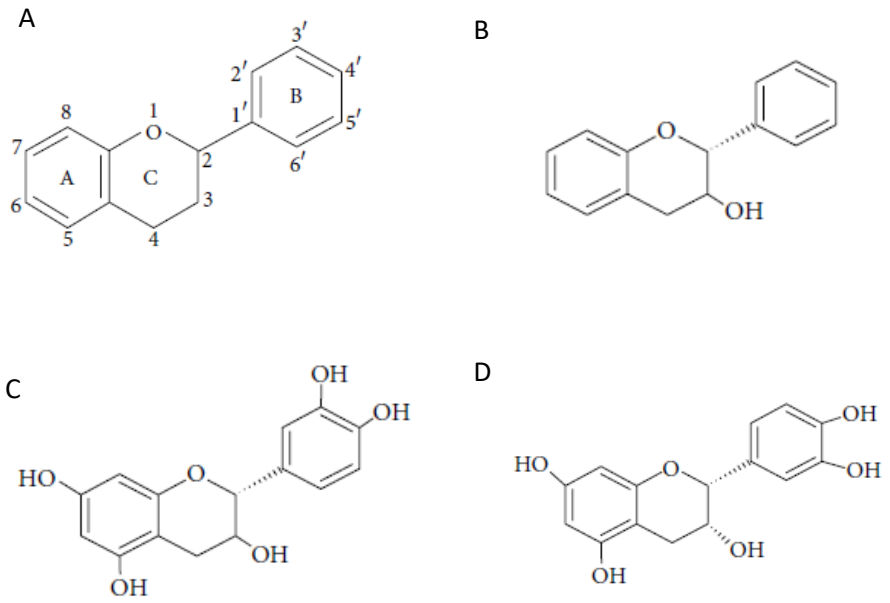

Figure 15: Chemical structures: A: basic structure of Flavonoids, B: chemical structure of flavanols, C: chemical structure of catechin, D: chemical structure of epicatechin [143].

Flavanols have attracted specific interest as they occur in high concentrations in several fruits and vegetables, teas, beans, red wines, and predominantly in cocoa products [171, 181]. Raw dried cocoa beans from the *Theobroma cacao* tree contain 12 - 18 % polyphenols (depending upon maturity of the bean, area of cultivation, climatic conditions, harvest season, and storage time after harvest [182]) of which 60 % are flavanol monomers (approximately 35 % is EC) and EC-based procyanidin oligomers [172, 183]. Furthermore, it was shown that cocoa has more phenolic phytochemicals, have greater positive effects on flow mediated dilation (FMD) and BP, and feature higher antioxidant activity compared to other flavonoid sources like teas and red wine [184, 185].

Cocoa flavanols

Special interest in cocoa raised from research on Kuna Indians. Despite increasing age, Kuna Indians living on the San Blas Islands off the coast of Panama have lower BP levels in contrast to migrated Kuna Indians to urban areas. In addition, they live longer and show fewer frequencies of myocardial infarction, stroke, cancer, and DM. Based on environmental

examinations, like stress, pollution, and diet (e.g. calcium, soluble fiber, Omega-3 fatty acids, protein, alcohol), it was demonstrated that their greater health levels were elicited by daily intake of at least 5 cups of cocoa, more specifically home-grown and Columbian cocoa powder, which are predominantly high in flavanols and procyanidins [186, 187]. Their daily dose is estimated at approximately 600 - 900 mg flavanols as well as other polyphenols [176, 188, 189]. Also, The Women's Health Study suggested lower cardiovascular deaths thanks to chocolate ingestion [190], The Zutphen Elderly Study indicated an inverse association of consumption of cocoa-containing foods with BP and 15-year cardiovascular and all-cause deaths [191], and The Stockholm Heart Epidemiology Program described a dose dependent reduction of cardiovascular mortality in acute myocardial infarct survivors by chocolate intake [192]. Note that apart from the Kuna observations where daily intake was estimated, the other observational studies did not consider the amount of flavanols ingestion. Although raw cocoa beans contain high levels of flavanols, the different manufacturing processes including fermentation, drying, roasting, alkalization, and conching significantly reduce flavanols content (up to 90 % loss in chocolate) [182, 193, 194]. Approximately 7.0 – 13.0 % polyphenols, 0.31 – 0.49 % C, and 0.35 - 1.6 8 % EC remained in cocoa liquor extractions produced in several countries [195]. The longer the fermentation, the greater the loss in polyphenols. Hence, there is a substantial difference in polyphenols profile between raw and processed cocoa beans, but also between processed available cocoa products on the market [194]. Therefore, before claiming any health properties, randomized controlled trials with clear indication of dose and composition of the interventional products are required.

So far, several randomized controlled trials have been executed with accordingly systematic reviews and meta-analyses, however, high heterogeneity between publications complicate and obstruct comparison, hence impede formulating unambiguous conclusions. Heterogeneity in research arises from differences in intervention (e.g. administered dose of flavanols and EC, 1 batch daily or split doses, pure/isolated flavanols versus processed flavanols in cocoa products, placebo formula) as well as varied study populations (e.g. healthy, hypertensive, elderly, young athletes, end stage renal disease, heart failure, postmenopausal women). Table 2 summarizes several systematic reviews and meta-analyses about the vascular health effects of cocoa in adults (references to randomized controlled trials can be found in these papers), which all underline heterogeneity across included studies so that caution is needed with interpretation of the results.

Because of this high heterogeneity between the performed trials, no strict guidelines considering the amount of intake (flavanols, EC, C) required for the beneficial vascular health effects in specific populations are formulated yet. Though, in 2012, The European Food Safety Authority published a health claim stating that cocoa flavanols help to preserve endothelium-dependent vasodilation maintaining normal blood flow in the general population. To obtain the claimed health effect, cocoa flavanols should be taken in quantities of at least 200 mg daily (equals 10 g high flavanol dark chocolate or 2.5 g high-flavanol cocoa powder). This daily intake should be consumed in the context of a balanced diet [196]. However, this recommended dose is questioned by a review of Vlachojannis et al. (2016) implying that 100 mg EC (50 – 200 g chocolate) is needed for improvement in FMD and minimal 900 mg flavanols (100 – 500 g chocolate) is required for reducing BP levels. They reported that the effects of cocoa flavanols

may be patient-dependent [188]. Hence, there is need for robust standardized research into the effects of cocoa flavanols in different populations.

A major advantage of cocoa flavanols are the absence of detrimental side-effects (if not consumed in extreme high values, safety research in 2015 indicated well tolerated amounts up to at least 2000 mg cocoa flavanols daily for 3 months in healthy adults [197-199]). So far, in interventional trials, only some minor gastrointestinal complaints were reported [200]. For example, in one trial 1 out of 42 participants reported constipation when consuming cocoa with skimmed milk for 4 weeks [201]. However, the constipation was solved with greater fiber intake and as constipations may be triggered by several causes [202], it is questionable whether cocoa flavanols were really cause for this constipation.

Table 2: Several systemic reviews and meta-analyses summarizing the effects of cocoa on vascular effects.

First author + year of publication	Population	Intervention (duration)	Vascular health effect
Desch et al., 2010 [203]	Healthy, prehypertension or stage 1 hypertension, 297 subjects	Flavanol-rich cocoa (2 - 18 w)	‣ SBP ↓: - 4.5 mmHg (95% CI: -5.9; -3.2) ‣ DBP ↓: - 2.5 mmHg (95% CI: -3.9; -1.2) **Limitation**: significant statistical heterogeneity across included papers
Ebaditabar et al., 2020 [204]	Varied population, 794 subjects	Dark chocolate and flavonoids (within 2 hours, <1 – 12 w)	‣ FMD ↑: acute: 1.12 % (95% CI: 0.72; 1.53); chronic: 0,61 % (95% CI: 0.30; 0.93) **Note:** non-linear association between flavonoids/ dark chocolate ingestion and FMD (not significant) **Limitation:** wide intervention doses which can be contributed to the high heterogeneity
Ellinger et al., 2012 [205]	Varied population, 391 subjects	EC ingestion via cocoa products (2 – 18 w)	‣ SBP ↓: -4.6 mmHg (95% CI: -5.4; -3.9) ‣ DBP ↓: -2.0 mm Hg (95% CI: -2.4; -1.5) ‣ 25 mg EC/d: SBP ↓ with -4.1 mmHg (95% CI: -4.6; -3.6) and DBP ↓ of -2.0 mmHg (95% CI: -2.4; -1.5 mm Hg) **Note:** Bayesian approach, limited included papers in normotensive subjects **Limitation:** varied EC doses explain in part heterogeneity across included papers
Hooper et al., 2012 [206]	Healthy or at any risk of cardiovascular diseases but not critically ill, 1297 subjects	Chocolate, cocoa or flavan-3-ols (within 2 hours - 18 w)	‣ FMD ↑: acute: 3.19 % (95% CI: 2.04; 4.33); chronic: 1.34 % (95% CI: 1.00; 1.68) ‣ DBP ↓: chronic: -1.60 mmHg (95% CI -2.77; -0.43) ‣ MAP ↓: chronic: -1.64 mmHg (95% CI: -3.27; -0.01) ‣ LDL ↓: acute: -0.07 mmol/L (95% CI: -0.14; -0.00) ‣ HLD ↑: acute: 0.03 mmol/L (95% CI: 0.00; 0.06) ‣ IR (HOMA) ↓: -0.67 points (95% CI: -0.098; -0.36) ‣ Insulin sensitivity index ↑: 5.38 points (95% CI: 1.81; 8.95) ‣ Fating glucose, HbA1c, QUICKI, C-reactive protein, total cholesterol, SBP = **Note**: ↑ dose of EC induces greater FMD ↑, at least 50 mg EC/ day required for effect on BP **Limitation:** heterogeneity in biomarker results in some included papers
Jafari et al., 2020 [207]	Varied population, 403 subjects (acute effects) and 632 subjects (effects after chronic ingestion)	Cocoa/ chocolate (within 3 hours – 52 w)	‣ No effect on platelet count ‣ PWV ↓: acute: -0.27 m/s (95% CI: -0.50; -0.04); chronic: -0.33 m/s (95% CI: -0.43; -0.22) ‣ AI ↓: acute: -4.47% (95% CI: -7.48; -1.47); chronic: -4.50% (95% CI: -7.05; -1.94) **Note**: non-linear dose response of chronic chocolate intake on PWV nor on AI, subgroup analysis indicated study design, sex, dose, and duration of the intervention as potential sources of heterogeneity **Limitation**: heterogeneity across included papers
Lin et al., 2016 [208]	Varied population, 1131 subjects	Cocoa flavanol (2 – 52 w)	‣ Triglycerides ↓: -0.10 mmol/L (95% CI: -0.16; -0.04)

			▸ HDL ↑: 0.06 mmol/L (95% CI: 0.02; 0.09) ▸ Fasting insulin ↓: -2.33 mIU/mL (95% CI: -3.47; -1.19) ▸ IR (HOMA) ↓: -0.93 points (95% CI: -1.31; -0.55) ▸ QUICKI ↑: 0.03 (95% CI: 0.01: 0.05) ▸ Insulin sensitivity index ↑: 2.54 (95% CI: 0.63, 4.44) ▸ C-reactive protein ↓: -0.83 mg/dL (95% CI: -0.88; -0.77) ▸ Vascular cell adhesion molecule ↑: 85.6 ng/mL (95% CI: 16.0; 155) ▸ LDL, total cholesterol, triglycerides, glucose = **Limitation**: heterogeneity of cocoa flavanol intervention across included papers
Ried et al., 2017 [200]	Adults with or without hypertension, 1084 subjects	Cocoa/ chocolate (2 - 18 w)	▸ SBP ↓: -1.76 mmHg (95% CI: -3.09; -0.43) ▸ DBP ↓: -1.76 mmHg (95% CI: -2.57; -0.94) **Note**: influence of baseline BP: greater effect in hypertensive, no effect in normotensive **Limitation**: findings are limited by heterogeneity across included papers (cannot be explained by subgroup analyses involving age, study duration, flavanol content, and blinding)
Shrime et al., 2011 [209]	Varied adult population, 1106 subjects	Flavonoid rich cocoa, chronic (2 – 18 w)	▸ SBP ↓: -1.63 mm Hg (95% CI: -0.13; -3.12) ▸ FMD ↑: 1.53 % (95% CI: 0.6; 2.40) ▸ LDL ↓: -0.077 mmol/L (95% CI: -0.0044; -0.149) ▸ HDL ↑: 0.046 mmol/L (95% CI: 0.0028; 0.089) ▸ IR (HOMA)↓: -0.94 points (95% CI: -0.59; -1.29) ▸ DBP, pulse, total cholesterol, triglycerides, C-reactive protein, fasting glucose, Insulin sensitivity index, QUICKI = **Note**: non-linear dose-response relationship between flavonoid rich cocoa and FMD, maximum effect at 500 mg/d **Limitation**: significant heterogeneity among included papers
Sun et al., 2019 [210]	Varied population, 730 subjects	Cocoa/ chocolate (1 – 12 w)	▸ FMD ↑: 1.17 % (95% CI: 0.76; 1.57) **Note**: non-linear dose-response association (inverted U-shape) between CF and FMD **Limitation:** heterogeneity across included papers
Taubert et al., 2007 [211]	Normotensive or hypertensive, 173 subjects	Chocolate (2 w)	▸ SBP ↓: -4.7 mmHg (95% CI: -7.6; -1.8) ▸ DBP ↓: -2.8 mmHg (95% CI: -4.8; -0.8) **Limitation**: residual statistical heterogeneity across included cocoa-papers

Data are presented with their 95% confidence interval (95% CI); ↓ represents a decrease; ↑ represents an increase; = represents no effect; AI= augmentation index; d= day; DBP= diastolic blood pressure; EC= epicatechin; FMD= flow mediated dilation test; HDL= high density lipoprotein (cholesterol); IR (HOMA)= insulin resistance measured via homeostatic model assessment; LDL= low density lipoprotein (cholesterol); PWV= pulse wave velocity; QUICKI= quantitative insulin sensitivity check index; SBP= systolic blood pressure; w= weeks.

Bioavailability of cocoa flavanols

The last 2 decades, in the context of cardiovascular health, research on cocoa flavanols (CF), flavanols extracted from the cocoa bean from the *Theobroma cacao* tree, has emerged to gain insight in their structure and function in parallel with their mechanism of action. The absorption and metabolism of flavonoids depend on their chemical structure [212].

CF monomers EC and C, but also oligomers up to pentameric procyanidins are rather stable during gastric transit [213]. Though, it is suggested that in the acidic gastric environment a portion of oligomers (trimers to hexamers) are hydrolyzed into monomers and dimers, hereby increasing the potential for their absorption in the small intestine [214]. Here, 22 – 55 % of EC and C compared to only 0.5 % for dimers and trimers are absorbed. Monomeric forms have a low molecular weight and so reach higher levels in plasma, with EC having greatest bioavailability. Absorption of EC into the intestinal tract leads to rapid metabolization by enterocytes and hepatocytes. This metabolization includes glucuronidation (uridine-5'-diphosphate glucuronosyl-transferases), sulfation (sulfo-transferases), and methylation (catechol-O-methyltransferases). Hence, glucuronides, sulfates and/ or methyl conjugates are the compounds in the bloodstream and reach the target organs. EC metabolites can cross the gut barrier and are widely spread via absorption in liver, testes, and lymphoid organs, like thymus, spleen, and mesenteric lymphoid nodes. In addition EC metabolites can cross the blood-brain barrier, hereby inducing effects at cerebral level [215]. The few procyanidins that cross the intestinal barrier are transported via the portal vein to the liver and here transformed into metabolites through glucorination, sulfation, and methylation. The non-absorbed procyanidins elicit a quite local effect at the gastrointestinal tract [194, 216, 217]. (Figure 16)

EC and its metabolites reach maximal plasma levels around 2 hours after ingestion and are excreted after 2 – 4 hours. Although rather large ranges were reported, around 21 – 30 % of the ingested dose is urinary excreted over a period of 24 hours, of which more than 80 % within 8 – 10 hours [218, 219].

In line with Vlachojannis et al. (2016) [188] who suggested a patient-dependent effect of CF, Manach et al. (2005) [218] hypothesized inter-individual differences in absorption of flavonoids due to particular polymorphisms for intestinal enzymes or transporters. Furthermore, the non-absorbed EC and the major part of procyanidins reach the colon where they are largely metabolized into various phenolic acids by the microbiota [220]. The microbial metabolites appear in the circulation and urine 6 – 48 hours post intake [219, 221]. Based on ex vivo studies, it is suggested that these metabolites could exert positive vascular effects [222-224]. Therefore, one could hypothesize that inter- (and maybe intra-) individual differences in the microbiome might influence CF effects. However, so far, research on this topic is limited. As CF undergo extensive transformations during metabolism, which could differ inter-individually, in vivo, but especially in vitro effects of CF have to be interpreted with caution.

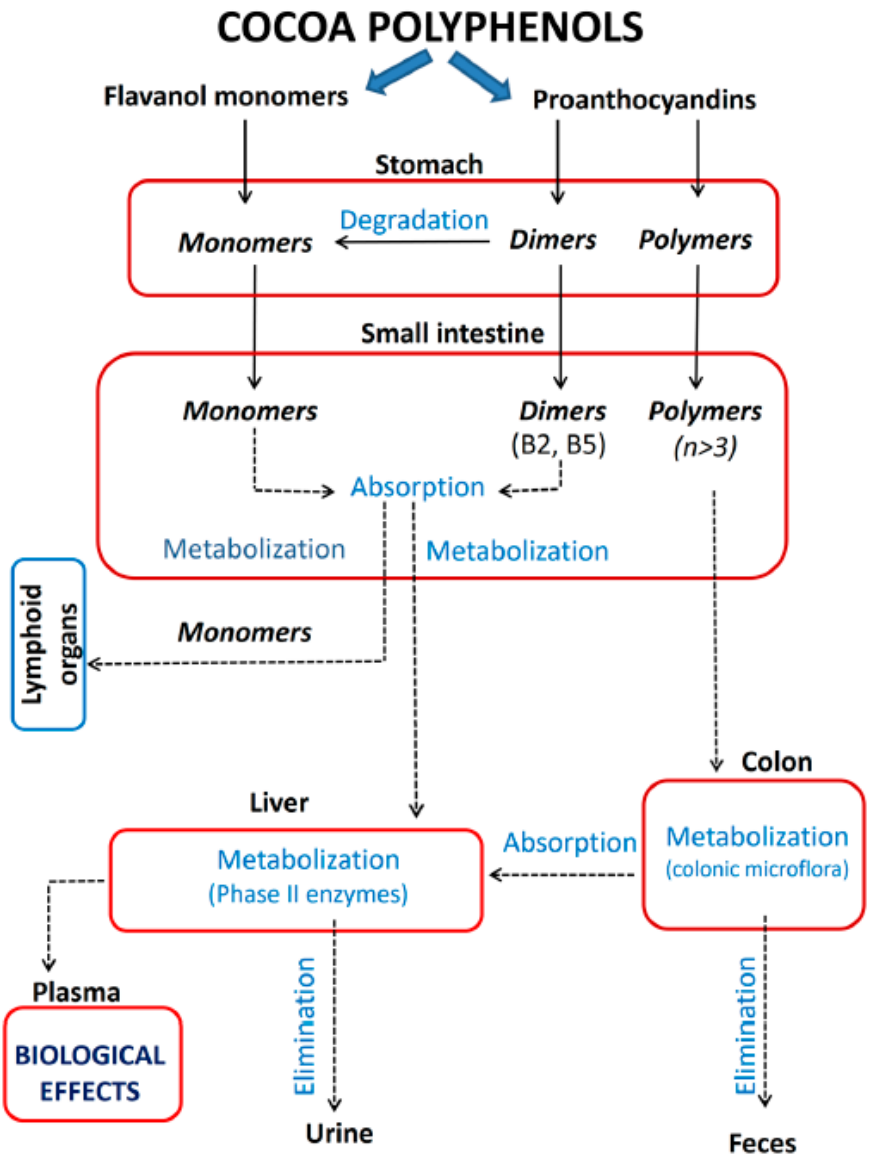

Figure 16: Pharmacokinetic scheme of cocoa polyphenols [217].

Mechanism of action of cocoa flavanols

CF show promising chemoprotective, neuroprotective, antioxidant, and cardioprotective properties [225]. The latter two are of main interest in this research project. CF could positively affect endothelial function [176, 226] and BP [200, 211], and may have an antiplatelet effect [227, 228], improve glucose and lipid metabolism [208, 229], and influence various inflammatory processes [216, 217, 230]. (Figure 17)

The exact mechanisms for these promising vasculoprotective actions of CF are not fully elucidated yet, but based on varied discovering in vitro, animal, and human research, following mechanisms are proposed or strongly indicated: A key mechanism of CF that has

been widely acknowledged is the potential of CF to increase NO bioavailability and -activity with subsequent vasodilatory effects, hence improving vascular function and BP levels [176, 231-234]. Greater NO levels through CF ingestion can be attained via higher production of NO due to greater eNOS activation [235], preservation of intracellular arginine pool via limiting arginase activity [236], and antioxidant properties limiting NO-loss through peroxynitrite ($ONOO^-$) formation [237]. The antioxidative actions are obtained via inhibition of lipid peroxidation [238-241], inhibition of NOX (NADPH oxidases) [242] and other enzymes related to oxidative stress [243], and can partly be explained by their chemical structure, which can scavenge free radicals and chelate redox-active metals: a catechol group on B-ring (Figure 10 C and D), phenolic quinoid tautomerism, and delocalization of electrons [244-246]. Additionally, CF may activate one of the major antioxidant defense responses, the nuclear factor erythroid 2-related factor 2 signaling pathway [247]. Note, some studies in humans did not demonstrate a change in oxidative stress with CF [248]. It is plausible that in pathological conditions when oxidative stress is increased, the antioxidant effects of CF are greater expressed [249]. It is also indicated that CF could increase parasympathetic tone, hence improve sympathovagal balance [217, 250, 251].

Moreover, CF could decrease the vasoconstrictor endothelin-1 causing even greater vasodilation [233, 252]. CF could modulate the renin-angiotensin-aldosterone system through inhibition of angiotensin converting enzyme activity [253-256] and thereby lower the prooxidant effects of angiotensin II. CF could also inhibit NF-kB, cyclo-oxygenase-2, and inducible NOS activation resulting in lower production of pro-inflammatory markers like interleukin 2, interleukin 6, interleukin 8, and tumor necrosis factor-α. In line, CF could decrease biomarkers of endothelial inflammations like intracellular and vascular cell adhesion molecules [216]. Furthermore, as mitochondrial dysfunction is an early marker of endothelial dysfunction and might contribute to the onset of cardiovascular disease, increasing research has been performed on this matter and illustrated the potential of chronic EC ingestion to stimulate mitochondrial function and biogenesis [257-259].

Albeit still debated, the abovenamed effects seem to be mainly induced, at least in part, by EC, the most abundant flavanol in cocoa. After cocoa ingestion, plasma EC levels are relatively high compared to C and procyanidins and solely EC, not C, have been related to greater FMD [217, 260-262].

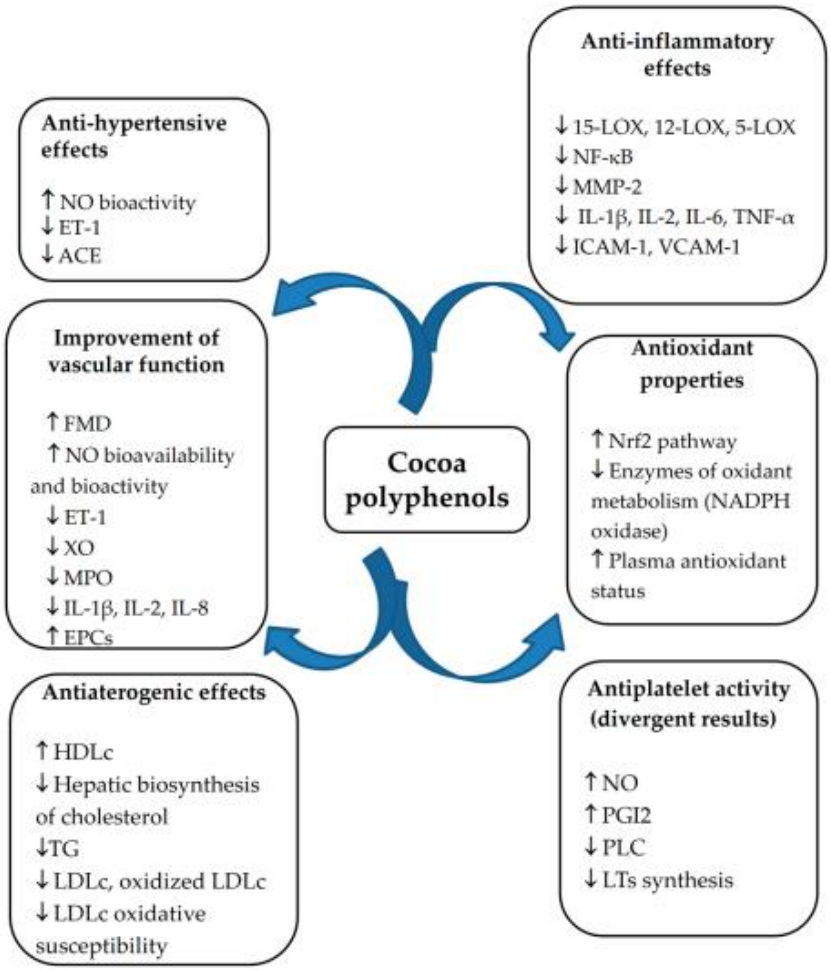

Figure 17: Theoretical background for major mechanisms of the beneficial vascular health effects of cocoa flavanols.

ACE= angiotensin-converting enzyme; EPCs= endothelial progenitor cells; ET-1= endothelin 1; FMD= flow-mediated dilation; HDLc= high-density lipoprotein-cholesterol; ICAM= intercellular adhesion molecule; IL= interleukin; LDLc= low-density lipoprotein-cholesterol; LOX= lipooxygenase; LTs= leukotrienes; MMP-2= matrix metalloproteinase 2; MPO= myeloperoxidase; NADPH= reduced nicotinamide adenine dinucleotide phosphate; NF-kB= nuclear factor kappa-light-chain-enhancer of activated B cells; NO= nitric oxide; Nrf2= nuclear factor erythroid-related factor 2; PGI2= prostaglandin I2; PLC= phospholipase C; TG= triglycerides; TNF= tumor necrosis factor; VCAM= vascular cell adhesion molecule; XO= xanthine oxidase [217].

As diabetic vascular complications are mainly caused by increasing ROS production and the resulting NO depletion and as increasing research shows promising results towards the vasculoprotective effects of CF through increasing NO bioavailability- and activity and via upregulating antioxidant activity and reducing ROS production, it is assumable that CF are promising nutraceuticals for limiting the increasing vascular burden of T2DM. However, so far, limited research with inconsistent results have been performed in this specific population.

3. Aims and outline of this research

In this doctoral project, we have performed research into the possible protective properties of CF on vascular health in patients with T2DM. Starting from evidence based on previous research on the vascular effects of CF in different populations (e.g. healthy, hypertensive, DM) together with the theoretical background of CF and the mechanisms explaining the onset of diabetic vascular complications, we hypothesized that CF could induce beneficial vascular health benefits in patients with T2DM (Figure 18).

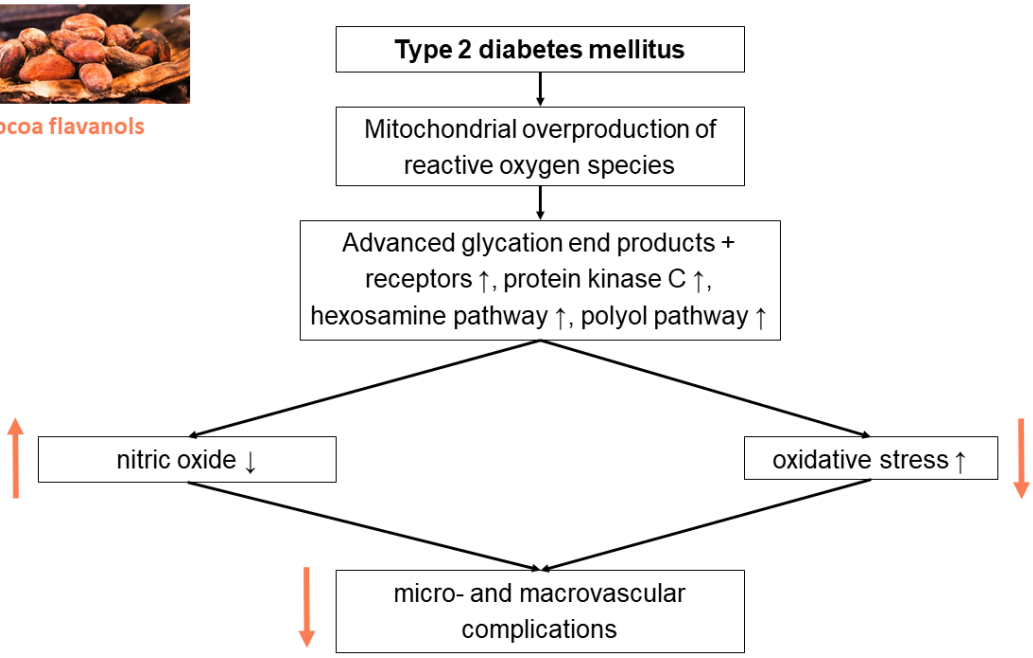

Figure 18: Theoretical mechanism of action of cocoa flavanols to prevent or delay the onset of diabetic vascular complications.

***Aim 1**: To investigate the evidence for CF-induced vascular health properties in patients with DM.*

T2DM is a complex metabolic disorder with high risk for developing vascular complications. These complications are mainly caused by ROS production and the resulting NO depletion. CF are natural substances, which are regarded to reduce ROS and increase NO bioavailability and –activity. Therefore, CF could be regarded as promising nutraceuticals to prevent or delay the onset of diabetic vascular complications. However, only few reports investigated these effects in patients with T2DM with inconsistent results.

Therefore, we published a **systematic review and meta-analysis** to provide a summary and main effect of the already examined CF-induced vascular health properties in patients with DM. This paper is entitled: 'Evaluation of blood pressure lowering effects of cocoa flavanols in diabetes'.

***Aim 2**: To setup a robust, standardized, clearly described trial protocol.*

Based on the systematic review and meta-analysis, we concluded that only very few research with marked clinical and methodological heterogeneity have been performed so far. Hence, there is high need for standardized research with a robust, clearly described protocol before conclusions on the possible CF-induced vascular health effects in patients with DM could be formulated.

An acute interventional study using capsules containing a flavanol rich cocoa extract with an adequate placebo is the first step for profoundly testing. Possible confounding factors like level of physical activity and glycemic control are taken into account. In addition, as hypertension is a very common comorbidity in patients with T2DM (> 60 % of all patients with T2DM [110]) and as little is known so far concerning possible interferences between antihypertensive drugs (AHD) and CF actions especially in patients with DM, possible influence on CF effects of beta-blockers, ACEi, or ARB are considered.

We published a **protocol paper** in which our acute, randomized, double-blinded, placebo-controlled cross-over trial was clearly described. This paper is entitled: 'Acute effects of cocoa flavanols on blood pressure and peripheral vascular reactivity in type 2 diabetes mellitus and essential hypertension: A protocol for an acute, randomized, double-blinded, placebo-controlled cross-over trial'.

***Aim 3**: To investigate the acute effects of CF on peripheral vascular reactivity in patients with T2DM.*

Subsequent to the setup of the protocol paper, we executed the described **acute, randomized, double-blinded, placebo-controlled cross-over trial**. However, due to the COVID-pandemic, measurements were forced to be cancelled for several months and recruitment was substantially hindered afterwards. Therefore, the initially wanted sample size in each group could not be reached, proper matching of participants between groups was not feasible anymore, and the group containing non-diabetic individuals with essential hypertension taking beta-blockers had to be excluded. Although the rather small sample size in each group reduces power, this paper has a robust methodology and provides interesting points of consideration that should be taken into account in future research. The paper reporting on the results is submitted and is entitled: 'Acute effects of cocoa flavanols on blood pressure and peripheral vascular reactivity in type 2 diabetes mellitus and essential hypertension'.

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Chapter 2: Original research publications

Tanghe, A., et al., *Evaluation of blood pressure lowering effects of cocoa flavanols in diabetes mellitus: a systematic review and meta-analysis.* Journal of Functional Foods, 2021. 79: p. 104399.

Tanghe, A., et al., *Acute Effects of Cocoa Flavanols on Blood Pressure and Peripheral Vascular Reactivity in Type 2 Diabetes Mellitus and Essential Hypertension: A Protocol for an Acute, Randomized, Double-Blinded, Placebo-Controlled Cross-Over Trial.* Frontiers in cardiovascular medicine, 2021. 8: p. 152.

Tanghe, A., et al., *Acute effects of cocoa flavanols on blood pressure and peripheral vascular reactivity in type 2 diabetes mellitus and essential hypertension*. Frontiers in cardiovascular medicine. *(submitted)*

Evaluation of blood pressure lowering effects of cocoa flavanols in diabetes mellitus: A systematic review and meta-analysis.

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Evaluation of blood pressure lowering effects of cocoa flavanols in diabetes mellitus: A systematic review and meta-analysis

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ABSTRACT

In healthy people, cocoa-derived flavanols (CF) improve blood pressure (BP). This meta-analysis investigates whether CF also affect BP in diabetic patients. PubMed, Web of Science, and Embase were consulted to retrieve eligible randomized controlled trials. A random-effects model and the Grades of Recommendation, Assessment, Development and Evaluation (GRADE)-approach were used for analyses and quality of evidence respectively. Of 267 citations, 11 trials were identified, studying either type 2 diabetic populations only (subgroup A) or type 2 diabetic patients plus non-diabetic subjects with increased cardiovascular risk (subgroup B1) or type 1 plus type 2 diabetic patients (subgroup B2). Mid/long-term CF consumption decreased BP slightly, however, only reaching statistical significance for diastolic BP in subgroup B1 (-1.89 mmHg, 95% CI: −3.24, −0.54, I^2 = 55%). Considerable heterogeneity between studies and low quality of evidence caused poor quality evidence of minimal effects of CF ingestion on BP in diabetic patients.

1. Introduction

According to the European Food Safety Authority (EFSA), flavanols derived from the seeds of *Theobroma cacao*, the cocoa bean, help to preserve endothelium-dependent vasodilation in healthy populations, if ingested in quantities exceeding 200 mg cocoa-derived flavanols (CF)/day. This equals 10 g high-flavanol dark chocolate or 2.5 g high-flavanol cocoa powder (EFSA Panel on Dietetic Products et al., 2012). However, it is unclear to what extent CF also enhance vasodilation and other vascular functions in people with increased cardiovascular risk, such as hypertension and diabetes mellitus (DM).

Flavanols are natural substances from the flavonoid family, a class of polyphenols (Manach et al., 2004), which can be found in cocoa products, but also in several fruits, beans, teas, and red wines (Arts et al., 2000; Manach et al., 2004).

In vitro- and animal studies, as well as reports from healthy volunteers have, indeed, suggested that CF improve cardiovascular health by enhancing endothelial function (Engler et al., 2004; Schroeter et al., 2006), inhibiting angiotensin converting enzymes (Actis-Goretta et al., 2006; Persson et al., 2011), lowering blood pressure (BP) (Ried et al., 2017; Taubert et al., 2007), influencing various inflammatory processes (Goya et al., 2016), and preventing platelet aggregation (Bordeaux et al., 2007; Hermann et al., 2006). Epicatechin, a highly active monomeric form of CF, is believed to be mainly responsible for these vascular effects, although this is still debated (Aprotosoaie, Miron, et al., 2016; Rodriguez-Mateos et al., 2018; Schroeter et al., 2010).

Abbreviations: BP (SBP – DBP), Blood Pressure (Systolic – Diastolic); BMI, Body Mass Index; CF, Cocoa-derived flavanols; CI, Confidence Interval; DM (T1DM – T2DM), Diabetes Mellitus (type 1 Diabetes Mellitus - type 2 Diabetes Mellitus); GRADE, Grades of Recommendation, Assessment, Development and Evaluation; I^2, Heterogeneity; NO, Nitric Oxide; RCT, Randomized-controlled trial.

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In addition, CF are suggested to act as antioxidants (Keen et al., 2005). In vivo, CF increase plasma antioxidant capacity (Rein et al., 2000) and reduce lipid peroxidation in humans (Wiswedel et al., 2004). CF also increase bioavailability of nitric oxide (NO) (Heiss et al., 2003; Heiss et al., 2005), by inhibiting endothelial Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase and by enhancing endothelial Nitric Oxide Synthase (eNOS) production and activity (Schewe et al., 2008).

The latter is of particular interest. In type 1 and type 2 DM (T1DM and T2DM respectively), NO-depletion is considered crucial in the development of DM-associated hypertension and vascular complications (Giacco et al., 2010; Honing et al., 1998). Therefore, CF could potentially influence the development and/or progress of DM-associated vascular complications in particular; given the high world-wide prevalence of DM and its associated vascular complications, this could have a serious preventive and/or therapeutic impact.

Yet, little research has been performed specifically on CF-induced vascular benefits in DM; the available reports study relatively small samples, have divergent study designs, and yield inconclusive results (Ayoobi et al., 2017; Dicks et al., 2018; Mellor et al., 2010; Rynarzewski et al., 2019). Considering the theoretical background, the potential impact, and the promising results in healthy and hypertensive subjects (Cooper et al., 2008; Hooper et al., 2008; Ried et al., 2017), we therefore performed a *meta*-analysis to evaluate the evidence for an effect of CF on BP reduction and/or improvement of vascular function in patients with T1DM and/or T2DM. We only focused on CF and only randomized-controlled trials were considered for inclusion.

2. Materials and methods

This *meta*-analysis was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2015) and was registered in PROSPERO, a database of systematic review protocols (registration number: CRD42018112229, https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=112229).

2.1. Literature sourcing

Keywords, Medical Subject Headings (MeSH) terms, and synonyms were inserted in 3 electronic databases (PubMed, Web of Science, and Embase) to identify potentially relevant studies published up to August 13th, 2020. The search terms included: diabetes mellitus, chocolate, cocoa flavanols, epicatechin, catechin, and vascular functioning (e.g. vascular stiffness, vascular resistance, blood pressure, blood circulation, and endothelial function) (Table S1). A manual search in reference lists of the included studies was also conducted. No limitations on language or date of publication were set. All search strategies are presented in Table S2.

2.2. Study selection

As described in PROSPERO and our literature search (Tables S1 and S2), eligible studies included randomized controlled trials (RCT) investigating vascular effects of CF administration, regardless of duration of intake, in patients with all types of DM. Citations were excluded if no full report of original research was published (e.g. protocols, letters, and guidelines) or if the full text was unavailable. After removal of duplicates, 2 researchers (KVW, AT) screened titles and abstracts, and subsequently full texts independently (κ-coefficient 0.94). In case of disagreement the authors deliberated until consensus was reached.

As is customary, we considered a *meta*-analysis of 4 or less publications to be unreliable. Because parameters of vascular function yielded less than 4 publications, we only focused on BP as a primary outcome. Thus, we could analyze 11 papers with comparable study populations (patients with DM) and intervention (mid/long-term administration of flavanols extracted from the cocoa bean only).

2.3. Data extraction

The following data were extracted from the included papers: (1) author, year of publication, and study design, (2) study population (intervention versus control group), (3) relevant information concerning the flavanol intervention (form and content/ day), (4) relevant information concerning the control treatment, (5) frequency of ingestion (single dose versus split-doses), (6) time (duration of intervention), (7) method of BP measurement, (8) miscellaneous information, and (9) effect on BP. These were outlined in an evidence table by 2 researchers (KVW, AT), independently. A third researcher (PC) adjudicated in case of disagreement.

A subdivision was made based on the populations studied in each paper, i.e. either T2DM patients only (subgroup A, 5 papers (Ayoobi et al., 2017; Curtis et al., 2013; Dicks et al., 2018; Mellor et al., 2010; Rostami et al., 2015)), non-diabetics plus T2DM combined (subgroup B1, 4 papers (Desideri et al., 2012; Gutiérrez-Salmeán et al., 2016; Mastroiacovo et al., 2015; Sorond et al., 2013)), or non-diabetics, T1DM, and T2DM combined (subgroup B2, 2 papers (Desch et al., 2010; Monagas et al., 2009)). If provided, the percentage of each type included in each paper is outlined in the evidence table (Table 1).

2.4. Risk of bias and quality of evidence

To assess the risk of bias within studies, the revised Cochrane risk-of-bias tool for randomized trials (RoB 2) was used. Five different domains on potential biases were evaluated independently by 2 researchers (PC, AT) and, in case of disagreement, by a third researcher (AVG): bias arising from (1) the randomization process, (2) deviations from intended interventions, (3) missing outcome data, (4) measurement of the outcome, and (5) selection of the reported result. Each domain was rated as 'low risk', 'some concerns' or 'high risk', based on signaling questions and on the associated Cochrane guidelines (Higgins et al., 2019). Afterwards, an overall risk of bias could be adjudicated for each publication by summation of the ratings on each domain. In case of a crossover (CO)-study, the signaling questions of each domain were adapted according to the Cochrane guidelines (Higgins et al., 2019).

No citation was excluded for the analyses based on risk of bias.

Finally, the strength of the body of evidence was assessed using the Grades of Recommendation, Assessment, Development and Evaluation (GRADE)- approach (Higgins et al., 2019). Publication bias was evaluated in total sample of included papers using the Egger's test (Egger et al., 1997) in R (version.string R version 3.6.1, 2019–07-05), however, also visually in subgroups through funnel plots.

2.5. Data synthesis and analysis

Data synthesis was performed by one researcher (AT), supervised by another researcher (AVG). Review Manager (RevMan) (Computer program, Version 5.3, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) was used for data analyses and creation of Figs. 2, 3, 4, and 5 and Supplementary Figures S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, and S15. A random-effects model was chosen a priori since not all papers had a study population of solely patients with DM and we anticipated that not all interventional or placebo supplementations would be similar in dose, substance, and form.

Standardization of the mean difference or the mean standard deviation was not necessary, since all studies reported SBP and DBP values in mmHg. Treatment effects of CF on outcomes were calculated from differences in mean changes (calculator in RevMan and Cochrane guidelines and formulae (Higgins et al., 2019)) within treatment groups and implementing hedges g to account for smaller study samples. Values pre- and post-intervention were analyzed separately except for 1 study (Monagas et al., 2009), who did not report baseline values for each

Table 1
Table of evidence and characteristics of included studies.

Paper /Study Design	Study Population		Flavanol Intervention		Control treatment	Frequency	time	BP assessment[1]	Miscellaneous	Effect on BP[2]
	Intervention group	Control group	Form	Flavanol content/ day						
Subgroup A: T2DM only										
Ayoobi et al., 2017* (Ayoobi et al., 2017) RCT - SB	• T2DM for 4.1 ± 0.3 y • 14 F, 7 M • Age: 50.6 ± 1.6 y	• T2DM for 3.8 ± 0.3 y • 13 F, 10 M • Age: 50.7 ± 1.6 y	30 g 84% dark chocolate	no information	no intervention	1x /d	8 w	sitting; after 10 min rest; frequency not specified	• oral anti-DM drugs only • no info on anti-HT drugs • HbA1c not reported	• SBP ↓ (-7.2 ± 1.6 mmHg) • DBP ↓ (-6.3 ± 1.7 mmHg)
Curtis et al., 2013 * (Curtis et al., 2013)RCT- DB	• T2DM for 5.0 y (median, 95% CI: 4.9; 9.2) • 47 F • Age: 62.1 ± 0.7 y • HbA1c: 7.1 ± 0.1%	• T2DM for 5.0 y (median, 95% CI: 4.4; 7.2) • 46 F • Age: 63.0 ± 0.8 y • HbA1c: 7.3 ± 0.2%	27 g flavonoid enriched chocolate	• flavan-3-ols: 850 mg • EC: 90 mg • Isoflavones: 100 mg	placebo chocolate (no info on flavanol content)	2x /d (lunch + evening)	52 w	• Aortic central BP, Ambulatory BP • 2 h, 10 min intervals	• postmenopausal women only • insulin allowed • anti-HT drugs allowed	No change
Dicks et al., 2018 (Dicks et al., 2018)RCT- DB	• T2DM for 6.7 ± 1,4 y • 10 F, 7 M • Age: 65.6 ± 2.6 y • HbA1c: 6.4%	• T2DM for 7.2 ± 1.0 y • 7 F, 11 M • Age: 62.8 ± 1.6 y • HbA1c: 6.5%	5 × 0.5 g cocoa powder capsules	• FL: 207.5 mg, • EC: 40.4 mg • C: 13.6 mg	5 × 0.5 g pure microcrystalline cellulose	3 in morning, 2 in evening	12 w	2 (3) measurements, 1–2 min intervals; fasting state	• all subjects had HT • oral drugs only	No change
Mellor et al., 2010 * (Mellor et al., 2010)CO– DB	• T2DM for 18.0 ± 1.4 *m* • 5 F, 7 M • Age: 68 y (median, range 42–71) • HbA1c: 6.4 ± 0.2%	cross-over	45 g high polyphenol chocolate, 85% cocoa solids	• EC: 16.6 mg	low polyphenol chocolate (<2 mg EC)	3x /d	8 w	sitting; after 10 min rest; frequency not specified; fasting state	• oral drugs only	No change
Rostami et al., 2015* (Rostami et al., 2015) RCT- DB	• T2DM for 7.5 ± 0.8 *m* • 20 F, 12 M • Age: 58.7 ± 1.6 y • HbA1c: 7.2 ± 0.2%	• T2DM for 7.9 ± 0.7 *m* • 16 F, 12 M • Age: 57.2 ± 1.5 y • HbA1c 7.6 ± 0.2%	25 g dark chocolate, 83% cocoa solids	• flavonoids: 450 mg	white chocolate (no flavonoids)	1x /d	8 w	sitting; after 10 min rest; 2 measurements	• all subjects had HT • oral drugs only	• SBP ↓ (-6.6 ± 1.9 mmHg) • DBP ↓ (-4.9 ± 1.9 mmHg)
Subgroup B1: mixed population with T2DM										
Desideri et al., 2012 (Desideri et al., 2012) RCT- DB	• Elderly, mild cognitive impairment, 2 groups a) high FL dose: 16 F, 14 M; Age: 71.2 ± 0.9 y; 16% T2DM; b) intermediate FL dose: 17 F, 13 M; Age: 71.3 ± 0.8 y; 20% T2DM	• Elderly, mild cognitive impairment, 1 group c) low FL dose:14 F, 16 M; Age: 71.0 ± 0.8 y; 23% T2DM	Cocoa drink with intermediate OR high FL content	• High: • FL: 993 mg • EC: 185 mg • C: 62 mg • Other: 41 g caffeine, 458 mg theobromine • Intermediate: • FL: 520 mg • EC: 95 mg • C: 35 mg • Other: 44 g caffeine, 429 mg theobromine	Cocoa drink with low FL content • FL: 48 mg • EC: 5 mg • C: 8 mg • Other: 46 g caffeine, 400 mg theobromine	1x /d (morning)	8 w	sitting; after 15 min rest, 4 measurements, non-fasting state	• 70% of high, 73% of intermediate, and 77% of low FL had HT • oral drugs only • no info on duration of DM or HbA1c	• High FL: SBP ↓ (-10.0 ± 0.6 mmHg) ; DBP ↓ (-4.8 ± 0.3 mmHg) • Intermediate FL SBP ↓ (-8.2 ± 0.6 mmHg) ; DBP ↓ (-3.4 ± 0.4 mmHg) • Low FL: SBP ↓ (-1.4 ± 1.0 mmHg) ; DBP ↓ (-0.9 ± 0.6 mmHg) • Effect of High > intermediate > low (p = 0.0018 for SBP and 0.007 for DBP)
Gutiérez-Salmeán et al., 2016 (Gutiérrez-Salmeán et al., 2016)RCT- DB	• Hyper-triglyceridemia • 20 F/M • Age: 18–55 y	• Hyper-triglyceridemia • 10 F/M • Age: 18–55 y	Hard gelatin capsules	• EC: 100 mg	Hard gelatin capsules, inactive placebo	2x /d 2 capsules lunch-dinner (30 min before meal)	4 w	frequency not specified	• insulin use, HT, and use of betablockers were exclusion criteria • % DM not specified	No change

(*continued on next page*)

Table 1 (*continued*)

Paper /Study Design	Study Population		Flavanol Intervention		Control treatment	Frequency	time	BP assessment[1]	Miscellaneous	Effect on BP[2]
	Intervention group	Control group	Form	Flavanol content/ day						
									• no info on duration of DM or HbA1c	
Mastroiacovo et al., 2015 (Mastroiacovo et al., 2015) RCT- DB	• Elderly, cognitive intact, 2 groups: a) high FL dose:18 F, 12 M; Age: 70.0 ± 0.9 y; 16% T2DM; b) intermediate FL dose: 19 F, 11 M; Age: 68,7 ± 0.7 y; 16% T2DM	• Elderly, cognitive intact, 1 group: c) low FL dose:16 F, 14 M; Age: 70.0 ± 0.8 y; 23% T2DM	Cocoa drink with intermediate OR high FL content	• High: • FL: 993 mg • EC: 185 mg • C: 62 mg • Other: 41 g caffeine, 458 mg theobromine • Intermediate: • FL: 520 mg • EC: 95 mg • C: 35 mg • Other: 44 g caffeine, 429 mg theobromine	Cocoa drink with low FL content • FL: 48 mg • EC: 5 mg • C: 8 mg • Other: 46 g caffeine, 400 mg theobromine	1x /d (morning)	8 w	sitting; after 15 min rest, 4 measurements, non-fasting state	• 53% of high, 63% of intermediate and 50% of low FL had HT • oral drugs only • no info on duration of DM or HbA1c	• High FL: SBP ↓ (-7.8 ± 0.6 mmHg) ; DBP ↓ (-4.8 ± 0.4 mmHg) • Intermediate FL SBP ↓ (-6.8 ± 0.6 mmHg) ; DBP ↓ (-3.2 ± 0.4 mmHg) • Low FL: SBP ↓ (-1.6 ± 1.1 mmHg) ; DBP ↓ (-1.6 ± 0.7 mmHg) • Effect of High > intermediate > low ($p < 0.0001$ for SBP and DBP)
Sorond et al., 2013 (Sorond et al., 2013) RCT-DB	• Elderly • 31 F, 29 M • Age: 72.9 ± 0.7 y • 53.3% T2DM	Equals intervention	FL-rich cocoa powder + water	• FL: 1218 mg	FL-poor cocoa powder + water; • FL: 26 mg /d	2x /d	4 w	median of 3 values	• 90% had HT • no info on anti-DM or anti-HT drugs • no info on duration of DM or HbA1c	No change
Subgroup B2: mixed population with T1DM and T2DM										
Desch et al., 2010 (Desch et al., 2010) RCT-SB	• CV-risk • 3 F, 40 M • Age: 65.2 ± 1.2 y • 30% DM T1 or T2	• CV-risk • 17 F, 31 M • Age: 66.8 ± 1.1 y • 46% DM T1 or T2	25 g dark Chocolate	• EC: 21 mg • C: 7 mg • Other: 12 mg caffeine, 105 mg theobromine	6 g dark chocolate • EC: 5 mg • C: 1.7 mg • Other: 3.1 mg caffeine, 26.4 mg theobromine	1x /d, 2 h post-dinner	12 w	• 24-h BP, 30-min intervals	• 99% use anti- HT drugs • insulin allowed • no info on duration of DM or HbA1c	• SBP ↓ in both groups (25 g/d: −2.8 ± 1.1 mmHg; 6 g/d: −3.4 ± 0.5 mmHg), NS difference between 2 groups • DBP ↓ only in control group (6 g/ d: −1.8 ± 0.4 mmHg), NS difference between 2 groups
Monagas et al., 2009 (Monagas et al., 2009) CO-SB	• DM or CV-risk factors • 23 F, 19 M • Age: 69.7 ± 1.8 y	cross-over	2 × 20 g cocoa powder + 250 ml skim milk	• EC: 46.08 mg • C: 10.41 mg • Other: 0.44 g theobromine	Skim milk, 500 ml/d	2x /d	4 w	3 measurements	• no info on anti-DM or anti-HT drugs • % DM not specified • no info on duration of DM or HbA1c	No change

Data are expressed in mean ± SEM unless described differently. Subgroup A = papers investigating diabetic populations only; subgroup B1 = papers investigating mixed populations, i.e. type 2 diabetic plus non-diabetic; subgroup B2 = papers investigating mixed populations, i.e. type 1 and/or 2 diabetic plus non-diabetic.

* papers performed in a geographical area in which dietary average flavanol intake is high (>400 mg/day); [1]all blood pressures were measured using automated sphygmomanometers; [2]only significant changes for blood pressure are reported; BP = blood pressure (systolic SBP, diastolic DBP); C = catechins; CI = confidence interval; CO = crossover trial; CV = Cardiovascular; d = day; DB = double blinded trial; DM = Diabetes Mellitus (T1 = type 1, T2 = type2); EC = epicatechins; FL = flavanols; h = hours; HT = hypertension; min = minutes; m = months; NS = non-significance; RCT = Randomized Controlled Trial; rep = repetitions; S = significance; SB = single blinded trial (only investigators are blinded); w = weeks; y = year

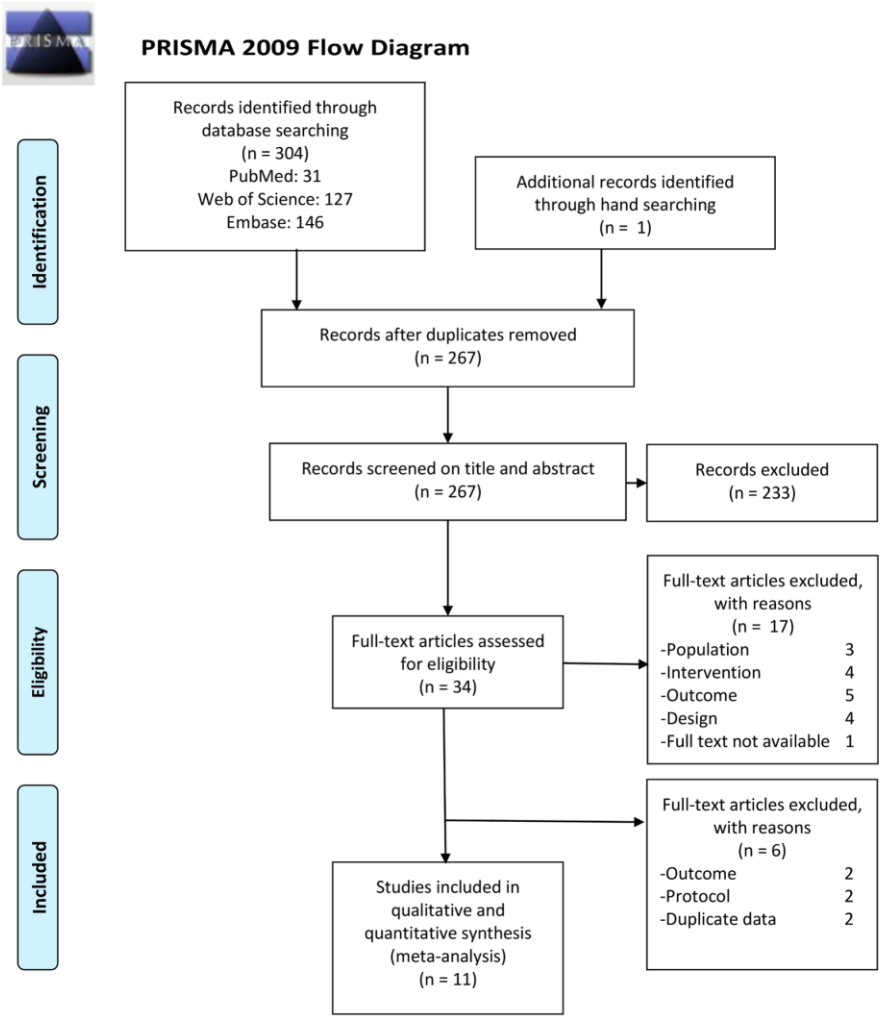

Fig. 1. Flow diagram showing the study selection process.

group. If certain information was not provided in an included paper, we followed Cochrane guidelines (Higgins et al., 2019) and used the calculator in RevMan to calculate the required data. When studies did not describe mean treatment effect scores and related standard deviations/ standard errors (Desch et al., 2010; Dicks et al., 2018; Gutiérrez-Salmeán et al., 2016; Mellor et al., 2010; Monagas et al., 2009; Sorond et al., 2013), effect scores were obtained by subtracting the final mean from the baseline mean and related standard deviations were computed using formulae provided by the Cochrane guidelines (Higgins et al., 2019). Since all correlation coefficients were above 0.5, we had to impute standard deviations for changes from baseline when not reported. Spearman correlation coefficients were used because these are the most conservative. Furthermore, 2 studies reported on 3 different intervention groups (Desideri et al., 2012; Mastroiacovo et al., 2015). Data of these interventions were combined as defined in the Cochrane handbook (Higgins et al., 2019).

The included publications were parallel RCT, apart from 2 CO-studies (Mellor et al., 2010; Monagas et al., 2009). Hence, 'generic inverse variance' was applied in RevMan as a method of analysis (Curtin et al., 2002; Elbourne et al., 2002).

If SBP and DBP were not reported separately (Balzer et al., 2008; Ramirez-Sanchez et al., 2013), or if relevant data could not be calculated (Ayoobi et al., 2017), corresponding authors were contacted by e-mail. In case of no answer, papers were excluded. In addition, if papers described a mixed population of whom only a certain percentage had T1DM or T2DM (Desch et al., 2010; Desideri et al., 2012; Gutiérrez-Salmeán et al., 2016; Mastroiacovo et al., 2015; Monagas et al., 2009; Sorond et al., 2013), corresponding authors were contacted by e-mail to obtain separate results for non-diabetic and diabetic subjects, with subdivision depending on type of DM. In case of no answer, the publication was included but data of the population (non-diabetic and diabetic subjects) was used in its entirety to evaluate the effect of CF.

Treatment effects of mid/long-term intake of CF on DBP and SBP were analyzed in the total group and then separately in each subgroup: subgroup A (T2DM only), B1 (non-diabetic plus T2DM), and B2 (non-diabetic, T1DM, and T2DM). To further examine which factors might explain possible heterogeneity in these results, the following additional subgroup analyses were performed on the total of included papers: split-dose versus single dose administration, dose of epicatechins administered/ day, composition of the placebo formula, BP at baseline, sex, age,

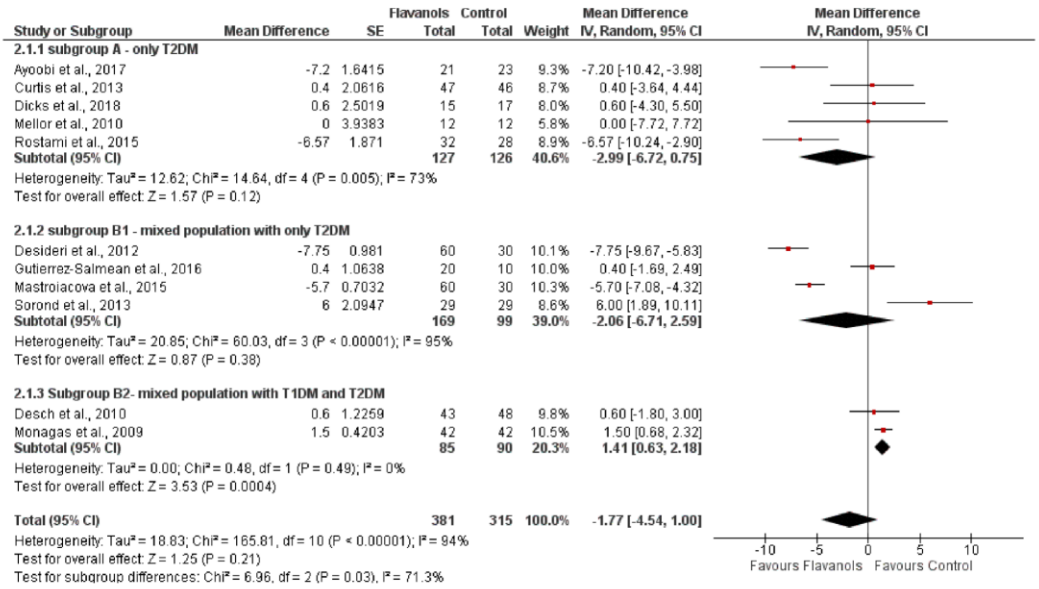

Fig. 2. Forest plot Systolic Blood Pressure. Solid diamonds represent the pooled estimates for each subgroup (A, B1 and B2) and in total; horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup A = papers investigating diabetic populations only; subgroup B1 = papers investigating mixed populations, i.e. type 2 diabetic plus non-diabetic; subgroup B2 = papers investigating mixed populations, i.e. type 1 and/or 2 diabetic plus non-diabetic.

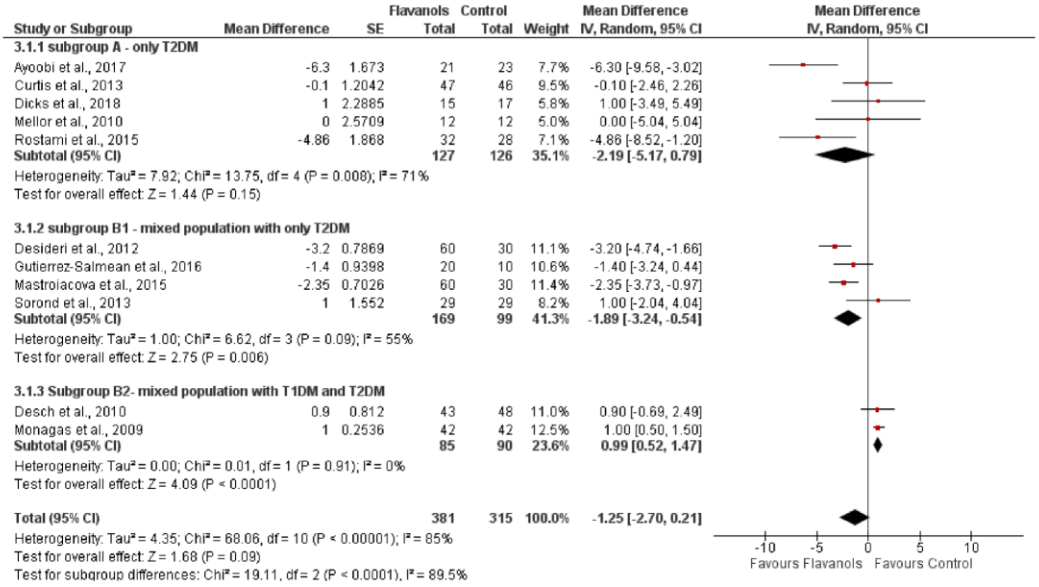

Fig. 3. Forest plot Diastolic Blood Pressure. Solid diamonds represent the pooled estimates for each subgroup (A, B1 and B2) and in total; horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup A = papers investigating diabetic populations only; subgroup B1 = papers investigating mixed populations, i.e. type 2 diabetic plus non-diabetic; subgroup B2 = papers investigating mixed populations, i.e. type 1 and/or 2 diabetic plus non-diabetic.

body mass index (BMI), and geographical differences in average daily flavanol intake. The cut-off point for age was based on the conventional definition of 'elderly' (Orimo et al., 2006) and the cut-off point for geographical differences in average daily flavanol intake was based on a report identifying regions with high (>400 mg) versus low (<400 mg) dietary flavanol ingestion (Escobar-Cévoli et al., 2017). Subgroup analyses based on percentage of cocoa, total daily dose of CF or medication use were not possible because data were either lacking or too heterogenous to analyze. For each analysis, the level of statistical heterogeneity (I^2, < 40%: might not be important and ≥ 75%: considerable

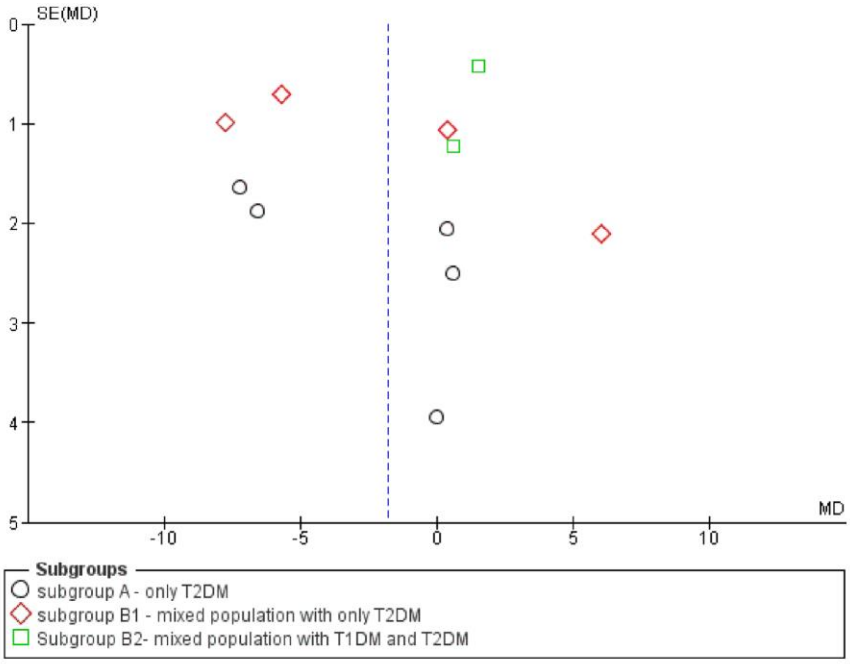

Fig. 4. Funnel plot Systolic Blood Pressure . The x-axis represents the mean difference; the y-axis represents the standard error of the mean difference; black open circles = subgroup A = papers investigating diabetic populations only; red open diamonds = subgroup B1 = papers investigating mixed populations, i.e. type 2 diabetic plus non-diabetic; green open squares = subgroup B2 = papers investigating mixed populations, i.e. type 1 and/or 2 diabetic plus non-diabetic.

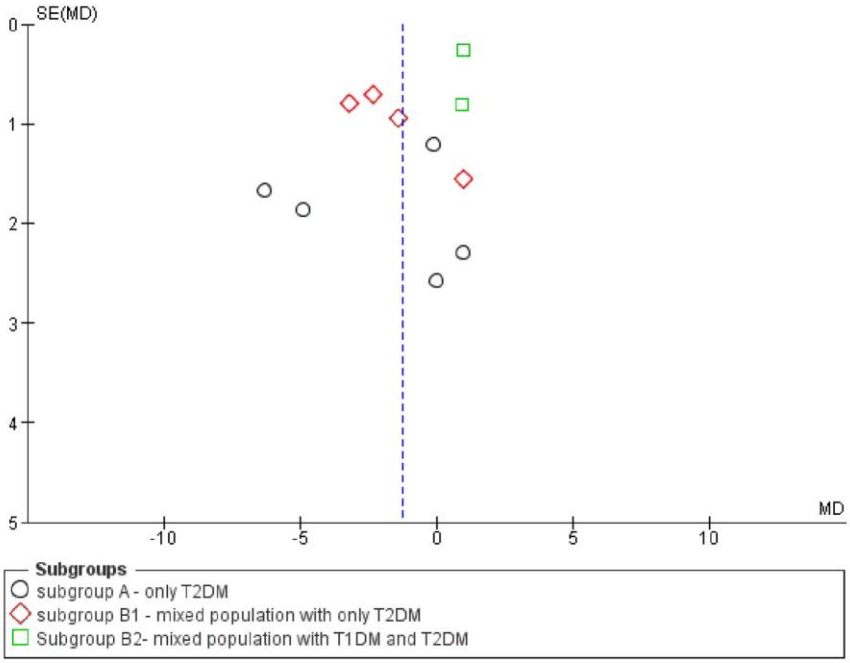

Fig. 5. Funnel plot Diastolic Blood Pressure. The x-axis represents the mean difference; the y-axis represents the standard error of the mean difference; black open circles = subgroup A = papers investigating diabetic populations only; red open diamonds = subgroup B1 = papers investigating mixed populations, i.e. type 2 diabetic plus non-diabetic; green open squares = subgroup B2 = papers investigating mixed populations, i.e. type 1 and/or 2 diabetic plus non-diabetic.

heterogeneity (Higgins et al., 2019)) was described. The level of statistical significance was set at an alpha-level below 0.05. Results are reported and depicted in Forest plots (mean difference and 95% CI).

3. Results

266 papers were identified for screening after literature search and 1 was additionally included after a manual search (Mastroiacovo et al., 2015) (Fig. 1). 233 citations were excluded after screening on title and abstract, and another 17 after subsequent screening on full text. 6 additional reports were excluded: 2 for not providing separate data on SBP and DBP (Balzer et al., 2008; Ramirez-Sanchez et al., 2013), 2 for presenting BP data that were also published elsewhere (Curtis et al., 2012; Haghighat et al., 2013), and 2 for investigating one-time (versus mid/long-term) CF administration (Basu et al., 2015; Rynarzewski et al., 2019).

3.1. Characteristics of eligible studies

All papers were published between 2009 and 2018 (Table 1). Sample sizes ranged from 12 − 60 in the intervention groups and from 10 − 48 in the control groups. All but one study (Curtis et al., 2013) included both sexes. Except in one publication (Gutiérrez-Salmeán et al., 2016), the mean age of each study population was > 50 years old and the greater part of subjects used antihypertensive (Curtis et al., 2013; Desch et al., 2010; Desideri et al., 2012; Dicks et al., 2018; Mastroiacovo et al., 2015; Mellor et al., 2010; Rostami et al., 2015) and antidiabetic drugs (Ayoobi et al., 2017; Curtis et al., 2013; Desch et al., 2010; Desideri et al., 2012; Dicks et al., 2018; Mastroiacovo et al., 2015; Mellor et al., 2010). Only in subgroup A, papers reported mean disease duration (7 months − 7 years) and mean baseline HbA1c values (6.4% − 7.6%) of the patients with T2DM. In other subgroups, these data were absent.

The intervention and placebo formulae varied considerably between the different studies as to the durations of CF intake (4 weeks − 1 year), as well as the CF-containing products used, and the administered amounts and frequencies. CF was administered in the form of dark chocolate (24–45 g), cocoa powder (2.5–58 g) or capsules, containing 207.5 mg – 993 mg flavanols and/or 16.6 mg – 185 mg epicatechins per day; they were given in one batch (Ayoobi et al., 2017; Desch et al., 2010; Desideri et al., 2012; Mastroiacovo et al., 2015; Rostami et al., 2015) or distributed over the day (Curtis et al., 2013; Dicks et al., 2018; Gutiérrez-Salmeán et al., 2016; Mellor et al., 2010; Monagas et al., 2009; Sorond et al., 2013). Placebo formulae were either not defined (Gutiérrez-Salmeán et al., 2016), or consisted of either no intervention (Ayoobi et al., 2017), only milk (Monagas et al., 2009), capsules with microcrystalline cellulose (Dicks et al., 2018), white (Rostami et al., 2015) or placebo (Curtis et al., 2013) chocolate, flavanol-poor chocolate (<2 mg epicatechin) (Mellor et al., 2010), flavanol-poor cocoa powder (26 mg flavanols (Sorond et al., 2013) or 48 mg flavanols and 5 mg epicatechin (Desideri et al., 2012; Mastroiacovo et al., 2015)) or a minute quantity of dark chocolate (6 g containing 5 mg epicatechin) (Desch et al., 2010).

Finally, very little information was provided on time of BP measurement and time between ingestion of antihypertensive medication and BP assessment. In addition, the methods of BP measurements varied between the included papers: 2 averaged two measurements (Dicks et al., 2018; Rostami et al., 2015), 4 assessed the mean of 3–4 times (Desideri et al., 2012; Mastroiacovo et al., 2015; Monagas et al., 2009; Sorond et al., 2013), 1 measured for 2 h with 10 min intervals (Curtis et al., 2013), 1 measured for 24 h with 30 min intervals (Desch et al., 2010), and 3 did not specify the frequency (Ayoobi et al., 2017; Gutiérrez-Salmeán et al., 2016; Mellor et al., 2010). Timing of the measurements was only reported by 4 citations, of which 2 measured in fasting state (Dicks et al., 2018; Mellor et al., 2010) and 2 in non-fasting state (Desideri et al., 2012; Mastroiacovo et al., 2015).

3.2. Risk of bias of eligible studies

The major reason for scoring papers as 'high risk' (Ayoobi et al., 2017; Curtis et al., 2013; Gutiérrez-Salmeán et al., 2016; Monagas et al., 2009; Sorond et al., 2013) or 'some concerns' (Desch et al., 2010; Dicks et al., 2018; Mellor et al., 2010; Rostami et al., 2015) was based on a negative score on domain 2 (bias due to deviations from intended interventions) and/or domain 5 (bias due to selection of the reported results) (Table S3). If the placebo differed visibly from the CF source (e.g. white chocolate versus dark chocolate (Rostami et al., 2015)), the impossibility of blinding participants and researchers led to a negative score on domain 2. Moreover, most trials were not analyzed in accordance with a pre-specified plan, which was finalized before unblinded outcome data were available for analysis (domain 5).

Based on the overall GRADE assessment (Table S4), the quality of the body of evidence appeared to be low (Schünemann et al., 2013). This appraisal can partly be explained by inconsistencies in results between studies (Table S4, Figs. 2 and 3). In addition, the funnel plot for DBP is rather symmetrical, but the funnel plot for SBP seems relatively asymmetrical, which could indicate publication bias (Figs. 4 and 5). However, based on the Egger's test, no publication bias is present in total sample of included studies, nor for SBP ($p = 0.50$), nor for DBP ($p = 0.06$).

3.3. SBP

No statistically significant effect on SBP was seen in subgroup A, subgroup B1 or in group A + B together (Fig. 2): mean treatment effect was –2.99 mmHg in subgroup A (95% CI : −6.72, 0.75, $I^2 = 73\%$, 127 participants in the intervention group and 126 participants in the control group), −2.06 mmHg in subgroup B1 (95% CI : −6.71, 2.59, $I^2 = 95\%$, 169 participants in the intervention group and 99 participants in the control group), and −1.77 mmHg in group A + B together (95% CI: −4.54, 1.00, $I^2 = 94\%$, 381 participants in the intervention group and 315 participants in the control group).

In subgroup B2 a statistically significant increase in SBP was observed (mean treatment effect + 1.41 mmHg, 95% CI : 0.63, 2.18, $I^2 = 0\%$, 85 participants in the intervention group and 90 participants in the control group).

3.4. DBP

CF induced a statistically significant decrease in DBP (Fig. 3) in subgroup B1 (mean treatment effect −1.89 mmHg; 95% CI : −3.24, −0.54, $I^2 = 55\%$, 169 participants in the intervention group and 99 participants in the control group).

There was no statistically significant effect of CF on DBP in either subgroup A (mean treatment effect −2.19 mmHg, 95% CI : −5.17, 0.79, $I^2 = 71\%$, 127 participants in the intervention group and 126 participants in the control group) or in group A + B together (mean treatment effect −1.25 mmHg, 95% CI: −2.70, 0.21, $I^2 = 85\%$, 381 participants in the intervention group and 315 participants in the control group).

Similarly to SBP, an increase in DBP in subgroup B2 was indicated (mean treatment effect + 0.99 mmHg, 95% CI : 0.52, 1.47, $I^2 = 0\%$, 85 participants in the intervention group and 90 participants in the control group).

3.5. Additional subgroup analyses

3.5.1. SBP

We performed additional subgroup analyses to assess the influence of various variables on our outcomes. These indicated that the effect of CF on SBP could have been influenced by single versus split-dose CF ingestion, the amount of epicatechins administered/ day, BP at baseline, sex, and usual dietary CF content. In studies administrating CF as a single dose, SBP decreased by 5.28 mmHg (95% CI : −8.15, −2.41, $I^2 = 87\%$), whereas a split-dose administration even increased SBP by 1.42

mmHg (95% CI : 0.26, 2.58), $I^2 = 20\%$) (Figure S1). Epicatechin content of 16 – 46 mg/ day, induced an increase of SBP by 1.37 mmHg (95% CI : 0.61, 2.14, $I^2 = 0\%$), while daily doses of epicatechins between 90 and 185 mg did not affect SBP (mean treatment effect: −3.36, 95% CI : −7.20, 0.47, $I^2 = 93\%$) (Figure S2). When at least 50% of all subjects in each paper had systolic hypertension (≥140 mmHg) at baseline, CF lowered SBP by 5.14 mmHg (95% CI: −7.51, −2.78, $I^2 = 69\%$), whereas no effect was observed when <50% had elevated SBP's at baseline (mean treatment effect + 0.23 mmHg 95% CI : −2.20, 2.65, $I^2 = 85\%$) (Figure S3). Furthermore, papers with a study population of >60% female participants showed a decrease of 5.04 mmHg (95% CI : −7.65, −2.42, $I^2 = 69\%$), whereas in trials with equal sex distribution (mean treatment effect −0.06 mmHg, 95% CI : −5.33, 5.21, $I^2 = 95\%$) no impact of CF on SBP was identified (Figure S4).

Subgroup analysis based on the geographical area in which the studies were performed indicated that in areas in which average daily flavanol ingestion normally exceeds 400 mg (e.g. Iran, UK, and Poland), CF tended to lower blood pressure by 3.86 mmHg (95% CI : −7.92, 0.20, $I^2 = 72\%$, $p = 0.06$). Contrastingly, in areas in which no more than 400 mg of flavanols are generally ingested daily (e.g. Germany, Mediterranean countries, and US), CF administration did not influence SBP (mean treatment effect: −0.99, 95% CI: −5.00, 3.01, $I^2 = 96\%$) (Figure S5).

The equilibration of intervention and placebo formula with caffeine and theobromine, age or BMI, did not seem to influence the effect of CF on SBP (Figures S6, S7, and S8).

3.5.2. DBP

Similarly, subgroup analyses showed that CF effect on DBP could have been influenced by single dose versus split-dose CF administration, the amount of epicatechins administered/ day, and sex, but in addition also by age, but not by dietary flavanol use. In studies with single CF doses, DBP dropped by 2.82 mmHg (95% CI : −5.00, −0.65, $I^2 = 83\%$), whereas split-dose studies showed no effect on DBP (mean treatment effect + 0.38 mmHg, 95% CI : −0.57, 1.33, $I^2 = 26\%$) (Figure S9). Epicatechin content ranging from 90 to 185 mg/ day decreased DBP by 1.99 mmHg (95% CI : −3.15, −0.83, $I^2 = 44\%$), while daily doses of epicatechins ranging from 16 to 46 mg increased DBP by 0.98 mmHg (95% CI : 0.51, 1.45, $I^2 = 0\%$) (Figure S10). Sex, again, appeared relevant: CF lowered DBP in papers with a study population of >60% female participants, −3.08 mmHg (95% CI: −5.45, −0.71, $I^2 = 72\%$), but not with equal sex distribution (mean treatment effect −0.20 mmHg, 95% CI : −2.58, 2.18, $I^2 = 85\%$) (Figure S11). Papers studying ages below 65 years found decreases in DBP by 2.87 mmHg (95% CI : −5.52, −0.23, $I^2 = 75\%$), as opposed to publications on mean ages above 65 years, which observed no effect on DBP (mean treatment effect: −0.41 mmHg, 95% CI: −2.11, 1.29, $I^2 = 86\%$) (Figure S12).

The equilibration of intervention and placebo formula with caffeine and theobromine, BMI or geographical differences in dietary flavanol use, did not seem to influence the effect of CF on DBP (Figures S13, S14, and S15). Since all participants in each study had normal ranges for DBP at baseline, the influence of baseline elevated DBP could not be assessed in a subgroup analysis.

4. Discussion

The aim of this systematic review and *meta*-analysis was to identify whether CF affect BP and/or vascular function in patients with DM. The paucity of reports (<4 publications on comparable vascular functions), however, confined us to the effects on BP only. Our analysis shows low quality of evidence due to risk of bias, inconsistency and heterogeneity among the publications, and imprecision of the available reports (GRADE). At best, there are weak indications of slight improvement in SBP and DBP after mid/long-term CF ingestion. Possibly, CF effects are greater when they contain at least 90 mg of epicatechin, when given in a single dose, and when subjects are female, younger and hypertensive. However, as mentioned, no definite conclusions can be drawn, neither positive nor negative.

4.1. Effects of cocoa flavanols (CF) on blood pressure (BP)

Our *meta*-analysis suggests that CF reduces DBP, but not SBP, by ~ 1–2 mmHg. This is compatible with the postulated mechanism of action: if, indeed, CF effects are largely achieved through increased NO bioavailability (Aprotosoaie, Miron, et al., 2016; Schewe et al., 2008) (see above), this may primarily affect peripheral vascular resistance (Cooke & Dzau, 1997) and hence DBP. SBP is predominantly associated with cardiac output rather than peripheral vascular resistance (Bouman et al., 2008). Nonetheless, SBP also showed a slight decrease of 1–3 mmHg, but this did not reach statistical significance.

Although small, these changes may not be irrelevant (Cook et al., 1995; Stamler, 1991; Whelton et al., 2002). In general populations aged 35 to 64 years, reductions of 2 mmHg in DBP and 2–5 mmHg in SBP decreased mortality by 3–7% (Stamler, 1991) and the risk of developing diastolic hypertension, coronary heart diseases and stroke/transient ischemic attack by 17%, 6%, and 15% respectively (Cook et al., 1995). Of note, in our *meta*-analysis, 4 out of 11 studies found statistically significant reductions in SBP of ≥ 5.70 mmHg and in DBP of ≥ 2.35 mmHg (Ayoobi et al., 2017; Desideri et al., 2012; Mastroiacovo et al., 2015; Rostami et al., 2015).

4.2. Heterogeneity

As mentioned, however, there was marked clinical and methodological heterogeneity between the citations, both in the actual intervention (administered dose, daily frequency, and the nature of both intervention and placebo formulae), and in the population characteristics (sex, BMI, age, the stage of disease (concerning DM, hypertension or other cardiovascular conditions), use of antihypertensive/ antidiabetic medication, type of DM, and geographical location). All of these aspects may have influenced the outcomes.

4.2.1. Heterogeneity in intervention

In order to address this heterogeneity, we performed various subgroup analyses where possible. These suggested that SBP and DBP decreased with one-time daily, but not split- dose administration. This is not surprising considering the absorption time and half-life of CF: epicatechin concentrations reach their maximum 2 to 3 h after ingestion (Baba et al., 2000; Richelle et al., 1999). However, conclusions must be drawn with caution: possibly, the timing of the split-doses relative to the timing and technique of the BP measurement (single measurement, multiple measurements, 24-hour blood pressure monitoring) could have affected the effective (peak) plasma dose at the time of BP measurement and therefore the actual outcomes as well as their comparability.

This comparability is further challenged by the composition of intervention and placebo formulae. Some, but not all studies equilibrated their placebo formula to the intervention: in order to isolate CF effects from other vaso-active compounds of cocoa, they added theobromine and/or caffeine to the placebo formula (Aprotosoaie, Luca, et al., 2016; Echeverri et al., 2010). Although our subgroup analysis did not identify a statistical effect of equilibration, given the paucity of studies analyzed, we cannot exclude that equilibration clouded (i.e. underestimated) overall effects.

In addition, CF composition and dose varied; since it was not even reported in all studies, we directed our subgroup analysis on epicatechin content, which, interestingly, was reported much more precisely. Only 90 mg of epicatechins/ day or more lowered BP. Although, again, this conclusion must be drawn with caution given the limitations of our *meta*-analysis in general and the subgroup analyses in particular (see below 4.3), it underlines the relevance of the (sub)content of the administered CF. A significant fraction of flavanols, including epicatechin and its oligomeric forms (procyanidins), are metabolized by colonic microbiota before absorption (Del Rio et al., 2013) into

biologically active metabolites. Ex vivo, but not in vivo human studies, suggest that these metabolites could exert vascular effects in their own right, among others by reducing endothelial oxidative stress (Álvarez-Cilleros et al., 2018; Álvarez-Cilleros et al., 2018; Fernandez-Millan et al., 2014). However, the clinical relevance of the latter is unclear (Rodriguez-Mateos et al., 2018).

Furthermore, the studies did not correct for baseline dietary flavanol content, which was only assessed in 2 citations (Desideri et al., 2012; Mastroiacovo et al., 2015). We therefore performed a subgroup analysis based on geographical location of the studies as a next best option. Somewhat to our surprise, CF seemed to lower BP ($p = 0.06$) in high-flavanol areas (average daily ingestion of the general population > 400 mg, e.g. Iran, UK, and Poland (Escobar-Cévoli et al., 2017)), but not in low flavanol areas (average daily ingestion < 400 mg, e.g. Germany, Mediterranean countries, and US). Again, the meaning of this outcome is unclear, since geographical area was not the only difference between the trials, and, as mentioned, most results were not corrected for individual daily flavanol intake.

4.2.2. Heterogeneity in study population

Not surprisingly, the heterogeneity of the baseline characteristics of the investigated populations further challenges the comparability of the results. Our subgroup analyses did not identify effects of BMI, in contrast to sex and age: CF seemed more effective in lowering SBP and DBP in women than in men. This is compatible with previous reports on sex differences in the regulation of vascular tone (De Angelis et al., 2004; Thompson & Khalil, 2003), and might be a concept worth considering in future research and perhaps clinical practice. Similarly, DBP, but not SBP, decreased in people under, but not over 65 years of age. Ried et al. (2017) report similar findings, using a cut-off point of 50 years; the authors postulate that the structural arterial changes in the elderly subdue vascular reactivity to physiological stimuli (Ried et al., 2017).

Nevertheless, CF effects in general seem more pronounced in the presence of vascular dysfunction (Aprotosoaie, Miron, et al., 2016; Kerimi & Williamson, 2015). For instance, a *meta*-analysis in non-diabetic subjects showed better CF-induced BP reduction in hypertensive than pre-hypertensive people, whereas there was no effect in normotensive people (Ried et al., 2017). We therefore investigated the effect of baseline hypertension in the study populations of our *meta*-analysis. Although mean BP was described as normal in all publications (120–140/ 70–85 mmHg), almost all studies reported the use of anti-hypertensive medication in several subjects, and standard deviations of BP were relatively high (up to 17 for baseline-SBP and 10 for baseline-DBP (Mellor et al., 2010)). Hence, we may conclude that a considerable fraction of the participants had essential hypertension (SBP $\geq$ 140 and DBP $\geq$ 90 (Hypertension. (13 september, 2019)) at baseline. And indeed, our subgroup analyses suggested stronger BP lowering effects in presence of baseline systolic hypertension; analysis for DBP was, unfortunately, not statistically feasible.

We could not correct for the use of antihypertensive drugs per se, again due to data heterogeneity. Nonetheless, this could be relevant considering their intrinsic vasoactive effects which might have amplified or annihilated CF effects. The same is true for antidiabetic drugs and insulin, which may stimulate NO synthase by themselves (Ferrannini & Cushman, 2012) and therefore affect the outcome, and for factors on which little or no information was given, such as the duration of DM and the presence of diabetic vascular complications.

4.3. Limitations

As mentioned, our *meta*-analysis was limited by the small amount of publications, the relatively small sample size of each and the considerable clinical and methodological heterogeneity amongst the papers, even on important clinical aspects and confounders. We describe these different aspects and their possible influence on the outcomes in our discussion. The subgroup analyses, which we performed in order to gain more insight into these factors, were based on rather arbitrary cut-off points and were binary by design. Other cut-offs and/or non-binary correction for confounding factors could perhaps have yielded different results. Combined with the rather high risk of bias and inconsistency, this results in low quality of evidence.

Therefore, we feel that all results, particularly those of the subgroup analyses should be interpreted with caution; at best, they indicate directions on which future research could be based.

5. Conclusion

Although CF seem to be promising nutraceuticals with potential beneficial effects on vascular health in the general population, the clinical evidence corroborating this theoretical notion is very weak in DM.

Our *meta*-analysis, restricted to BP effects, suggests that, at best, there is weak evidence for a reduction of DBP, but not SBP, by 1–2 mmHg, after mid/long-term CF administration; these effects seem stronger in female, younger and hypertensive people, when CF is ingested in 1 daily batch, and when epicatechin content is high enough. However, the marked heterogeneity among the available small studies challenges the drawing of unequivocal conclusions. Nevertheless, the world-wide prevalence of DM and the associated cardiovascular morbidity warrant further exploration of the possible role CF might play in its therapy or prevention. Especially given the proven, consistent CF effects in healthy and hypertensive people as well as the theoretical background CFs influence on NO bioavailability, we highly recommend further research using larger sample sizes and correction for the above-mentioned confounders.

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CRediT authorship contribution statement

Anouk Tanghe: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Elsa Heyman:** Conceptuialization, Writing - review & editing. **Karsten Vanden Wyngaert:** Investigation, Validation, Writing - review & editing. **Ans Van Ginckel:** Formal analysis, Investigation, Methodology, Validation, Writing - review & editing. **Bert Celie:** Conceptualization, Writing - original draft. **Ernst Rietzschel:** Conceptualization, Writing - review & editing. **Patrick Calders:** Conceptualization, Investigation, Methodology, Project administration, Supervision, Visualization, Writing - original draft. **Samyah Shadid:** Conceptualization, Project administration, Supervision, Visualization, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104399.

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Table S1 Eligibility criteria

Selection criteria	Inclusion criteria	Exclusion criteria
Population	diabetes mellitus, humans	animals, cells
Intervention	cacao, catechin, cocoa, cocoa flavanol, epicatechin, chocolate	other sources of flavonoids like tea and wine
Comparison	/	/
Outcome	vascular stiffness, vascular resistance, peripheral resistance; vasodilation; vasodilatation; vasorelaxation; vasoconstriction; blood circulation; regional blood flow; blood pressure; arterial pressure; systolic pressure; diastolic pressure; blood vessels; endothelium; endothelial function	
Study Design	randomized-controlled studies	all articles that are no randomized-controlled studies

Table S2 Search strategy

Database	Search query	# hits	# hits After deduplication
PubMed	("Diabetes Mellitus"[Mesh] OR diabetes mellitus OR diabetes OR “diabetic patients”) AND (“cacao” [Mesh] OR “catechin” [Mesh] OR epicatechin OR Cacao OR Catechin OR Cocoa OR Cocoa Flavanol OR Epicatechin OR Chocolate) AND (“Vascular Stiffness” [Mesh] OR “Vascular Resistance” [Mesh] OR “Vasodilation” [Mesh] OR “Vasoconstriction” [Mesh] OR “Regional Blood Flow” [Mesh] OR “Blood Circulation” [Mesh] OR “Blood Pressure” [Mesh] OR “Arterial Pressure” [Mesh] OR “Blood Vessels” [Mesh] OR “Endothelium” [Mesh] OR Vascular Stiffness OR Vascular Resistance OR Peripheral Resistance OR Vasodilation OR Vasodilatation OR Vasorelaxation OR Vasoconstriction OR Blood Circulation OR Regional Blood Flow OR Blood pressure OR Arterial Pressure OR Systolic Pressure OR Diastolic Pressure OR Blood Vessels OR Endothelium OR Endothelial Function) AND (randomized controlled trial[pt] OR randomized controlled trials as topic[mh] OR random allocation [mh] OR double-blind method[mh] OR single-blind method[mh] OR random*[tw] OR "Placebos"[Mesh] OR placebo[tiab] OR ((singl*[tw] OR doubl*[tw] OR trebl*[tw] OR tripl*[tw]) AND (mask*[tw] OR blind*[tw] OR dumm*[tw]))) NOT review	31	267
Web of Science	(TS=(("Diabetes Mellitus"[Mesh] OR diabetes mellitus OR diabetes OR “diabetic patients”) AND (epicatechin OR Cacao OR Catechin OR Cocoa OR Cocoa Flavanol OR Epicatechin OR Chocolate) AND (Vascular Stiffness OR Vascular Resistance OR Peripheral Resistance OR Vasodilation OR Vasodilatation OR Vasorelaxation OR Vasoconstriction OR Blood Circulation OR Regional Blood Flow OR Blood pressure OR Arterial Pressure OR Systolic Pressure OR Diastolic Pressure OR Blood Vessels OR Endothelium OR Endothelial Function))) AND DOCUMENT TYPES: (Article)	127	

	Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, ESCI Timespan=All years	
Embase	('diabetes mellitus'/exp OR 'diabetes' OR 'diabetes mellitus' OR 'diabetic' OR 'diabetic patient'/exp OR 'diabetes mellitus patient' OR 'diabetes patient' OR 'diabetic patient' OR 'diabetic people' OR 'diabetic person' OR 'diabetic subject') AND ('cacao'/exp OR 'theobroma cacao' OR 'cacao' OR 'cacao tree' OR 'cocao' OR 'cocoa' OR 'cocoa tree' OR 'chocolate'/exp OR 'chocolate' OR 'catechin'/exp OR '3 cyanidanol' OR '3, 3`, 4`, 5, 7 flavanpentol' OR 'catechin' OR 'catechinic acid' OR 'catechuic acid' OR 'catergen' OR 'cathergene' OR ' ciandiol' OR 'ciandiol 3' OR 'cianidanol' OR 'cianidanol monohydrate' OR 'cianidanol tetrahydrate' OR 'cianidol' OR 'cyanidanol' OR 'cyanidanol 3' OR 'cyanidol' OR 'd catechin' OR 'dextro catechine' OR 'dextro cyanidanol 3' OR 'kb 53' OR 'epicatechin'/exp OR 'epicatechin' OR 'procyanidin'/exp OR 'procyanidin' OR 'procyanidine') AND ('vascular disease'/exp OR 'angiopathy' OR 'blood vessel disease' OR 'blood vessel disorder' OR 'disturbance, vascular' OR 'hemangiopathy' OR 'ischaemic vasculopathy' OR 'ischemic vasculopathy' OR 'macroangiopathy' OR 'vascular disease' OR 'vascular diseases' OR 'vascular disorder' OR 'vascular disturbance' OR 'vascular pathology' OR 'vasculopathia' OR 'vasculopathy' OR 'vascular function'/exp OR 'cardiovascular system'/exp OR 'cardiovascular system' OR 'cardiovascular tract' OR 'circulatory system' OR 'vascular system' OR 'endothelium cell'/exp OR 'cell, endothelium' OR 'endothelial cell' OR 'endothelial cells' OR 'endothelial lining cell' OR 'endothelium cell' OR 'endothelium lining cell' OR 'littoral cell' OR 'endothelial dysfunction'/exp OR 'endothelial disease' OR 'endothelial diseases' OR 'endothelial disorder' OR 'endothelial disorders' OR 'endothelial dysfunction' OR 'endothelial dysfunctions' OR 'endothelium disease' OR 'endothelium diseases' OR 'endothelium disorder' OR 'endothelium disorders' OR 'endothelium dysfunction' OR 'endothelium dysfunctions' OR 'endothelium'/exp OR 'endothelial junction' OR 'endothelium' OR 'flow-mediated dilation test'/exp OR 'fmd	146

technique' OR 'fmd test' OR 'flow-mediated dilatation assessment' OR 'flow-mediated dilatation technique' OR 'flow-mediated dilatation test' OR 'flow-mediated dilation assessment' OR 'flow-mediated dilation technique' OR 'flow-mediated dilation test' OR 'blood pressure'/exp OR 'blood pressure' OR 'blood tension' OR 'intravascular pressure' OR 'normotension' OR 'pressure, blood' OR 'vascular pressure' OR 'vasodilatation'/exp OR 'blood vessel dilatation' OR 'dilatation, blood vessel' OR 'vascular dilation' OR 'vaso dilatation' OR 'vasodilatation' OR 'vasodilatation reflex' OR 'vasodilation' OR 'vasodilation test' OR 'vasodilator system' OR 'vasorelaxation' OR 'vasoconstriction'/exp OR 'vasoconstriction' OR 'vasoconstrictor reflex' OR 'vasoconstrictor test' OR 'vessel constriction' OR 'arterial stiffness'/exp OR 'aorta stiffness' OR 'aortic stiffening' OR 'aortic stiffness' OR 'aortic wall stiffening' OR 'aortic wall stiffness' OR 'arterial stiffening' OR 'arterial stiffness' OR 'arterial wall stiffening' OR 'arterial wall stiffness' OR 'artery stiffening' OR 'artery stiffness' OR 'artery wall stiffening' OR 'artery wall stiffness' OR 'vascular stiffness' OR 'vascular resistance'/exp OR 'blood flow resistance' OR 'blood vessel resistance' OR 'peripheral resistance' OR 'peripheral vascular resistance' OR 'total peripheral resistance' OR 'vascular resistance' OR 'vascular vessel resistance' OR 'venous resistance') AND (([randomized controlled trial]/lim OR [controlled clinical trial]/lim OR randomized) AND controlled AND trial AND topic OR randomized) AND controlled AND 'trial'/exp OR 'randomization'/exp OR randomisation OR randomization OR 'random allocation' OR 'double blind procedure'/exp OR 'double blind method' OR 'double blind study' OR 'double blind studies' OR 'double blind procedure' OR 'single blind procedure'/exp OR 'single blind method' OR 'single blind study' OR 'single blind studies' OR 'placebo'/exp OR random*:ti,ab,kw OR sham:ti,ab,kw OR placebo*:ti,ab,kw OR (((singl* OR doubl*) NEXT/1 (blind* OR dumm* OR mask*)):ti,ab,kw) OR (((tripl* OR trebl*) NEXT/1 (blind* OR dumm* OR mask*)):ti,ab,kw))

Table S3. Risk of bias of eligible studies

Paper	Dom. 1	Dom. 2a	Dom. 2b	Dom. 3	Dom. 4	Dom. 5	Overall ROB
subgroup A - only T2DM							
Ayoobi et al., 2017 (Ayoobi et al., 2017)	low	/	Some concerns	Low	High	Some concerns	high
Curtis et al., 2013 (Curtis et al., 2013)	low	/	Some concerns	Low	Low	high	high
Dicks et al., 2018 (Dicks et al., 2018)	low	/	low	Low	Low	Some concerns	Some concerns
Mellor et al., 2010 (Mellor et al., 2010)	Some concerns	Some concerns	/	Low	Low	low	Some concerns
Rostami et al., 2015 (Rostami et al., 2015)	low	/	Some concerns	Low	Low	Some concerns	Some concerns
subgroup B1 - mixed population with T2DM							
Desideri et al., 2012 (Desideri et al., 2012)	low	low	/	Low	Low	low	low
Gutiérrez-Salmeán et al., 2016 (Gutiérrez-Salmeán et al., 2016)	low	low	/	Low	Low	high	high
Mastroiacovo et al., 2015 (Mastroiacovo et al., 2015)	low	low	/	Low	Low	low	low
Sorond et al., 2013 (Sorond et al., 2013)	low	Some concerns	/	Low	Low	high	high
subgroup B2 - mixed population with T1DM and T2DM							
Desch et al., 2010 (Desch et al., 2010)	low	/	Some concerns	Low	Some concerns	Some concerns	Some concerns
Monagas et al., 2009 (Monagas et al., 2009)	low	low	/	Low	Low	high	high

Each domain and overall Risk of bias (ROB) was rated as 'low risk', 'some concerns' or 'high risk'. Domain 1= bias arising from the randomization process; domain 2= bias due to deviations from intended interventions (a= effect of assignment to intervention (in case of an intention-to-treat

effect), b= effect of adhering to intervention (in case of a per-protocol effect)); domain 3= bias due to missing outcome data; domain 4= bias in measurement of the outcome; domain 5= bias in selection of the reported result; Dom.= domain; subgroup A= papers investigating diabetic populations only; subgroup B1= papers investigating mixed populations, i.e. type 2 diabetic plus non-diabetic; subgroup B2= papers investigating mixed populations, i.e. type 1 and/or 2 diabetic plus non-diabetic.

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Table S4. Quality Assessment of Outcome (GRADE)

Outcome	# studies, design (n)	Quality of evidence factors						Overall quality of evidence (GRADE)	importance
		ROB	Inconsistency (statistical test, I^2)	Indirectness	Imprecision (sample size)	Publication bias	Other factors and upgrading		
subgroup A - only T2DM									
SBP	4 RCT (229), 1 CO (12)	VS.[a]	S. (p=0.005; I^2=73%)	NS.	S. (229 + 12)	undetected[b,c]	Upgrading: dose-response relation	Low	Important but not critical
DBP	4 RCT (229), 1 CO (12)	VS.[a]	S. (p=0.008; I^2=71%)	NS.	S. (229 + 12)	Undetected[b,c]	Upgrading: dose-response relation	Low	Of limited importance
subgroup B1 - mixed population with T2DM									
SBP	4 RCT (268)	S.[d]	VS. (p<0.00001; I^2= 95%)	S.[e,f]	S. (268)	undetected[b,c]	Upgrading: dose-response relation	Low	Important but not critical
DBP	4 RCT (268)	S.[d]	S. (p=0.09; I^2= 55%)	S.[e,f]	S. (268)	Undetected[b,c]	Upgrading: dose-response relation	Low	Of limited importance
subgroup B2 - mixed population with T1DM and T2DM									
SBP	1 RCT (91), 1 CO (42)	S.[g]	NS. (p=0.76; I^2= 0%)	S.[e,f]	S. (91+42)	undetected[b,c]	Upgrading: dose-response relation	Low	Important but not critical
DBP	1 RCT (91), 1 CO (42)	S.[g]	NS. (p=0.96; I^2= 0%)	S.[e,f]	S. (91+42)	undetected[b,c]	Upgrading: dose-response relation	Low	Of limited importance

[a]effect of assignment and effect of adhering to intervention; [b]based visually on funnel plot; [c]based on Egger's test (all included papers together); [d]effect of assignment to intervention; [e]very important differences in population; [f]differences in intervention or comparison sufficient to make a difference in outcome; [g]effect of adhering to intervention; CO= cross-over; DBP= diastolic blood pressure; n= number of participants; NS.= Not Serious; RCT= randomized controlled trial; ROB= risk of bias; S.= serious; SBP= systolic blood pressure; subgroup A= papers investigating diabetic populations only; subgroup B1= papers investigating mixed populations, i.e. type 2 diabetic plus non-diabetic; subgroup B2= papers investigating mixed populations, i.e. type 1 and/or 2 diabetic plus non-diabetic; VS.= very serious.

Figures S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, and S15: Forest plots subgroup analyses

Study or Subgroup	Mean Difference	SE	Flavanols Total	Control Total	Weight	Mean Difference IV, Random, 95% CI
1.1.1 1 dose daily						
Ayoobi et al., 2017	-7.2	1.6415	21	23	18.3%	-7.20 [-10.42, -3.98]
Desch et al., 2010	0.6	1.2259	43	48	20.4%	0.60 [-1.80, 3.00]
Desideri et al., 2012	-7.75	0.981	60	30	21.5%	-7.75 [-9.67, -5.83]
Mastroiacova et al., 2015	-5.7	0.7032	60	30	22.6%	-5.70 [-7.08, -4.32]
Rostami et al., 2015	-6.57	1.871	32	28	17.2%	-6.57 [-10.24, -2.90]
Subtotal (95% CI)			**216**	**159**	**100.0%**	**-5.28 [-8.15, -2.41]**
Heterogeneity: Tau² = 9.00; Chi² = 31.47, df = 4 (P < 0.00001); I² = 87%						
Test for overall effect: Z = 3.61 (P = 0.0003)						
1.1.2 several doses daily (split-dose)						
Curtis et al., 2013	0.4	2.0616	47	46	7.5%	0.40 [-3.64, 4.44]
Dicks et al., 2018	0.6	2.5019	15	17	5.2%	0.60 [-4.30, 5.50]
Gutierrez-Salmean et al., 2016	0.4	1.0638	20	10	22.2%	0.40 [-1.69, 2.49]
Mellor et al., 2010	0	3.9383	12	12	2.2%	0.00 [-7.72, 7.72]
Monagas et al., 2009	1.5	0.4203	42	42	55.7%	1.50 [0.68, 2.32]
Sorond et al., 2013	6	2.0947	29	29	7.3%	6.00 [1.89, 10.11]
Subtotal (95% CI)			**165**	**156**	**100.0%**	**1.42 [0.26, 2.58]**
Heterogeneity: Tau² = 0.45; Chi² = 6.22, df = 5 (P = 0.29); I² = 20%						
Test for overall effect: Z = 2.40 (P = 0.02)						
Test for subgroup differences: Chi² = 17.99, df = 1 (P < 0.0001), I² = 94.4%						

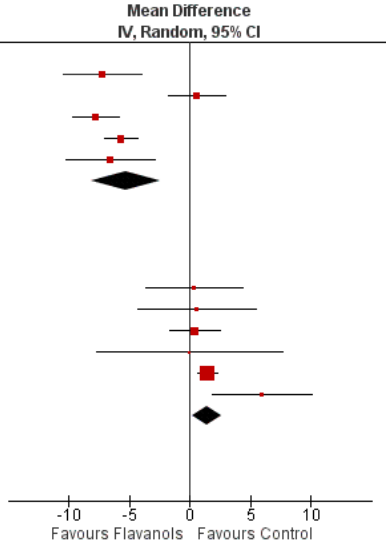


Figure S1**.** Forest plot Systolic Blood Pressure – 1 dose daily versus several doses daily (split-dose)

Solid diamonds represent the pooled estimates for each subgroup (1 dose daily, several doses daily (split-dose)); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (1.1.1)= included trials in which the intervention is administered 1 time daily; subgroup 2 (1.1.2)= included trials in which the intervention is administered throughout the day in split-doses.

Study or Subgroup	Mean Difference	SE	Weight	Mean Difference IV, Random, 95% CI
2.1.1 16-46 mg epicatechins/ day				
Desch et al., 2010	0.6	1.2259	10.2%	0.60 [-1.80, 3.00]
Dicks et al., 2018	0.6	2.5019	2.4%	0.60 [-4.30, 5.50]
Mellor et al., 2010	0	3.9383	1.0%	0.00 [-7.72, 7.72]
Monagas et al., 2009	1.5	0.4203	86.4%	1.50 [0.68, 2.32]
Subtotal (95% CI)			**100.0%**	**1.37 [0.61, 2.14]**
Heterogeneity: Tau² = 0.00; Chi² = 0.71, df = 3 (P = 0.87); I² = 0%				
Test for overall effect: Z = 3.51 (P = 0.0004)				
2.1.2 90-185 mg epicatechins/ day				
Curtis et al., 2013	0.4	2.0616	21.3%	0.40 [-3.64, 4.44]
Desideri et al., 2012	-7.75	0.981	26.0%	-7.75 [-9.67, -5.83]
Gutierrez-Salmean et al., 2016	0.4	1.0638	25.8%	0.40 [-1.69, 2.49]
Mastroiacova et al., 2015	-5.7	0.7032	26.9%	-5.70 [-7.08, -4.32]
Subtotal (95% CI)			**100.0%**	**-3.36 [-7.20, 0.47]**
Heterogeneity: Tau² = 13.75; Chi² = 40.73, df = 3 (P < 0.00001); I² = 93%				
Test for overall effect: Z = 1.72 (P = 0.09)				
Test for subgroup differences: Chi² = 5.63, df = 1 (P = 0.02), I² = 82.2%				

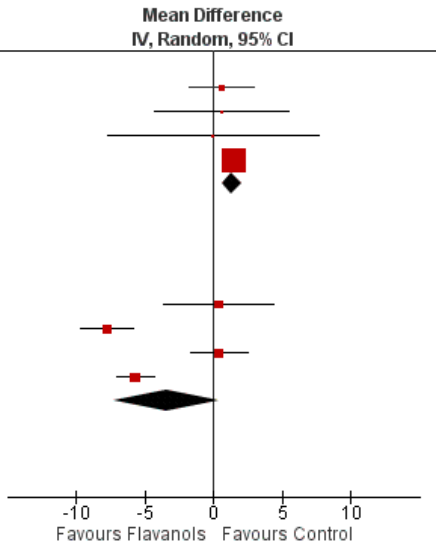


Figure S2**.** Forest plot Systolic Blood Pressure – daily amount of epicatechins in intervention

Solid diamonds represent the pooled estimates for each subgroup (16 - 46 mg epicatechins/ day, 90 - 185 mg epicatechins/ day); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (2.1.1)= included trials in which 16 - 46 mg epicatechins/ day is consumed as intervention; subgroup 2 (2.1.2)= included trials in which 90 - 185 mg epicatechins/ day is consumed as intervention. Ayoobi et al. (2017) (Ayoobi et al., 2017), Rostami et al. (2015) (Rostami et al., 2015), and Sorond et al. (2013) (Sorond et al., 2013) did not mention the amount of epicatechins/ day in the intervention formula and are therefore not included in this subgroup analysis.

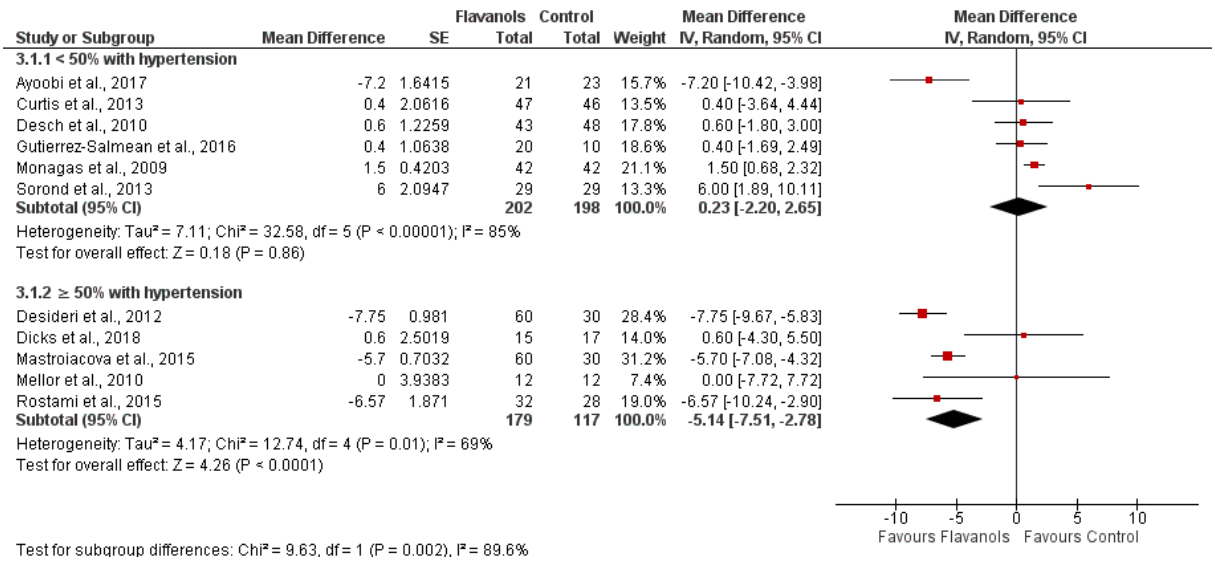


Figure S3**.** Forest plot Systolic Blood Pressure – Blood Pressure at baseline

Solid diamonds represent the pooled estimates for each subgroup (less than 50% of participants per study have hypertension at baseline, at least 50% of participants per study have hypertension at baseline, based on reported 95% confidence interval of baseline blood pressure); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (3.1.1)= included trials in which less than 50% of the participants have hypertension at baseline; subgroup 2 (3.1.2)= included trials in which at least 50% of the participants have hypertension at baseline.

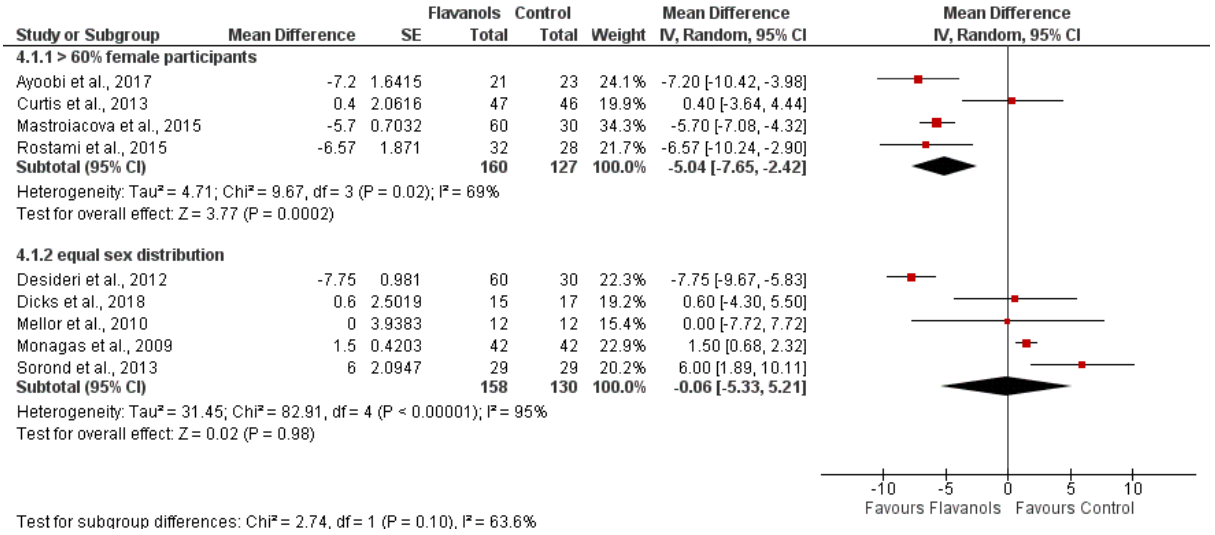


Figure S4**.** Forest plot Systolic Blood pressure – sex

Solid diamonds represent the pooled estimates for each subgroup (> 60% female participants, equal sex distribution); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (4.1.1)= included trials in which more than 60% of participants are female; subgroup 2 (4.1.2)= included trials with equal sex distribution. Gutiérrez-Salmeán et al. (2016) (Gutiérrez-Salmeán et al., 2016) did not mention the amount of female and male participants and is therefore not included in this subgroup analysis. Desch et al. (2010) (Desch et al., 2010) was the only trial in which more than 60% of participants are male and is therefore not included in this subgroup analysis.

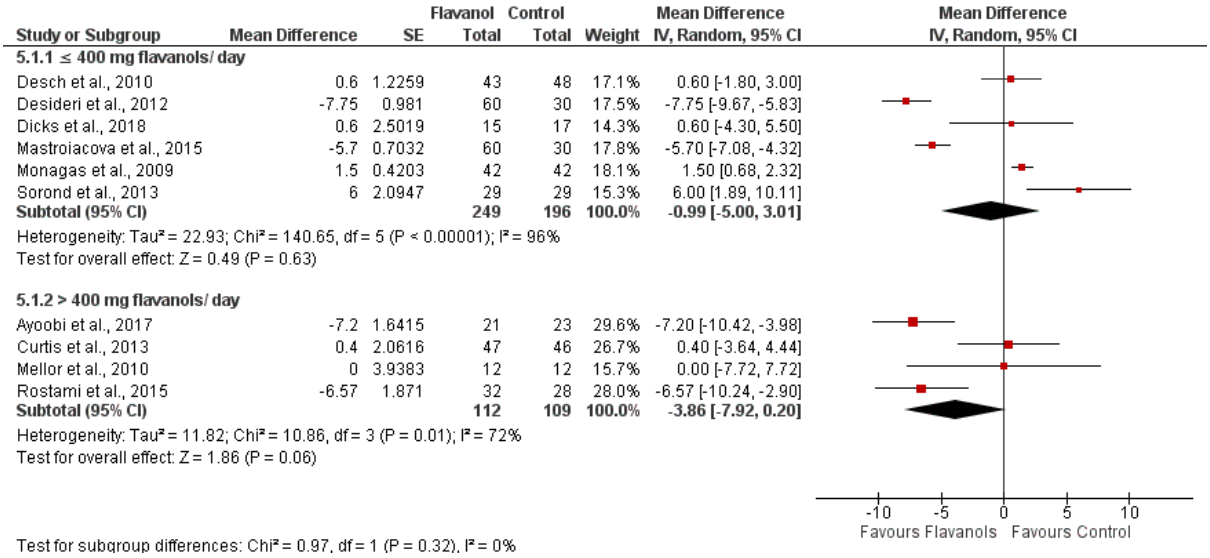


Figure S5**.** Forest plot Systolic Blood Pressure – geographical differences in average daily flavanol intake

Solid diamonds represent the pooled estimates for each subgroup (average daily flavanol intake of maximal 400 mg, average daily flavanol intake of more than 400 mg); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (5.1.1)= included trials executed in a geographical area with an average daily flavanol intake of maximal 400 mg; subgroup 2 (5.1.2)= included trials executed in a geographical area with an average daily flavanol intake above 400 mg. Since too little is known so far about the average daily flavanol intake in Asian countries, Gutiérrez-Salmeán et al. (2016) (Gutiérrez-Salmeán et al., 2016) is not included in this subgroup analysis.

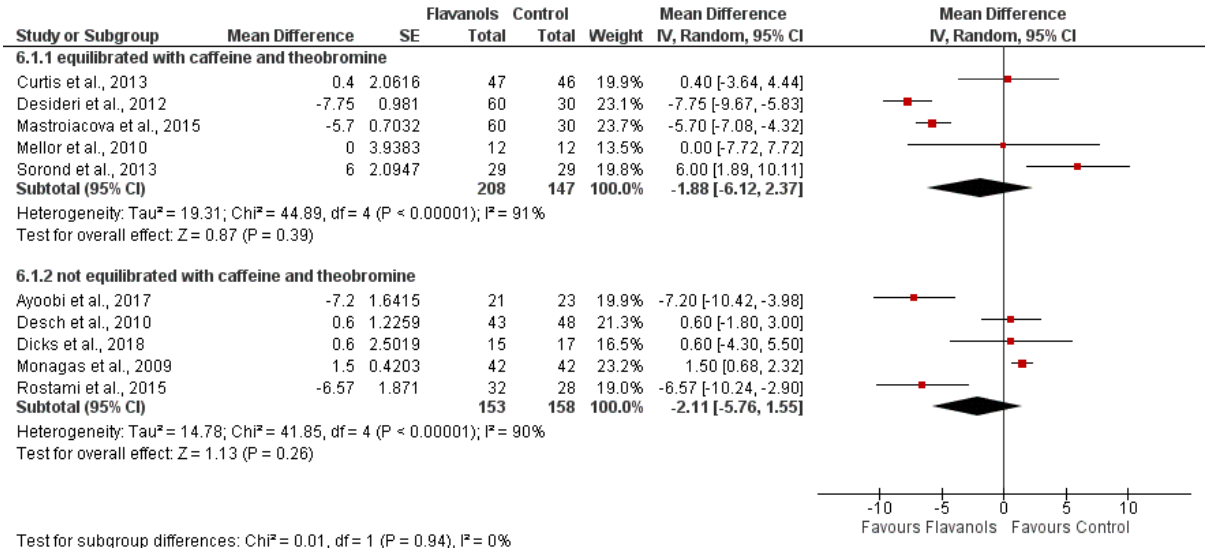
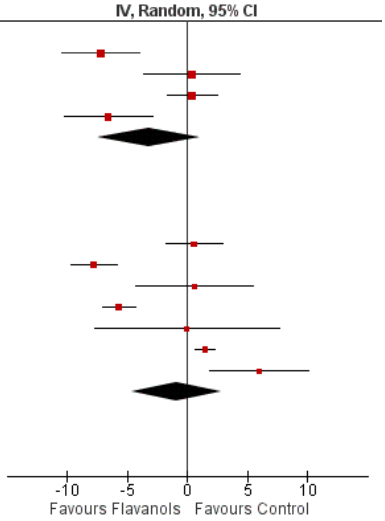


Figure S6**.** Forest plot Systolic Blood Pressure – placebo-formula

Solid diamonds represent the pooled estimates for each subgroup (interventional and placebo formula are equilibrated with caffeine and theobromine, interventional and placebo formula are not equilibrated with caffeine and theobromine); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (6.1.1)= included trials in which interventional- and placebo-formula are equilibrated with caffeine and theobromine; subgroup 2 (6.1.2)= included trials in which interventional- and placebo-formula are not equilibrated with caffeine and theobromine. Gutiérrez-

Salmeán et al. (2016) (Gutiérrez-Salmeán et al., 2016) did not mention composition of the placebo and is therefore not included in this subgroup analysis.

Study or Subgroup	Mean Difference	SE	Flavanols Total	Control Total	Weight	Mean Difference IV, Random, 95% CI
7.1.1 < 65 years old						
Ayoobi et al., 2017	-7.2	1.6415	21	23	25.2%	-7.20 [-10.42, -3.98]
Curtis et al., 2013	0.4	2.0616	47	46	23.3%	0.40 [-3.64, 4.44]
Gutierrez-Salmean et al., 2016	0.4	1.0638	20	10	27.4%	0.40 [-1.69, 2.49]
Rostami et al., 2015	-6.57	1.871	32	28	24.1%	-6.57 [-10.24, -2.90]
Subtotal (95% CI)			**120**	**107**	**100.0%**	**-3.20 [-7.48, 1.09]**
Heterogeneity: Tau² = 16.32; Chi² = 22.28, df = 3 (P < 0.0001); I² = 87%						
Test for overall effect: Z = 1.46 (P = 0.14)						
7.1.2 ≥ 65 years old						
Desch et al., 2010	0.6	1.2259	43	48	15.4%	0.60 [-1.80, 3.00]
Desideri et al., 2012	-7.75	0.981	60	30	15.8%	-7.75 [-9.67, -5.83]
Dicks et al., 2018	0.6	2.5019	15	17	12.9%	0.60 [-4.30, 5.50]
Mastroiacova et al., 2015	-5.7	0.7032	60	30	16.1%	-5.70 [-7.08, -4.32]
Mellor et al., 2010	0	3.9383	12	12	9.7%	0.00 [-7.72, 7.72]
Monagas et al., 2009	1.5	0.4203	42	42	16.3%	1.50 [0.68, 2.32]
Sorond et al., 2013	6	2.0947	29	29	13.8%	6.00 [1.89, 10.11]
Subtotal (95% CI)			**261**	**208**	**100.0%**	**-0.90 [-4.67, 2.87]**
Heterogeneity: Tau² = 22.43; Chi² = 140.70, df = 6 (P < 0.00001); I² = 96%						
Test for overall effect: Z = 0.47 (P = 0.64)						

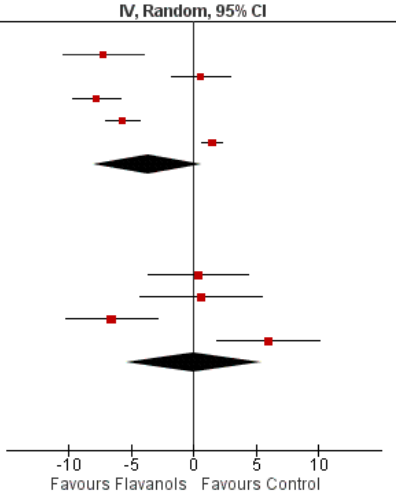


Test for subgroup differences: Chi² = 0.62, df = 1 (P = 0.43), I² = 0%

Figure S7. Forest plot Systolic Blood Pressure – age

Solid diamonds represent the pooled estimates for each subgroup (below 65 years old, at least 65 years old); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (7.1.1)= included trials in which the mean age of the participants was below 65 years; subgroup 2 (7.1.2)= included trials in which the mean age of the participants was at least 65 years.

Study or Subgroup	Mean Difference	SE	Flavanols Total	Control Total	Weight	Mean Difference IV, Random, 95% CI
8.1.1 < 30 kg/m²						
Ayoobi et al., 2017	-7.2	1.6415	21	23	18.8%	-7.20 [-10.42, -3.98]
Desch et al., 2010	0.6	1.2259	43	48	19.7%	0.60 [-1.80, 3.00]
Desideri et al., 2012	-7.75	0.981	60	30	20.1%	-7.75 [-9.67, -5.83]
Mastroiacova et al., 2015	-5.7	0.7032	60	30	20.5%	-5.70 [-7.08, -4.32]
Monagas et al., 2009	1.5	0.4203	42	42	20.8%	1.50 [0.68, 2.32]
Subtotal (95% CI)			**226**	**173**	**100.0%**	**-3.66 [-8.00, 0.69]**
Heterogeneity: Tau² = 23.45; Chi² = 142.04, df = 4 (P < 0.00001); I² = 97%						
Test for overall effect: Z = 1.65 (P = 0.10)						
8.1.2 ≥ 30 kg/m²						
Curtis et al., 2013	0.4	2.0616	47	46	25.3%	0.40 [-3.64, 4.44]
Dicks et al., 2018	0.6	2.5019	15	17	23.7%	0.60 [-4.30, 5.50]
Rostami et al., 2015	-6.57	1.871	32	28	25.9%	-6.57 [-10.24, -2.90]
Sorond et al., 2013	6	2.0947	29	29	25.1%	6.00 [1.89, 10.11]
Subtotal (95% CI)			**123**	**120**	**100.0%**	**0.05 [-5.37, 5.47]**
Heterogeneity: Tau² = 26.04; Chi² = 20.52, df = 3 (P = 0.0001); I² = 85%						
Test for overall effect: Z = 0.02 (P = 0.99)						

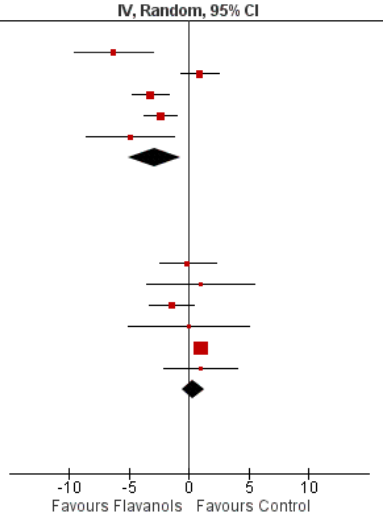


Test for subgroup differences: Chi² = 1.09, df = 1 (P = 0.30), I² = 8.4%

Figure S8. Forest plot Systolic Blood Pressure – body mass index (BMI)

Solid diamonds represent the pooled estimates for each subgroup (BMI below 30 kg/m², BMI of at least 30 kg/m²); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (8.1.1)= included trials in which the mean BMI of the participants was below 30 kg/m²; subgroup 2 (8.1.2)= included trials in which the mean BMI of the participants was at least 30 kg/m². Mellor et al. (2010) (Mellor et al., 2010) and Gutiérrez-Salmeán et al. (2016) (Gutiérrez-Salmeán et al., 2016) did not mention the mean BMI or both mean weight and mean height and are therefore not included in this subgroup analysis.

Study or Subgroup	Mean Difference	SE	Flavanols Total	Control Total	Weight	Mean Difference IV, Random, 95% CI
9.1.1 1 dose daily						
Ayoobi et al., 2017	-6.3	1.673	21	23	16.3%	-6.30 [-9.58, -3.02]
Desch et al., 2010	0.9	0.812	43	48	22.6%	0.90 [-0.69, 2.49]
Desideri et al., 2012	-3.2	0.7869	60	30	22.8%	-3.20 [-4.74, -1.66]
Mastroiacova et al., 2015	-2.35	0.7026	60	30	23.4%	-2.35 [-3.73, -0.97]
Rostami et al., 2015	-4.86	1.868	32	28	14.9%	-4.86 [-8.52, -1.20]
Subtotal (95% CI)			**216**	**159**	**100.0%**	**-2.82 [-5.00, -0.65]**
Heterogeneity: Tau² = 4.80; Chi² = 24.22, df = 4 (P < 0.0001); I² = 83%						
Test for overall effect: Z = 2.54 (P = 0.01)						
9.1.2 several doses daily (split-dose)						
Curtis et al., 2013	-0.1	1.2042	47	46	12.8%	-0.10 [-2.46, 2.26]
Dicks et al., 2018	1	2.2885	15	17	4.2%	1.00 [-3.49, 5.49]
Gutierrez-Salmean et al., 2016	-1.4	0.9398	20	10	18.6%	-1.40 [-3.24, 0.44]
Mellor et al., 2010	0	2.5709	12	12	3.4%	0.00 [-5.04, 5.04]
Monagas et al., 2009	1	0.2536	42	42	52.7%	1.00 [0.50, 1.50]
Sorond et al., 2013	1	1.552	29	29	8.4%	1.00 [-2.04, 4.04]
Subtotal (95% CI)			**165**	**156**	**100.0%**	**0.38 [-0.57, 1.33]**
Heterogeneity: Tau² = 0.38; Chi² = 6.78, df = 5 (P = 0.24); I² = 26%						
Test for overall effect: Z = 0.79 (P = 0.43)						

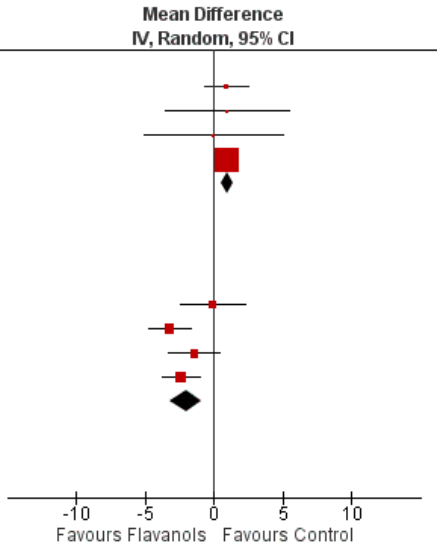


Test for subgroup differences: Chi² = 6.98, df = 1 (P = 0.008), I² = 85.7%

Figure S9. Forest plot Diastolic Blood Pressure – 1 dose daily versus several doses daily (split-dose)

Solid diamonds represent the pooled estimates for each subgroup (1 dose daily, several doses daily (split-dose)); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (9.1.1)= included trials in which the intervention is administered 1 time daily; subgroup 2 (9.1.2)= included trials in which the intervention is administered throughout the day in split-doses.

Study or Subgroup	Mean Difference	SE	Weight	Mean Difference IV, Random, 95% CI
10.1.1 16-46 mg epicatechins/ day				
Desch et al., 2010	0.9	0.812	8.7%	0.90 [-0.69, 2.49]
Dicks et al., 2018	1	2.2885	1.1%	1.00 [-3.49, 5.49]
Mellor et al., 2010	0	2.5709	0.9%	0.00 [-5.04, 5.04]
Monagas et al., 2009	1	0.2536	89.3%	1.00 [0.50, 1.50]
Subtotal (95% CI)			**100.0%**	**0.98 [0.51, 1.45]**
Heterogeneity: Tau² = 0.00; Chi² = 0.16, df = 3 (P = 0.98); I² = 0%				
Test for overall effect: Z = 4.10 (P < 0.0001)				
10.1.2 90-185 mg epicatechins/ day				
Curtis et al., 2013	-0.1	1.2042	16.9%	-0.10 [-2.46, 2.26]
Desideri et al., 2012	-3.2	0.7869	28.3%	-3.20 [-4.74, -1.66]
Gutierrez-Salmean et al., 2016	-1.4	0.9398	23.3%	-1.40 [-3.24, 0.44]
Mastroiacova et al., 2015	-2.35	0.7026	31.5%	-2.35 [-3.73, -0.97]
Subtotal (95% CI)			**100.0%**	**-1.99 [-3.15, -0.83]**
Heterogeneity: Tau² = 0.61; Chi² = 5.39, df = 3 (P = 0.15); I² = 44%				
Test for overall effect: Z = 3.37 (P = 0.0007)				



Test for subgroup differences: Chi² = 21.77, df = 1 (P < 0.00001), I² = 95.4%

Figure S10. Forest plot Diastolic Blood Pressure – daily amount of epicatechins in intervention

Solid diamonds represent the pooled estimates for each subgroup (16 - 46 mg epicatechins/ day, 90 - 185 mg epicatechins/ day); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (10.1.1)= included trials in which 16 - 46 mg epicatechins/ day is consumed as intervention; subgroup 2 (10.1.2)= included trials in which 90 - 185 mg epicatechins/ day is consumed as intervention. Ayoobi et al. (2017) (Ayoobi et al., 2017), Rostami et al. (2015) (Rostami et al., 2015), and Sorond et al. (2013) (Sorond et al., 2013) did not mention the amount of epicatechins/ day in the intervention formula and are therefore not included in this subgroup analysis.

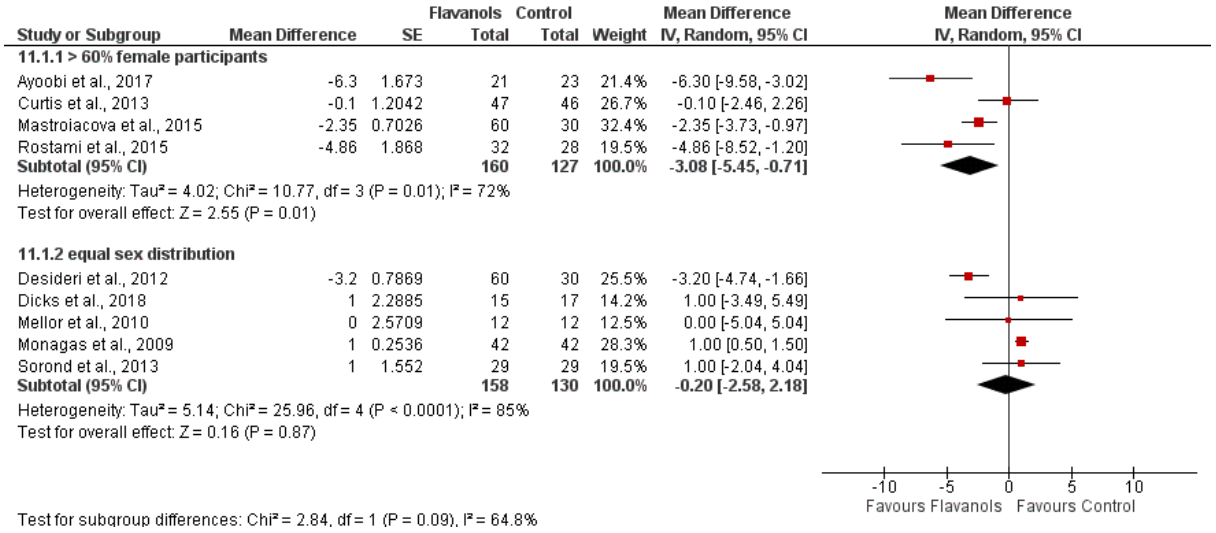


Figure S11. Forest plot Diastolic Blood Pressure – sex

Solid diamonds represent the pooled estimates for each subgroup (> 60% female participants, equal sex distribution); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (11.1.1)= included trials in which more than 60% of participants are female; subgroup 2 (11.1.2)= included trials with equal sex distribution. Gutiérrez-Salmeán et al. (2016) (Gutiérrez-Salmeán et al., 2016) did not mention the amount of female and male participants and is therefore not included in this subgroup analysis. Desch et al. (2010) (Desch et al., 2010) was the only trial trials in which more than 60% of participants are male and is therefore not included in this subgroup analysis.

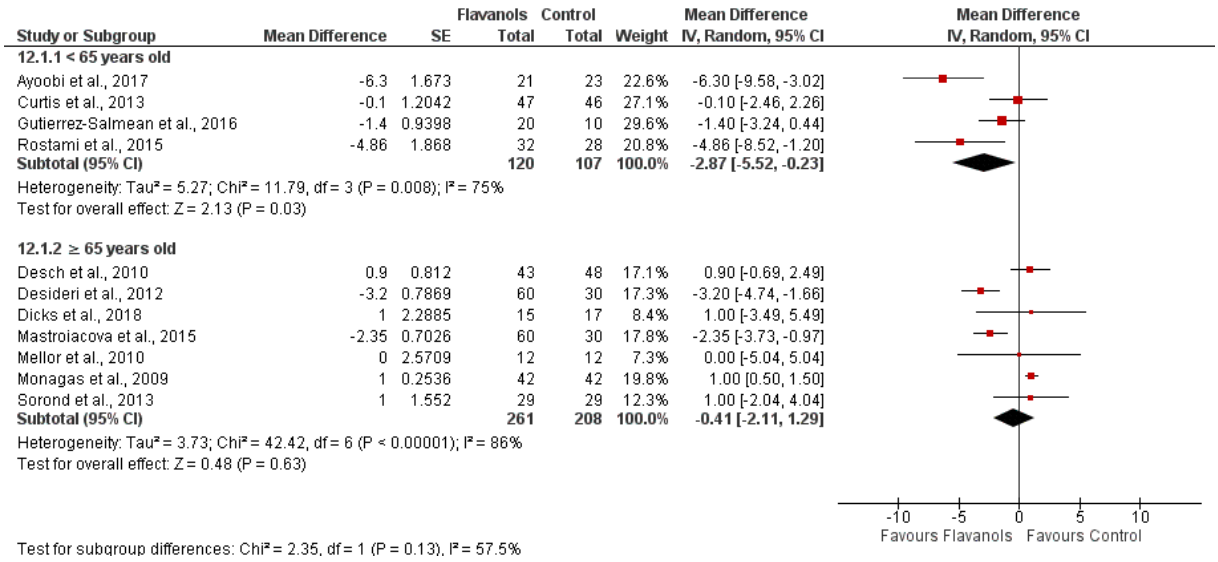


Figure S12. Forest plot Diastolic Blood Pressure – age

Solid diamonds represent the pooled estimates for each subgroup (below 65 years old, at least 65 years old); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (12.1.1)= included trials in which the mean age of the participants was below 65 years; subgroup 2 (12.1.2)= included trials in which the mean age of the participants was at least 65 years.

Study or Subgroup	Mean Difference	SE	Flavanols Total	Control Total	Weight	Mean Difference IV, Random, 95% CI
13.1.1 equilibrated with caffeine and theobromine						
Curtis et al., 2013	-0.1	1.2042	47	46	20.1%	-0.10 [-2.46, 2.26]
Desideri et al., 2012	-3.2	0.7869	60	30	27.7%	-3.20 [-4.74, -1.66]
Mastroiacova et al., 2015	-2.35	0.7026	60	30	29.4%	-2.35 [-3.73, -0.97]
Mellor et al., 2010	0	2.5709	12	12	7.5%	0.00 [-5.04, 5.04]
Sorond et al., 2013	1	1.552	29	29	15.3%	1.00 [-2.04, 4.04]
Subtotal (95% CI)			**208**	**147**	**100.0%**	**-1.44 [-2.98, 0.09]**
Heterogeneity: Tau² = 1.59; Chi² = 9.40, df = 4 (P = 0.05); I² = 57%						
Test for overall effect: Z = 1.84 (P = 0.07)						
13.1.2 not equilibrated with caffeine and theobromine						
Ayoobi et al., 2017	-6.3	1.673	21	23	18.1%	-6.30 [-9.58, -3.02]
Desch et al., 2010	0.9	0.812	43	48	24.3%	0.90 [-0.69, 2.49]
Dicks et al., 2018	1	2.2885	15	17	14.0%	1.00 [-3.49, 5.49]
Monagas et al., 2009	1	0.2536	42	42	26.8%	1.00 [0.50, 1.50]
Rostami et al., 2015	-4.86	1.868	32	28	16.7%	-4.86 [-8.52, -1.20]
Subtotal (95% CI)			**153**	**158**	**100.0%**	**-1.33 [-3.74, 1.09]**
Heterogeneity: Tau² = 5.60; Chi² = 27.79, df = 4 (P < 0.0001); I² = 86%						
Test for overall effect: Z = 1.08 (P = 0.28)						
Test for subgroup differences: Chi² = 0.01, df = 1 (P = 0.94), I² = 0%						

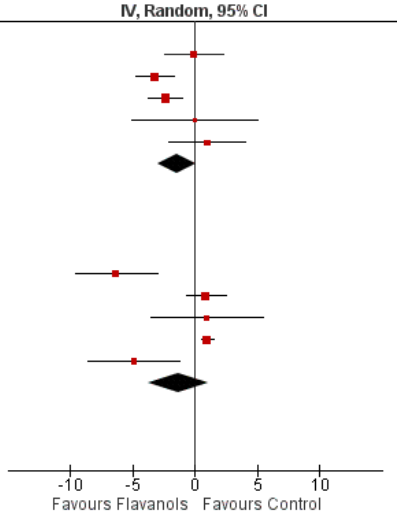


Figure S13. Forest plot Diastolic Blood Pressure – placebo-formula

Solid diamonds represent the pooled estimates for each subgroup (interventional and placebo formula are equilibrated with caffeine and theobromine, interventional and placebo formula are not equilibrated with caffeine and theobromine); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (13.1.1)= included trials in which interventional- and placebo-formula are equilibrated with caffeine and theobromine; subgroup 2 (13.1.2)= included trials in which interventional- and placebo-formula are not equilibrated with caffeine and theobromine. Gutiérrez-Salmeán et al. (2016) (Gutiérrez-Salmeán et al., 2016) did not mention composition of the placebo and is therefore not included in this subgroup analysis.

Study or Subgroup	Mean Difference	SE	Flavanols Total	Control Total	Weight	Mean Difference IV, Random, 95% CI
14.1.1 < 30 kg/m²						
Ayoobi et al., 2017	-6.3	1.673	21	23	15.4%	-6.30 [-9.58, -3.02]
Desch et al., 2010	0.9	0.812	43	48	20.5%	0.90 [-0.69, 2.49]
Desideri et al., 2012	-3.2	0.7869	60	30	20.6%	-3.20 [-4.74, -1.66]
Mastroiacova et al., 2015	-2.35	0.7026	60	30	21.0%	-2.35 [-3.73, -0.97]
Monagas et al., 2009	1	0.2536	42	42	22.5%	1.00 [0.50, 1.50]
Subtotal (95% CI)			**226**	**173**	**100.0%**	**-1.71 [-3.98, 0.55]**
Heterogeneity: Tau² = 5.86; Chi² = 57.62, df = 4 (P < 0.00001); I² = 93%						
Test for overall effect: Z = 1.48 (P = 0.14)						
14.1.2 ≥ 30 kg/m²						
Curtis et al., 2013	-0.1	1.2042	47	46	32.0%	-0.10 [-2.46, 2.26]
Dicks et al., 2018	1	2.2885	15	17	18.3%	1.00 [-3.49, 5.49]
Rostami et al., 2015	-4.86	1.868	32	28	22.8%	-4.86 [-8.52, -1.20]
Sorond et al., 2013	1	1.552	29	29	26.9%	1.00 [-2.04, 4.04]
Subtotal (95% CI)			**123**	**120**	**100.0%**	**-0.69 [-3.17, 1.80]**
Heterogeneity: Tau² = 3.57; Chi² = 6.92, df = 3 (P = 0.07); I² = 57%						
Test for overall effect: Z = 0.54 (P = 0.59)						
Test for subgroup differences: Chi² = 0.36, df = 1 (P = 0.55), I² = 0%						

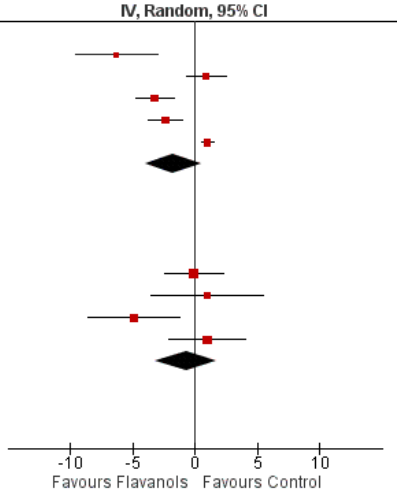


Figure S14. Forest plot Diastolic Blood Pressure – body mass index (BMI)

Solid diamonds represent the pooled estimates for each subgroup (BMI below 30 kg/m², BMI of at least 30 kg/m²); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (14.1.1)= included trials in which the mean BMI of the participants was below 30 kg/m²; subgroup 2 (14.1.2)= included trials in which the mean BMI of the participants was at least 30 kg/m². Mellor et al. (2010) (Mellor et al., 2010) and Gutiérrez-Salmeán et al. (2016) (Gutiérrez-Salmeán et al., 2016) did not mention the mean BMI or both mean weight and mean height and are therefore not included in this subgroup analysis.

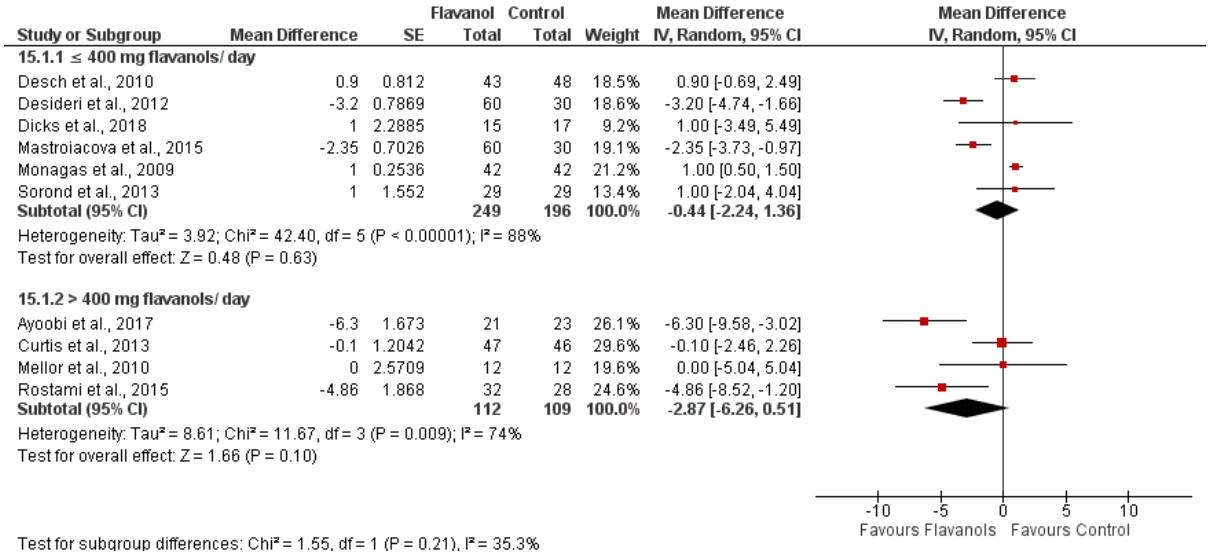


Figure S15**.** Forest plot Diastolic Blood Pressure – geographical differences in average daily flavanol intake

Solid diamonds represent the pooled estimates for each subgroup (average daily flavanol intake of maximal 400 mg, average daily flavanol intake of more than 400 mg); horizontal lines represent 95% confidence interval; red dots represent point estimate of each study; I^2 and p values for heterogeneity and subgroup differences are shown. Subgroup 1 (15.1.1)= included trials executed in a geographical area with an average daily flavanol intake of maximal 400 mg; subgroup 2 (15.1.2)= included trials executed in a geographical area with an average daily flavanol intake above 400 mg. Since too little is known so far about the average daily flavanol intake in Asian countries, Gutiérrez-Salmeán et al. (2016) (Gutiérrez-Salmeán et al., 2016) is not included in this subgroup analysis.

References

Ayoobi, N., Jafarirad, S., Haghighizadeh, M. H., & Jahanshahi, A. (2017). Protective Effect of Dark Chocolate on Cardiovascular Disease Factors and Body Composition in Type 2 Diabetes: A Parallel, Randomized, Clinical Trial. *Iranian Red Crescent Medical Journal, 19*(8).

Desch, S., Kobler, D., Schmidt, J., Sonnabend, M., Adams, V., Sareban, M., Eitel, I., Blüher, M., Schuler, G., & Thiele, H. (2010). Low vs. higher-dose dark chocolate and blood pressure in cardiovascular high-risk patients. *American journal of hypertension, 23*(6), 694-700. https://doi.org/10.1038/ajh.2010.29

Gutiérrez-Salmeán, G., Meaney, E., Lanaspa, M. A., Cicerchi, C., Johnson, R. J., Dugar, S., Taub, P., Ramírez-Sánchez, I., Villarreal, F., Schreiner, G., & Ceballos, G. (2016). A randomized, placebo-controlled, double-blind study on the effects of (-)-epicatechin on the triglyceride/HDLc ratio and cardiometabolic profile of subjects with hypertriglyceridemia: Unique in vitro effects. *International journal of cardiology, 223*, 500-506. https://doi.org/10.1016/j.ijcard.2016.08.158

Mellor, D. D., Sathyapalan, T., Kilpatrick, E. S., Beckett, S., & Atkin, S. L. (2010). High-cocoa polyphenol-rich chocolate improves HDL cholesterol in Type 2 diabetes patients. *Diabetic medicine, 27*(11), 1318-1321. https://doi.org/10.1111/j.1464-5491.2010.03108.x

Rostami, A., Khalili, M., Haghighat, N., Eghtesadi, S., Shidfar, F., Heidari, I., Ebrahimpour-Koujan, S., & Eghtesadi, M. (2015). High-cocoa polyphenol-rich chocolate improves

blood pressure in patients with diabetes and hypertension. *ARYA atherosclerosis, 11*(1), 21-29. https://www.ncbi.nlm.nih.gov/pubmed/26089927

Sorond, F. A., Hurwitz, S., Salat, D. H., Greve, D. N., & Fisher, N. D. (2013). Neurovascular coupling, cerebral white matter integrity, and response to cocoa in older people. *Neurology, 81*(10), 904-909. https://doi.org/10.1212/WNL.0b013e3182a351aa

Table S5. PRISMA-checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3,4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	4,5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5 + Table S1
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Table S2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5,6

Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6, 9
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6, 8, 9
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	7
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	7, 8, 9
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	8, 9, 10

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	7
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	9,10
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	10 + Fig.1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	10, 11, 12 + Table 1
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	12 + Table S3
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	13, 14

Synthesis of results	21	Present the main results of the review. If meta-analyses are done, include for each, confidence intervals and measures of consistency.	13, 14
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	12,13 + Table S4
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	14, 15, 16
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	16 + 17,18,19,20
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	20, 21
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	21, 22
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	32, 33

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: **www.prisma-statement.org**.

Acute effects of cocoa flavanols on blood pressure and peripheral vascular reactivity in type 2 diabetes mellitus and essential hypertension: A protocol for an acute, randomized, double-blinded, placebo-controlled cross-over trial.

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Acute Effects of Cocoa Flavanols on Blood Pressure and Peripheral Vascular Reactivity in Type 2 Diabetes Mellitus and Essential Hypertension: A Protocol for an Acute, Randomized, Double-Blinded, Placebo-Controlled Cross-Over Trial

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Introduction: Patients with type 2 diabetes mellitus are at high risk to develop vascular complications resulting in high morbidity and mortality. Cocoa flavanols are promising nutraceuticals with possible beneficial vascular effects in humans. However, limited research is currently available on the vascular effects in a diabetic population with inconsistent results. Possible reasons for this inconsistency might be heterogeneity in the given intervention (dose per time and day, single dose vs. split-dose, placebo formula) and the studied population (blood pressure at baseline, duration of diabetes, use of vasoactive antihypertensive and antidiabetic drugs, sex). Therefore, we aimed to develop a randomized, double-blinded, placebo-controlled cross-over trial to investigate whether cocoa flavanols have an acute impact on blood pressure and vascular reactivity in patients with type 2 diabetes with and without arterial hypertension.

Methods and Analysis: We will include participants in four groups: (i) patients with type 2 diabetes without arterial hypertension, (ii) patients with type 2 diabetes with arterial hypertension and 1 antihypertensive drug, (iii) non-diabetic participants with essential hypertension and 1 antihypertensive drug, and (iv) healthy controls. All participants will complete the same protocol on both testing days, consuming high-flavanol cocoa extract (790 mg flavanols) or placebo. Macrovascular endothelial function (flow-mediated dilation) and blood pressure will be measured before and after capsule ingestion. Forearm muscle vasoreactivity (near-infrared spectroscopy)

and brachial artery blood flow (echo-doppler) will be assessed in response to a dynamic handgrip exercise test after capsule ingestion. Data will be analyzed with a random intercept model in mixed models.

Clinical Trial Registration: www.Clinicaltrials.gov, identifier: NCT03722199.

Keywords: type 2 diabetes, cocoa flavanols, vascular reactivity, blood pressure, muscular oxygenation, antihypertensive drugs

INTRODUCTION

Nitric Oxide, produced by the endothelial cells, is of crucial importance for general vascular health. It triggers relaxation of the vascular smooth muscle cells through accumulation of intracellular cyclic guanosine monophosphate (cGMP) (1–5).

Type 2 diabetes (T2DM) is the most prevalent type [90% (6, 7)] of diabetes mellitus, a highly prevalent disorder [estimated at 425 million people worldwide in 2017 and is expected to be 629 million in 2045 (8)] and poses a challenge to global health. It is characterized by chronic hyperglycemia, which increases oxidative stress. Free radicals easily bind with and simultaneously deactivate nitric oxide to form peroxynitrite. Both high amounts of oxidative stress and nitric oxide depletion increase the risk for developing micro- (retinopathy, nephropathy, and neuropathy) and macrovascular (cardiovascular, cerebrovascular, and peripheral artery diseases) complications (9, 10). These complications decrease quality of life and increase the global burden of T2DM in terms of health care costs, morbidity [hypertension is present in >60% of all patients with T2DM (11)], and even mortality (12–15). The past years researchers have been investigating products to limit or delay the onset of diabetic vascular complications with special attention for non-pharmacological approaches to counter polypharmacy.

Flavanols are promising nutraceuticals from the flavonoid family, a class of polyphenols (16), which can be found in several fruits, beans, teas, red wine, but predominantly in cocoa products (16, 17). Especial interest goes to flavanols derived from the seeds of the cocoa bean (*Theobroma cacao*), cocoa flavanols (CF), as they have higher antioxidant activity and more phenolic compounds (18). The increased attention for the effects of cocoa originates from research on the Kuna Indians. In contrast to migrated Kuna Indians to urban areas, Kuna Indians living on the San Blas Islands off the coast of Panama show low blood pressure (BP), even with increasing age, and have lower frequency of diabetes mellitus, cancer, and cardiovascular diseases (19). Causal research for this cardiovascular protection focused on environmental factors including nutrition and revealed that island-dwelling Kuna drink daily more than five cups of cocoa with high concentrations of flavanols and procyanidins (19–21). Starting from evidence based on further research on the vascular effects of CF, the European Food Safety Authority (EFSA) stated that CF help to preserve endothelium-dependent vasodilation in healthy populations, if taken in quantities exceeding 200 mg CF daily. This equals 10 g high-flavanol dark chocolate or 2.5 g high-flavanol cocoa powder (22).

The mechanisms of action of these CF are still debated. It is believed that they improve endothelial function (23), decrease BP (24), ameliorate insulin sensitivity (25–27), influence various inflammatory processes (28), and prevent platelet aggregation (29, 30) *via* antioxidant properties (31, 32), increasing nitric oxide bioavailability (33, 34), and inhibition of the angiotensin-converting-enzyme activity (35, 36). These effects seem to be induced, at least in part, by epicatechin, a highly active monomeric form of CF (37, 38).

It could be presumed that populations with T2DM could benefit from the intake of CF as it potentially reduces cardiovascular risk. CF would indeed improve both endothelial function and insulin sensitivity and so influence cardiovascular as well as metabolic disorders (39). Nonetheless, limited research with a high degree of inconsistency due to large heterogeneity (dose per time or day, acute or chronic intake, single dose vs. split-dose, placebo formula, and characteristics of population e.g., sex, BP at baseline, and use of vasoactive drugs like insulin and antihypertensives) is reported.

As presented in **Table 1**, at the moment, six studies investigated the effect of chronic CF intake (8 weeks−1 year) on vascular function (40–45) in patients with T2DM. Only two showed a statistically and clinically relevant decrease in BP (40, 45) and only two indicated a statistically improvement of endothelial function (41, 42) (**Table 1**). The heterogeneity of results about mid- to long-term effects of CF in patients with diabetes had been recently approached through a systematic review and meta-analysis (48). This paper indeed shows low quality of evidence of slight improvements in BP after mid/long-term CF ingestion. However, risk of bias, imprecision of the publications, and inconsistency and heterogeneity among the reports are reported and could be cause for the lack of a definite conclusion. This meta-analysis ultimately highlights the need for further research with a robust methodology taking into account possible confounding factors like hypertension at baseline and intake of BP lowering medication. Antihypertensive drugs, which were never considered in these papers on chronic effects of CF in patients with T2DM, have indeed a great impact on vasoreactivity (49) and may hence interfere with the effects of CF.

The first step for profoundly testing these possible confounding factors would lie in the design of acute protocols applied to several well-characterized (particularly hypertension and its medications) groups of patients with T2DM. Up to

Abbreviations: ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; BB, beta-blockers; BP, blood pressure (SBP, systolic blood pressure; DBP, diastolic blood pressure); CF, cocoa-derived flavanols; dHGE, dynamic handgrip exercise; FMD, flow-mediated dilation; NIRS, Near-infrared spectroscopy; T2DM, type 2 diabetes mellitus.

TABLE 1 | Characteristics of chronic and acute trials examining the effect of cocoa flavanols in patients with type 2 diabetes.

References	Population (sex intervention group)/(sex control group)	Medication	Intervention		Control		Frequency and duration	Vascular assessment
			Form	Flavanol content/d	Form	Flavanol content/d		
Chronic trials								
Ayoobi et al. (40)	T2DM (14F + 7M)/(13F + 10M)	Oral anti-DM drugs only, no information on anti-HT drugs	30 g 84% dark chocolate	No information	No intervention	No intervention	1x/d, 8 w	SBP↓, DBP ↓, NO =, angiotensin II =
Balzer et al. (41)	T2DM (13F + 8M)/(16F + 4M)	Insulin allowed, anti-HT drugs allowed	Cocoa powder + 250 mL water	3 × 321 mg FL, 3 × 57.8 mg EC	Cocoa powder + 250 mL water	3 × 25 mg FL, 3 × 4.5 mg EC, matched for theobromine and caffeine	3x/d, 30 d	FMD ↓, MAP =, HR =
Curtis et al. (42)	T2DM, postmenopausal (47F)/(46F)	Insulin allowed, anti-HT drugs allowed	2 × 13.5 g flavonoid enriched chocolate	850 mg flavan-3-ols, 90 mg EC	2 × 13.5 g placebo chocolate	Matched for macronutrient content	2x/d (lunch + evening), 52 w	SBP =, DBP =, MAP =, PP = (PP variability ↓), CCA-IMT =, PWV ↓, AI =, ACE =, NO =, ET-1 =
Dicks et al. (43)	T2DM + HT (10F + 7M)/(7F + 11M)	Oral anti-DM drugs only, anti-HT drugs allowed	5 × 0.5 g cocoa powder capsules	207.5 mg flavanols, 40.4 mg EC, 13.6 mg C	5 × 0.5 g pure microcrystalline cellulose	No flavanols	3 in morning, 2 in evening, 12 w	SBP =, DBP=
Mellor et al. (44)	T2DM (5F + 7M)/(cross-over)	Oral anti-DM drugs only, anti-HT drugs allowed	3 × 15 g high polyphenol chocolate, 85% cocoa solids	16.6 mg EC	3 × 15 g low polyphenol chocolate, no non-fat cocoa solids	<2 mg EC, matched for macronutrient content	3x/d, 8 w	SBP =, DBP =
Rostami et al. (45)	T2DM + HT (20F + 12M)/(16F + 12M)	Oral anti-DM drugs only, anti-HT drugs allowed	25 g dark chocolate, 83% cocoa solids	450 mg flavonoids	White chocolate	No flavonoids	1x/d, 8 w	SBP ↓, DBP ↓
Acute trials								
Balzer et al. (41)	T2DM (2F + 8M)/(cross-over)	Insulin allowed, anti-HT drugs allowed	Cocoa powder + 250 mL water	*High*: 963 mg FL, 203 mg EC, 50.8 mg C *Medium*: 371 mg FL, 78.9 mg EC, 19.7 mg C	Cocoa powder + 250 mL water	75 mg FL, 16.8 mg EC, 4.2 mg C, matched for theobromine and caffeine	1x/d, 1, 2, 3, 4, 6 h post-intake	FMD ↓
Basu et al. (46)	T2DM + obese, (14F + 4M)/(cross-over)	Oral anti-DM drugs only, anti-HT drugs allowed	Cocoa powder + warm water, intake with a high-fat breakfast	480 mg FL, 40 mg EC, 18 mg C	Flavanol-free placebo powder + warm water, intake with a high-fat breakfast	<0.1 mg FL, not matched for theobromine or caffeine	1x/d, 30 min, 1, 2, 4, 6 h post-intake	SBP =, DBP =, large artery elasticity ↓, small artery elasticity =
Mellor et al. (47)	T2DM (1 postmenopausal F + 9M)/(cross-over)	Oral anti-DM drugs only, no information on anti-HT drugs	13.5 g high polyphenol chocolate + 200 mL water	3.5% polyphenols	13.5 g low polyphenol chocolate	0.9% polyphenols, identical formulation as intervention chocolate	1x/d, 3 h post-intake	Reactive hyperemia peripheral arterial tonometry ↓, endothelial serum adhesion molecules =

The exact quantity of flavanols and/or epicatechin of the supplement is indicated only when this information is available in the paper; Effects in intervention group are expressed through: ↑, significant increase; ↓, significant decrease; =, no change; ACE, angiotensin-converting enzyme; AI, augmentation index at 75 heart rate; BP, blood pressure (systolic SBP, diastolic DBP); C, catechins; CCA-IMT, intima-media thickness of the common carotid artery; d, day; EC, epicatechins; ET-1, endothelin-1; F, female; FL, flavanols; FMD, flow-mediated dilation; h, hours; HR, heart rate; HT, hypertension; M, male; MAP, mean arterial pressure; min, minutes; NO, nitric oxide; PP, pulse pressure; PWV, pulse wave velocity; T2DM, Type 2 Diabetes Mellitus; w, weeks; y, year.

now, three studies evaluated the vascular effects of acute CF supplementation in patients with T2DM (41, 46, 47). Two detected a significant improvement of endothelial function (41, 47) and one indicated a decrease in large artery elasticity (46) (**Table 1**). However, the same way as in the chronic studies, the three acute studies either gave no information about antihypertensive drugs or allowed the use of antihypertensive treatment (different types are reported) without considering this treatment as a possible confounding factor in the analyses (**Table 1**). To our knowledge, only four studies investigated the effect of CF when combined with antihypertensive drugs (different types are reported) in non-diabetic hypertensive adults (50, 51), in non-diabetic heart transplant recipients (52), and in non-diabetic adults with congestive heart failure (53). These showed a supplementary effect of cocoa intake on BP (50, 51) and/or endothelial function (50, 52, 53).

In addition, together with the heart, the micro- and macrocirculation determine the hemodynamics of the circulating system (54). It is important to study both systems simultaneously, but apart from one report (46) none of these studies in patients with T2DM investigated the effect of CF simultaneously in both of these vascular beds (i.e., micro and macrovascular beds) (**Table 1**).

In this study, we will take into account these results, assumptions, and points of heterogeneity as will be explained in this paper. In addition, this study will investigate the impact of CF on both micro- and macrovascular functions. As CF would increase nitric oxide, we hypothesize more effect on macrovessels as microvessels are also dependent on other vasodilators like prostaglandins. However, as little is known so far, our research will provide novel insights on this matter. Moreover, acute protocols will help to be sure of the efficacy of the dose chosen, to know which of the vascular (either micro or macrovascular) beds would be more impacted, and to identify whether some patients would be non-responders because of their medications. Hence, since our study has a robust methodology, it may act as a sort of "pilot" for the setup of long-term trials.

This acute, randomized, double-blinded, placebo-controlled cross-over study aims to investigate whether a single intake of a high dose of CF (790 mg flavanols) induces an improvement in endothelial function (primary outcome), a reduction in BP, and an enhancement in muscle vasoreactivity and oxygenation in patients with T2DM compared to healthy controls.

Because hypertension is a common comorbidity in patients with T2DM and as little is known so far concerning possible interferences between antihypertensive drugs and CF actions especially in patients with diabetes mellitus, this study will additionally investigate possible influence of beta-blockers (BB), angiotensin converting enzyme inhibitor (ACEi), or angiotensin receptor blocker (ARB) in diabetic persons with arterial hypertension and non-diabetic persons with essential hypertension treated with these drugs.

METHODS AND ANALYSIS

Study Setting and Organization

The trial is a single center study and will be executed at the Ghent University Hospital (Belgium). All participants will complete the same protocol on both test days, however consuming a different type of capsules in a randomized order: high-flavanol cocoa extract or placebo. The flavanols used in this trial were extracted from the cocoa bean. A wash-out period of minimal 3 days and maximal 2 weeks is provided. The first measurement was conducted on October 5th, 2018 and since the COVID-19 pandemic forced to cancel all measurements for about 6 months, the end date of the study was postponed and is estimated in June 2021.

Participants

Eligibility Criteria

All subjects are divided in four groups: (i) patients with T2DM with arterial hypertension (with BB, $n = 5$/ACEi, $n = 5$/ARB, $n = 5$) ($n = 15$), (ii) patients with T2DM without arterial hypertension ($n = 10$), (iii) non-diabetic patients with essential arterial hypertension (BB, $n = 10$/ACEi, $n = 10$/ARB, $n = 10$) ($n = 30$), and (iv) healthy participants (control group; $n = 20$).

Inclusion and exclusion criteria are summarized in **Table 2**.

Recruitment and Screening

Subjects are recruited by endocrinologists and other medical specialists, general practitioners, dieticians and investigator's acquaintances. Flyers are distributed in the Ghent University Hospital and by pharmacists and physiotherapists. In addition, the essential information for possible participants of our study is disseminated on media and social media.

Patient and Public Involvement

No patient or patient advisor was/is involved with study design, recruitment or conduct.

Intervention

Study Protocol

As depicted in **Figure 1**, patients will arrive at the lab (Ghent University Hospital) at 8 o'clock after an overnight fast of minimal 8 h. Blood samples will be collected and participants will be evaluated for body composition [weight, height, skinfolds, and fat% (bio-electrical impedance analyzer)] and BP, followed by a standardized breakfast with negligible flavonoid and nitrate/nitrite amounts, in accordance to guidelines of a dietician (**Table 3**), and consumed within 15 min. Participants have to choose 1 formula of breakfast which will be consumed at both visits. After 15 min rest period, a baseline Flow-Mediated Dilation (FMD) measurement will be performed, followed by the ingestion of CF-enriched capsules (2.5 g of cocoa extract which contains 790 mg flavanols of which 150 mg epicatechin, *Naturex©, France*) or placebo (maltodextrin and an equivalent dose of theobromine and caffeine compared to the CF-enriched capsules) (**Table 4**). The dose of CF given in this study is based on the research of Balzer et al. (41) who investigated the effect of different doses of CF (high dose = 963 mg CF of which 203 mg epicatechin, medium dose = 371 mg CF of which 78.9 mg epicatechin, and low dose (control) = 75 mg CF of which 16.8 mg epicatechin), closely matched for theobromine and caffeine, on FMD in 10 patients with T2DM. The equilibration of theobromine and caffeine, both vasoactive compounds of cocoa (38, 56, 57), in the different interventions is a very important

TABLE 2 | In- and exclusion criteria for participants in this study.

Inclusion criteria
Male and female; age: 18–85 years; BMI: 20–40 kg/m²
(i) Patients with T2DM without arterial hypertension: at least 5 years T2DM [HbA1c ≥6.5%, glucose (fasting) ≥126 mg/dl, glucose (not fasting): ≥200 mg/dl, defined by the American Diabetes Association (ADA) (55)]
(ii) Patients with T2DM with arterial hypertension: at least 5 years T2DM [HbA1c ≥6.5%, glucose (fasting) ≥126 mg/dl, glucose (not fasting): ≥200 mg/dl, defined by the American Diabetes Association (ADA) (55)] AND at least 1 year arterial hypertension taking BB, ACEi or ARB (optionally combined with diuretics)
(iii) Patients with essential arterial hypertension: at least 1 year arterial hypertension taking BB, ACEi or ARB (optionally combined with diuretics), matched by age, sex, and BMI with subjects with T2DM
(iv) Healthy controls: taking no medication except for contraceptive drugs, matched by age, sex, and BMI with subjects with T2DM
Exclusion criteria
Smoking habits: current smoking; smoking history of more than 30 years or pack years are higher than years of smoking cessation
Alcohol consumption: more than 10 units per week
Additional systemic disorders: chronic inflammatory disease, active cancer
Microvascular complications: retinopathy, nephropathy, peripheral sensory(motor), or autonomic neuropathy
Macrovascular complications: cardiovascular and respiratory diseases: heart failure NYHA class 3 and 4, uncontrolled arrhythmias or angora, documented peripheral arterial disease or experienced heart attack, active or chronic recurrent vasculitis, severe to very severe chronic pulmonary diseases (GOLD stage III and IV)
Neurological diseases: cerebrovascular accident, transient ischemic attack, reversible ischemic neurological deficit or stenosis >50% by doppler
Other: important (and relevant) musculoskeletal disorders; factors that impede the execution of the dynamic handgrip exercise test; pregnancy; known cognitive impairment (such as dementia, intellectual disability), language barriers
Medication: medication directly influencing endothelial function except for insulin or antihypertensive drugs; nitric oxide-containing medication; phosphodiesterase type 5-inhibitors

T2DM, type 2 diabetes mellitus; BMI, body mass index; BB, beta blocker; ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.

quality of this study and will ensure to test isolated vascular effects of CF.

The investigator will ensure all capsules are taken in properly. After ingestion, the participants will remain seated for 70 min. Thereafter, blood samples will be collected and a second BP and FMD assessment followed by a dynamic handgrip exercise (dHGE) test with simultaneous near-infrared-spectroscopy (NIRS) monitoring and blood flow measurements will be executed. Venous blood samples will be drawn before and after the dHGE test.

All measurements post intake will be performed within 2 h as highest circulating concentrations of flavanols are found between 90 and 120 min after consumption (58, 59).

Participants will fill in questionnaires (once) to evaluate factors which may impact vascular reactivity [i.e., daily physical activity [International Physical Activity Questionnaire—long version (60)], flavanols-intake [a self-designed questionnaire about the frequency of flavanol-enriched food intake (16, 17, 61)], sleep behavior (Epworth Sleepiness Scale, STOP-BANG), and autonomic function (Autonomic Symptom Profile-COMPASS). We will also assess quality of life and general health status (36-Item Short Form Health Survey, World Health Organization Quality of Life questionnaire-BREF, and only for patients with T2DM Diabetes Quality Of Life questionnaire). In addition to the questionnaires, each participant's physical activity levels (62) and glycemic excursions and variability (63) will be objectively measured during 1 usual week using accelerometry and a Continuous Glucose Monitoring System, respectively.

Guidelines for Participants

Subjects will be asked not to participate in other trials from 3 weeks before start of this study to 1 week after the second study day. Supplement or vitamin consumption that could interfere with the mechanisms of action of flavanols have to be suspended for at least 4 weeks prior start of the study. In addition, to minimize flavanol intake before each study day, participants will be asked to follow guidelines (16, 17, 61):

- 3 days before each study day, participants will be asked to drink maximal two cups of tea per day, to avoid red wine or cider, to refrain from eating chocolate (in any form), beans, and rhubarb and to consume maximal two small portions of fruits (piece, juice, or jam) or one portion in combination with 10 g nuts.
- Twenty-four hours before each study day, participants will be asked to refrain from vigorous physical activity (apart from daily movements as climbing stairs, biking to the train station, walking to the car etc.), alcohol or caffeine containing drinks (e.g., coffee, cola, and tea). They have to consume the same meal the evening before both study days.
- Eight hours before the actual start of the study, participants will be asked to fasten (no food or drink intake, apart a small amount of water) and, importantly, to take in their medication as usual (identical dose as prescribed by their physician). Antihypertensive medication need to be taken in exactly 2 h before actual start of the study. Antidiabetics must be taken in at home or at the lab during breakfast depending on type of the drug. Metformin, SGLT-2 inhibitors, and GLP-1 analoga may be taken in at home or at the lab and all sulfonylurea drugs must be taken in at the lab during breakfast. For insulin, the long-acting insulin must be taken in at home and the short-acting insulin must be taken in at the lab during breakfast.

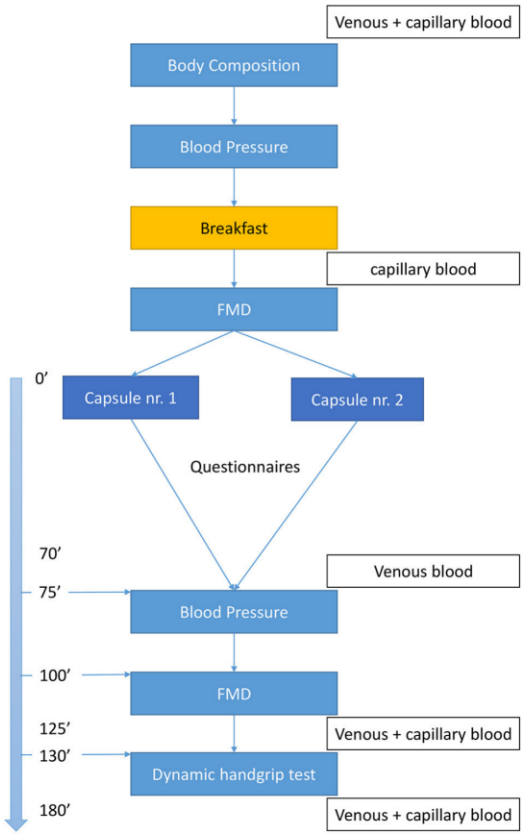

FIGURE 1 | Flowchart. FMD, flow-mediated dilation test; capillary blood, finger prick to measure capillary glycaemia (only patients with T2DM).

TABLE 3 | Standardized breakfast formulas.

Breakfast formulas	Composition	Ingredients
Formula 1	Energy: 418 Kcal Proteins: 21.7 g (21%) Carbohydrates: 61.4 g (59%) Fat: 8.8 g (20%)	60 g cereals (Special K, Kellogg's) 200 g semi-skimmed milk 125 g semi-skimmed cottage cheese
Formula 2	Energy: 386 Kcal Proteins: 19.3 g (21%) Carbohydrates: 49.5 g (53%) Fat: 10.8 g (26%)	90 g light brown bread 15 g butter (Halvarine, Blue Band) 17 g cream cheese (La vache qui rit) 15 g jam (reduced sugars) 125 g low-fat yogurt
Formula 3	Energy: 499 Kcal Proteins: 23.3 g (19%) Carbohydrates: 53.9 g (44%) Fat: 20.0 g (37%)	90 g light brown bread 200 g semi-skimmed milk 15 g butter (Halvarine, Blue Band) 15 g jam (reduced sugars) 30 g cheese (Gouda, Hollandic)
Formula 4	Energy: 501 Kcal Proteins: 22.4 g (18%) Carbohydrates: 58.6 g (48%) Fat: 17.6 g (33%)	90 g light brown bread 15 g butter (Halvarine, Blue Band) 25 g gingerbread (reduced sugars) 30 g cheese (Gouda, Hollandic) 125 g low-fat yogurt

TABLE 4 | Nutrient content of the capsules.

Nutrient content	8 CF-enriched capsules	6 capsules with placebo
Total cocoa extract (g)	2.5	0
Total flavanols (mg)	794	0
Epicatechin (mg)	149	0
Catechin (mg)	30	0
Caffeine (mg)	23	24
Theobromine (mg)	179	180
Maltodextrin (mg)	928	1.956

Amount is presented for total amount taken and not for each capsule. CF, Cocoa flavanols.

Blinding and Randomization

In this cross-over study, every participant will receive CF-enriched and placebo capsules. Randomization of capsules will be done by sealed envelopes and type of capsules will be indicated by number 1 or number 2. Each participant may choose 1 envelope at the first study day. Both types of capsules have an identical look and taste. Hence, participants will be blinded to their group allocation. Furthermore, since type of capsules will be identified by numbers, outcome assessors and personnel involved in data collection and data analysis will be blinded to participants' group allocation throughout the entire trial.

Data Management

All researchers, outcome assessors, data collector, data manager, data entry personnel, and statistician will receive special training regarding the standard procedure and data management. During the recruitment period, our data collector will record the baseline characteristics of participants in case report forms and all data will be checked by the data manager.

Study data will be pseudonymized, collected, and managed using REDCap, a secure, GDPR-proofed, web-based software platform designed to support data capture for research studies, providing (1) an intuitive interface for validated data capture, (2) audit trails for tracking data manipulation and export procedures, (3) automated export procedures for seamless data downloads to common statistical packages, and (4) procedures for data integration and interoperability with external sources (64, 65).

Solely two main investigators (endocrinologist and study responsible) will have the authority to decode the pseudonymized data. Concerning obtained data of FMD measurements, all clips will be saved in dicom-format and stored at a central server (University Ghent).

Adverse Events and Safety Monitoring

Adverse events after intake will be described as it is monitored by oral interrogation during the study day and by e-mail if participants experience some side-effects. So far, no adverse effects were defined in literature. However, in the article of Monagas et al. (66) 1 out of 42 included subjects reported

constipation during the intake of cocoa concomitant with 250 mL skim milk for 4 weeks, which was solved by increasing fiber intake.

Outcome Measures

Primary Outcome Measure

Flow-Mediated Dilation

FMD is a non-invasive technique and the gold standard to measure endothelial function. The FMD measurement will be performed at the brachial artery, at the non-dominant side, following a standardized protocol described by Thijssen et al. (67). An ultrasound system (GE, Vivid 7) with a linear probe (12L, 7–10 MHz) will be used to assess the brachial artery diameter based upon longitudinal images with the lumen–intima interface visualized on both (anterior and posterior) walls. The arterial occlusion will be performed *via* a sphygmomanometer cuff (Hokanson, SC5^{TM}, 6 × 83 cm), placed 5–10 cm below the elbow, inflated and held between 220 and 240 mmHg (68, 69) for 5 min. For stability, the probe will be hold tight by a stand-off probe support. To assess the effect of the reactive hyperemia, the brachial artery will be visualized pre-occlusion for 90 s (mean of three individual clips comprising each 30 s) and post-occlusion for 4 min with additional 15 s before cuff release. Although it is recommended to perform an FMD measurement in fasting state (67), this is not feasible since the entire protocol lasts around 5 h. However, the measurements will be done 15 and 150 min after food intake (standardized breakfast at each visit). Boundaries for diameter measurement will be identified automatically by means of a boundary tracking software (Quipu, diastolic phase) and optically controlled by a single, independent and blinded investigator. When tracking is impaired, the investigator will restore this tracking with maintenance of region of interest. Data obtained from false tracking or altered region of interest will be removed from analysis. FMD measurement is a challenging technique and has a significant learning curve. At least 100 scans supervised by a specialist were done prior to the start of this study.

Secondary Outcome Measure

Blood Pressure

BP measurements will be carried out at the dominant side, in sitting position, after 3 min of rest by an automatic device (Tango+, SunTech Medical). Heart rate and systolic and diastolic BP will be assessed for 21 min with an interval of 3 min, pre- and post-capsules intake. During the recording, participants will be asked to remain calm and silent and refrain from drinking. Systolic and diastolic BP, as well as mean arterial pressure, pulse pressure, and heart rate will be used in analysis.

Dynamic Handgrip Exercise Test With Near-Infrared-Spectroscopy

A maximal dHGE will be performed with simultaneous NIRS (OxiplexTS, ISS, Champaign, IL, USA) monitoring to assess the oxygenation and vasoreactivity at the level of arterioles and capillaries of Musculus flexor digitorum superficialis, Musculus flexor carpi ulnaris, and Musculus flexor carpi radialis. A reliable handgrip exercise protocol, specifically designed for patient populations using NIRS measurements, will be carried out according to Celie et al. (70). In order to minimize bias and to work with relative values, an arterial occlusion of the ipsilateral upper arm will be performed by a pneumatic cuff (Hokanson, SC5^{TM}, 6 × 83 cm) inflated and held between 220 and 240 mmHg for 5 to maximal 7 min, until maximal deoxygenation (hemoglobin and myoglobin) values are reached (71). In this study, the maximal voluntary contraction will be calculated by means of two attempts with 10 s in between. The exercise test, consisting of 2-min periods of an incremental cyclic contractions protocol (1 s contraction, 1 s relaxation) followed by 1-min rest periods, will start at 20% of the maximal voluntary contraction and increase by 10% of the maximal voluntary contraction each step until exhaustion. Total hemoglobin [sum of deoxy- and oxyhemogblin, which reflects the change in regional blood volume (72)], deoxyhemoglobin and –myoglobin, and saturation will be assessed according to Celie et al. (70).

Blood flow through the brachial artery *via* echo-doppler will be measured according to Celie et al. (71) (same transducer and linear probe as used for the FMD measurement) at baseline (before cuff occlusion), immediately after cuff occlusion, and in the periods of rest after each increasing step.

Blood Analyses

Levels of uric acid (photometry, Architect c16000, Abbott), vitamin C (colorimetric method, R-biopharm/Roche reagent, Indiko Plus, Thermo Fisher), and glucose (photometry, Architect c16000, Abbott) will be measured at each study day in whole blood (anticoagulant-free tubes). Plasma and serum derived from EDTA and anticoagulant-free tubes will be centrifuged (3,500 g for 10 min at 10°C) and thereafter stored at −80°C together with a cryovial whole blood in the medical biobank of the Ghent University Hospital (Bioresource center Ghent, Gent, Belgium, ID: BE 71067049) (73). At the end of the study, cholesterol (photometry, Architect c16000, Abbott), high density lipoproteins (photometry, Architect c1600, Abbott), triglycerides (photometry, Architect c16000, Abbott), high sensitive C-reactive protein (particle-enhanced immunonephelometry, BN II, Siemens), vitamin E (liquid/liquid extraction followed by detection *via* UPLC-DAD), free fatty acids (enzymatic colorimetry, Cobas 8000 e801, Roche Diagnostics), and free insulin (Cobas e801 Roche, ECLIA) will be determined in serum, while hemoglobin A1c (exchange chromatography—Tosoh HLV-723 G8) and haptoglobin (Behring Nephelometer analyzer II), a marker of iron metabolism (74), will be determined in whole blood. All samples will be analyzed at the lab of clinical biology of Ghent University Hospital.

Additional Outcome Measures

In each participant, physical activity [steps per day and moderate-to-vigorous physical activity (MVPA)] will be evaluated with an accelerometer [Actigraph wGT3X-BT, dominant side, hip (75)] and glycemic excursions (percentages of time in hyper- or hypoglycemic ranges and time in range), risk for hypo- and hyperglycemia (Low and High Blood Glucose Indexes, LBGI and HBGI), and glycemic variability [standard deviation, coefficient of variation, and mean amplitude of glucose excursion (MAGE)]

(76) by continuous interstitial glucose measurement (blind mode, IPro2, Medtronic, abdomen) for 1 week, immediately after the second study day. During this week, the above-mentioned restrictions concerning food intake and physical activity are not applicable and participants are encouraged to maintain their normal daily habits. Additionally, participants are asked to fill in a diary concerning their food intake and their physical activities for each day.

Statistical Analyses

Power and Sample Size Calculation

Sample size calculations are based on previous research focusing on interventional studies with FMD measurements (77, 78) who established that in cross-over trials at least 20–30 participants are required for a minimal detectable change of 1.5–2% in FMD (80% power, alpha-level = 0.05). Sample size calculation was done by SAS power and sample size, indicating a minimum of nine participants in each group (41). Data will be analyzed taking into account volunteer- and methodology-related factors (79).

Data-Analysis Plan

Effects of CF ingestion on primary and secondary outcome measures will be evaluated within and between groups. Data will be analyzed using a random intercept model in mixed models. Fixed effects will be the group, the supplementation (cocoa flavanols vs. placebo), the time (pre- vs. post-intake of capsules) during each visit for repeated measurements, as well as group × supplementation interaction and time × supplementation interactions. We will also consider the order of both visits in a first intent and this factor will be kept in analyses only if significant. As all groups are matched by age, sex, and BMI, only medication intake, level of physical activity, glycemic excursions, risk for hypo- or hyperglycemia, and glycemic variability will be considered as covariates when analyzing data. In case of drop-outs or missing data, the participant is still included in data analysis providing one out of two visits is completed.

Results will be presented as mean ± standard deviation with its corresponding 95% confidence interval. Level of significance will be set at $p < 0.05$. Data analysis will be conducted using IBM SPSS statistics version 26.

DISCUSSION

In recent years, there has been an increased attention to polyphenols and their beneficial effects on vascular health. Several studies have been carried out in healthy participants to assess this. However, the studies, acute as well as chronic, on patients with T2DM with a high risk for vascular complications are scarce and show inconsistent results. Possible reasons for this inconsistency might be heterogeneity in the given intervention (dose, duration, source), the studied population (duration of diabetes, severity of comorbidities, BP at baseline, sex), and possibly used medication (vasoactive antihypertensive and antidiabetic drugs). Hence, there is a high need for more acute and chronic research concerning this topic in this population.

This work will provide novel data helpful for the development of strategies in the nutritional education of particularly vulnerable populations, given their high risk for developing cardiovascular disease, including non-pharmacological therapies and strategies that employ lifestyle modification. This intervention might also have implications for the preparation of recommendations in clinical practice guidelines and quality improvement programs aimed at the care of patients with T2DM.

ETHICS STATEMENT

Study procedures were approved by the ethical committee of Ghent University Hospital on April 5th, 2018 (STUDY B670201835660). To enquire all participants and to ensure making an informed decision to participate, each subject will receive an informed consent that first is explained orally by an investigator and thereafter sent by e-mail providing the opportunity to reread this form and to ask questions afterwards. The informed consent contains all details of the research (background, aims, and possible risks or advantageous) and is written in Dutch for good understanding. In case of important protocol modifications, all participants will be informed by e-mail. The results will be submitted to an international peer-reviewed journal and presented at scientific conferences. They will be disseminated through digital science communication platforms, including academic social media, to extend its outreach and usefulness.

AUTHOR CONTRIBUTIONS

AT, BC, SS, ER, JO, KR, EH, and PC were involved in the methodological design and drafting of the trial protocol. AT, SS, ER, PC, and EH have overall responsibility for the design, conduct, and decision to submit for publication. BC, JO, and KR are co-researchers. BC designed the dynamic handgrip exercise test and set up the plan for analysis. JO and KR designed the flow-mediated dilation measurement and set up the plan for analysis. AT, ER, SS, PC, and EH recruit participants into the study. AT, PC, and EH drafted the manuscript. All authors will contribute to data interpretation, conclusions, and dissemination and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.602086/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table S1. Participant timeline

		Study day x*			Study day y*			
Time point	Enrollment Before first study day	Pre-allocation T_{-1}	Allocation T_0	Post-allocation T_1	Pre-allocation T_{-1}	Allocation T_0	Post-allocation T_1	After second study day
Enrollment								
Eligibility screen	x							
Informed consent	x							
Interventions (depending on number in sealed envelope)								
Capsules with cocoa flavanols			x					
Capsules with placebo						x		
Assessments								
Baseline variables: Body mass index, venous blood sample		x			x			
Flow-mediated dilation measurement		x		x	x		x	
Blood pressure measurement		x		x	x		x	
Dynamic handgrip exercise test with Near-infrared spectroscopy				x			x	
Additional measurements: Accelerometer and continuous glucose monitoring system								x

**: the intake of the capsules is randomized so study day x or study day y can be the first or the second study day.*

Acute effects of cocoa flavanols on blood pressure and peripheral vascular reactivity in type 2 diabetes mellitus and essential hypertension.

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Acute effects of cocoa flavanols on blood pressure and peripheral vascular reactivity in type 2 diabetes mellitus and essential hypertension.

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Keywords: Type 2 diabetes, cocoa flavanols, vascular reactivity, blood pressure, antihypertensive drugs

Abstract

Background: Type 2 diabetes mellitus (T2DM) is associated with high a risk of vascular complications, among others due to increased oxidative stress and nitric oxide depletion. Cocoa flavanols (CF) increase nitric oxide availability and activity and decrease oxidative stress and therefore exert well-established beneficial vascular effects in non-diabetic subjects.

In T2DM, these effects have only been scarcely studied, yielding inconsistent results. We performed a study to assess effects on vascular reactivity of a single-dose of CF in T2DM and whether certain antihypertensive drugs, as commonly used in T2DM, may modulate these effects.

Material and Methods: Non-diabetic and T2DM participants were studied after administration of both CF-enriched capsules (790 mg CF) and placebo in randomized order. Fasting blood samples, blood pressure (BP), and arterial vasoreactivity (flow-mediated dilation, FMD) were assessed before capsules ingestion. BP and FMD assessments were repeated 70 minutes after capsules ingestion, followed by evaluation of muscle microvascular reactivity through a dynamic handgrip strength test (near-infrared spectroscopy). Age, waist-to-hip ratio, BP at baseline, and use of antihypertensive drugs were regarded as covariates in a Mixed Models analysis (random intercept).

Results: 24 non-diabetic and 11 T2DM subjects participated in this trial. Single CF ingestion did not have any effect on any parameter. However, independent of type of capsules ingested, a decrease in diastolic BP and an increase in the change in brachial artery diameter (pre vs. post occlusion) were detected in the non-diabetic group, while they remained unchanged in the T2DM group (p=0.01 and 0.07 for time x group interaction respectively). The diastolic BP decreased by 3 mmHg (95% CI: -4.0; -2.0) and difference in brachial artery diameter increased by 0.06 mm (95% CI: 0.01; 0.12).

Conclusion: No beneficial effects were detected of a single dose of CF on vascular reactivity parameters in T2DM, but surprisingly neither in non-diabetic participants. We discuss various possibilities for the lack of effects. Since the impact of CF on vascular function can be non-linear, more standardized randomized studies, testing different CF doses would be required to assess possible vascular benefits of CF administration in different populations.

1 Introduction

Type 2 Diabetes mellitus (T2DM) and the cardiovascular morbidity and mortality associated with it represent an increasing worldwide health burden. The predicted rise in prevalence severely challenges economic and health care resources, such that there is an equally increasing need for cheap and readily available interventions (1-5). If effective, nutraceuticals, i.e. foods or compounds occurring in foods exerting medical or health benefits, might be worth considering for this purpose (6).

In the context of T2DM, flavonoids, and more specifically flavanols with its most abundant monomeric form epicatechin (EC), are of particular interest. These nutraceuticals found in, among others, cocoa, black tea, berries and several other natural foods (7, 8), are not only suggested to exert vasoprotective effects (9, 10), but the physiological processes involved coincide with those implicated in the pathophysiology of T2DM related micro- and macrovasculopathies.

In T2DM, β-cell dysfunction and/or insulin resistance lead to chronic hyperglycemia (2, 11). This, in turn, increases oxidative stress, inflammation, and orthosympatic activity, and, more importantly, limits availability of nitric oxide (NO), resulting in the above-mentioned micro- and macrovascular dysfunctions (12).

Flavanols derived from cocoa (CF) increase NO bioavailability and -activity by enhancing endothelial NO synthase production and activity, as well as by reducing oxidative stress through the inhibition of endothelial nicotinamide adenine dinucleotide phosphate oxidase (13, 14). Moreover, CF are suggested to inhibit endothelin-1, a strong vasoconstrictor (15), and to inhibit angiotensin-converting enzyme activity (16-19).

Indeed, in vitro, animal, and human research has suggested CF-induced amelioration of endothelial function (20-22) and blood pressure (BP) (23, 24). In addition, epidemiological studies have linked lower BP levels and decreased cardiovascular as well as all-cause mortality to cocoa consumption (25-27). Moreover, several randomized control trials and meta-analyses implicated beneficial vascular properties of CF in different study populations (22-24, 28-30). This prompted the European Food Safety Authority to publish a health claim in 2012, stating that daily intake of 200 mg CF, concomitant with a normal, balanced diet, helps to protect the compliance of blood vessels preserving a normal blood flow in the general population (31).

However, it is not clear to what extent this recommendation might be extrapolated to T2DM patients. In a recent meta-analysis, we found that evidence supporting the latter is weak at best: little, very heterogeneous research has produced inconsistent results only (32). We found weak evidence that mid/long-term CF ingestion decreased diastolic BP (DBP), but not systolic BP (SBP), by approximately 1 – 2 mmHg only. These effects were suggested to be greater in female, younger, and hypertensive adults, when EC content is at least 90 mg, and when CF is ingested in 1 daily batch. Also, the few trials examining acute CF effects are heterogeneous and inconsistent (33-35).

To distinguish whether the lack of results represents a lack of effectiveness of CF, or merely a lack of homogeneity in study methods and populations, we designed a basic protocol, using standardized measurements in a circumscript population using CF extracts in capsule form and placebo capsules in a cross-over study design.

We aimed to investigate whether single ingestion of a high dose of CF induced an improvement in endothelial function (primary outcome), a reduction in BP, and/or an enhancement in muscle vasoreactivity in patients with T2DM compared to non-diabetic controls. This study also examined possible confounding effects of the use of antihypertensive drugs (36), specifically angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB), as these drugs are frequently used in T2DM subjects (36) and have intrinsic vasoactive effects.

We hypothesized that CF ingestion would improve micro- and macrovascular parameters in both non-diabetic and T2DM participants; however, stronger effects were expected in patients with T2DM and in participants taking antihypertensive drugs, as they need to reduce levels of oxidative stress and increase NO bioavailability.

2 Material and methods

As our research group recently published a protocol-paper (37) describing the exact protocol of this acute, randomized, double-blinded, placebo-controlled cross-over study, only the main methodological parts are highlighted in this report.

This trial was approved by the ethical committee of Ghent University Hospital (April 2018) and was registered at clinicaltrials.gov (ID: NCT03722199). Written consent was obtained from all subjects before enrollment. Recruitment was done by spreading out flyers in Ghent and announcements on (social) media. Volunteers came from different parts in Flanders, Belgium. Data collection was performed from October 5th, 2018 until March 20th, 2021. However, since the COVID-19 pandemic forced to cancel all measurements for approximately 6 months and substantially hindered participation afterwards, data collection was strongly impeded in the last 12 months.

2.1 Participants

We considered two groups of participants (men and women, aged 18 to 85 years) for inclusion: (1) people with T2DM without micro- or macrovascular complications for at least 5 years (American Diabetes Association's definition (38)), with and without arterial hypertension and (2) subjects without T2DM, with and without essential hypertension. Hypertensive subjects were only allowed to use ACEi or ARB, optionally combined with diuretics.

Exclusion criteria included active smoking or a history of smoking, either in the past 5 years or having more than 30 packyears, alcohol use (more than 10 units per week), chronic inflammatory diseases, active cancer, microvascular (retinopathy, nephropathy, neuropathy) or macrovascular (cardiovascular, cerebrovascular, peripheral artery diseases) diseases, neurological diseases, pregnancy, known cognitive impairment (e.g. dementia), language barrier (other than Dutch, French or English), musculoskeletal disorders that could interfere with the outcomes of the measurements, and use of NO-containing medication or phosphodiesterase type 5-inhibitors.

2.2 Trial protocol

The study was designed as an acute randomized, double-blinded, placebo-controlled cross-over study. As presented in Figure 1, a standardized protocol was followed for 2 identical test days with type of capsules as the only difference: CF-enriched capsules (2.5 g of cocoa extract containing 790 mg flavanols, 149 mg of which EC, Naturex SA, France) or placebo (maltodextrin and an equivalent dose of theobromine and caffeine compared to the CF-enriched capsules). The exact composition of each type of capsules was reported previously (37).

After an overnight fast (at least 8 hours), blood samples [lipid profile, free fatty acids, hemogobinA1c (HbA1c), glucose, insulin, vitamin A, E, C, uric acid, C-reactive protein, and haptoglobin] were collected and body composition was assessed. Immediately after, BP was recorded for 20 minutes, in 3 minutes intervals (Tango+, SunTech Medical), followed by a standardized breakfast (composition of meal: 18 - 21% protein, 20 - 37% fat, 44 - 59% carbohydrates, and 386 - 501 Kcal (37); division of choices are listed in Table S1).

30 minutes after the start of the breakfast, a flow-mediated dilation (FMD)-test (GE, Vivid 7) was performed, after which either CF or placebo capsules were ingested (randomized order). After a 70-minutes resting period during which questionnaires (Autonomic Symptoms Profile

Questionnaire, Epworth Sleepiness Scale) were filled out, a second BP measurement and FMD-test were executed. Subsequently, a dynamic handgrip exercise test (starting at 20 % of maximal voluntary contraction and 10 % increase each step, performed until exhaustion) with simultaneous monitoring of total hemoglobin (THb) variations via near-infrared spectroscopy (NIRS; OxiplexTS, ISS, Champaign, IL, USA) was completed.

In patients with T2DM, several capillary glycemic measurements were performed throughout the test day for safety reasons (no measurements during hypo- or clinically significant hyperglycemia (>250 mg/dL)).

Participants were asked to follow specific guidelines concerning physical activity and CF-low food and drink consumption starting from 3 days prior to each test day, as described previously (37).

Immediately following the second test day, participants wore an accelerometer (Actigraph wGT3X-BT, dominant side, hip (39)) and a Continuous Glucose Monitoring System (blind mode, IPro2, Medtronic, abdomen) for 7 consecutive days.

Study data was pseudonymized, collected, and managed using REDCap (40, 41). Blood samples were stored in the medical biobank of the Ghent University Hospital (Bioresource center Ghent, Gent, Belgium, ID: BE 71067049) (42).

2.3 Statistical analyses

Sample size calculations based on the reported results of Balzer et al. (2008) (33) were done by SAS power and sample size. At least 9 subjects in each group were mandatory for 85 % power based on the primary outcome.

To assess differences in baseline characteristics between both visits within and between each group (non-diabetic versus T2DM), Paired-T-tests or Wilcoxon signed-rank tests and independent T-tests or Mann Whitney U-tests were executed depending on the distribution of data. Both groups included patients with arterial hypertension using antihypertensive drugs (AHD). Therefore, we also further analyzed differences between and within groups based on the use of AHD. The same statistical tests were executed depending on the distribution of data; however, in the subgroups of the T2DM group only non-parametric tests were used because of the small sample size.

Differences between non-diabetic and T2DM subjects concerning physical activity level by accelerometry and usual glycemic profile by continuous glucose monitoring were assessed via independent T-test or Mann Whitney U-tests, depending on the distribution of data.

For the analysis of CF effects on the macrovascular reactivity (FMD-test) and BP, a random intercept in mixed models was used with the following fixed effects: group (T2DM, non-diabetic), supplementation (CF-enriched, placebo), time of measurement during each visit (pre-, post-intake of capsules), as well as time x supplementation interaction, time x group interaction, and time x supplementation x group interaction. Differences between pre and post intake were calculated with a post-hoc Sidak test if the model was significant or showed a tendency. Age, waist-to-hip ratio, and systolic BP (SBP) at baseline were added as covariates; however, SBP at baseline was not inserted for analyses of SBP, mean arterial pressure, and

pulse pressure as they already include SBP. P-values and estimated coefficients, calculated by mixed models, were reported when a significant influence of covariates was detected.

For the analysis of CF effects on microvascular reactivity (dynamic handgrip exercise test results), a random intercept in mixed models was used as well, with the same covariates, but with other fixed effects because this test was only executed once at each visit: group (T2DM, non-diabetic), supplementation (CF-enriched, placebo), time of measurement during exercise test (increasing exercise steps with 10 % of maximal voluntary contraction), as well as group x supplementation interaction, time x supplementation interaction, time x group interaction, and time x supplementation x group interaction.

Subsequently, a cofactor indicating the use of AHD was inserted to the models to analyze whether the use of AHD influences the observed effects.

In case of dropouts or missing data, the participant was still included in data analysis providing one out of two visits was completed. Level of significance was set at $p < 0.05$ for all data analyses apart from the comparison of baseline characteristics between subgroups (based on the use of AHD) where level of significance was set at $p < 0.025$. Data analyses were conducted using IBM SPSS statistics version 26.

3 Results

3.1 Participants' characteristics

11 T2DM and 24 non-diabetic subjects were studied (Table 1). Of these individuals, 15 non-diabetic participants and 4 T2DM participants had essential hypertension and used AHD. Characteristics did not differ between subgroups of T2DM subjects, neither between subgroups of non-diabetic subjects (Tables S2, S3, and S4). However, age, waist-to-hip ratio (WHR), and SBP were significantly higher in the 11 T2DM vs. 24 non-diabetic participants recruited (Table 1).

No statistical differences were detected for subjects' characteristics between both visits (data not reported).

Fasting blood results are listed in Table 2. HbA1c and fasting glucose were significantly higher in the T2DM group, LDL- and total cholesterol were significantly higher in the non-diabetic group, and antilipemic drug use was considerably lower. Only minimal differences for fasting blood results between subgroups of T2DM subjects, and between subgroups of non-T2DM subjects were detected (data of subgroups separately are presented in Supplementary Table S5).

Physical Activity levels were similar in both groups (Table 3). As expected, time in hyperglycemia (> 180 mg/dL, > 250 mg/dL), day-to-day glycemic excursions and variability were significantly higher in the T2DM participants. However, variability indexes suggested relatively stable glycemic values in T2DM participants (43).

Data in subgroups based on the use of AHD (within non-diabetic or T2DM) were not significantly different (data of 4 groups separately are presented in Supplementary Table S6).

3.2 Results of primary and secondary measurements

All data of one complete visit with CF ingestion had to be excluded because a participant (within the non-diabetic group) vomited after breakfast consumption. Similarly, data of two pre-CF FMD-measurements were excluded because of unreliable tracing of diameter boundaries of the arteria brachialis (participants within the T2DM group). For all models with significant or almost significant (tendency) interactions, residuals were Gaussian. The impact of covariates on a tested model is only reported when significant.

3.2.1 Macrovascular reactivity: Flow-mediated dilation test (FMD) – primary outcome (Table 4)

No effects of CF were detected on FMD, baseline brachial artery diameter (BAD) or peak BAD in either group. For the difference in BAD (peak BAD minus baseline BAD) however, an over-time increase in the non-diabetic group was found independent of capsules ingestion, which tended (p= 0.07) to differ from the T2DM group (61.7 µm; 95% CI: 6.5; 116.9 in non-diabetic vs. -32.5 µm; 95% CI: -118.9; 53.9 in T2DM group) (Figure 2). This tendency disappeared when the use of AHD was inserted as a cofactor. WHR significantly influenced baseline BAD (the higher WHR, the higher the BAD; e= +1.8; p= 0.02) and peak BAD (the higher WHR, the higher the peak BAD; e= +1.8; p=0.02). Hyperglycemia (~200 mg/dL) was present in 2 T2DM persons just prior to the FMD measurement pre capsules ingestion (placebo, participants using AHD) and in 3 T2DM persons just before the FMD measurement post capsules ingestion (placebo and CF, varied participants, with and without the use of AHD) (data not shown).

3.2.2 Blood Pressure assessment – secondary (macrovascular) outcome (Table 4)

No effect of CF was observed for SBP, mean arterial pressure, pulse pressure or heart rate. However, for DBP, an over-time decrease was found in the non-diabetic group after breakfast and independent of capsules ingestion (-3 mmHg, 95% CI: -4.0; -2.0 in non-diabetic vs. -0.3 mmHg; 95% CI: -1.8; 1.2 in T2DM group) (Figure 3). DBP was influenced by age (the higher the age, the lower the DBP; e= -0.2; p= 0.02) and SBP at baseline (the higher the SBP at baseline, the higher the DBP; e= +0.5; p< 0.001).

3.2.3 Microvascular reactivity: Dynamic handgrip strength test with near-infrared spectroscopy – secondary outcome (Table 5, Figure 4)

During the dynamic handgrip strength test, no interaction effect between exercise intensity, type of capsules ingested, and/ or group was found for THb values, a factor reflecting the change in regional blood volume (44). However, when the cofactor ‘use of AHD’ was added to the model, a significant interaction effect reflecting higher THb levels during the entire exercise test was detected in the non-diabetic individuals without the use of AHD after placebo ingestion (p< 0.001). THb was inversely correlated with age (e= -2.7; p= 0.01).

4 Discussion

We aimed to assess whether the positive vascular effects of CF, as suggested in non-diabetic individuals, also occur in T2DM patients. In this acute, randomized, double-blinded, placebo-controlled cross-over study design, we found no additional effect of CF compared to placebo. The small (-3 mmHg), but clinically relevant (45-47) decrease in DBP and the increased difference in BAD (+61.7 µm) we measured, occurred with both CF and placebo capsules, and only in the non-diabetic group.

The absence of CF effects on vascular dynamic parameters might partly be explained by the small sample size. We based our power calculation on the study of Balzer et al. (2008) (33); however, this study used a cocoa drink instead of purified CF capsules as the CF source, as well as a different placebo CF composition, which might have caused a biased calculation. Other possible reasons for a lack of effect include (1) the 'acute' study design (one single CF ingestion compared to split doses throughout the day or ingestion for several days or weeks), (2) other methodological choices (e.g. post-prandial as opposed to fasting assessment) or (3) the choice of the CF source (purified CF capsules compared to chocolate beverages or bars). Furthermore, certain subject characteristics (such as age, fasting blood results, drug use) might explain the divergent results compared to other studies. In our study, for matching reasons between groups, our non-diabetic participants were relatively older than in previous publications, had a higher weight with a mean BMI of 26.4 ± 4.5 $kg.m^{-2}$ and the prevalence of insulin resistance was relatively high. This caused an overlap with the T2DM group, which might also partly account for a lack of difference between the T2DM and non-T2DM group.

The selection of T2DM individuals was specifically designed to be strict, especially concerning the absence of both macro- and microvascular conditions, in order to limit heterogeneity in our study population and to be able to address the presence of diabetes mellitus/ chronic hyperglycemia as a pure, specific variable. This might, however, have reduced the detectability of possible CF effects, since these are reportedly more pronounced in persons with a certain level of vascular dysfunction (48-50). Perhaps in a T2DM population with vascular complications, CF effects would have been considerably more pronounced.

Nonetheless, we expected to find some vascular improvement from CF administration, if only in the non-diabetic group, and if only in FMD parameters, based on previous publications and especially considering the Health Claim of the European Food Safety Authority for the general population (31).

However, in this respect it should be noted that the studies, upon which the above-mentioned Health Claim was based, were quite heterogenous. Not only did patient characteristics (e.g. sex, age, BMI, BP, smoking behavior) vary considerably, but in addition, there was a marked methodological heterogeneity in both interventional and control formulas: the former varied from beverages with milk or cold/ hot water to chocolate bars, and contained different amounts of CF as well as non-flavanol cocoa compounds. The latter varied from white chocolate, low CF dosed compounds, compounds unmatched for non-flavanol components of cocoa to having no control at all.

This complicated comparability, not only amongst the studies, but also in relation to our study. For instance, when only looking at the analyzed acute trials, 9 out of 11 papers report on subjects between 25 and 55 years old (14, 21, 51-57). In comparison, our population was aged 59.5 years (controls) and 66.7 years (T2DM group). So far, it is unclear to what extent this might explain the differences from our study: although age impacts vascular compliance

(58), and therewith perhaps direct post- CF vascular reactivity, reports on age-dependent CF effects on vascular reactivity are contradictory. For instance, BP reducing effects of cocoa products have been suggested to be greater in younger compared to older adults in some studies [CF effect in persons aged below compared to above 50 (23) and 65 years old (32)], but others have suggested the opposite (24). However, these reports were based on long-term CF administration, and it is unclear to what extent these effects can be extrapolated to single CF ingestion.
For FMD, we found one study suggesting that single CF ingestion exerts greater acute (60 – 120 minutes) effects in older (> 57.5 years) compared to younger adults (< 57.5 years), whereas chronic (2 - 84 days) CF effects were not age-specific (22). Our results showed no effect of age as a covariate.

As mentioned, other methodological challenges concerning the Health Claim involve the variation in intervention and placebo formulas in the papers analyzed. The varied use of chocolate introduces carbohydrates (e.g. sugar) and milk (whole milk or processed in chocolate), which may alter CF activity, effects on gastric emptying and substance uptake et cetera and so complicate the comparability with our study, but also between previous publications (59-65). Moreover, several studies did not report on the exact amount of CF and/ or did not control for non-flavanol components in cocoa, such as caffeine and theobromine (66-68).

Theobromine and caffeine are both methylxanthines with intrinsic vascular impact; theobromine being a rather weak adenosine receptor antagonist (69, 70). It has been suggested that adding theobromine increases EC as well as CF effects on FMD (49), via its endothelium-independent vasodilating effects through cyclic nucleotide phosphodiesterases inhibition (71). Our study matched CF and placebo capsules for theobromine and caffeine content, although the theobromine levels in our capsules (180 mg) might have been too low to exert synergistic effects with CF or EC, which is possibly seen by Balzer et al. (2008). They showed an increase of FMD through single consumption of a cocoa drink containing comparable doses of CF and EC as in our study (371 - 963 mg, 78.9 – 203.0 mg respectively), but higher doses of theobromine (575.6 – 586.2 mg) (72).

It is also worth mentioning that the Health Claim is solely dedicated to CF and not to other interfering components (e.g. caffeine and theobromine). Nevertheless, in our opinion, further research on synergistic, antagonistic, and/or supplementary actions with other cocoa components is required before drawing definite conclusions on the effects of cocoa ingestion on vascular health, and on the contribution of CF per se hereon.

Furthermore, it should be noted that the Health Claim is based on papers performing a standardized FMD technique. Although it is recommended to measure FMD in a fasting state (73), this was not feasible in our study for practical reasons. It is possible that the consumed breakfast influenced our results. In the general healthy population, a decrease in FMD was demonstrated 1 hour after glucose ingestion and was restored within 4 hours (74). This period of impaired endothelial function is in accordance with the postprandial rise in glucose levels, 1 to 3 hours. Compared to healthy individuals, T2DM subjects have a delayed peak of insulin levels which could possibly explain, in part, the observed differences between our non-diabetic vs. T2DM group (75). As described, in the T2DM group, with and without the use of AHD, a few patients had a glycemia of around 200 mg/dL before an FMD test. In general, only few researchers examined postprandial CF effects, however, compared to everyday life,

a postprandial state is the most physiological situation as we live in this state most of the day (74).

As our study provided an intervention containing 790 mg CF, which is more than the recommended dose of 200 mg daily in the Health Claim, the impact of person characteristics as well as the impact of varied interventional and placebo formulas may not be ignored in cocoa research. Hence, we would plead for a nuanced interpretation of the current literature and the Heath Claim concerning the beneficial vascular effects of CF.

In conclusion, despite the paucity of effects of CF ingestion on peripheral micro- and macrovascular reactivity in our study, the congruent involvement of nitric oxide in both CF effects and the pathophysiology of vascular T2DM complications make us reluctant to dismiss these cheap and easily accessible compounds as valuable nutraceuticals.

As demonstrated, we believe the matter to be too complex to draw straight-forward conclusions based on current reports. If vascular effects of CF are to be studied further, one would preferably need to correct for all vasoactive components present in CF, use purified CF instead of chocolate formulas and report on the entire composition of interventional formulas with consumed foods or drinks. These and the other methodological aspects should also be considered when interpreting reports on CF.

Finally, we would like to mention that the recruitment of eligible participants was challenging by both the strict in- and exclusion criteria, but the COVID-pandemic hindered recruitment dramatically. Therefore, we could not complete our groups and we could not perform all analyses as explained in our registered trial protocol and reported in our protocol-paper (37).

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

AT, BC, SS, ER, JOR, EH, and PC were involved in the methodological design and drafting of the trial protocol. AT is a PhD-student, SS, ER, PC, and EH are the medical and non-medical principal investigators, who have overall responsibility for the design, conduct and decision to submit for publication. BC, JS, EL, and JOR are co-researchers. BC designed the dynamic handgrip exercise test and set up the plan for analysis. JOR designed the flow-mediated dilation measurement and set up the plan for analysis. JS helped with the statistical analyses. AT, ER, SS, PC, and EH recruited participants into the study. AT executed all measurements. AT, PC, SS, and EH drafted the manuscript. All authors read and approved the final manuscript**.** All authors contributed to data interpretation and conclusions and approved the final version.

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9 Abbreviations

ACEi = angiotensin-converting enzyme inhibitor; AHD= antihypertensive drugs; ARB = angiotensin receptor blocker; BAD= brachial artery diameter; BP = blood pressure (SBP, systolic blood pressure; DBP, diastolic blood pressure); CF = cocoa-derived flavanols; FMD = flow-mediated dilation; NIRS= Near-infrared spectroscopy; NO= nitric oxide; non-DM= participants without diabetes mellitus; T2DM = type 2 diabetes mellitus; THb= total hemoglobin; WHR= waist-to-hip ratio.

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11 Figures

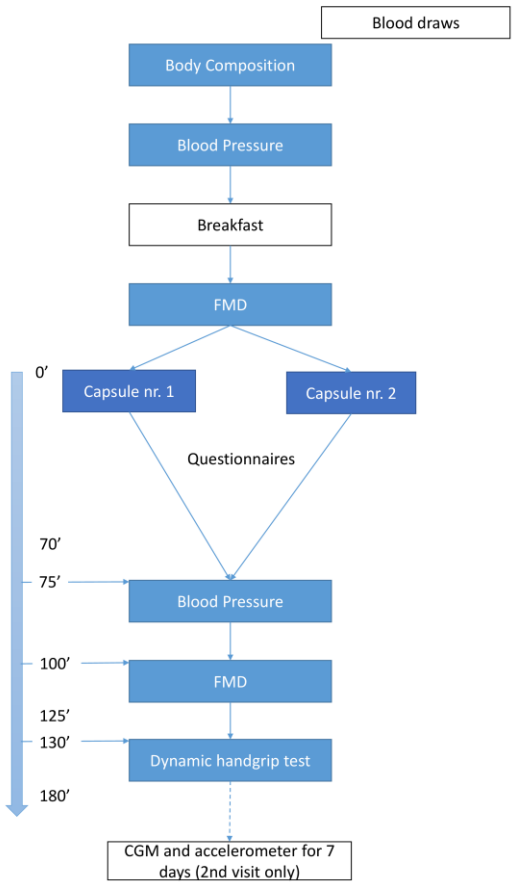

Figure 1: Flowchart.

Legend: FMD= flow-mediated dilation test; CGM= continuous glucose monitoring system.

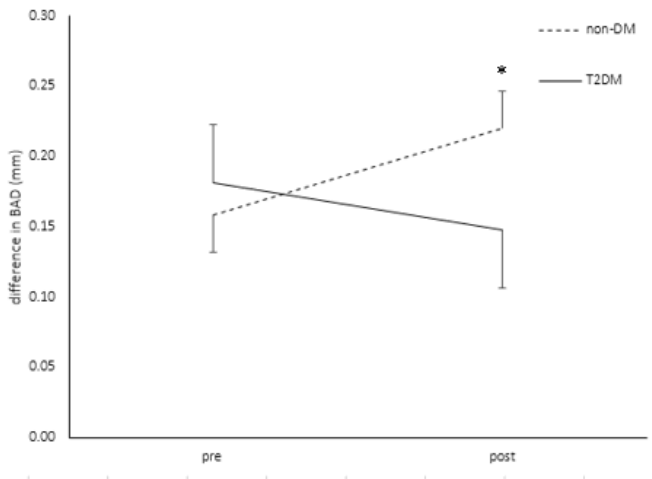

Figure 2: Difference in brachial artery diameter (BAD) in response to breakfast and capsules ingestion (cocoa flavanols and placebo combined) in both groups.

Legend: Data are means ± SE. Main effects from mixed models when significant: Time x group interaction for the difference in BAD (tendency, P = 0.07); Post-hoc analyses: no significant pairwise group differences, but a significant pairwise time difference in the non-diabetic group (p = 0.03;); difference in BAD= BAD post cuff inflation minus BAD pre cuff inflation.*

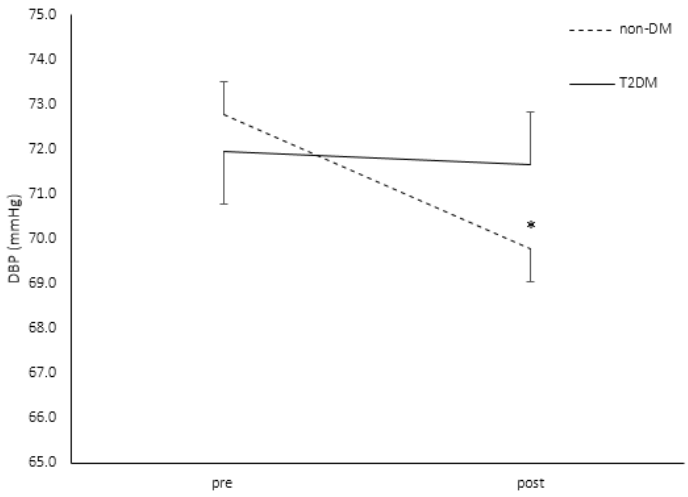

Figure 3: Diastolic blood pressure (DBP) in response to breakfast and capsules ingestion (cocoa flavanols and placebo combined in both groups).

Legend: Data are means ± SE. Main effects from mixed models when significant: Time x group interaction, P = 0.01; Post-hoc analysis: no significant pairwise group differences, but a significant pairwise time difference in the non-diabetic group (p<0.001;).*

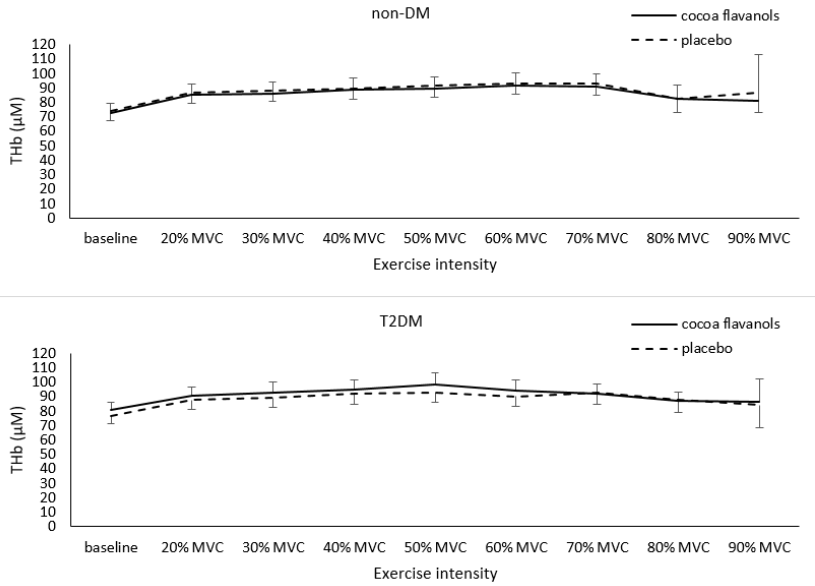

Figure 4: Muscle microvascular reactivity to the dynamic exercise test in both groups.

Legend: Data are means ± SE. MVC= maximal voluntary contraction; THb= total hemoglobin.

12 Tables

Table 1: Participants' Characteristics

	Non-DM	T2DM
N	24	11
Sex (♀/♂)	14/10	4/7
Age (years)	59. 5 ± 5.5 [48 – 69]	66.7 ± 6.1 [57 – 78]*
Weight (kg)	77.1 ± 14.1 [53.2 – 98.3]	77.6 ± 13.7 [57.3 – 99.8]
BMI (kg.m^{-2})	26.4 ± 4.5 [18.2 – 35.5]	26.2 ± 3.0 [21.1 – 31.9]
Fat mass (%)	31.9 ± 9.1 [15.2 – 47.6]	29.7 ± 5.6 [21.5 – 37.7]
Waist-to-hip ratio	0.91 ± 0.06 [0.78 – 1.02]	0.95 ± 0.11 [0.65 – 1.04]*
Baseline SBP (mmHg)	116.0 ± 10.9 [99.0 – 141.5]	125.1 ± 14.7 [91.2 – 143.7]*
Baseline DBP (mmHg)	73.5 ± 7.5 [60.3 – 89.0]	75.1 ± 8.2 [58.8 – 88.0]
Baseline Mean arterial pressure (mmHg)	87.7 ± 8.4 [73.7 – 103.4]	91.8 ± 10.1 [69.6 – 106.6]
HbA1c (%)	5.8 ± 0.3 [5.1 – 6.5]	6.9 ± 0.7 [6.2 – 8.5]
Duration diabetes (years)	NA	9.3 ± 5.5 [5 – 23]
Antihyperglycemic drugs (n):	NA	10
Metformin		9
Sulfonylurea		2
DPP4- inhibitors		0
GLP-1- RA		2
SGLT-2		1
Insulin		1
Duration of hypertension (years)	7.5 ± 5.5 [2 – 20]	9.0 ± 3.2 [6 – 13]
Antihypertensive drugs (n)(%):	15 (62.5%)	4 (36.4%)
ACEi	11	1
ARB	4	3
Lipid-lowering drugs (n)(%):	5 (20.8 %)	7 (63.6 %)*
HMG-CoA reductase inhibitors	4	7
Fibrates	1	0
History of smoking (years)	3.3 ± 6.7 [0 – 25]	8.8 ± 14.7 [0 – 40]
Autonomic profile[a]:		
Orthostatic intolerance (/40)	2.2 ± 5.0 [0 – 16]	2.5 ± 5.7 [0 – 16]
Vasomotor function (/5)	0.2 ± 0.7 [0.0 – 3.3]	0.3 ± 0.8 [0.0 – 2.5]
Daytime sleepiness[b] (/24)	7.3 ± 3.6 [2 – 17]	5.7 ± 2.1 [3 – 9]

*Data: means ± SD with [range] or frequencies at first visit; [a]data obtained through the Autonomic Symptoms Profile Questionnaire; [b]data obtained through the Epworth Sleepiness Scale; DPP4= dipeptidylpeptidase-4; GLP-1-RA=glucagon-like peptide-1- receptor agonist; HMG-CoA= 3-hydroxy-3-methylglutaryl-coenzyme A; SGLT-2= sodium glucose-cotransporter 2 inhibitors; *= p-value < 0.05 for difference between T2DM and non-diabetic.*

Table 2: Fasting blood results

	Non-DM		T2DM	
	CF	placebo	CF	placebo
Glucose (mg/dL)	97.0 ± 8.0	96.2 ± 9.1	127.7 ± 20.6*	127.4 ± 17.0*
Insulin (mU/L)	10.3 ± 6.6	9.8 ± 5.3	9.5 ± 6.1	9.5 ± 6.0
HOMA[a]	2.4 ± 1.6	2.4 ± 1.4	3.1 ± 2.1	3.0 ± 1.8
QUICKI[b]	0.35 ± 0.03	0.35 ± 0.03	0.34 ± 0.04	0.34 ± 0.04
Triglycerides (mg/dL)	109.9 ± 38.4	106.9 ± 42.1	135.9 ± 72.2	112.1 ± 41.3∫
FFA (mmol/L)	0.55 ± 0.16	0.54 ± 0.20	0.57 ± 0.16	0.55 ± 0.15
HDL-cholesterol (mg/dL)	57.8 ± 18.7	57.9 ± 14.8	52.6 ± 14.4	53.8 ± 17.3
LDL-cholesterol (mg/dL)[c]	126.2 ± 35.8	124.2 ± 36.4	89.7 ± 31.5*	92.6 ± 30.7*
Total cholesterol (mg/dL)	206.0 ± 47.5	203.5 ± 45.9	169.5 ± 39.5*	168.8 ± 41.3*
Uric acid (mg/dL)	4.7 ± 0.9	4.9 ± 1.3	5.1 ± 1.1	5.2 ± 1.0
CRP (mg/dL)	2.3 ± 3.4	2.8 ± 4.6	1.8 ± 1.0	1.5 ± 0.6
Vitamin C (mg/dL)	0.7 ± 0.3	0.7 ± 0.4	0.5 ± 0.3*	0.6 ± 0.3
Vitamin A (µg/dL)[d]	67.5 ± 13.1		74.4 ± 15.2	
Vitamin E (mg/dL)[d]	1.3 ± 0.3		1.2 ± 0.3	
Haptoglobin (g/L)[d]	1.0 ± 0.4		1.2 ± 0.6	

*Data: means ± SD or frequencies; [a]calculated via fasting insulin x fasting glucose / 22.5; [b]calculated via 1/(log(insulin)+log(glucose)); [c]calculated via the Friedewald Formula; [d]only measured once, at first visit; CRP= C-reactive protein; FFA= free fatty acids; HDL= high density lipoprotein; LDL= low density lipoprotein. ∫= significant difference between both visits (p<0.05); *= significant difference between both groups (p<0.05).*

Table 3: Accelerometry and Continuous Glucose Monitoring

	Non-DM	T2DM
Accelerometry:		
Wearing time (minutes/day)	874.9 ± 16.5 [830.9 – 891.4]	842.2 ± 50.9 [721.6 – 889.3] $
Valid days (days/week)	6.8 ± 0.5 [5 – 7]	6.6 ± 0.9 [4 – 7]
Step counts (n/day)	7521.1 ± 2690.3 [3217.4 – 13600.6]	6593.8 ± 2279.1 [3154.3 – 10338.0]
Moderate (minutes/week)	316.6 ± 219.5 [24.0 – 939.0]	186.4 ± 145.2 [0.0 – 456.0] $
Vigorous (minutes/week)	6.3 ± 14.2 [0.0 – 54.0]	6.5 ± 17.4 [0.0 - 58.0]
Very vigorous (minutes/week)	0.8 ± 3.5 [0.0 – 17.0]	1.3 ± 4.2 [0.0 – 14.0]
MVPA (minutes/week)	323.7 ± 225.9 [24.0 – 941.0]	194.2 ± 149.3 [0.0 – 456.0] $
Continuous Glucose Monitoring System:		
Glycemic excursions:		
% time in range (70-180 mg/dL)	97.1 ± 3.4 [87.4 – 100.0]	83.9 ± 14.0 [56.8 – 98.3]*
% time in hypoglycemic range <70 mg/dL	1.1 ± 1.8 [0.0 – 6.7]	0.7 ± 2.2 [0.0 – 7.0]*

% time in hyperglycemic range		
>180 mg/dL	1.8 ± 3.0 [0.0 – 12.6]	15.4 ± 14.1 [1.7 – 43.2]*
>250 mg/dL	0.2 ± 0.4 [0.0 – 1.7]	1.4 ± 1.8 [0,0 – 5,4]*
Area under curve (mg/dL/day)		
Below 70 mg/dL	133.8 ± 236.0 [0.0 – 872.5]	143.1 ± 451.5 [0.0 – 1428.1]*
Above 180 mg/dL	681.9 ± 1208.2 [0.0 - 3928.3]	6635.8 ± 6653.6 [220.9 – 21230.8]*
Above 250 mg/dL	76.9 ± 276.8 [0.0 - 1333.6]	720.5 ± 1087.7 [0.0 – 3004.7]*
Glycemic variability:		
Standard deviation (mg/dL)	19.7 ± 8.2 [11.2 – 39.8]	32.7 ± 9.9 [21.1 – 48.3]*
Coefficient of variation (%)[a]	17.4 ± 6.8 [9.9 – 35.7]	22.9 ± 7.0 [16.3 – 38.1] *
MAGE (mg/dL)	45.1 ± 21.1 [22.3 – 97.7]	79.4 ± 23.7 [49.5 – 111.6]*
Low blood glucose index	0.62 ± 0.48 [0.07 – 2.01]	0.24 ± 0.52 [0.00 – 1.72]*
High blood glucose index	0.62 ± 0.66 [0.05 – 2.75]	3.50 ± 2.29 [1.36 – 8.36] *
Data sufficiency (%)	99.9 ± 0.4 [98.2 – 100.0]	99.9 ± 0.1 [99.6 -100.0]
Days CGM worn (n)	5.9 ± 0.4 [4 - 6]	6.0 ± 0.0 [6 – 6]

*Data: means ± SD and [range]; [a]calculated by mean blood glucose divided by standard deviation; MAGE= mean amplitude of glycemic excursion; MVPA= moderate to vigorous physical activity; *= significant difference between groups (p<0.05, $ for tendency).*

Table 4: Results of examinations for macrovascular beds

	Non-DM		T2DM	
	CF	placebo	CF	placebo
Macrovascular reactivity:				
FMD-test: diameter of brachial artery (BAD)				
Baseline BAD (mm)				
Before capsule	3.8 ± 0.1 [2.7 – 5.3]	3.8 ± 0.1 [2.6 – 5.3]	3.9 ± 0.2 [2.8 – 4.6]	4.1 ± 0.2 [2.7 – 4.7]
Post capsule	3.9 ± 0.1 [2.8 – 5.3]	3.8 ± 0.1 [2.7 – 5.2]	4.1 ± 0.2 [2.6 – 5.1]	4.1 ± 0.3 [2.4 – 5.2]
Peak BAD (mm)				
Pre intake	4.0 ± 0.1 [2.9 – 5.6]	3.9 ± 0.1 [2.8 – 5.3]	4.2 ± 0.2 [3.1 – 5.0]	4.2 ± 0.2 [2.8 – 5.4]
Post intake	4.1 ± 0.2 [3.0 – 5.8]	4.1 ± 0.1 [2.8 – 5.3]	4.3 ± 0.2 [2.8 – 5.3]	4.3 ± 0.2 [2.6 – 5.5]
Difference BAD(µm) (peak – baseline)				
Pre intake	158.0 ± 25.7 [-135.0 – 383.0]	161.3 ± 25.5 [-60.0 – 560.0]	260.5 ± 91.0 [22.0 – 884.0]	165.6 ± 56.1 [-11.0 – 684.0]
Post intake	189.0 ± 32.9 [-60.0 – 610.0]	244.4 ± 35.8 [40.0 – 630.0]	132.7 ± 29.7 [-38.0 – 257.0]	171.7 ± 30.4 [-50.0 – 308.0]
Difference[a]	61.7 ± 27.8∫		-32.5 ± 43.5 $	
FMD (%) [((peak BAD – baseline BAD))/ baseline BAD) x 100]				
Pre intake	4.3 ± 0.8 [-3.0 – 11.9]	4.6 ± 0.9 [-2.0 ± 18.9]	4.9 ± 1.4 [0.5 ± 14.9]	4.0 ± 1.2 [-0.2 ± 14.4]
Post intake	4.8 ± 0.8 [-1.8 – 13.9]	6.7 ± 1.0 [0.9 ± 16.8]	3.3 ± 0.8 [-1.0 ± 7.5]	4.6 ± 1.0 [-1.1 ± 10.8]
Blood pressure (BP) and heart rate				
SBP (mmHg)				
Pre intake	114.1 ± 2.6 [89.2 – 144.5]	114.4 ± 2.4 [94.7 – 141.5]	122.3 ± 3.7 [91.2 -136.8]	123.5 ± 4.5 [94.0 – 143.7]
Post intake	110.4 ± 2.3 [87.8 – 133.5]	110.3 ± 2.1 [93.5 – 131.0]	119.6 ± 3.1 [91.5 – 129.8]	121.8 ± 3.5 [95.3 – 137.3]

DBP (mmHg)				
Pre intake	71.7 ± 1.7 [54.3 – 85.2]	72.4 ± 1.6 [60.3 – 89.0]	73.4 ± 1.9 [58.8 – 81.2]	74.0 ± 2.6 [60.0 – 88.0]
Post intake	69.2 ± 1.6 [51.2 – 84.2]	68.8 ± 1.4 [58.2 – 83.7]	73.2 ± 1.6 [60.5 – 78.2]	73.7 ± 1.8 [62.3 – 81.5]
Difference[a]	-2.98 ± 0.51∫		-0.29 ± 0.78*	
Mean arterial pressure [DBP + (1/3 x Pule pressure)]				
Pre intake	85.8 ± 1.9 [65.9 – 101.7]	86.4 ± 1.8 [71.8 – 103.4]	89.7 ± 2.5 [69.6 – 99.7]	90.5 ± 3.2 [71.3 – 106.6]
Post intake	82.9 ± 1.7 [63.4 – 99.7]	82.6 ± 1.6 [70.7 – 97.3]	88.6 ± 2.0 [70.8 – 94.8]	89.7 ± 2.3 [73.3 – 100.1]
Pulse pressure (mmHg) (SBP – DBP)				
Pre intake	42.4 ± 1.3 [34.8 – 64.2]	41.9 ± 1.2 [31.7 – 57.2]	48.9 ± 2.2 [32.3 – 56.3]	49.4 ± 2.3 [34.0 – 64.8]
Post intake	41.3 ± 1.1 [33.0 – 53.5]	41.5 ± 1.1 [31.5 – 51.7]	46.4 ± 1.9 [31.0 – 52.5]	48.1 ± 2.2 [33.0 – 60.3]
Heart rate (bpm)				
Pre intake	62.1 ± 1.6 [48.7 – 80.5]	64.5 ± 1.6 [50.8 – 79.7]	65.0 ± 3.5 [47.8 – 85.2]	63.6 ± 2.7 [51.3 – 77.7]
Post intake	66.4 ± 1.7 [53.5 – 83.0]	67.3 ± 1.7 [49.2 – 86.3]	70.5 ± 3.6 [54.7 – 92.8]	67.5 ± 2.9 [52.5 – 82.7]

*Data are expressed as mean ± SE [range]; differences between pre and post intake were calculated with a post-hoc Sidak test if the model was significant or showed a tendency. Any time x supplementation x group interaction was significant, nor with the cofactor use of AHD; any time x supplementation interaction was significant, nor with the cofactor use of AHD; *= significant time x group interaction, also with the cofactor use of AHD (p<0.05; $ for tendency); ∫= significant difference pre vs post capsules ingestion. [a]Since differences were independent of the type of capsules ingestion, data of CF and placebo were combined.*

Table 5: Results of examinations for microvascular beds

	Non-DM				T2DM			
	Without AHD		**With AHD**		**Without AHD**		**With AHD**	
	CF	placebo	CF	placebo	CF	placebo	CF	placebo
Microvascular reactivity:								
Muscle vasoreactivity to exercise								
THb (µM)								
Baseline								
	63.1 ± 5.7	66.9 ± 7.8	77.4 ± 9.4	77.9 ± 9.3	81.3 ± 6.8	76.7 ± 6.2	79.2 ± 11.5	75.3 ± 9.9
	[41.6 – 88.2]	[34.8 – 111.4]	[21.5 – 148.4]	[21.1 – 149.4]	[68.0 – 110.1]	[53.3 – 101.4]	[51.0 – 102.1]	[53.7 – 101.8]
Maximal								
	83.9 ± 7.7	90.9 ± 10.5	101.5 ± 12.4	102.3 ± 11.5	102.4 ± 8.7	93.6 ± 8.1	101.8 ± 16.3	100.7 ± 13.7
	[59.8 – 114.9]	[53.4 – 140.3]	[25.9 – 187.5]	[30.1 – 185.9]	[86.5 – 140.5]	[66.0 – 122.5]	[65.6 – 134.0]	[73.1 – 138.4]
Difference (maximal – baseline)								
	20.8 ± 2.5	24.0 ± 4.2*	24.1 ± 4.2	24.5 ± 3.1	21.1 ± 2.2	17.0 ± 2.5	22.6 ± 6.3	25.5 ± 3.9
	[13.5 – 34.0]	[12.4 – 55.3]	[4.4 – 64.6]	[6.9 – 53.2]	[14.1 – 30.3]	[6.5 – 24.6]	[13.1 – 40.7]	[19.4 – 36.7]

*Data are expressed as mean ± SE [range]; THb= total hemoglobin; only a significant group x supplementation x cofactor interaction for THb; *= significant difference between type of capsules within subgroup (p< 0.001).*

Supplementary Material

Table S1: Choice of breakfast-formulae

	Non-DM		T2DM	
	Without AHD	**With AHD**	**Without AHD**	**With AHD**
Formula 1: cereals	1	1	0	0
Formula 2: yoghurt	4	4	3	1
Formula 3: milk	1	4	3	1
Formula 4: gingerbread	3	6	1	2

Data: frequencies; AHD= antihypertensive drugs; formula 1= 60 g cereals (Special K, Kellogg's), 200 g semi-skimmed milk, 125 g semi-skimmed cottage cheese; formula 2= 90 g light brown bread, 15 g butter (Halvarine, Blue Band), 17 g cream cheese (La vache qui rit), 15 g jam (reduced sugars), 125 g low-fat yoghurt; formula 3= 90 g light brown bread, 200 g semi-skimmed milk, 15 g butter (Halvarine, Blue Band), 15 g jam (reduced sugars), 30 g cheese (Gouda, Hollandic); formula 4= 90 g light brown bread, 15 g butter (Halvarine, Blue Band), 25 g gingerbread (reduced sugars), 30 g cheese (Gouda, Hollandic), 125 g low-fat yoghurt. For nutrient composition we refer to our published protocol-paper (1).

Table S2: Characteristics of participants

	Non-DM		T2DM	
	Without AHD	**With AHD**	**Without AHD**	**With AHD**
N	9	15	7	4
Sex (♀/♂)	5/4	9/6	3/4	1/3
Age (years)	59.7 ± 5.3	59.3 ± 5.8	67.7 ± 5.9	65.0 ± 7.0
Weight (kg)	75.8 ± 13.1	77.8 ± 15.0	74.6 ± 15.8	82.9 ± 8.1
BMI (kg/m²) [range]	25.7 ± 3.9	26.8 ± 5.0	25.5 ± 3.5	27.5 ± 1.8
	[20.9 – 32.7]	[18.2 – 35.5]	[21.1 – 31.9]	[24.9 – 29.0]
Fatmass (%)	31.6 ± 9.7	32.1 ± 9.1	29.7 ± 5.8	29.6 ± 6.0
Waist-to-hip ratio	0.90 ± 0.06	0.91 ± 0.07	0.92 ± 0.14	0.98 ± 0.02
Baseline SBP (mmHg) [range]	115.4 ± 10.7	116.4 ± 11.3	121.2 ± 17.0	131.9 ± 6.7
	[99.0 – 132.3]	[100.2 – 141.5]	[91.2 – 143.7]	[125.3 – 137.8]
Baseline DBP (mmHg) [range]	72,0 ± 6,7	74.4 ± 8.0	73.7 ± 9.4	77.6 ± 5.8
	[61.3 – 85.3]	[60.3 – 89.0]	[58.8 – 88.0]	[72.7 – 84.5]
Baseline mean arterial pressure (mmHg)	86.5 ± 7.8	88.4 ± 8.9	89.5 ± 11.8	95.7 ± 5.0
[range]	[74.3 – 101.0]	[73.7 – 103.4]	[69.6 – 106.6]	[90.3 – 102.3]
HbA1c (%)	5.7 ± 0.4	5.9 ± 0.2	7.1 ± 0.7	6.7 ± 0.5
Duration diabetes (years) [range]	NA	NA	9.3 ± 6.4	9.3 ± 4.3
			[5 – 23]	[6 – 15]
Antihyperglycemic drugs (n)	NA	NA	**6**	**4**

-Metformin			5	4
-Sulfonylurea			1	1
-DPP4-inhibitors			0	0
-GLP-1-RA			1	1
-SGLT-2			1	0
-Insulin			1	0
Duration of hypertension (years)[range]	NA	7.5 ± 5.5 [2 – 20]	NA	9.0 ± 3.2 [6 – 13]
Antihypertensive drugs (n)(%)	NA	**15** (62.5%)	NA	**4** (36.4%)
-ACEi		11		1
-ARB		4		3
Lipid-lowering drugs (n)(%)	**2**	**3**	**4**	**3**
-HMG-CoA reductase inhibitors	1	3	4	3
-Fibrates	1	0	0	0
History of smoking (n)	2.2 ± 5.1	3.9 ± 7.6	10.2 ± 16.6	6.3 ± 12.5
Autonomic profile[a]				
-Orthostatic intolerance	2.7 ± 5.3	1.9 ± 5.0	1.7 ± 4.5	4.0 ± 8.0
-Vasomotor function	0.0 ± 0.0	0.3 ± 0.9	0.1 ± 0.3	0.6 ± 1.2
Daytime sleepiness[b]	5.9 ± 3.0	8.2 ± 3.8	6.3 ± 2.4	4.8 ± 1.0

Data: means ± SD or frequencies at first visit; [a]data obtained through the Autonomic Symptoms Profile Questionnaire; [b]data obtained through the Epworth Sleepiness Scale; DPP4= dipeptidylpeptidase-4; GLP-1=glucagon-like peptide-1-receptor agonist; HMG-CoA= 3-hydroxy-3-methylglutaryl-coenzyme A; SGLT-2= sodium glucose-cotransporter 2 inhibitors. No differences between subgroups of T2DM subjects, neither between subgroups of non-diabetic subjects.

Table S3: Autonomic Symptoms Profile Questionnaire

ID	Ortostatic	Vaso-motor	Secreto-motor	Gastro-intestinal	Bladder	Pupillo-motor	Total
Non-DM without AHD							
ngnb101	0	0	6.42	10.68	0	1.65	18.75
ngnb103	0	0	2.14	0.89	0	2.64	5.67
ngnb204	0	0	6.42	0	0	0	6.42
ngnb205	0	0	2.14	0	0	0.33	2.47
ngnb206	0	0	2.14	0.89	1.11	0.66	4.8
ngnb207	0	0	0	2.67	3.33	1.65	7.65
ngnb108	12	0	0	5.34	1.11	0	18.45
ngnb109	0	0	6.42	1.78	0	1.98	10.18
ngnb111	12	0	0	2.67	0	0.99	15.66
Non-DM with AHD							
ngac201	12	0	0	0.89	0	1.32	14.21
ngac102	0	0	2.14	0	1.11	1.65	4.9
ngac104	0	0	0	4.45	0	0	4.45
ngac105	16	1.66	2.14	0.89	2.22	2.64	25.55
ngac206	0	0	0	3.56	1.11	1.65	6.32
ngac107	0	0	2.14	0.89	1.11	0	4.14
ngac108	0	0	2.14	0	1.11	2.64	5.89
ngac109	0	0	0	3.56	0	1.32	4.88
ngac110	0	0	2.14	0	0	0.99	3.13
ngac211	0	0	0	0.89	1.11	1.32	3.32
ngac212	0	0	0	3.56	0	0	3.56
ngab101	0	3.32	8.56	0.89	2.22	2.97	17.96
ngab202	0	0	2.14	1.78	0	0	3.92
ngab203	0	0	2.14	1.78	2.22	0.99	7.13
ngab104	0	0	2.14	0.89	0	0.66	3.69
T2DM without AHD							
dmac201	0	0	8.56	3.56	3.33	0	15.45
dmnb101	0	0	4.28	1.78	0	1.65	7.71
dmnb103	12	0.83	4.28	2.67	3.33	1.32	24.43
dmnb204	0	0	0	2.67	0	0	2.67
dmnb105	0	0	8.56	8.01	0	2.31	18.88
ngnb202	0	0	2.14	0	1.11	0.99	4.24
dmnb207	0	0	2.14	3.56	0	0.99	6.69
T2DM with AHD							
dmab201	16	0	2.14	2.67	4.44	1.98	27.23
dmab202	0	0	0	5.34	0	1.32	6.66
dmab103	0	2.49	6.42	4.45	1.11	1.32	15.79
dmac202	0	0	0	0.89	0	0	0.89

Scoring of questionnaire was done via Sletten et al. (2012) (2), each column represents the total score of the participants within each domain and the last column represents the sum of

the scores of all domains multiplied with their Cronbach α coefficient; AHD= antihypertensive drugs.

Table S4: Epworth Sleepiness Scale

ID	ESS_1	ESS_2	ESS_3	ESS_4	ESS_5	ESS_6	ESS_7	ESS_8	ESS_total
Non-DM without AHD									
ngnb101	1	3	1	1	2	0	1	0	9
ngnb103	0	3	1	2	2	0	1	0	9
ngnb204	0	0	1	0	1	0	0	0	2
ngnb205	1	2	1	1	2	0	1	0	8
ngnb206	0	1	0	1	1	0	0	0	3
ngnb207	2	3	1	0	2	0	1	0	9
ngnb108	1	1	0	0	3	0	0	0	5
ngnb109	0	1	0	0	1	0	0	0	2
ngnb111	2	3	0	0	1	0	0	0	6
Non-DM with AHD									
ngac201	2	0	0	0	2	0	0	0	4
ngac102	2	2	2	2	2	1	2	2	15
ngac104	3	3	0	1	3	0	0	0	10
ngac105	2	2	0	0	2	0	0	0	6
ngac206	1	0	0	3	3	0	2	0	9
ngac107	0	3	1	0	2	0	3	0	9
ngac108	3	3	2	2	2	1	2	2	17
ngac109	1	1	0	0	2	0	1	0	5
ngac110	1	2	0	1	3	0	0	0	7
ngac211	1	2	0	missing	3	0	2	0	8
ngac212	0	2	0	0	3	0	0	0	5
ngab101	missing	missing	missing	missing	missing	missing	missing	missing	missing
ngab202	1	3	0	1	2	0	1	0	8
ngab203	1	2	0	0	2	0	2	0	7
ngab104	1	0	0	0	2	0	2	0	5
T2DM without AHD									
dmac201	3	2	1	1	2	0	0	0	9
dmnb101	1	3	1	missing	3	0	0	0	8
dmnb103	0	0	0	0	3	0	0	0	3
dmnb204	0	2	1	0	2	0	1	0	6
dmnb105	0	3	0	0	3	0	1	0	7
ngnb202	0	1	0	1	1	0	0	0	3
dmnb207	2	3	0	0	3	0	0	0	8
T2DM with AHD									
dmab201	0	2	0	0	2	0	0	0	4
dmab202	missing	2	0	1	2	0	1	0	6
dmab103	1	2	0	0	1	0	0	0	4
dmac202	0	2	0	0	2	0	1	0	5

Scoring of questionnaire was done via Sander et al. (2016) (3); *AHD= antihypertensive drugs; ESS_x= represents number of question within the questionnaire 'Epworth Sleepiness Scale'; ESS_total= represents total score on the questionnaire, calculated via sum of each ESS_x.*

Table S5: Fasting blood results

	Non-DM				T2DM			
	Without AHD		With AHD		Without AHD		With AHD	
	CF	placebo	CF	placebo	CF	placebo	CF	placebo
Glucose (fasting) (mg/dL)	94.2 ± 9.1	90.0 ± 8.0 ∫	98.8 ± 6.8	100.5 ± 7.4*	125.9 ± 19.0	124.6 ± 10.9	131.0 ± 25.7	132.3 ± 26.0
Insulin (mU/L)	9.0 ± 7.7	7.9 ± 5.3	11.1 ± 5.8	11.1 ± 5.2	8.0 ± 7.1	7.6 ± 5.3	12.2 ± 2.7	12.9 ± 6.1
HOMA[a]	2.2 ± 2.0	1.8 ± 1.3	2.6 ± 1.4	2.8 ± 1.3	2.6 ± 2.5	2.4 ± 1.7	3.9 ± 1.0	4.1 ± 1.6
QUICKI[b]	0.35 ± 0.03	0.36 ± 0.03	0.34 ± 0.02	0.33 ± 0.02	0.35 ± 0.05	0.35 ± 0.04	0.31 ± 0.01	0.31 ± 0.02
Uric acid (mg/dL)	4.5 ± 0.9	4.6 ± 1.0	4.8 ± 1.0	5.0 ± 1.4	4.9 ± 1.3	5.0 ± 1.2	5.3 ± 0.7	5.5 ± 0.7
Triglycerides (mg/dL)	107.3 ± 36.4	92.6 ± 24.3	111.4 ± 40.7	116.1 ± 49.0	115.4 ± 48.7	104,4 ± 36.0	171.6 ± 99.8	125.6 ± 52.1
FFA (nmol/L)	0.57 ± 0.12	0.48 ± 0.10	0.54 ± 0.19	0.59 ± 0.23	0.66 ± 0.12	0.63 ± 0.11	0.43 ± 0.11	0.42 ± 0.08*
HDL-cholesterol (mg/dL)	56.7 ± 12.6	56.1 ± 12.8	58.5 ± 22.0	59.1 ± 16.2	51.2 ± 15.7	52.5 ± 19.2	55.1 ± 13.6	56.1 ± 15.9
LDL-cholesterol (mg/dL)[c]	121.2 ± 30.1	121.9 ± 28.7	129.2 ± 39.6	125.7 ± 41.6	96.5 ± 36.8	97.6 ± 38.1	78.0 ± 17.7	83.8 ± 8.5
Total cholesterol (mg/dL)	199.4 ± 38.0	196.6 ± 36.9	210.0 ± 53.2	208.0 ± 51.7	170.7 ± 48.2	171.0 ± 50.6	167.4 ± 23.3	165.0 ± 23.4
CRP (mg/dL)	1.1 ± 0.6	2.2 ± 2.9	3.0 ± 4.1*	3.1 ± 5.5	1.6 ± 0.6	1.7 ± 0.6	2.1 ± 1.6	1.2 ± 0.5
Vitamin C (mg/dL)	0.6 ± 0.3	0.7 ± 0.4	0.7 ± 0.3	0.7 ± 0.3	0.7 ± 0.3	0.7 ± 0.3	0.4 ± 0.1	0.4 ± 0.2
Vitamin A (µg/dL)[d]	61.8 ± 9.7		70.9 ± 14.0		74.5 ± 16.6		74.2 ± 14.8	
Vitamin E (mg/dL)[d]	1.2 ± 0.2		1.3 ± 0.4		1.2 ± 0.3		1.2 ± 0.3	
Haptoglobin (g/L)[d]	0.8 ± 0.3		1.1 ± 0.4		1.2 ± 0.7		1.2 ± 0.5	

*Data: means ± standard deviation (SD) or frequencies at first visit unless specified differently; [a]calculated via fasting insulin (mU/L) x fasting glucose (mg/dL) / 405; [b]calculated via 1/(log(insulin (mU/L))+log(glucose (mg/dL))); [c]calculated via the Friedewald Formula; [d]only measured once, at first visit; AHD= antihypertensive drugs; CF= cocoa flavanols; CRP= C-reactive protein; FFA= free fatty acids; HDL= high density lipoprotein; LDL= low density lipoprotein; *= differences between subgroups (p<0.025); ∫= significant difference between both visits (p<0.025).*

Table S6: Results of additional outcome measurements

	Non-DM		T2DM	
	Without AHD	**With AHD**	**Without AHD**	**With AHD**
Accelerometry:				
Wearing time (minutes/day)	867.5 ± 21.4	879.4 ± 11.3	826.6 ± 64.8	869.5 ± 25.3
Valid days (days/week)	6.9 ± 0.3	6.8 ± 0.6	6.9 ± 0.4	6.2 ± 1.5
Step counts (n/day)	7677.7 ± 2041.1	7427.1 ± 3079.7	6593.9 ± 2239.0	6593.6 ± 2699.6
Moderate (minutes/week)	356.2 ± 178.5	292.8 ± 243.6	194.9 ± 136.0	171.5 ± 181.3
Vigorous (minutes/week)	6.7 ± 14.3	6.1 ± 14.7	8.6 ± 21.8	3.0 ± 5.4
Very vigorous (minutes/week)	0.1 ± 0.3	1.1 ± 4.4	0.0 ± 0.0	3.5 ± 7.0
MVPA (minutes/week)	363.0 ± 181.5	300.1 ± 251.9	203.4 ± 140.8	178.0 ± 184.6
Continuous Glucose Monitoring System:				
Glycemic excursions:				
% time in range (70-180 mg/dL)	96.3 ± 4.6	97.6 ± 2.6	89.7 ± 10.6	70.5 ± 12.8
% time in hypoglycemic range				
<70 mg/dL	1.4 ± 2.2	1.0 ± 1.5	0.0 ± 0.0	2.3 ± 4.1
% time in hyperglycemic range				
>180 mg/dL	2.3 ± 4.1	1.4 ± 2.2	10.3 ± 10.6	27.2 ± 16.2
>250 mg/dL	0.2 ± 0.6	0.1 ± 0.2	0.9 ± 1.2	2.5 ± 2.6
Area under curve (mg/dL/minute)				
Below 70mg/dL	0.1 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.6
Above 180 mg/dL	0.7 ± 1.2	0.3 ± 0.6	3.0 ± 3.4	8.4 ± 5.5
Above 250 mg/dL	0.1 ± 0.3	0.0 ± 0.1	0.2 ± 0.4	1.2 ± 1.1
Glycemic variability:				
Standard deviation (mg/dL)	20.5 ± 10.0	19.3 ± 7.3	29.4 ± 8.9	40.3 ± 8.7
Coefficient of variation (%)[a]	18.1 ± 8.1	16.9 ± 6.1	21.1 ± 5.4	27.0 ± 9.8
MAGE (mg/dL)	46.8 ± 23.9	44.0 ± 20.1	71.8 ± 22.3	97.0 ± 19.6
Low blood glucose index	0.7 ± 0.6	0.5 ± 0.4	0.1 ± 0.1	0.6 ± 1.0
High blood glucose index	0.7 ± 0.9	0.6 ± 0.5	2.7 ± 1.6	5.4 ± 2.8
Data sufficiency (%)	100.0 ± 0.0	99.8 ± 0.5	99.9 ± 0.1	99.8 ± 0.2
Days CGM worn (n)	6.0 ± 0.0	5.8 ± 0.6	6.0 ± 0.0	6.0 ± 0.0

Data: means ± standard deviation (minimum – maximum); [a]calculated by mean blood glucose divided by standard deviation; AHD= antihypertensive drugs; MAGE= mean amplitude of glycemic excursions; MVPA= moderate to vigorous physical activity. No significant differences between subgroups of T2DM subjects, neither between subgroups of non-diabetic subjects.

References

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Part 3: General discussion

1. Summary of main findings

This doctoral project aimed to provide an overview of the current State of The Art regarding potential vascular health benefits of CF in patients with T2DM. In addition, we described the results of an acute, randomized, double-blinded, placebo-controlled cross-over trial, performed to increase the insight in this topic in this specific population.

Our published systematic review and meta-analysis illustrated that previous research into the effects of CF in patients with DM are quite heterogeneous concerning the intervention (administered dose, daily frequency, and the nature of both intervention and placebo formulae), the population characteristics [sex, BMI, age, the stage of disease (concerning DM, hypertension or other cardiovascular conditions), use of antihypertensive/ antidiabetic medication, type of DM, and geographical location], and the examined outcomes (both method of measurement as measurement as such). As little research has been performed so far on the vascular effects of CF in this population, we could only perform a meta-analysis on BP analyses.
We concluded that, although the theoretical background of mechanisms of actions of CF rise promising assumptions towards CF-induced vascular health properties in DM, the clinical evidence is rather weak. Taking into account high heterogeneity across included publications, at best, a reduction of 1 – 2 mmHg of DBP, but not SBP, would be induced after mid/long-term CF ingestion. Based on subgroup analyses, these effects seem stronger in female, younger, and hypertensive adults, when CF is ingested in 1 daily batch, and when EC content is high enough, i.e. at least 90 mg.

As the weak evidence is based on limited research with high heterogeneity, which is a major limitation, more homogenous research is required before conclusions or health recommendations can be formulated.
As a first step towards robust testing of the possible vascular effects of CF in patients with T2DM, we setup an acute, randomized, double-blinded, placebo-controlled cross-over study with a standardized, clear described trial protocol, intervention, and population.

Based on this study, no beneficial CF-induced peripheral vascular health effects were detected after single CF ingestion, neither in patients with T2DM, nor in non-diabetic volunteers. However, independent of the type of capsules ingested (CF-enriched or placebo), DBP decreased over time (after breakfast and capsules ingestion) in the non-diabetic group compared to the T2DM group. Furthermore, the assessed differences in brachial arterial diameter over time (after breakfast and capsules ingestion, independent of type) tended to differ between both groups, with a significant increase in the non-diabetic group.

Because the differences over time (starting from baseline until after breakfast and capsules ingestion; equals a 3 – 4 hours period) were independent of the type of capsules, one might assume that this was caused by the breakfast consumed. However, because of several limitations like the small sample size, and hence altered statistical analyses due to COVID-pandemic induced restrictions, caution is needed when interpreting these results.

2. Discussion of results

The first aim of this doctoral research was to provide evidence of vascular CF effects in patients with DM. The meta-analyses showed only a small reduction of DBP after mid/long-term CF ingestion in populations with an increased cardiovascular risk, including T2DM. Hence, no strong conclusions concerning mid/long-term CF intake in patients with DM could be formulated. The meta-analysis, however, revealed considerable heterogeneity between the papers studying CF-induced vascular benefits in DM. We therefore setup a robust, standardized trial protocol to gain insight in potential vascular CF effects in T2DM patients as our second aim. In this trial protocol capsules containing a pure cocoa extract would be ingested to avoid influence of additional substances in cocoa powder and chocolate, like sugar and fat. Also, other confounding variables like age, sex, BP at baseline, and ingested EC dose were taken into account as suggested by our and other meta-analyses. The execution of this trial protocol and analyses of the obtained results to investigate acute effects of single CF ingestion on peripheral vascular reactivity in patients with T2DM was our third aim. In this study, no CF-induced micro- or macrovascular health benefits could be detected in non-diabetic or T2DM subjects. Hence, based on this doctoral research, we could not formulate conclusions concerning potential vascular health benefits of CF in T2DM patients.

However, based on the vascular health properties of flavonoids and previous research indicating vascular health benefits of CF, we believe that cocoa are promising nutraceuticals. Nevertheless, we doubt that solely CF are responsible for inducing vascular health benefits as cocoa products also contain other vasoactive compounds that need to be considered. The following discussion will explain why.

First, all different compounds of cocoa and chocolate are presented to underline the variety of substances and to highlight that cocoa contains also other vasoactive substances than CF (section **2.1**). Second, reported evidence of vascular health effects of cocoa products based on previous publications are debated in order to further explain why we think that demonstrated effects might not be solely caused by CF. We discuss the described causal link between ingestion of cocoa products and vascular health based on observational studies (section **2.2.1**) and we analyze administered interventions and study designs/type of interventional studies (section **2.2.2**). Third, the critical considerations on previous reports are transferred to our setup trial protocol and other methodological considerations are formulated to better explain the lack of effect in our study (section **2.3**).

2.1. Cocoa compounds

Raw cocoa beans consist for 50 – 57 % of cocoa butter: palmitic acid (C16:0), oleic acid (C18:1), linolenic acid (C18:3), and particularly stearic acid (C18:0). The other, non-fat part consists of around 26 % fibers, 20 % protein (globulins, prolamin, glutelin, and particularly albumin), 16 % starch, 5 % ash, and 33 % other compounds like polyphenols, methylxanthines (theobromine, caffeine, and theophylline), biogenic amines (e.g. serotonine), minerals (magnesium, iron, copper, zinc, natrium, calcium, phosphorus, sodium, selenium and potassium), and vitamins (E, B6, B12, D, E, K) [1-4]. In raw dried cocoa beans, 12 - 18 % are

polyphenols: 29 – 38 % catechins (mainly EC), 58 – 38 % pro(antho)cyanidins, 4 % anthocyanidins, and the remaining % are flavones, polyphenolic acids, stilbenes, and caffeoyl-conjugates [5, 6]. To form chocolate, cocoa solids, cocoa butter, sugar, and lecithin (emulsifier) are the main ingredients. Dark, milk or white chocolate vary upon their amount of cocoa nibs, cocoa butter, and milk fat [7, 8].

The percentage of cocoa mainly indicates the amount of each nutrient in chocolate. The more cocoa solids, the less carbohydrates (sugars), but the higher the fat content in total [7]. Although cocoa and dark chocolate have more saturated than non-saturated fat, the greater part of saturated fat is stearic acid (C18:0), which is neutral without cholesterolemic or atherogenic properties and their effects on lipids might be comparable with unsaturated fatty acids that exert cardiovascular protection [1, 7, 9-11]. Furthermore, the more cocoa beans in chocolate, the more minerals are retained, which are important for vascular functionality [12-16]. Calcium, magnesium, phosphorus, and potassium have the highest levels in varied types of cocoa products [8].

Hence, cocoa products have a complex composition and all its compounds need to be looked at for explaining vascular health properties [17]. Several factors like bean maturity, plant genotype, geographical area with its climatic conditions, and manufacturing processes impact the polyphenols profile, but also the content of nutrients in cocoa products [5, 8, 18]. Most studies investigating the health effects of cocoa products only focus on cocoa polyphenols and neglect or underestimate the possible impact of other vasoactive substances [14, 19, 20]. It is possible that the presence of certain nutrients provoke synergistic effects with CF and so entail vascular protection. It is already suggested that the intake of theobromine together with EC exert greater effects [21, 22]. However, lack of representation of entire composition of interventions in randomized controlled trials impedes precise comparison of interventions between publications [23].

2.2. Evidence based on previous publications

Increasing research indicate vascular health benefits of cocoa products and link these health properties to the presence of cocoa flavonoids, especially CF and its most abundant monomeric form EC [24-26]. Nevertheless, regarding the complex composition of cocoa products, the presence of different vasoactive compounds, and the sometimes inconsistent results of examinations on cocoa products-induced vascular health, it is questionable whether solely CF are responsible for the exerted vascular improvements. Observational studies and randomized controlled trials reporting vascular health effects through cocoa products ingestion are discussed.

2.2.1. Observational studies

Observational studies identify an association between cocoa ingestion and BP-lowering and cardiovascular protection [27-33]. Nevertheless, for all observational studies, certain confounders have to be taken into account, such as the distinction between types of chocolate (e.g. white or milk and dark chocolate with its minimal % cocoa solids depending on country-regulations [34] and the polyphenols profile of the cocoa beans depending on several factors like bean genotype and manufacturing processes), consumption of other foods with

antioxidant and other vascular health properties (e.g. fruits and vegetables), the accuracy of reported amounts of cocoa products consumption by participants, and level of physical activity and daily caloric food intake [17]. Hence, the formulation of an unambiguous causal link between cocoa flavonoids, more specifically CF, intake and vascular health based on observational studies is difficult and might be inadequate.

2.2.2. Randomized controlled trials

Also randomized controlled trials and meta-analyses illustrated vascular health properties of CF in varied populations (see Table 2 in introduction) [35-38]. Interventional studies have the advantage that they describe CF doses instead of self-reported amounts of cocoa products with unknown composition. However, it would be more informative if the entire composition instead of solely CF and/or EC doses are listed to gain insight in complementary/ synergistic/ antagonistic vascular effects of other vasoactive cocoa compounds with CF. To underline the required attention for entire composition of the intervention and additional food intake, we discuss the effects on vascular systems of individual cocoa compounds as well as their interactions, followed by the influence of a control intervention and combined food intake. Besides, we emphasize the important distinction between in vitro-, animal-, and human studies.

Pure epicatechin ingestion

Epicatechin, especially the stereochemical (-)-epicatechin (EC), is regarded to be, at least in part, responsible for the cardiovascular health effects of flavanol-containing foods [24-26]. Animal studies showed that EC would increase NO bioavailability through antioxidant properties and upregulation of eNOS. Furthermore, a decrease in plasma endothelin-1 concentrations and a lower expression of proinflammatory and proatherogenic markers was demonstrated [39]. However, only limited research (1 dose vs. 4 week-period) have examined the vascular effects of pure EC administration in humans with inconsistent results [39-45]. Because of limited data with rather small sample sizes and quite large heterogeneity in studied population, caution is required for interpretation of these publications [39].

It is suggested that EC exerts greater effects through the pharmacokinetic and pharmacological interactions with other cocoa flavonoids and compounds [39]. Therefore, possibly higher doses of pure EC are needed for inducing a beneficial vascular effect and possibly vascular effects are attenuated without the interactions with other vasoactive cocoa compounds. Also, Dower et al. (2015) illustrated a plateau effect for endothelial function after 4 weeks of pure EC ingestion so that the duration of trials should be considered when formulating conclusions [41]. Further studies are required to gain insight into the pure and combined effects of EC in cocoa products.

Methylxanthines

A certain part of cocoa products comprises methylxanthines, predominantly theobromine and to a lesser extent caffeine (~10-fold lower [46]). Methylxanthines provoke several vascular effects such as antagonism of adenosine receptors, mobilization of calcium, modulation of γ-aminobutyric acid-A receptor actions, and inhibition of cyclic nucleotide phosphodiesterases leading to higher cyclic AMP and cyclic GMP levels, hence inducing vasodilation [47]. Compared to caffeine, theobromine is a rather weak adenosine receptor antagonist (2 - 3 fold lower affinity to A1 and A2 receptors) and provoke limited central stimulations [4, 48-52]. In

the raw cocoa bean, around 2.4 - 4 % is theobromine and 0.2 - 1 % is caffeine [5, 6, 47]. In chocolate liquor, in commercial cocoas, and commercial sweet chocolates, contents are lower comprising around 0.46 - 1.22 % for theobromine and 0.07 - 0.21 % for caffeine [53]. Hence, albeit debated, concentrations of methylxanthines are dependent on manufacturing processes and type of cocoa product [4, 21, 46, 54]. Although rather small amounts of theobromine and caffeine are present in cocoa products, the order of bioavailability in human plasma has shown to be theobromine > caffeine > EC > C > procyanidins [55], so that possible influence of methylxanthines-induced effects may not be ignored.

It has been shown that theobromine exerts vascular health effects independent of flavanols, providing sufficiently high doses. A single ingestion of 700 mg pure theobromine decreased BP in healthy female subjects aged 51 ± 12.7 years [56]. Conversely, another study indicated no effect of a single theobromine ingestion (250 mg, 500 mg, and 1000 mg) on BP in younger healthy adults (23.3 ± 3.5 years old); however, a dose-dependent increase of heart rate was shown [57]. In healthy men and women between 40 and 70 years old, 850 mg pure theobromine for 4 weeks increased HDL cholesterol [58]. They suggested that theobromine plays the key role for increasing HDL-cholesterol in cocoa. However, another study in apparently healthy men and women (60 ± 6 years old) showed no effect on HDL cholesterol with 500 mg theobromine/ day for 4 weeks [59]. As this latter study provided lower doses, one could suggest a dose-response effect of theobromine for inducing improvements in lipid-profile.

Similarly, caffeine exerts vascular effects. One hour after single administration of 300 mg caffeine in young healthy men an increase in SBP (6.0 ± 6.0 mmHg) and DBP (2.6 ± 3.1 mm Hg), but no effect on heart rate or baseline blood flow in the forearm was detected. However, this dose increased the blood flow response to acetylcholine in the forearm, suggestively via increased NO production [60]. As explained, caffeine is an antagonist of adenosine receptors which induce vasodilation. Hence, the vasoconstricting properties of caffeine may explain the increase in BP. Furthermore, a previous study in isolated rat aorta showed that caffeine would increase endothelial NO synthesis via inhibition of cyclic guanosine monophosphate degradation and via endoplasmic reticulum released Ca^{2+} through stimulation of the ryanodine-sensitive Ca^{2+} channel [61]. A balance of vasodilatory and vasoconstrictive properties of caffeine may regulate vascular function [60]. A meta-analysis reported that 295 – 750 mg pure caffeine ingestion for 7 - 84 days increased SBP (4.16 mm Hg; 95% CI; 2.13; 6.20) and DBP (1.22 mmHg; 95% CI: 0.52; 1.92) without an effect on heart rate in healthy or hypertensive subjects. Moreover, the higher the caffeine dose (≥ 450 mg/day), the higher the increase in SBP [62]. Until now, effects of caffeine and caffeine containing products on human health have been extensively researched [52]. Note that, as reported previously, shown detrimental or protective effects of caffeine containing products, like coffee, have to be interpreted with caution as also other compounds of these products may exert these effects rather than caffeine alone [63]. The European Food Safety Authority claimed that in the general population, a single dose of 200 mg caffeine (equals around 3 mg/kg body weight for a 70-kg adult) or varied doses from varied sources up to 400 mg caffeine/ day (equals around 5.7 mg/kg body weight for a 70-kg adult) may be consumed without adverse cardiovascular health effects [64].

Although theobromine and caffeine are both methylxanthines, their vascular effects differ. Martinez-Pinilla et al. (2015) suggested that their different half-life could explain, at least in part, this discrepancy in effect. They also indicated that the combination of theobromine and caffeine in cocoa may exert methylxanthines-induced benefits without reported side effects of caffeine [65]. However, to our knowledge, the combined effects of these methylxanthines are not yet thoroughly investigated, but could gain insight in the mechanisms of action of cocoa. Flavanol enriched cocoa with increased theobromine content (340 mg flavanols, 24 mg EC, 979 mg theobromine, 10.2 mg caffeine) for 3 weeks increased 24-hours ambulatory SBP and heart rate, decreased central SBP, decreased augmentation index, and augmented pulse wave velocity in healthy adults (age 62 ± 4.5 years), whereas flavanol enriched cocoa with normal theobromine content (305 mg flavanols, 25 mg EC, 106 mg theobromine, 10.4 mg caffeine) only increased pulse wave velocity [66]. Since caffeine concentrations were similar in both flavanol enriched cocoa test drinks with varied theobromine content, the induced effects, besides increased pulse wave velocity, could have been provoked by theobromine. Furthermore, as already described, it has been shown that 850 mg pure theobromine induced an increase in HDL cholesterol. Interestingly, in contrast, the cocoa intervention with lower theobromine doses (325 mg flavanols and 150 mg theobromine) and the cocoa intervention with higher theobromine doses (325 mg flavanols, 1000 mg theobromine) did not provoke an increase in HDL cholesterol [58]. It is a strong limitation that the amounts of caffeine in the different interventions were not reported. Until now, theobromine has received substantially few attention in comparison with cocoa polyphenols and caffeine so that further research is required for gaining insight into the beneficial or detrimental effects of theobromine and the required doses. Also, interactions between cocoa compounds are assumed because, like indicated, the combined intake of EC and theobromine would exert greater effects. Hence, also for theobromine and caffeine, required doses could differ between pure and combined ingestion. Note that cocoa contains the highest concentrations of theobromine of all foods [67].

Placebo/ control intervention

The lack or presence of an effect of cocoa products might also be explained by chosen placebo/ control intervention, which can comprise varied supplementations. When participants in the control group consume nothing [68] or white chocolate [69-72], blinding is rather impossible. Blinding of participants and researchers decrease possible bias [73] and so increase strength and credibility of the study. In addition, as white chocolate does not contain a cocoa extract, vasoactive compounds like polyphenols but also theobromine and caffeine are absent. Hence, as reported by others, it is questionable whether the demonstrated effects of dark chocolate compared to white chocolate are solely caused by flavanols [71]. Also, white chocolate comprises more sugar and sweeteners than dark/ milk chocolate [74], so that we, together with other researchers [74], do not consider this as an appropriate control.

An often used placebo/ control intervention is chocolate/ cocoa powder with low doses of CF. Depending on manufacturing processes, cocoa can contain more or less flavonoids, but, as already explained, also more or less nutrients. Studies evaluating high versus low doses of CF should describe the entire composition of the interventions for transparency.

Interventions were sometimes only matched for fat, carbohydrates, and proteins, but not for methylxanthines contents [75], or all nutrients apart from methylxanthines contents were described [76, 77]. This again raises the question whether the vascular health properties of

cocoa were solely induced by flavanols [78]. In our published meta-analysis, we examined the possible influence of equilibration of intervention formulae with methylxanthines but our subgroup analysis did not indicate a statistical effect [79]. However, due to paucity of included papers, high heterogeneity between analyzed publications, and because predominantly examined in diabetic subjects, influence cannot be excluded based on these subgroup analyses. Also, we only looked at equilibration with methylxanthines, whereas also other vasoactive compounds were present. More research using intervention formulae with equilibrated vasoactive compounds are needed to gain insight. Our executed randomized controlled trial is already a good example.

Desideri et al. (2012) analyzed the effect of a cocoa drink with 3 different flavanol doses [993 mg (high), 520 mg (intermediate), and 48 mg (low)] for 8 weeks. They reported total composition of interventions and explained that the high and intermediate doses were made with a flavanol-rich cocoa powder, whereas the low dose was made with a highly processed, alkalized cocoa powder. Both the high and intermediate doses induced a decrease in SBP and DBP with the greatest reduction after high dose ingestion [80]. Since only flavanol doses varied between interventions, one could assume a flavanol-induced effect. However, when considering the manufacturing processes, it is unclear how the 3 interventions were closely matched on non-flavanol compounds. Did they use the cocoa powder (flavanol-rich or highly processed and alkalized) as basis and added non-flavonoid nutrients afterwards in order to match formulae? Even though this seems the most appropriate procedure, it is questionable whether this manufactured composition might be transferrable to daily dietary intake. When a lot of non-flavonoid nutrients have to be added, one could question whether ingestion of cocoa products are the best way for attaining these levels, considering its fat and sugar content, especially in certain populations like obese and DM patients.
Similarly, Balzer et al. (2008) tested the acute effects of single ingestion of 3 different doses of CF (963 mg, 371 mg, and 75 mg CF), closely matched on non-flavanol compounds, and detected a dose-dependent improvement of endothelial function in patients with T2DM. Next, he also executed a sub chronic trial where T2DM subjects ingested 2 different doses of CF (321 mg 3 times daily and 25 mg 3 times daily), closely matched on non-flavanol compound, for 30 days and only found an improvement in FMD through the high dose [81]. Unfortunately, Balzer et al (2008) did not report the manufacturing processes. We tried to contact both corresponding authors to gain insight in their used interventions. Only one corresponding author [80] replied but was unaware of the procedure and redirected us to Mars Inc, however, without success.

It is not our purpose to fight the assumed beneficial effects of CF. In contrast, we are convinced that CF induce vascular improvements. However, we want to increase attention for other vasoactive compounds in cocoa because, depending on the amount, these non-flavanol compounds might have synergistic/ complementary/ antagonistic vascular effects with CF. Due to considerable variability in interventions between publications, comparison is complicated. We underline that more homogeneous research investigating individual and combined effects of different vasoactive compounds of cocoa are required.

Combined food intake
Not only the intervention as such, but also the products that are ingested together with cocoa have to be considered because they might counter, strengthen or mask the CF-provoked

vascular benefits. It has been shown that carbohydrates may increase the extent and rate of flavanol absorption [82, 83]. In agreement, Schramm et al. (2003) investigated the effect of lipids, proteins, and carbohydrates on CF metabolism in healthy subjects and detected solely an influence of carbohydrates causing an increased CF uptake [82].

However, it is also reported that the sugar content should be taken into account in research as this may blunt the vascular health effects of CF. Faridi et al. (2008) examined the acute effects of a single ingestion of sugar-free cocoa (805 mg flavanols, 48 mg EC, 436 mg theobromine, 28.1 mg caffeine, sweetened with vanillin, acesulfame-potassium, and aspartame), a sugared cocoa (805 mg flavanols, 48 mg EC, 436 mg theobromine, 28.1 mg caffeine, 90.6 g sugar), or a placebo with neglectable amounts of cocoa (9 mg flavanols, without EC, theobromine or caffeine, no information on sugar content) in overweight adults. They found a significant cocoa-induced improvement in FMD with a greater improvement in the sugar-free cocoa compared to the sugared cocoa (5.7 ± 2.6 % and 2.0 ± 1.8 % respectively). For BP, only a reduction was detected in the sugar-free cocoa compared to the cocoa-free placebo (systolic BP: -2.1 ± 7.0 mmHg compared with 3.2 ± 5.6 mmHg; diastolic BP: -1.2 ± 8.7 mmHg compared with 2.8 ± 5.6 mmHg) [84]. The same interventions were tested chronically (6 weeks) by Njike et al. (2011), who also showed a CF-evoked increase in FMD in overweight adults and presented a greater, however not significantly different, increase in the sugar-free cocoa compared to a sugar-sweetened cocoa (2.4 %; 95% CI: 1.5; 3,2 and 1.5 %; 95% CI: 0.6; 2.4 respectively). No effect on BP was detected [85]. A Cochrane review examining the sub chronic effects of CF ingestion (at least 2 weeks) on BP indicated that publications using more than 10 g of sugar showed smaller CF-induced BP reduction in healthy participants, some with hypertension (systolic BP: -2.52 mmHg; 95% CI: -4.74; -0.31 compared to -1.12 mmHg; 95% CI: -7.08; 4.85; diastolic BP: -2.34 mmHg; 95% CI -4.19; -0.50 compared to -1.32 mmHg; 95% CI: -4.70; 2.06) [86]. It is possible that the sugar and/ or fat contents in cocoa products counteract the mechanisms of CF, however, it is also plausible that the human body is more responsive to the sugar and/or fat intake than to CF. Moreover, one should consider the studied population. The daily intake of extra amounts of fat and sugar in obese and DM patients for example requires caution.

Furthermore, although conflicting data, the consumption of milk together with CF (whole milk or processed in chocolate) could decrease total antioxidant capacity and could limit CF absorption. It is hypothesized that milk proteins might bind with cocoa polyphenols and in turn limit uptake in the gastrointestinal tract [87-89]. Further studies investigating the possible confounding of milk on CF effects should consider fat content in milk and measurement methods of antioxidant capacity [87, 90].

Also fiber content should be considered. It has been reported in the literature that an important function of dietary fibers is to transport polyphenols through the gastrointestinal tract [91]. Around 50 % of total polyphenolics transverse the small intestine linked to dietary fiber. So the more dietary fibers ingested, the better the flavanols are transported through the gastrointestinal tract, which might influence the amount of CF absorbed in the small intestine and hence the amount of metabolite formation.

Results of CF ingestion together with or short after a meal should be interpreted with caution. Glucose and fat intake can impair endothelial function and so inhibit CF-induced FMD and BP

improvements [22, 92]. Furthermore, glucose and fat are main energy sources that are immediately used or stored evoking both short- and long-term effects that should be considered when analyzing acute and chronic effects of cocoa products ingestion [22]. In young healthy volunteers, flavanol-rich dark chocolate ingestion (447 mg EC) for 3 days reduced wave reflections, increased FMD, and prevented the increase in endothelin-1 and 8-iso-PGF2a levels after an oral glucose tolerance test (hyperglycemic state) [72]. In T2DM patients, without other cardiovascular risk factors than T2DM, single cocoa ingestion (960 mg polyphenols, 480 mg CF, 40 mg EC, 220 mg theobromine, and 21 mg caffeine) with a high-fat fast-food breakfast lowered large artery elasticity, mitigated postprandial dyslipidemia and inflammation, but had no impact on BP or small artery elasticity [93, 94]. In T2DM patients, with presence of other cardiovascular risk factors like hypertension, hyperlipidemia, coronary artery disease and/or vascular damage, Rynarzewski et al. (2019) did not find a CF-induced effect on postprandial BP or postprandial glucose and lipid metabolism through administration of CF-enriched capsules (40.4 mg EC, 52.5 mg theobromine, and 5.0 mg caffeine) and a diabetic-suitable breakfast [95].

In comparison to our executed trial in which participants ingested the intervention (CF-enriched or placebo capsules) 45 – 60 minutes after start of the breakfast for practical reasons, an influence of this breakfast is plausible as all the breakfast formulae contained at least 1 milk source, i.e milk, cheese, and/ or yoghurt and more than 10 g sugar (will be further discussed in section **2.3,** Critical methodological considerations on our setup trial protocol).

Relevance of in vivo studies

The setup of a randomized controlled trial taking into account all these points of consideration are a challenge. However, research in vivo is necessary as the metabolism of flavanols is quite complex and partly unknown. After CF consumption, around 22 – 55 % of monomeric CF (EC and C) are metabolized via the gastrointestinal tract and are transformed into varied EC metabolites through glucuronidation, sulfation, and methylation [6, 96-98]. In contrast to in vitro studies, in vivo research showed loss of $O_2^{\bullet-}$-scavenging properties of EC upon methylation [99]. Furthermore, the other part of monomeric CF and poly- and oligomers (90 – 95 % of total polyphenols [100, 101]) are broken down by the gut microbiota into biologically active metabolites, like valerolactones, valeric acids, and acetic acids, with in vitro suggested potential further health effects [102-105]. Research in humans is limited and showed conflicting results [106]. It is also demonstrated that cocoa polyphenols have a bidirectional interaction with the gut microbiota with various health outcomes as recently reviewed elsewhere [100]. More in vivo examinations and research into the bioactivity of the varied intestinal and colonic metabolites of CF is required to gain insight in the vasoactive mechanisms of cocoa.

Animal versus human

Not only in vitro, but also in vivo animal studies have to be interpreted with prudence. It is obvious that in smaller animals, lower cocoa doses are required for inducing an effect compared to humans; however, it is questionable whether a multiplication of a dose would exert similar effects. Furthermore, one should consider toxicity in animals. The European Food Safety Authority stated that certain theobromine levels are harmful and even toxic for animals in contrast to humans [51]. Also, the tolerable caffeine content in small animals like rats is much lower compared to humans [64].

We believe that cocoa has great potential as a nutraceutical with promising vascular health benefits; however, we want to underline its complexity. Homogeneous, standardized human research reporting the entire composition of intervention formulae as such, but also consumed food or drinks together with the interventions are required.

2.3. Critical methodological considerations on our setup trial protocol

Based on the critical considerations on observational studies and randomized controlled trials, we think that our setup trial in which participants ingested a single pure cocoa extract is a good example and a first step for gaining insight in the vascular properties of cocoa. Although we did not find an acute vascular effect of this cocoa extract in T2DM or non-diabetic participants, we do not exclude vascular health benefits of cocoa. Some points that have to be considered and may (partly) explain lack of effect of our robust, standardized study are described.

2.3.1. Intervention formulae

As indicated, isolated and combined research of vasoactive compounds of cocoa are needed. In our study, we tested the effect of 790 mg CF and 150 mg EC on the vascular reactivity. Both types of interventional capsules were equilibrated with the vasoactive compounds caffeine and theobromine (Table 1).

Table 3: nutrient content of the capsules

	8 CF-enriched capsules	6 capsules with placebo
Total cocoa extract (g)	2.5	0
Total flavanols (mg)	794	0
Epicatechin (mg)	149	0
Catechin (mg)	30	0
Caffeine (mg)	23	24
Theobromine (mg)	179	180
Maltodextrin (mg)	928	1 956

Amount is presented for total amount taken and not for each capsule. CF: Cocoa flavanols

The CF dose in our trial was based on the study of Balzer et al. (2008) [81] who reported an effect of acute and chronic CF ingestion on FMD in patients with T2DM. However, their interventions contained 570 – 580 mg theobromine, which is around 400 mg more compared to our supplementation. As it is suggested that a dose-response relationship for theobromine exist and since a study indicated that 150 mg theobromine is too less for inducing an effect on lipid profile, it is possible that our administered theobromine dose was too low. We could have increased our levels of theobromine, however it is reported that high doses of theobromine (≥ 500 mg daily) increase gastrointestinal complaints [21].

Not only the content of theobromine, but also the content of other vasoactive substances differed when comparing the entire composition of the intervention of Balzer et al (2008) [81]

with our supplementations. Unfortunately, because of force majeure, we do not know yet the entire composition of our capsules, for example the amounts of other flavonoids and magnesium.

Furthermore, we used capsules as intervention, whereas the subjects of Balzer et al. (2008) [81] ingested the different CF doses via 18 g cocoa powder mixed with 250 mL of distilled water. As this cocoa drink comprised 54 % (w/w) non-fat milk powder and additionally around 15 g sugars, 27 g carbohydrates, 15 g proteins, and 3 - 4.5 g total fat, it is questionable whether the effects (and thus dose) reported in Balzer et al. (2008) could be extrapolated to ingestion of a pure cocoa-extract through capsules. However, although our subjects ingested capsules containing a pure cocoa extract, 45 – 60 minutes before intake, participants administered a breakfast comprising 50 – 61 g carbohydrates, 19 – 23 g proteins, and 9 – 20 g fat. In addition, all 4 breakfast formulae contained at least 1 milk source, i.e. milk, yoghurt, or cheese and besides 1 formula providing 10.2 g mono- and disaccharides and 37.2 g polysaccharides, the other 3 formulae provided 1.7 g mono- and disaccharides and 36.9 g polysaccharides. As explained above (section **2.2**, *combined food intake*), this administered breakfast prior capsules ingestion could have influenced potential CF- and in general cocoa-induced vascular effects.

Besides, there are several differences concerning the uptake and metabolism of cocoa compounds via chocolate bars or drinks (as used in most studies) compared to capsules containing a cocoa extract for isolated testing (as used in our study). Peak plasma EC levels arise around 2 - 3 hours post capsules or chocolate ingestion [96, 97, 107, 108]. Conversely, theobromine reaches its plasma peak values 3 hours post capsules ingestion and 2 hours post chocolate ingestion with the latter reaching higher plasma concentrations. Caffeine concentrations in plasma peaks after 0.5 hours post capsules ingestion, but is delayed to 1.5 – 2 hours post chocolate ingestion, with the latter reaching lower maximal plasma levels [109]. Hence, since we used capsules in our trial, plasma caffeine levels peaked presumably already 30 minutes post capsules intake, which was before vascular measurements (BP, FMD, handgrip strength test) post-intake were resumed (70 minutes rest period post capsules ingestion, see protocol-paper [110]). However, dependent on the half-life of caffeine, which is generally between 2.5 – 4.5 hours in adults [63, 111], certain amounts could have been present when vascular assessments post intake were executed. Nevertheless, our intervention capsules contained only 23 mg caffeine (24 mg in placebo), which is a quite low dose [56, 60, 62]. Furthermore, plasma theobromine levels peaked presumably around 3 hours post capsules ingestion; however, the FMD test (primary outcome) was already performed 2 hours after capsules ingestion and the protocol trial was terminated 3 hours post capsules ingestion (see protocol-paper [110]).
Here we did not take into account the plausible influence of the breakfast formulas (in our study, the capsules were ingested around 1 hour after start of the breakfast) or plausible influence of altered absorption due to specific disorders like T2DM patients with autonomic neuropathy in varies parts of the gastrointestinal tract (in our study, strict exclusion criteria, including diabetic vascular complications, were formulated).
Nevertheless, in publications using chocolate as intervention, peak plasma concentrations of caffeine, theobromine, and EC would all arise around 2 hours post intake. Consider that bioavailability of theobromine and caffeine are higher compared to EC [55].

Lastly, our two types of interventions differed in amount of maltodextrin, added as a diluent. Maltodextrin is a hydrolysis product of starches, a low-nutritive carbohydrate, a saccharide polymer comprising D-glucose compounds with an energy value of 4 kcal/g (equals 16 kJ/g) [112, 113]. Maltodextrin is considered a fat- and calorie-decreasing sweetener (dextrose equivalency of < 20) and is often used in food products and pharmaceutical industry via tablets or powder applications [113, 114]. To our knowledge, no other studies investigating the effects of CF or theobromine and caffeine have used maltodextrin in placebo/ control formula..

Independent of cocoa, tablets with maltodextrin (450 mg) were used as a placebo to investigate the effect of 450 mg green tea (containing EC, 240 mg C) or 450 mg sour tea (containing flavonoids, 250 mg anthocyanin) for 6 weeks on BP and lipid profile in healthy adult men. SBP was significantly decreased after sour tea ingestion compared to maltodextrin; however, no other differences between these 3 supplementations were detected [116]. In comparison, our added maltodextrin levels are 2 – 4 times higher. Unfortunately, to our knowledge, no specifications of interventions other than reported here were described so that further interpretation and comparison with our intervention is impeded.

Carbohydrate administration increase glycemia and so insulin production with vasodilating properties [117]. Hence, one could assume vasodilation following maltodextrin consumption. Even though our placebo formula contain almost a double content of maltodextrin compared with the flavanol enriched capsules, the added doses are rather low (< 2 g). So far, limited research has been executed to analyze possible influence of maltodextrin on vascular systems. Despite the increasing burden to participate (volunteers would have to come to the lab 3 times), a third interventional formula only containing maltodextrin (equal amount as used in the placebo formula) could have gained insight in vascular properties of these small amounts of maltodextrin.

2.3.2. Measurement methods

We chose an FMD test to examine the effects of CF on endothelial function. Here we measured non-invasively the diameter of the arteria brachialis at baseline and post occlusion. The rationale is that an increase in luminal blood flow and internal wall shear stress will result in higher endothelial NO production, hence vasodilation [118, 119]. As it is acknowledged that CF increase bioavailability and -activity of NO [39], FMD seemed a proper measurement method for assessing CF-induced endothelial effects. Note that this is a challenging technique and is highly operator dependent [118, 119]. A training by a specialist in this technique was provided to ensure technical validation and good reproducibility. Also a software with automated tracking (together with optical control) was used for data analyses, as recommended [118]. However, the intra- and inter-session coefficient of variation of the relative peak change in diameter (% FMD) was 23 % and 32 % respectively, which is comparable with coefficients of variation described in other research [120]. Hence, a limiting factor of FMD tests is the rather broad noise and should be considered.

Furthermore, it is recommended to perform an FMD test in fasting state. We were aware of this recommendation; however, since our trial protocol lasted for around 5 hours after an

overnight fast, this was unfeasible. We decided to provide a breakfast immediately before the FMD test. For standardization, subjects were asked to consume the breakfast within 15 minutes with thereafter 15 minutes of rest, followed by an FMD test. In practice it took another 10 minutes to prepare the participant, to find the arteria brachialis, and to record the baseline diameter for several minutes before the actual FMD test could be performed. Hence, the FMD test was executed when glucose levels were rising and almost maximal (postprandial glucose peak arises around 60 minutes after the start of a meal [121]). Hyperglycemia provokes an endothelial dysfunction [92], so that one could assume that an improvement in FMD after capsules ingestion (equals around 2 hours and 45 minutes after start of breakfast) is due to the restoring of glycemia instead of capsules activity. In our results we found a tendency for the time x group interaction for difference in brachial arterial diameter (see results of original paper; *submitted*), which could be explained by a difference in response to the breakfast. In general, compared to healthy individuals, T2DM patients show a delayed insulin peak so that postprandial glucose excursions are inadequately controlled [121]. In our study, a few T2DM patients, with and without use of AHD, had a glycemia of around 200 mg/dL before an FMD test. Considering our small sample size, this delayed postprandial rise in glucose should certainly be considered as this could have impacted our results. Note that capillary glucose was assessed prior each FMD test in T2DM subjects, but that the positioning of the participant and the monitoring of the baseline brachial artery diameter required another 5 - 10 minutes before start of the FMD test. Therefore, a continuous glucose monitoring system measuring glycemia during the study day would have been more appropriate for gaining insight on this hypothesis. The impact of postprandial glucose on CF effects requires further investigation, especially in T2DM patients.

2.3.3. Population

As reported by Vlachojannis et al. (2016), an appropriate CF dose could be dependent on several personal characteristics like age, hypertension, and obesity [17]. Therefore, the characteristics of the included participants (e.g. age, BP at baseline, sex, BMI, features of DM) should be considered as well and are discussed.

Considering influence of age

The age range for inclusion in our study was 18 – 85 years; however, as diagnosis of T2DM is mostly given above 45 years of age and for proper matching between groups, rather older samples in both groups were expected. Also, since subjects were asked to come twice to the University hospital of Ghent, retired people were more willingly to participate. However, age impacts vascular compliance and vascular reactivity to physiological stimuli [35]. The impact of CF on vascular reactivity is still debated. Our recent published meta-analysis (DM patients and persons with an increased cardiovascular risk) [122] and the meta-analysis of Ried et al (2017) (healthy persons with or without hypertension) [35] showed that the older the participant, the fewer effects on BP were detected through chronic CF ingestion (at least 2 weeks). Conversely, Jafarnejad et al. (2020) (varied populations combined) reported greater effects on BP in elderly (≥ 65 years) compared to middle aged (≥ 45 years) persons after chronic cocoa ingestion (at least 4 weeks) [123]. To our knowledge, no influence of age on acute BP-lowering effects through CF have been investigated so far. For FMD, CF ingestion would exert greater acute effects in older adults (> 57.5 years) compared to younger adults (< 57.5 years), while chronic effects were shown to be significant in both young (< 50 years) and older (> 50 years) adults (varied populations combined) [124]. In our original research paper,

we added age as a covariate and only found an influence of age on DBP reflecting a decrease in DBP with increasing age. However, several limitations like the small sample size require caution for interpretation.

Considering influence of BP at baseline
Two recent meta-analyses reported a CF-provoked reduction of SBP in hypertensive subjects but not in normotensive subjects after at least 2 – 4 weeks of CF ingestion [35, 123]. A stronger BP-lowering effect in persons with an elevated SBP was also suggested through subgroup analyses in our recent published meta-analysis (DM patients and persons with an increased cardiovascular risk) [122]. In our study, non-diabetic and T2DM subjects had rather normal BP values which might partly explain the lack of an effect on BP through CF ingestion. However, it should be reminded that our study had an acute design and that 15/ 24 non-diabetic and 4/ 11 T2DM participants used ACEi or ARB.

Considering influence of BMI
It was previously suggested that increased insulin resistance, which is often present in overweight/ obese individuals, attenuates the vascular effects of cocoa products instead of BMI as such [86]. Therefore, higher doses of CF or other cocoa-compounds could be required in those individuals. Davison et al. (2008) showed BP-lowering effects 2 hours post ingestion of cocoa powder comprising 451 mg CF, 337 mg theobromine, and 18 mg caffeine mixed with water in middle aged, overweight/ obese individuals [125]. In a quite comparable population, Faridi et al. (2008) demonstrated a decrease in BP, but also an increase in FMD 2 hours post consumption of dark chocolate administration containing 821 mg CF, 21.5 mg EC, 525 mg theobromine, and 44 mg caffeine [84]. Note that CF and/or theobromine doses were higher in the cited studies compared to our intervention and that only Faridi et al. (2008) did not match their control intervention with theobromine and caffeine. It is plausible that required doses of CF and other cocoa-compounds should be expressed in amounts/ kg body weight instead of 1 dose fits all.

Considering influence of sex
Our published meta-analysis suggested a greater effect of CF on BP in DM women compared to DM men [122]. In agreement, West et al. (2014) demonstrated a lower arterial stiffness after 4 weeks of dark chocolate ingestion in middle-aged overweight women, but this could be partly due to their higher baseline values [126]. The meta-analysis of Jafari et al. (2021) (varied populations combined) on the acute and chronic effects of cocoa on arterial stiffness and platelet count reported that next to study design, dose, and duration of supplementation, sex was the potential source for heterogeneity [127]. These results are not surprising considering the reported sex differences in the regulation of vascular tone [128, 129]. Therefore, in our study, not only the small sample size as such, but also the inclusion of both sexes increased heterogeneity. If known in advance that recruitment of eligible participants would be so difficult, testing within 1 sex would have been an appropriate choice. More research examining sex-differences on CF-induced vascular effects is required.

Considering influence of vascular complications
The lack of an effect in our study might also be explained through the health status of our T2DM participants. As suggested in previous research, CF-provoked vascular effects may be emphasized in persons with a certain level of dysfunction [22, 130, 131], so that it might be

plausible that any possible vascular effect might have been more pronounced in people with more vascular complications. Balzer et al. (2008) reported that all T2DM participants in the acute trial had a history of a coronary artery disease. They did not report on absence/ presence of other diabetic vascular complications [81]. However, to avoid bias based hereon and to limit heterogeneity, we consciously chose to exclude T2DM subjects with complications. Nevertheless, the absence/ presence of vascular complications was not objectively measured but questioned through a screening questionnaire in which they had to indicate their complications. It should be considered that T2DM individuals are very heterogeneous. As explained in the introduction of this dissertation, T2DM is a complex, chronic, progressive disease and is largely underdiagnosed. Therefore, patients' characteristics (e.g. HbA1c, diabetes duration, insulin, glucose, FFA) should be clearly described and presence of micro- and macrovascular diabetic complications should be reported in order to more adequately compare interventions and effects between studies.

Considering influence of fasting blood results

When comparing the fasting blood results between included non-diabetic and T2DM individuals, solely glucose values were higher in the T2DM group and cholesterol (total and LDL) were higher in the non-diabetic group. Other parameters like insulin, HOMA, QUICKI, triglycerides, and FFA were not significantly different between groups. Considering the age and BMI levels of all the tested participants and the drug use in the T2DM group, these comparable blood results might not be that surprising. However, the high insulin levels, HOMA, and QUICKI in the non-diabetic subjects might indicate insulin resistance in some individuals [132, 133]. Possibly, high insulin levels and insulin resistance could attenuate/ counteract the beneficial vascular impact of CF, theobromine, and/ or caffeine. If so, this might explain, in part, the lack of an effect of CF and placebo in our populations.

However, again heterogeneity between studies complicates comparison of patients' characteristics. Because of paucity of reports, influence of patients' characteristics on CF effects on microvascular reactivity would be unreliable and is therefore not discussed here.

2.3.4. Responders vs. non-responders

Vlachojannis et al. (2016) [17] stated that more research should focus on responders and non-responders for gaining insight. It was our purpose to perform subgroup analyses and to insert certain fasting blood parameters as covariates into our models, however, these additional analyses were impeded because of too small sample sizes.

Age, waist-to-hip ratio, and SBP at baselines were inserted as covariates into our statistical models. Because the little sample size impeded adding more covariates and hindered to perform subgroup analyses, we tried to compare included subjects and their observed individual results (separate case-studies) to gain insight in certain characteristics of potential responders and non-responders. Interpretation of these individual data requires caution and underlines the complexity of human interventional studies. Subject characteristics were too different to compare with observed effects: smoking history, medication intake (other than antihypertensive drugs), BMI, consumed breakfast at study day, daily coffee consumption,

daily chocolate consumption, alcohol use, physical activity levels, glycemic variability and excursions etc.

The spaghettiplots comprising the results of % FMD (primary outcome) are presented below in groups and subgroups and demonstrate the high heterogeneity in response to CF and placebo (Figures 19, 20, 21, 22).

Explanation of codes:

dm= diabetes (T2DM); ng= non-diabetic

ab= angiotensin receptor blocker; ac= Angiotensin converting enzyme inhibitor; nb= normal blood pressure

1= female: 2= male

e.g. dmab103= person with T2DM, using angiotensin receptor blocker, female, number 03 of that subgroup

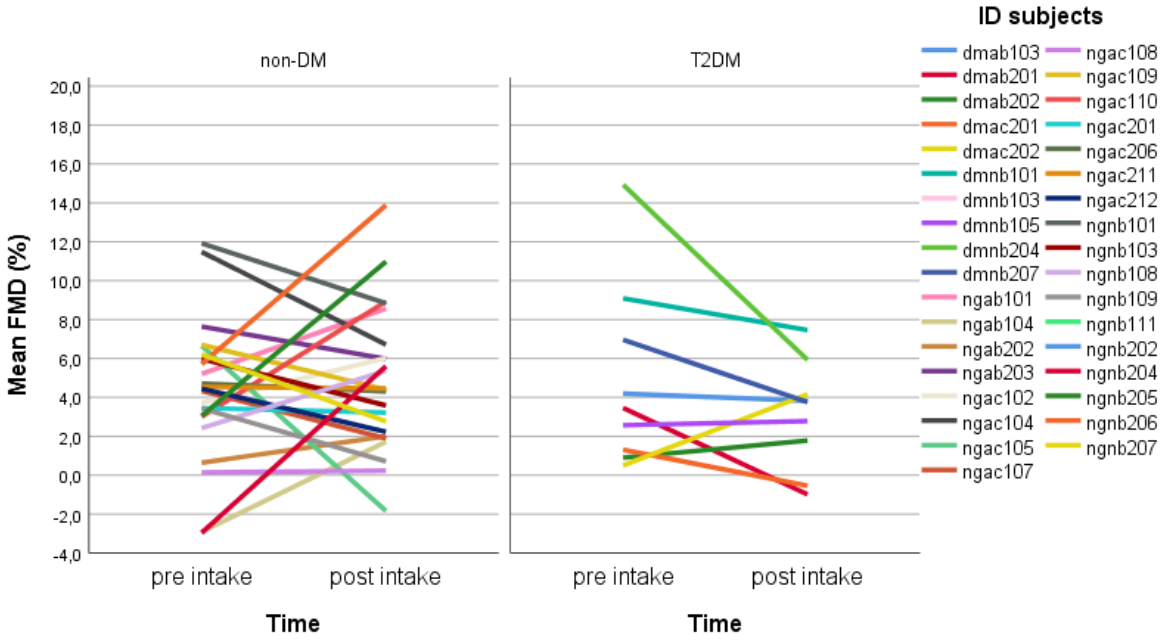

Figure 19: Individual response on **cocoa flavanols** ingestion on FMD.

FMD= flow-mediated dilation test; non-DM= participants without diabetes mellitus; T2DM= participants with type 2 diabetes mellitus.

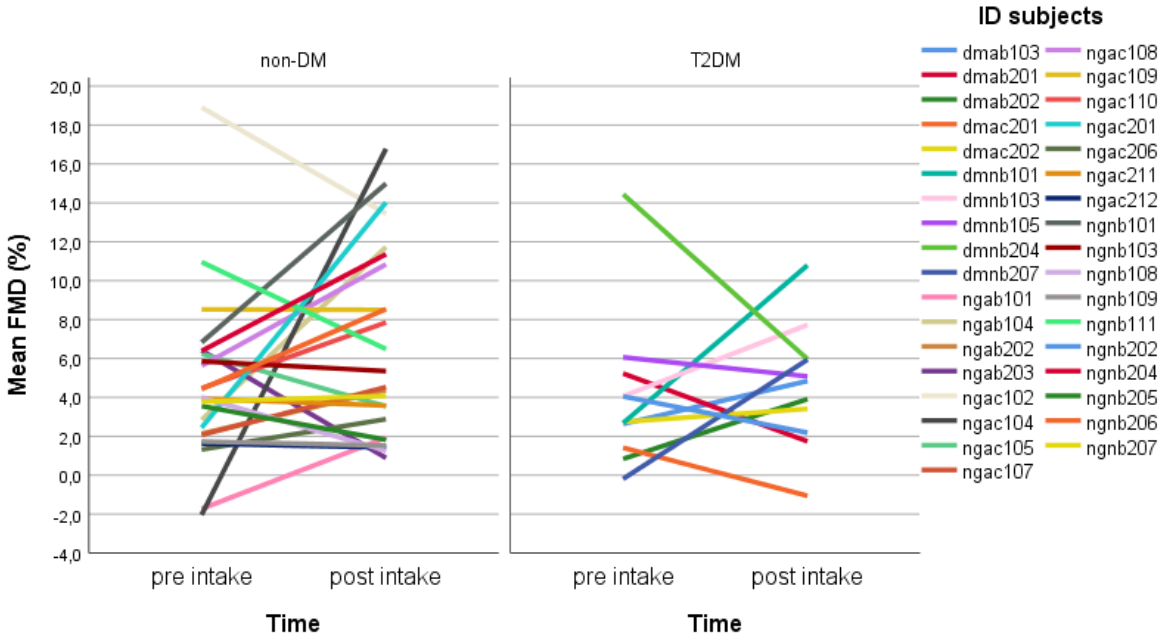

Figure 20: Individual response on **placebo** ingestion on FMD.
FMD= flow-mediated dilation test; non-DM= participants without diabetes mellitus; T2DM= participants with type 2 diabetes mellitus.

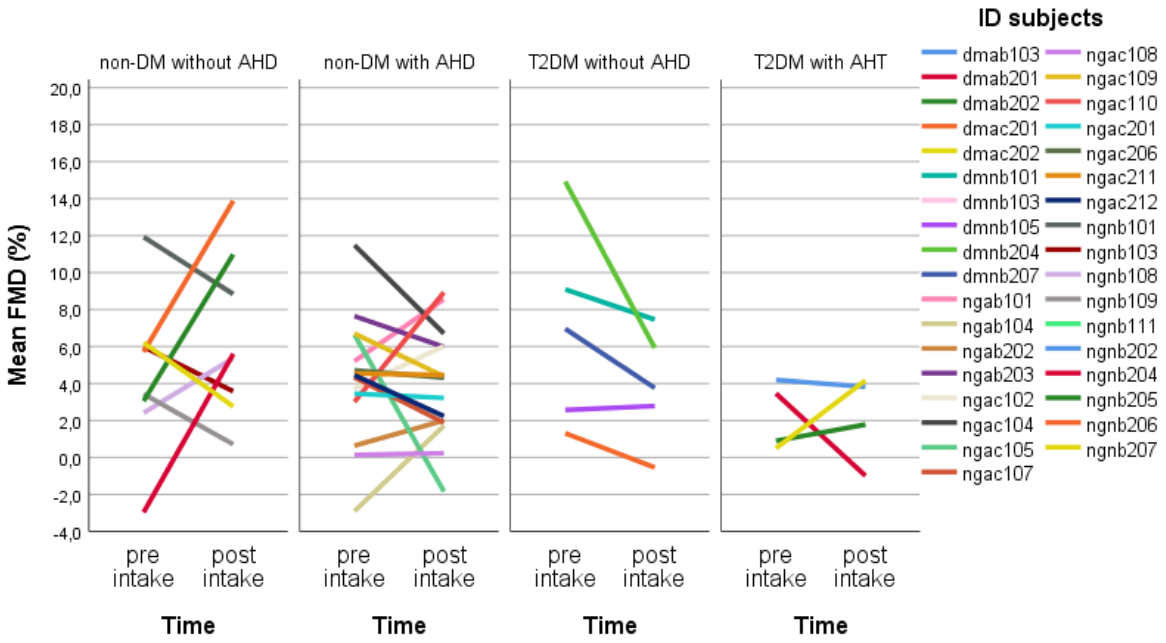

Figure 21: Individual response on **cocoa flavanols** ingestion on FMD.
AHD= antihypertensive drugs; FMD= flow-mediated dilation test; non-DM= participants without diabetes mellitus; T2DM= participants with type 2 diabetes mellitus.

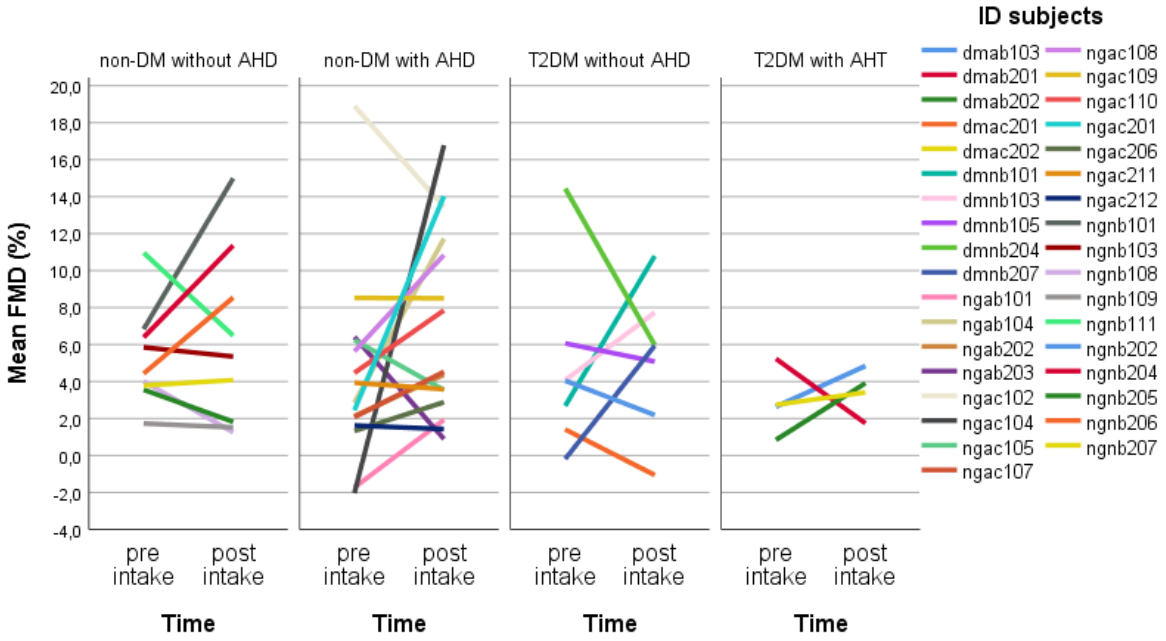

Figure 22: Individual response on **placebo** ingestion on FMD.
AHD= antihypertensive drugs; FMD= flow-mediated dilation test; non-DM= participants without diabetes mellitus; T2DM= participants with type 2 diabetes mellitus.

Hence, as shown in the figures and as could be assumed considering the lack of significant results, the varied responses on the interventions (in both CF-enriched and placebo capsules) together with the diverse patients characteristics [e.g. smoking history, medication intake (other than antihypertensive drugs), BMI, consumed breakfast at study day, daily coffee consumption, daily chocolate consumption] made it rather difficult to make suggestions about plausible influencing factors or responders and non-responders.

3. Clinical relevance and transfer to dietary intake

The clinical relevance of this doctoral research is that if CF increase NO and limit oxidative stress, hence exert beneficial vascular health effects in T2DM patients, cocoa could influence the development and/or progress of DM-associated hypertension and other vascular complications. As explained, these complications substantially result in higher morbidity and mortality rates, decrease quality of life, and increase health economic burden [134-138]. Since the direct and indirect costs of DM to the Belgian social security system accounted for 5.82 billion euros of which 94 % is related to treatment of complications (data from 2018) [139], this would have a serious positive impact.

Based on our randomized controlled trial, no acute CF-induced vascular effects were detected in T2DM patients, nor in non-diabetic subjects. However, because of several limitations like the small sample size, caution is needed for interpretation. Further research is certainly required before conclusions could be formulated.

Until now, we cannot provide a straight guideline for dietary intake of cocoa. The European Food Safety Authority published a health claim indicating daily doses of 200 mg CF [140]. A non-linear dose-response relationship of cocoa on FMD has been established [33, 124], but one's ideal dose remains a question [17]. A recent meta-analysis of epidemiological reports indicated that weekly intake of 45 g chocolate, independent of type of chocolate, is the most appropriate dose for reducing cardiovascular risk. Conversely, beneficial effects would be countered or even adverse effects could arise when weekly chocolate ingestion exceeds 100 g. However, an important limitation is the lack of identification of characteristics of chocolate (e.g. type) in the included papers [33]. A recent meta-analysis of randomized controlled trials stated that the greatest improvement in FMD was provoked with daily flavonoid doses of > 40 g (greatest effect in daily doses of 40 – 60 g) or with daily ingestion of 20 g dark chocolate. However, heterogeneity in flavonoid/ dark chocolate doses and populations were present [124]. As explained in the general discussion of this dissertation, it is difficult to make conclusion without clear description of composition and when high heterogeneity (e.g. based on dose, population) between included papers is present.

We can only recommend the moderate intake of dark chocolate over milk chocolate and certainly over white chocolate. Vlachojannis et al. (2016) indicated that for adequate levels of CF and EC, one would have to consume 125 – 300 g chocolate/ day [17]. However, as chocolate is an energy-dense food, over-consumption can cause weight gain and in general negative health outcomes [31, 36]. We think that, providing a proved ideal composition of cocoa products in future, a combined intake of cocoa products with other sources, like fruits and vegetables, to attain adequate levels of flavanols and EC is the best way for improving or maintaining vascular health, certainly when considering the sugar and fat content of chocolate.
Especially in certain populations like T2DM patients, the additional fat and sugar content in cocoa products have to be considered so that cocoa effects are not masked and to avoid detrimental consequences.

4. Strengths and limitations

This doctoral project has several strengths, but, as in all studies, also presents some shortcomings.

Our first aim was to examine the current State of The Art regarding potential CF-induced vascular health properties in T2DM. However, because of paucity of research on CF effects in DM individuals, our meta-analyses could only include few papers examining mid/long-term effects of CF ingestion on BP with high heterogeneity based on dose, population, and measurement methods. Therefore, no strong conclusions could be formulated and subgroup analyses had to be interpreted with caution.

Based on literature search, so far, only few research with high heterogeneity and inconsistent results had been performed to examine the vascular effects of CF in patients with DM. Hence, there is high need for homogeneous, standardized research with a robust protocol taking into account the limitations of these studies. Therefore, a major strength of this doctoral research was the clear formulation of the trial protocol for the acute, randomized, double-blinded, placebo-controlled cross-over study.

First, the clear description of our study population with strict in- and exclusion criteria ensured unambiguous testing reducing bias and heterogeneity based on patients characteristics (e.g. smoking, use of antihypertensive drugs, use of vitamins and supplements). However, this increased burden to find eligible participants. Moreover, as it is suggested that antioxidative properties of CF are greater expressed in pathological conditions, it is possible that CF-induced vascular effects would have been more pronounced in patients with vascular complications.

Second, the composition of the interventional capsules is both a strength and a limitation. Since caffeine and theobromine are vasoactive substances, the equilibration of these substances in both types of intervention is certainly a strength. Though, because the amount of maltodextrin differed, a second placebo containing solely maltodextrin would have been appropriate. Furthermore, until now, we do not know yet the entire composition of our intervention formulae. As explained above, we encourage future publications to describe entire composition, but unfortunately we were not able to report them ourselves.

Third, to limit bias, from 3 days prior start of the study participants were asked to follow guidelines concerning food and drink consumption (literature-based) and amount of physical activity. Not all trials have such guidelines, or do not report this in their publications; however, we strongly recommend such guidelines for standardization.

A strong limitation of our interventional study is the small sample size. As many other research, the COVID-pandemic substantially hindered our recruitment and testings as such. The study was initially planned to run until the end of March 2021, however, we decided to stop the study at the end of December 2020 since no testing had been executed from mid-March 2020, the first lock-down in Belgium. Hence, groups were not completed and not entirely matched. When formulating our trial protocol, we had a lot of ideas for subgroup analyses to gain insight and possibly explain the difference in responders and non-responders

to CF-provoked vascular effects. The use of AHD (varied types) together with cocoa products for example were, so far, only examined in 4 studies in non-diabetic hypertensive adults [141, 142], in non-diabetic heart transplant recipients [143], and in non-diabetic adults with congestive heart failure [144]. These reports indicated a supplementary effect of cocoa intake on BP [141, 142] and/or endothelial function [142-144]. As AHD is frequently used in T2DM patients and affect similar pathways as CF, further research into the vascular effects of a combined ingestion of CF and AHD in T2DM populations is certainly needed. Also the possible influence of antidiabetic drugs with CF ingestion, which could not be tested reliably in our trial, requires further research as many of these drugs activate similar pathways as CF [145, 146]. Furthermore, the potential influence of sex, age, and systolic BP at baseline on CF effects in DM subjects as suggested by our executed meta-analysis requires caution but could only be poorly tested in our study because of few power [122]. We also aimed to account for the influence of physical activity and glycemic control: as these impact vascular function they may influence the measured CF effects. However, instead of subgroup analyses based on these variables, they could only be regarded as patient characteristics. Furthermore, we had to exclude the group with subjects using beta-blockers, again due to Pandemic induced restrictions

Last, another limitation is the choice out of 4 different breakfast formulas. Since the amount of nutrients and the composition (e.g. type of milk source) varied, this increased heterogeneity. The varied fiber content for example, although small amounts, since it has been reported that dietary fiber improve the transport of polyphenols through the gastro-intestinal tract, this may have influenced the amount of CF absorbed in the small intestine, hence the amount of metabolite formation [91].

5. Future perspectives and considerations

Although no impact of our supplementations, nor the CF-enriched capsules, nor the placebo capsules, was detected in our randomized controlled trial, we believe that cocoa have great potential as a nutraceutical with promising vascular health benefits. However, we want to underline its complexity and the high need for more homogeneous, standardized research.

It is possible that our concentrations of varied cocoa compounds were not sufficiently high for inducing vascular effects in patients with T2DM. However, completion of the study is needed to confirm or reject this hypothesis. A larger sample size, inclusion of more complicated T2DM subjects, correction of varied confounding factors like level of physical activity and glycemic profile, and use of a 2nd placebo only containing maltodextrin would be appropriate before conclusions for use of our intervention in T2DM subjects could be formulated.

For future research, as opposed to solely focusing on CF, all vasoactive substances in cocoa, especially theobromine and caffeine levels because of their potential vascular health effects and their rather high bioavailability in cocoa [74], should be considered when analyzing the effects of cocoa products. Therefore, we recommend to setup testings with pure vasoactive cocoa compounds followed by combined testings of these vasoactive substances. Especially pure theobromine doses and its vascular effects should be further investigated as limited research has been performed so far. In addition, it is not clear yet what effects the combined intake of theobromine and caffeine exerts on vascular systems. Moreover, it is not known whether these effects are enlarged/ countered/ blunted when combined with flavonoids (e.g. CF) ingestion and other chocolate compounds like sugar, fat, and milk. Furthermore, we advise to avoid no intervention or white chocolate as a control/ placebo intervention, but to closely match interventions on vasoactive compounds apart from the studied substance(s). Also, future research should clearly describe the characteristics of their investigated population with extra attention for variables that impact vascular function like age, sex, level of physical activity, and glycemic variability and excursions.

For example, a first next study could be the execution of nearly the same design as explained in our trial protocol; however, using capsules containing only CF and EC levels, capsules with only theobromine levels, and capsules with only caffeine levels. Next, a similar study combining these types of capsules should be executed to test the synergistic/ complementary/ antagonistic effects. The theobromine content could be increased in the administered capsules so that the intervention is more comparable to the supplementation used in Balzer et al. (2008). Furthermore, T2DM patients with coronary artery diseases may be included, again to increase comparability with Balzer et al. (2008), and to perform subgroup analyses exploring influence of vascular complications on CF effects. Also, recruitment of younger participants is recommended. Furthermore, we advise to monitor glycemia during the measurements through continuous glucose monitoring systems. Last, to avoid influence of other consumed foods like a breakfast, it should be considered to split up our listed outcome parameters in separate trials. Although it is a strength of our study to simultaneously assess CF effects on micro- and macrovascular reactivity, this increases duration of the trial protocol and so complicates unambiguous testing of cocoa (e.g. because of breakfast

consumption and circadian influences). In a later phase, however, when knowledge on vascular cocoa effects would have increased, this could be very interesting.

When there will be clarity on the vasoactive effects and the required doses taking into account the potential synergistic/ complementary/ antagonistic effects of different cocoa compounds, other nutrients consumed together with cocoa (like milk, breakfast/meal), and the characteristics of the population, long-term randomized double blinded, placebo-controlled trials should be setup to formulate health recommendations for dietary, habitual cocoa products ingestion.

Furthermore, naturally occurring compositions of cocoa compounds should be considered, especially in long-term trials. If beneficial vascular effects are exerted by doses of cocoa compounds (e.g. CF, EC, theobromine, caffeine) that occur in cocoa products, intake of cocoa products could be recommended. However, when certain amounts of CF, EC, theobromine, caffeine, or other vasoactive compounds of cocoa need to be added to cocoa products for inducing maximal effects, it is questionable whether ingestion of cocoa products should be recommended for administration of these compounds. The sugar and fat content of several cocoa products may not be ignored, especially not in certain populations like obese and DM patients. Maybe cocoa products with saving manufacturing processes (to preserve as much as possible the ideal nutrient composition of the raw cocoa bean) and only small additions of sugar and fat to cover the bitterness could be a possible alternative? To avoid misunderstanding and to form clear health recommendations, another name than 'chocolate' could be invented.

At last, as described in the introduction, a major advantage of CF are the absence of adverse effects, apart from some gastrointestinal complaints in very few cases. In our study, 1 person vomited 50 minutes after ingestion of CF enriched capsules, but she already felt sick before capsules ingestion and suggested that the yoghurt administered with the breakfast was expired.

However, when excessive amounts of flavonoids are consumed (around 10 – 20 times of normal daily intake), there is increased risk for mutations, increased production of free radicals and thus higher levels of oxidative stress, and inhibition of key enzymes required for hormone metabolism [147]. Moreover, detrimental effects of flavan-3-ols in particular additionally include hemorrhage formation, contribution to hepatoxicity, estrogenic tumor formation, and gastroenteritis, antinutritive activity and weight loss, and change in pharmacokinetics of medications [6, 148]. Therefore, one should consider transfer from a study setting to daily dietary intake together with daily ingestion of other sources of flavonoids like tea and red wine [149, 150].

6. Final conclusion

One cannot ignore the increasing research demonstrating beneficial vascular effects of cocoa. There is strong evidence that cocoa products enhance endothelial function and moderate evidence for improving BP, lipid profile, and vascular stiffness in the general population. High heterogeneity between studies impedes formulation of strong conclusions [131]. Increasing NO leading to vasodilation and modulation of the renin-angiotensin-aldosterone system, but also anti-oxidative and anti-inflammatory actions of CF, theobromine, caffeine and/ or other cocoa compounds are, so far, the suggested main mechanisms for inducing these effects [60, 131, 151, 152].

There is certainly high need for further research into the acute and chronic effects of cocoa on vascular systems in varied, well described populations. Based on previous research and the theoretical background of cocoa, we believe that cocoa is a promising nutraceutical. However, we are convinced that most of the mechanisms of cocoa-induced health effects are still unknown. CF might play a major role in exerting vascular health benefits, but considering the high bioavailability in plasma of methylxanthines and the inconsistent results when focusing on CF or when pure EC is ingested, we doubt that solely CF are responsible for the provoked vascular health effects.

Considering the literature discussed in this dissertation, we think that the interaction of CF with methylxanthines and other flavonoids are necessary for its most optimal effect. Furthermore, additional substances like milk, fat, and sugar should be considered, the latter two especially in obese and DM individuals.

The lack of an effect of our intervention can be due to the small sample size, which is a major limitation. However, also the administered dose of cocoa-extract with its compounds could be insufficient. Maybe higher doses and/or another composition were needed for our population? Maybe the breakfast prevented the CF-induced vascular benefits? Comparison with other studies is impeded since very few information considering intervention and/ or population characteristics were provided in most papers. Also, taking into account the metabolism and bioavailability of cocoa compounds, caution is needed for comparison of effects induced by capsules containing a cocoa extract with effects of chocolate/ cocoa powder mixed with water or milk.

Hence, at the end of this dissertation, the question *'a chocolate a day, keeps the doctor away?'*, stays a (promising) question.

Title: eureKA!BOEM
Artist: Floris van Opstal (www.flowris.com)
Photo: Kristof Burm

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Chapter 4: Summary – samenvatting – résumé

1. Summary

Type 2 diabetes mellitus (T2DM) represents 90 – 95 % of all diabetes cases and is characterized by β-cell dysfunction and insulin resistance leading to hyperglycemia. Hyperglycemia increases oxidative stress, inflammation, and orthosympatic activity and limits bioavailability of nitric oxide (NO), resulting in micro- (nephropathy, neuropathy, retinopathy) and macrovascular (cerebrovascular, cardiovascular, and peripheral artery disease) complications. These complications result in higher morbidity and mortality rates, decrease quality of life, and increase health economic burden. Increasing physical activity and a more balanced, healthy food intake are the first-line management. Herein, the promising vascular health benefits of nutraceuticals, like flavonoids and more specifically flavanols, have gained interest.

Flavanols are natural substances present in several fruits, teas, red wines, beans, and predominantly in cocoa and are believed to beneficially affect human health. Based on epidemiological, in vitro-, animal-, and human studies, cocoa flavanols (CF) would have antioxidant properties, improve endothelial function, lower blood pressure (BP), and reduce inflammation. The mechanisms of action of CF are not yet completely understood, but it is believed that increasing NO bioavailability and –activity and antioxidative actions like inhibiting lipid peroxidation and nicotinamide adenine dinucleotide phosphate oxidase and scavenging free radicals play a key role.

So far, research into the potential beneficial vascular health properties of CF in patients with diabetes mellitus (DM) is limited and demonstrated inconsistent results. However, based on the pathophysiology of diabetic vascular complications and the believed mechanisms of action of CF, one could assume that CF would exert vascular protection in T2DM subjects. Therefore, this doctoral research investigated whether CF exert vascular health benefits in patients with T2DM through the following 3 aims: (1) examine the evidence for CF-induced vascular health properties in patients with DM, (2) setup of a robust, standardized, clearly described trial protocol, and (3) investigate the acute effects of CF on peripheral vascular reactivity in patients with T2DM via execution of the described acute, randomized, double-blinded, placebo-controlled cross-over trial.

First, we published a systematic review and meta-analysis on the vascular health effects of CF in patients with DM. We highlighted the need for more, robust, standardized research because of the high heterogeneity in administered intervention (dose, duration and frequency, nature of intervention), the studied population (age, sex, BMI, medical therapy, stage of disease), and measurement methods. Because of paucity of reports, we could only perform the meta-analysis on the mid/long-term effects of CF on blood pressure (BP) in patients with DM and mixed populations with increased cardiovascular risk. This meta-analysis indicated weak evidence for a reduction in diastolic BP (DBP) of, at best, 1 – 2 mmHg. No effect on systolic BP (SBP) was detected. Furthermore, CF effects on BP would be stronger in female, hypertensive, younger adults, providing a CF dose comprising at least 90 mg epicatechine (EC), and when ingested in 1 daily batch.

Second, the protocol paper illustrating our setup acute, randomized, double-blinded, placebo-controlled cross-over trial was published. Here we thoroughly described our protocol trial in which we take into account the limitations in previous studies. We believe that acute studies

in which subjects ingest a pure cocoa extract are the first step to gain insight in CF actions as possible confounding impact of additional fat, sugars, milk or other substances could mask/ counteract/ strengthen the effects of CF. We provided a clear description of the administered intervention, the studied population, and standardized measurement methods. We based our CF dose on another study who reported improvements of endothelial function in T2DM subjects after single CF ingestion. Moreover, as suggested by our meta-analysis, we aimed to perform subgroup analyses based on sex, age, BP at baseline, and use of antihypertensive drugs. Besides, both levels of physical activity and glycemic profile would be recorded as these factors may influence vascular systems. A major advantage and novelty of this study was the simultaneous analysis of micro- (dynamic handgrip strength test) and macrovascular (flow-mediated dilation test and blood pressure assessment) reactivity.

Last, after the formulated protocol trial was executed and results were analyzed, a third paper examining the acute effects of CF on peripheral vascular reactivity in patients with T2DM and/or essential hypertension was submitted (under embargo). Due to the COVID-pandemic, testings were forced to be cancelled for several months and recruitment was substantially hindered afterwards. Therefore, we could not complete our groups. Because of the small samples and thus to avoid overcorrection, only age, waist-to-hip ratio, and SBP at baseline were inserted as covariates since these variables differed between groups. Moreover, the potential influence of use of antihypertensive drugs was solely analyzed through insertion of a cofactor into the statistical models.

Based on this research, no effects of CF on micro- or macrovascular reactivity were detected. Independent of type of capsules ingested, this research demonstrated a decrease over time (from baseline until after breakfast and capsules ingestion) for DBP and an increase over time (from baseline just after breakfast ingestion until after capsules ingestion) for difference in diameter of arteria brachialis in the non-diabetic group (difference in diameter of arteria brachialis showed only a tendency for time x group interaction). Nevertheless, several limitations, like the small sample size, requires caution for interpretation.

Although we did not find an effect of CF, we believe that cocoa is a promising nutraceutical. However, we want to underline its complexity. Besides CF, **cocoa comprise other vasoactive compounds,** like theobromine and caffeine, with a higher bioavailability than CF. Nevertheless, so far, most studies only focused on CF content and levels of its most abundant monomeric form EC. Also the health claim published by the European Food Safety Authority reported solely minimal daily CF amounts to preserve vascular compliance. However, other studies have shown higher required doses and indicated that minimal CF doses for inducing vascular benefits are dependent on person characteristics, like, age, BMI, and BP. It is plausible that the inconsistency concerning minimal doses are not solely dependent on these characteristics, but are also provoked by different concentrations of other vasoactive cocoa compounds. Furthermore, studies investigating the administration of pure EC, thus without influence of other vasoactive compounds, reported inconsistent results. It is suggested that EC exert greater effects through the **pharmacokinetic and pharmacological interactions with other cocoa flavonoids and compounds**, like theobromine. Hence, considering the high bioavailability in plasma of other vasoactive cocoa compounds and the inconsistent results of

studies focusing on CF and trials with pure EC administration, it is questionable whether only CF are responsible for the provoked vascular health effects of cocoa.

In conclusion, based on this doctoral research, no conclusions about CF-induced vascular health effects in patients with T2DM could be formulated. However, our general discussion underlined important points of consideration. We believe that not only CF but the combination of CF with other vasoactive compounds of cocoa, like theobromine, are mainly responsible for the vascular health benefits. Future research investigating successively the effects of pure vasoactive cocoa compounds, followed by trials examining the combined intake of these compounds to analyze their synergistic/ antagonistic/ complementary effects in different, well described populations are required before conclusions and health recommendations could be formulated.

2. Samenvatting

90 – 95 % van alle personen met diabetes hebben type 2 diabetes, welke gekenmerkt wordt door dysfunctie van de β-cellen en insuline resistentie waardoor deze personen een te hoge bloedsuiker (hyperglycemie) hebben. Hyperglycemie verhoogt de oxidatieve stress, inflammatie, en activiteit van het orthosympathisch zenuwstelsel en beperkt de biologische beschikbaarheid van stikstofmonoxide leidend tot micro- (nefropathie, neuropathie, retinopathie) en macrovasculaire (cerebrovasculair, cardiovasculaire, en perifere vaataandoeningen) complicaties. Die complicaties leiden dan weer tot een toename in morbiditeit en mortaliteit, vermindering van de levenskwaliteit en verhoging van de kosten voor de gezondheidszorg. Meer fysieke activiteit en een evenwichtige, gezonde voeding vormen de eerstelijnsbehandeling. De aandacht voor nutraceutica, zoals flavanoïden en meer specifiek flavanolen, is toegenomen vanwege hun veelbelovende vasculaire voordelen.

Flavanolen zijn natuurlijke stoffen die aanwezig zijn in verschillende soorten fruit, thee, rode wijn, bonen en zijn voornamelijk te vinden in cacao. Flavanolen zouden een gunstige invloed hebben op de menselijke gezondheid. Op basis van epidemiologische, in vitro-, dierlijke en menselijke studies, zouden cacao flavanolen antioxiderende eigenschappen hebben, de endotheelfunctie verbeteren, de bloeddruk verlagen, en ontstekingen verminderen. De werkingsmechanismen van cacao flavanolen zijn tot nu toe nog niet geheel duidelijk, maar een verhoging van de biologische beschikbaarheid en activiteit van stikstofmonoxide en een antioxiderende werking zoals het remmen van vet-peroxidatie en nicotinamide adenine dinucleotide fosfaat oxidase en het opruimen/afvoeren van vrije radicalen zouden een belangrijke rol spelen.

Tot nu toe is onderzoek naar de mogelijke positieve vasculaire effecten van cacao flavanolen bij patiënten met diabetes mellitus beperkt en zijn de resultaten hiervan inconsistent. Echter, kijkend naar de pathofysiologie van vasculaire complicaties bij diabetes en de veronderstelde werkingsmechanismen van cacao flavanolen, zouden cacao flavanolen een vasculaire bescherming kunnen bieden bij personen met type 2 diabetes. Dit brengt ons bij het onderwerp van dit doctoraatsproject, namelijk het onderzoeken of cacao flavanolen effectief een voordelige impact hebben op de vasculaire systemen bij personen met type 2 diabetes. We onderzochten dit in 3 stappen: (1) de evidentie nagaan van vasculaire effecten van cacao flavanolen bij personen met diabetes, (2) een robuust, gestandaardiseerd, duidelijk beschreven onderzoeksprotocol opzetten, en (3) de acute effecten van cacao flavanolen op de perifere vasculaire reactiviteit bij patiënten met type 2 diabetes testen door de uitvoering van het beschreven acuut, gerandomiseerd, dubbel geblindeerd, placebogecontroleerde cross-over onderzoeksprotocol.

Eerst hebben wij een systematische review en meta-analyse gepubliceerd, waarin wij de noodzaak voor meer, robuust, gestandaardiseerd onderzoek onderlijnen vanwege de grote heterogeniteit in toegediende interventies (dosis, duur en frequentie, aard van de interventie), de bestudeerde populaties (leeftijd, geslacht, BMI, medische therapie, stadium van de ziekte), en de meetmethoden. Door het beperkt aantal gepubliceerde artikels, konden wij enkel een meta-analyse uitvoeren naar de halflange tot lange termijneffecten van cacao flavanolen op de bloeddruk bij personen met diabetes mellitus en gemengde populaties met een verhoogd cardiovasculair risico. Op basis van die meta-analyse toonden we een lage evidentie voor een daling van de diastolische bloeddruk aan van, in het beste geval, 1 - 2

mmHg. We vonden geen effecten op de systolische bloeddruk. De effecten van cacao flavanolen op de bloeddruk zouden sterker zijn bij vrouwen, bij hypertensieven, bij jongere volwassenen, als de CF dosis minimaal 90 mg epicatechinen bevat, en bij inname van één dagelijkse dosis.

Vervolgens werd het onderzoeksprotocol gepubliceerd waarin het acuut, gerandomiseerd, dubbel geblindeerde, placebogecontroleerd cross-over onderzoek grondig werd beschreven en waarbij rekening gehouden werd met de beperkingen in vorige studies. Wij geloven dat de eerste stap naar meer inzicht in de mogelijke effecten van cacao flavanolen bestaat uit acute studies waarbij de proefpersonen een zuiver cacao-extract innemen en waarbij dus mogelijke verstoring door extra vet, suikers, melk of andere stoffen die de effecten van CF zouden kunnen maskeren/tegengaan/versterken zoveel als mogelijk vermeden wordt. Wij hebben de toegediende interventie, de bestudeerde populatie en de gestandaardiseerde meetmethoden duidelijk beschreven. De dosis cacao flavanolen is gebaseerd op een andere studie die verbeteringen van de endotheelfunctie aantoonde bij proefpersonen met type 2 diabetes na eenmalige inname van cacao flavanolen. Bovendien beschreven we subgroep analyses op basis van geslacht, leeftijd, bloeddruk bij aanvang en gebruik van antihypertensiva, zoals aangegeven in de meta-analyse. Ook zouden zowel het niveau van lichamelijke activiteit als het glycemisch profiel worden geregistreerd, aangezien beide factoren een invloed kunnen hebben op de vasculaire systemen. Een groot voordeel en vernieuwing van dit onderzoek is ook dat de effecten op micro- (dynamische handknijptest) en macrovasculaire (een flow-gemedieerde vaatverwijding en meting van bloeddruk) reactiviteit tezamen bestudeerd zouden worden.

Tenslotte, na uitvoering van het onderzoeksprotocol en analyse van de onderzoeksresultaten, dienden we een derde artikel in waarin de acute effecten van cacao flavanolen op de perifere vasculaire reactiviteit bij personen met type 2 diabetes en/of essentiële hypertensie werden onderzocht (onder embargo). Als gevolg van de COVID-pandemie werden de testen voor een aantal maanden stilgelegd en was de rekrutering sterk gehinderd bij heropstart. Daarom konden wij onze verschillende onderzoeksgroepen niet vervolledigen. Vanwege de kleine steekproef en dus om overcorrectie te voorkomen, werden alleen leeftijd, taille-heup verhouding en systolische bloeddruk bij aanvang als covariaten opgenomen, omdat deze variabelen verschilden tussen de groepen. Bovendien werd de mogelijke invloed van het gebruik van antihypertensiva alleen geanalyseerd door het invoegen van een cofactor in de statistische modellen.

Op basis van dit onderzoek konden geen effecten van cacao flavanolen op micro- of macrovasculaire reactiviteit aangetoond worden. Onafhankelijk van het type capsule dat werd ingenomen, werd een daling over de tijd (van nuchtere baseline tot na inname van ontbijt en capsules) voor diastolische bloeddruk en een toename over de tijd (van baseline net na inname van het ontbijt tot na inname van capsules) voor verschil in diameter van arteria brachialis in de niet-diabetische groep aangetoond (verschil in diameter van arteria brachialis toonde enkel een tendens voor de tijd x groep interactie). Hoewel, bepaalde beperkingen, zoals de kleine steekproefgrootte, vragen voorzichtigheid bij de interpretatie.

Ondanks er geen effect van cacao flavanolen werd aangetoond in onze studie, zijn wij ervan overtuigd dat cacao een veelbelovend nutracueticum is. Maar, wij willen de complexiteit

hiervan benadrukken. Cacao bevat naast flavanolen nog **andere vasoactieve bestanddelen**, zoals theobromine en cafeïne, welke een hogere biologische beschikbaarheid hebben dan cacao flavanolen. Tot nu toe richtten de meeste studies zich op de ingenomen dosis van cacao flavanolen en van zijn meest voorkomende monomere vorm, epicatechine. Ook de gepubliceerde gezondheidsclaim van de Europese Autoriteit voor Voedselveiligheid vermeldde alleen minimale hoeveelheden cacao flavanolen per dag om de vasculaire compliantie te behouden. Uit andere studies is echter gebleken dat de vereiste dosissen hoger zijn en dat de minimale dosis van cacao flavanolen voor het induceren van vasculaire voordelen afhankelijk zou zijn van persoonskenmerken, zoals leeftijd, BMI en bloeddruk. Het is ook mogelijk dat de inconsistentie betreffende de minimale dosis van cacao flavanolen niet enkel afhankelijk is van deze kenmerken, maar ook veroorzaakt zou worden door verschillende concentraties van andere vasoactieve cacaobestanddelen. Bovendien toonden studies die pure epicatechines toedienden, dus zonder invloed van andere vasoactieve bestanddelen, inconsistente resultaten. Epicatechines zouden grotere effecten hebben bij **farmacokinetische en farmacologische interacties met andere cacao flavonoïden en bestanddelen**, zoals theobromine. Gezien de hoge biologische beschikbaarheid in plasma van andere vasoactieve cacaobestanddelen, de inconsistente resultaten van studies gericht op cacao flavanolen, en de niet eenduidige conclusies in studies met inname van pure epicatechines, betwijfelen wij of alleen cacao flavanolen verantwoordelijk zijn voor de vasculaire gezondheidseffecten van cacao.

Dus, op basis van dit doctoraal onderzoek konden we niet besluiten of cacao flavanolen wel of geen effect hebben op de vasculair systemen bij personen met type 2 diabetes, maar, op basis van onze algemene discussie zijn wij toch tot belangrijke en interessante bedenkingen gekomen die verder onderzoek vereisen. Wij geloven dat niet alleen cacao flavanolen, maar eerder de combinatie van cacao flavanolen met andere vasoactieve bestanddelen van cacao, zoals theobromine, verantwoordelijk zijn voor de vasculaire gezondheidsvoordelen van cacao. Toekomstig onderzoek zou eerst de zuivere effecten van verschillende vasoactieve bestanddelen van cacao moeten onderzoeken. Nadien zou een gecombineerde inname van die bestanddelen onderzocht moeten worden om synergetische/ antagonistische/ complementaire effecten te analyseren. Belangrijk is dat die proeven in verschillende, goed beschreven populaties gebeuren. Pas na al die onderzoeken zouden conclusies en gezondheidsaanbevelingen geformuleerd kunnen worden.

3. Résumé

Le diabète de type 2 représente 90 à 95 % de tous les cas de diabète et se caractérise par un dysfonctionnement des cellules β et une résistance à l'insuline qui provoquent l'hyperglycémie. L'hyperglycémie augmente le stress oxydatif, l'inflammation et l'activité orthosympathique et limite la biodisponibilité du monoxyde d'azote, lesquels favorisent les complications micro- (néphropathie, neuropathie, rétinopathie) et macrovasculaires (maladies cérébrovasculaires, cardiovasculaires et des artères périphériques). Ces complications sont des facteurs majeurs de morbidité et de mortalité, diminuent la qualité de vie et augmentent la charge économique liée à la santé. L'augmentation de l'activité physique et une alimentation plus équilibrée et saine constituent les soins primaires. Aux vues de leurs propriétés vasculaires prometteuses, l'intérêt pour les nutraceutiques, tels que les flavonoïdes et plus particulièrement les flavanols, s'est accru.

Les flavanols sont des substances naturelles, présentes dans plusieurs fruits, thés, vins rouges, haricots et principalement dans le cacao, qui auraient des effets bénéfiques sur la santé humaine. Sur la base d'études épidémiologiques, in vitro, animales et humaines, les flavanols du cacao auraient des propriétés antioxydantes, amélioreraient la fonction endothéliale, abaisseraient la tension artérielle et réduiraient l'inflammation. Les mécanismes d'action des flavanols du cacao ne sont pas encore complètement élucidés, mais on pense que l'augmentation de la biodisponibilité et de l'activité du monoxyde d'azote et des actions antioxydantes tels que l'inhibition de la peroxydation lipidique et de la nicotinamide adénine dinucléotide phosphate oxydase ainsi que la neutralisation des radicaux libres jouent un rôle clé.

Jusqu'à présent, la recherche concernant les effets vasculaires des flavanols du cacao chez les patients diabétiques est limitée et donne des résultats contradictoires. Cependant, sur la base de la physiopathologie des complications vasculaires diabétiques et des mécanismes d'action suspectés des flavanols du cacao, on pourrait supposer que les flavanols du cacao offrent une protection vasculaire aux personnes avec un diabète de type 2. Dans ce contexte, notre objectif était d'évaluer les bénéfices vasculaires pour les personnes diabétiques de type 2, en articulant le projet en trois étapes (1) examen des preuves des effets bénéfiques vasculaires des flavanols de cacao chez des patients diabétiques dans la littérature, (2) création d'un protocole de recherche robuste, standardisé et clairement décrit (étude randomisée, en double aveugle, contrôlée par placébo), et (3) mise en œuvre de ce protocole sur les effets d'une prise unique de flavanols de cacao sur la réactivité vasculaire périphérique chez des patients diabétiques de type 2.

Tout d'abord, nous avons publié une revue systématique et une méta-analyse, dans lesquelles nous soulignons le besoin de davantage de recherches robustes et standardisées en raison de la grande hétérogénéité des interventions administrées (dose, durée et fréquence, nature de l'intervention), des populations étudiées (âge, sexe, IMC, thérapie médicale, stade de la maladie) et des méthodes de mesure. A cause du nombre réduit d'articles publiés, nous n'avons pu réaliser qu'une méta-analyse sur les effets à moyen/long-terme des flavanols du cacao sur la tension artérielle chez les patients

diabétiques et des populations mixtes (comprenant des diabétiques) avec un risque cardiovasculaire élevé. Cette méta-analyse a mis en évidence des preuves faibles d'une réduction de la tension artérielle diastolique, tout au plus de 1 à 2 mmHg. Aucun effet n'a été détecté sur la tension artérielle systolique. De plus, les effets des flavanols du cacao sur la tension artérielle seraient plus marqués chez les femmes, les hypertendus et les jeunes adultes, à condition que la dose de flavanols comprenne au moins 90 mg d'epicatechine et qu'elle soit consommée en une seule prise par jour.

Ensuite, nous avons écrit et publié le protocole décrivant notre étude croisée, randomisée, en double aveugle et contrôlée par placebo, sur les effets d'une prise unique de flavanols du cacao sur la réactivité vasculaire de patients avec un diabète de type 2. Nous y avons décrit en détail notre protocole dans lequel nous prenons en compte les limitations des études précédentes. Nous pensons que les études aigues dans lesquelles les sujets consomment un extrait de cacao pur constituent une première étape pour obtenir des informations sur les effets des flavanols du cacao car des lipides et glucides, du lait ou d'autres substances additionnelles pourraient masquer, contrecarrer ou renforcer les effets des flavanols. Nous avons présenté une description claire de l'intervention administrée, de la population étudiée et des méthodes de mesure standardisées. La dose des flavanols du cacao est basée sur une autre étude qui a rapporté des améliorations de la fonction endothéliale chez des sujets diabétiques de type 2 après une ingestion d'une dose unique de flavanols du cacao. De plus, comme suggéré dans notre méta-analyse, nous avons voulu effectuer des analyses en sous-groupes en fonction du sexe, de l'âge, de la tension artérielle basale et de l'utilisation de médicaments antihypertenseurs. En outre, la mesure des niveaux d'activité physique et du profil glycémique habituels étaient planifiée car ces facteurs peuvent influencer les fonctions vasculaires. Un avantage majeur et une nouveauté de cette étude était l'analyse simultanée de la réactivité micro- (test dynamique de compression de la main) et macrovasculaire (test de dilatation médiée par le flux et évaluation de la tension artérielle).

Enfin, ce protocole a été réalisé et l'analyse et l'interprétation des résultats ont permis d'écrire un troisième article sur les effets à court terme des flavanols du cacao sur la réactivité vasculaire périphérique chez les patients diabétiques type 2 et/ou présentant une hypertension essentielle (article soumis, sous embargo). A cause de la pandémie de COVID, les expérimentations ont dû être annulées pendant plusieurs mois et par la suite, le recrutement a été considérablement entravé. Par conséquent, nous n'avons pas pu compléter les groupes initialement prévus. En raison du faible effectif et donc pour éviter une sur-correction, seuls l'âge, le rapport des circonférences taille sur hanche et la tension artérielle systolique basale ont été insérés comme covariables, car ces variables différaient entre les groupes. En outre, l'influence potentielle de l'utilisation de médicaments antihypertenseurs a été analysée par l'insertion d'un cofacteur dans les modèles statistiques.

Sur la base de cette recherche, aucun effet des flavanols du cacao n'a été détecté sur la réactivité micro- ou macrovasculaire. Indépendamment du type de gélules ingérées, cette recherche a montré une diminution de la tension artérielle diastolique (entre le moment

à jeun (basal) et après le petit déjeuner et l'ingestion des capsules) et une augmentation de la différence de diamètre de l'artère brachiale (du moment immédiatement après le petit déjeuner (basal) jusqu'à l'ingestion des capsules) dans le groupe non-diabétique (la différence de diamètre de l'artère brachiale montrait qu'une tendence). Néanmoins, plusieurs limitations, comme la petite taille de l'échantillon, exigent une certaine prudence quant à l'interprétation.

Bien que nous n'ayons pas trouvé d'effet des flavanols du cacao, nous pensons que le cacao représente un nutraceutique prometteur. Néanmoins, nous tenons à en souligner la complexité. Hormis les flavanols, **le cacao contient d'autres composants vasoactifs**, comme la théobromine et la caféine, dont la biodisponibilité est supérieure à celle des flavanols. Cependant, jusqu'à présent, la plupart des études ne se sont focalisées que sur la teneur en flavanols et les concentrations de sa forme monomère la plus abondante, l'epicatechine. De même, l'allégation de santé publiée par l'Autorité Européenne de Sécurité des Aliments ne mentionne que des quantités minimales quotidiennes des flavanols pour préserver la compliance vasculaire. D'autres études ont montré que les doses requises étaient plus élevées et que les doses minimales de flavanols pour induire des effets bénéfiques vasculaires dépendaient des caractéristiques des personnes, tels que l'âge, l'IMC et la tension artérielle. Il est plausible que l'incohérence concernant la dose minimale ne dépende pas uniquement de ces caractéristiques, mais soit également provoquée par différentes concentrations d'autres composants vasoactifs du cacao. En outre, les études administrant de l'epicatechine pure, donc sans influence d'autres composants vasoactifs, ont rapporté des résultats divergents. Il est suggéré que l'epicatechine exerce des effets plus prononcés grâce aux **interactions pharmacocinétiques et pharmacologiques avec d'autres flavonoïdes et composants du cacao**, tels que la théobromine. Par conséquence, compte tenu (1) de la biodisponibilité élevée dans le plasma d'autres composants vasoactifs du cacao, (2) des résultats incohérents des études axées sur les flavanols et (3) des conclusions divergentes des études sur l'administration d'epicatechine pure, on peut se demander si seuls les flavanols sont responsables des effets sur la santé vasculaire induits par le cacao.

En résumé, sur la base de cette recherche doctorale, aucune conclusion sur les effets des flavanols du cacao sur la santé vasculaire des patients diabétiques de type 2 n'a pu être formulée. Cependant, notre discussion générale souligne des points importants à prendre en considération. Nous pensons que non seulement les flavanols, mais aussi la combinaison des flavanols avec d'autres composants vasoactifs du cacao, comme la théobromine, sont principalement responsables des avantages pour la santé vasculaire. Des recherches futures étudiant successivement les effets des composants vasoactifs purs du cacao, suivies d'essais examinant la prise combinée de ces composants pour analyser leurs effets synergiques/ antagonistes/ complémentaires dans différentes populations bien décrites, sont nécessaires avant de pouvoir formuler des conclusions et des recommandations de santé.

Chapter 5: Curriculum vitae

1. About the author

Name Anouk Tanghe

Date of Birth April 5th, 1994

Education Secondary school: Regina Pacis (Hove), Sint-Gummariscollege (Lier)

1st grade: Latin

2nd grade: Latin-Mathematics 5h

3th grade: Sciences-Mathematics 6h

2006 – 2012

University education: University of Ghent

Master of Science in Rehabilitation Sciences and Physiotherapy
Main Subject Rehabilitation Sciences and Physiotherapy with Internal Diseases

(bachelor and master degree obtained with magna cum laude)

2012 – 2017

Doctoral education: University of Ghent and University of Lille

University of Ghent: Health Sciences

University of Lille: Sciences and Techniques of Physical and Sportive Activities

2017 – 2021

2. Personal reference list

Full papers in international journals with peer review

1. Vanden Wyngaert K, Van Craenenbroeck AH, Van Biesen W, Dhondt A, **Tanghe A**, Van Ginckel A, et al. The effects of aerobic exercise on eGFR, blood pressure and VO2peak in patients with chronic kidney disease stages 3-4: a systematic review and meta-analysis. PLoS One. 2018;13(9):e0203662.

2. **Tanghe A**, Heyman E, Wyngaert KV, Van Ginckel A, Celie B, Rietzschel E, et al. Evaluation of blood pressure lowering effects of cocoa flavanols in diabetes mellitus: a systematic review and meta-analysis. Journal of Functional Foods. 2021;79:104399.

3. **Tanghe A**, Celie B, Shadid S, Rietzschel E, Op't Roodt J, Reesink KD, et al. Acute Effects of Cocoa Flavanols on Blood Pressure and Peripheral Vascular Reactivity in Type 2 Diabetes Mellitus and Essential Hypertension: A Protocol for an Acute, Randomized, Double-Blinded, Placebo-Controlled Cross-Over Trial. Frontiers in cardiovascular medicine. 2021;8:152.

4. **Tanghe A,** Heyman E, Lespagnol E, Stautemas J, Celie B, Op 't Roodt J, Rietzschel E, Dias Soares D, Hermans N, Tuenter E, Shadid S*, Calders P* (*these authors contributed equally).

Acute Effects of Cocoa Flavanols on Blood Pressure and Peripheral Vascular Reactivity in Type 2 Diabetes Mellitus and Essential Hypertension. (will be submitted to Frontiers in cardiovascular medicine, *under embargo*)

National congress contributions

1. Belgian Nutrition Society (BNS) for the Annual BNS Meeting in Brussels (May 2019)

   The effects of flavonoids on blood pressure and vascular function in patients with diabetes mellitus: a Meta-Analysis

   Anouk Tanghe, Samyah Shadid, Elsa Heyman, Ernst Rietzschel, Bert Celie, Patrick Calders

   (Live, oral presentation)

International congress contributions

1. European and International Congress on Obesity (September 2020)

   Effects of a single dose of cocoa flavanols on blood pressure in patients with type 2 diabetes compared to non-diabetic people.

   Anouk Tanghe, Elsa Heyman, Ernst Rietzschel, Bert Celie, Samyah Shadid, Patrick Calders

   (poster presentation, online congress due to COVID-pandemic)

2. European Society for Clinical Nutrition and Metabolism (September 2020)

   Effects of a single dose of cocoa flavanols on blood pressure in patients with type 2 diabetes compared to healthy people.

   Anouk Tanghe, Elsa Heyman, Samyah Shadid, Ernst Rietzschel, Elodie Lespagnol, Bert Celie, Patrick Calders

   (poster presentation, online congress due to COVID-pandemic)

National symposia/ meetings

1. Presentation at the Research Day & Student Research Symposium UGhent (April 2019)

   Effects of flavanols on BP and vascular function in people with diabetes mellitus.

   Anouk SH Tanghe, Samyah Shadid, Bert Celie, Ernst Rietzschel, Ans Van Ginckel, Karsten Vanden Wyngaert, Patrick Calders

   (Live presentation)

2. Presentation at a postgraduate meeting (Lokaal overlegplatform) for endocirnilogists (June 2020)

   Acute effects of flavanols on peripheral vascular reactivity in patient with diabetes type 2, patients with essential hypertension, and healthy controls

   (Live online presentation due to COVID-pandemic)

3. Journée André Verbert (colloque des doctorants Biologie santé de Lille) (November 2020)

   Effects of a single dose of cocoa flavanols in blood pressure in patients with type 2 diabetes compared to healthy people.

   (video presentation due to COVID-pandemic)

3. Followed courses

1. How to get your paper accepted in a biomedical journal?
   - Cluster: Communication Skills
   - Organisation: Ghent University Doctoral School
   - Dates and venue: 13/2/2018, 27/2/2018, 6/3/2018 (3 x 2hrs)
2. Statistical analysis with the help of SPSS - Starters
   - Cluster: Research & Valorization
   - Organisation: Biostatistics Unit, University of Ghent
   - Dates and venue: 1/3/2018, 8/3/2018, 15/3/2018, 22/3/2018, 29/3/2018 (5 x 3hrs)
3. Advanced Academic English: Conference Skills - Academic Posters
   - Cluster: Communication Skills
   - Organisation: Ghent University Doctoral School
   - Dates and venue: 20/4/2018, 27/4/2018 (2x 3hrs)
4. Advanced Academic English: Writing Skills - Life Sciences and Medicine
   - Cluster: communication skills
   - Organisation: Ghent University Doctoral School – Dominique Neyt
   - Dates and venue: WEEKLY on Wednesday: starting 13 February, 2019 - ending 8 May, 2019 (10 x 2hrs)
5. Networking 2.0, discover your 'why' with Lego Serious Play
   - Cluster: career management
   - Organisation: Ghent University Doctoral School (teacher: braingain)
   - Dates and venue: 1/4/2019 (8hrs)
6. Conducting and Publishing a Systematic Review and Meta-Analysis
   - Cluster: Specialist course
   - Organisation: Het Kenniscentrum voor de GezondheidszorgGent
   - Dates and venue: Tuesday 19/11 + 26/11 + 3/12 + 10/12 + 27/12/2019 (5x 4hours)
7. PhD introduction day: How to stay sane during your PhD (7/2/2019)
8. A course to develop the skill to perform a Flow Mediated Dilatation (FMD)-measurement
   - Cluster: Specialist course
   - Organiser and teacher: Jos Op 't Roodt, specialist in FMD techniques

- Dates and venue: 20 contact hours

9. Module 10: Multilevel Analysis for Grouped and Longitudinal Data
   - Cluster: Specialist course
   - Organizer and teacher: IPVW-ICES, Prof. dr. Leoniek Wijngaards-de Meij
   - Dates and venue: Three consecutive full days: Wednesday April 7, Thursday April 8 and Friday April 9, 2021 from 9 am till 4 pm.

4. Teaching experience

I guided for 3 years master students (Rehabilitation Sciences: 1st and 2nd master, Medicine: 2nd and 3th master) to establish their master thesis.

I gave practical lessons for 3 years at students in 2nd year of the bachelor in Rehabilitation Sciences (physiotherapeutic actions at the upper and lower limb).

Chapter 6: Dankwoord

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