Cerebrovascular and peripheral vascular function in adults

Cerebrovascular and peripheral vascular function in adults: Effects of exercise training, soy nuts and inorganic nitrate

This research was performed at the department of Nutrition and Movement Sciences within the School of Nutrition and Translational Research in Metabolism (NUTRIM), as part of the Physiology of Human Nutrition (PHuN) research group. The research presented in this dissertation was part of a project organized and executed under the auspices of Top institute Food and Nutrition (TiFN), and was funded by the Netherlands Organization for Scientific Research (NWO). The study as described in Chapter 6 was partly funded by the Alpro Foundation.

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DISSERTATION

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CHAPTER 1

General introduction

Age is an important non-modifiable independent risk factor for developing cardiovascular disease (CVD), cognitive impairment, and dementia (1, 2). With advancing age, several modifiable traditional risk factors for these age-related conditions (e.g., hypertension, impaired glucose metabolism and dyslipidemia) play an important role (3). Reducing or even avoiding exposure to these risk factors by lifestyle modifications during aging results in a decrease in the development of CVD and cognitive impairment (4).

Cardiovascular disease

An estimated 17.9 million people died from CVD in 2016, which includes cerebrovascular (e.g., strokes) and coronary heart diseases (heart attacks), making it the leading cause of death worldwide (5). The underlying disease process that causes many forms of CVD is known as atherosclerosis. Atherosclerosis is a pathological process that involves deposition of lipid and cholesterol inside the lumen in the arterial walls which develops over many years. This will result in plaques, that make the blood vessel irregular, narrow, and stiffer. Early detection of CVD risk markers is important to prevent or attenuate the progression of the disease, as it may be reversable up to certain stages (6).

It is estimated that the risk for CVD can be reduced by 75% by addressing behavioral risk markers. Maintaining or incorporating a healthy diet and increasing physical activity offer the greatest potential of all known approaches for reducing the CVD risk in the general public (6). Traditional risk markers however often fail to explain the CVD risk reduction after a healthy lifestyle intervention. Vascular function markers address endothelial function and arterial stiffness, which are key mechanisms for the development in CVD (7). Most studies only investigated a specific part of the vasculature, while a complete picture of vascular function in the periphery and the brain is often lacking. Investigating these markers in different regions of vascular tree may therefore provide insight in the development and prevention of the age-related conditions.

Cognitive impairment

Around 50 million people worldwide are not able to remember, learn, concentrate, or make decisions in everyday life due to cognitive impairment (8, 9). This number is estimated to triple by 2050 (8, 9). Cognitive impairment ranges from mild to severe, starting with subjective cognitive decline a form of mild cognitive impairment, which is the self-reported experience of worsening or more frequent confusion or memory loss. If the decline in cognitive abilities is severe enough to interfere with daily life, it is defined as dementia. Cognitive impairment is the major cause of disability and dependency among older people worldwide. This has a devastating social impact on the individual and other relations, but also inflicts a heavy economic burden, which is estimated to rise to 2 trillion dollars annually by 2030 (8, 9). Moreover, cognitive impairment related deaths increased with 148% over the period from 1990 to 2016, which was the greatest increase of all diseases. In 2016 dementia was the fifth leading cause of death globally, accounting for 2.4 million deaths (5), mainly due population aging and growth.

The vasculature plays a critical role in the pathogenesis of many types of cognitive impairment, including vascular dementia. To maintain the brain's structural and functional integrity it requires constant supply of oxygen and nutrients, while metabolic waste products need to be removed through a complex network of blood vessels (10). However,

during aging the regulation of blood flow in the brain is affected, as global cerebral blood flow (CBF) decreases with approximately 0.45% to 0.50% per year in middle-aged and older adults (11). This decrease is associated with reduced cognitive performance (12).

Although no curative treatment exists, a healthy lifestyle, consisting of increased physical activity and a healthy diet, may delay the onset and the natural progression of the disease. In fact, physically active people were less likely to develop cognitive impairment (13). Based on previous literature, the World Health Organization (WHO) recommends at least 150 min of moderate-intensity aerobic physical exercise divided over several days for all adults to attenuate cognitive decline (8). Additionally, a healthy diet was associated with improved cognitive performance (14). Concerning individual foods and nutrients, consumption of fruit, vegetables and products, such as fish and nuts, containing high amounts of polyunsaturated fatty acids (PUFAs) were most consistently associated with reduced risk of dementia (8).

Vascular function and age-related health conditions

Different cerebrovascular and peripheral vascular function markers exist, which are associated with the risk to develop CVD and are increasingly acknowledged as important contributors to impaired cognitive performance and (12, 15). **Figure 1.1** provides a schematic overview of age-related cardiovascular disease and cognitive impairment which can progress to dementia with their common denominator vascular function. Lifestyle approaches may reduce the risk for developing these age-related conditions via changes in vascular function. CBF is a sensitive marker for cerebrovascular function, that can be measured using various techniques. Different peripheral vascular function markers have been associated with CVD risk and focus on several regions of the arterial tree, including central and peripheral vascular endothelial function, arterial stiffness, and retinal vascular structure. In addition, the more traditional cardiometabolic risk markers (e.g., blood pressure [BP], serum lipids, and glucose metabolism) can be determined. Due to

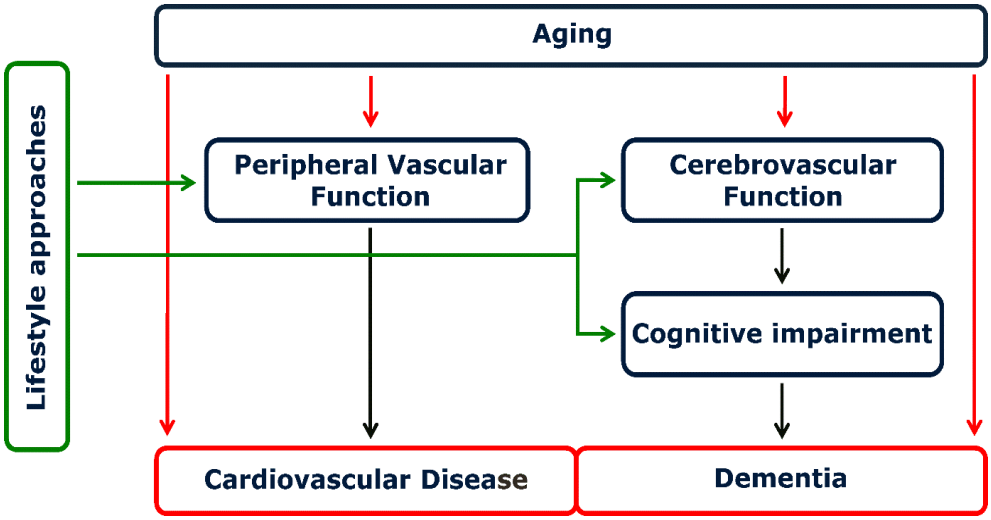

Figure 1.1. Schematic overview of the development of age-related cardiovascular disease and cognitive impairment which can progress to dementia with their common denominator vascular function.

technological advances, we can also measure blood pressure, glucose metabolism and activity at regular intervals during daily-life, which can be used to determine patterns and interactions. Although underlying mechanisms are unclear, it has been hypothesized that beneficial cerebrovascular effects play a role in the development of cognitive impairment. However, a limited number of well-executed trials has investigated the effect of healthy lifestyle factors on cerebrovascular function. Specifically, physical exercise training and dietary factors may reduce the risk for CVD and attenuate the age-related decline in cognitive performance (16-18).

Cerebrovascular function

The relatively high metabolic demand of the brain requires approximately 15% of the cardiac output, and the blood perfuses neurons in the brain via the carotid and vertebral arteries through a network of capillaries. Every neuron is supplied by its own capillary, resulting in a total length of approximately 600 km of vessels in the human brain. Autoregulation of the brain's blood vessels assures that blood flow matches the metabolic demands. This is particularly important as neurons do not have sufficient energy reserves and metabolic demands change rapidly. Additionally, high pressure and consequential microvascular damage have to be prevented. Functional impairment of these regulatory mechanisms plays a critical role in brain aging, which involves deterioration of brain structure, and the pathogenesis of cognitive impairment (10). Once the brain structure is affected, it cannot be restored, although functional beneficial effects are possible (19). Distinct deviation in regional CBF may be linked to the severity of impaired cognitive performance (20).

Multiple methods exist for the reliable assessment of CBF in humans. The most important are Positron Emission Tomography (PET), Magnetic Resonance Imaging (MRI), ultrasound, and Near-Infrared Spectroscopy (NIRS). The trials described in this thesis used MRI Arterial Spin Labeling (ASL) to quantify CBF, which is considered the non-invasive gold standard (21). This method uses radiofrequency pulses to endogenously label blood water that flows through the carotid and vertebral arteries. These arteries are labelled perpendicular based on an angiogram that is first acquired. Labelled images are obtained when the blood has reached the brain, while separate control images are acquired without prior labeling. The difference between control and labelled images provides a measure of labelled blood from arteries delivered to the tissue by perfusion. After calibration, this results in a three-dimensional CBF map of the brain (**Figure 1.2**). ASL MRI was extensively validated against other methods that used exogenous contrast agents, such as ^{15}O-labelled water positron emission tomography and has demonstrated good reproducibility (22). The ASL scans in this thesis followed the most recent recommendations by a consensus paper of the International Society for Magnetic Resonance in Medicine (22), which include pseudo-continuous ASL (PCASL). With this method, labeling occurs continuously as blood flows through a single labeling plane. After a post-labeling delay, the images are acquired with a spatial gap (**Figure 1.2A**). The PCASL sequence strategy provides superior labeling efficiency and the highest signal-to-noise ratio compared to other ASL methods (22).

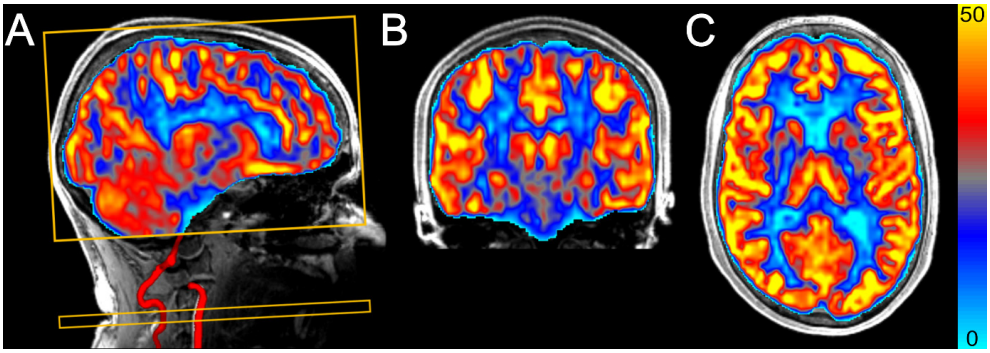

Figure 1.2. Example of perfusion-weighted image acquired using pseudo-continuous arterial spin labeling. The images show the cerebral blood flow in mL/100 g tissue/min (scale shown by color bar). (**A**) Sagittal slice including angiogram from vertebral and carotid artery, coronal and axial slice. The yellow rectangular boxes represent the imaging box and labeling plane perpendicular to the arteries. (**B**) Coronal slice. (**C**) Axial slice.

Peripheral vascular function

The endothelial monolayer connects the vasculature throughout the body, although the functional properties may differ between locations. The endothelium is a physical barrier, but also synthesizes and releases a wide range of active biological molecules that influence the function and structure of the arteries and surrounding tissues. One of the most important endothelial-derived molecules is nitric oxide (NO), which induces regional vasodilation in a paracrine fashion. Endothelial dysfunction in aging is largely due to inadequate NO bioavailability driven by increased oxidative stress and low-grade inflammation (23). Endothelial function of a muscular artery can be reliably assessed using the brachial flow-mediated dilation (FMD) test, which involves occlusion-induced hyperemia. Recently, carotid artery reactivity (CAR) in response to a cold pressor test has been proposed as a sensitive marker for endothelial function of a conduit artery in the central vasculature. The CAR response is determined by the balance between the effect of catecholamines released during the cold pressor on endothelial vasodilation and constriction of the smooth muscle cells. The change in arterial diameter during the FMD and CAR has been associated with the risk on CVD (24-26), but represent different mechanisms.

Increased arterial stiffness also contributes independently and significantly to the pathophysiology of age-related increases in CVD risk (27). Arterial stiffness in the central vasculature can be measured along the aorta by measuring the carotid-to-femoral pulse wave velocity ($PWV_{c\text{-}f}$) (28). Structural changes to the arterial wall combined with functional changes in vascular smooth muscle tone may contribute to the elevated arterial stiffness (23). Moreover, local carotid stiffness can be measured by using the beat-to-beat diameter change imaged with high frequency ultrasound. Carotid macrovascular structure can also be analyzed using these images, as it provides a direct view of the intimal and medial layers of the of the carotid arterial wall. The carotid intima-media thickness (cIMT) is considered as a marker of the early stages of atherosclerosis (29). Finally, fundus photography can be used to quantify the diameters of the retinal microvasculature, which are also associated with CVD (30).

Cognitive performance

The capabilities required to learn, think, reason, remember, solve problems, make decisions, and pay attention are referred to as cognitive performance. To determine how a healthy lifestyle affects cognitive performance, a battery of valid, reliable, and sensitive measures is required. Preferably, measures of cognitive performance should be valid surrogates for neural processes underlying major cognitive domains that correspond to challenges of everyday life. Many tests have been developed to determine performance in the main cognitive domains, including attention and psychomotor speed, executive function and memory (31). Tests of psychomotor speed relate to the ability to process relevant information rapidly and efficiently, as well as accurately and appropriately, which is classically assessed with a reaction time task with multiple choices. Executive function is involved in complex thinking processes and includes planning and response inhibition, which can be assessed with a variety of tests of which the Stroop test is the best known. Memory involves learning and recall of for example visual information. Tests usually involve images which need to be remembered. In this thesis we used the Cambridge Neuropsychological Test Automated Battery (CANTAB), which is a computerized, standardized, validated and sensitive method to detect in clinical trials changes in response to exercise and dietary interventions (32).

Glucose metabolism

Impaired glucose metabolism is a major comorbidity which is associated with CVD. Insulin is a key hormone that regulates cellular metabolism of many tissues. Imbalance in glucose metabolism caused by insulin resistance can induce changes in systemic lipid metabolism, due to inflammation and excessive availability of energy-rich substrates, which results in hepatic and intestinal lipoprotein overproduction leading to hypertriglyceridemia. The elevated concentration of triglyceride-rich lipoproteins may cause a decrease in the functionality of high density lipoproteins (HDL) (33) and a shift towards small dense low density lipoproteins (LDL) particles (34). This state of dyslipidemia relates to many metabolic disturbances such as an increased oxidative stress and increased inflammatory responses, which results in cell damage and contributes to atherosclerotic plaque formation (35). The hyperinsulinemic-euglycemic clamp is the gold standard for measuring whole-body insulin resistance, but is expensive, time consuming and difficult to perform. Alternatively, the oral glucose tolerance test (OGTT) is a validated test which includes ingestion of a standardized amount of glucose to determine how quickly it is cleared from the blood. Blood samples are taken at regular intervals to assess glucose and insulin concentrations, which can be used derive indexes for muscle and hepatic insulin sensitivity (36) and can be affected differently by an intervention (e.g. physical activity for muscle insulin sensitivity (37)).

Brain insulin-sensitivity

A decreased response to insulin in the brain is called brain insulin resistance, which is a shared pathological feature of metabolic and cognitive disturbances in obesity, type 2 diabetes mellitus and dementia (38). In the central nervous system glucose transport is independent of insulin, as glucose can enter the brain by diffusing across the blood-brain-barrier. Brain insulin action has multiple behavioral and metabolic effects, influencing eating behavior, peripheral metabolism and cognitive performance (38). Several insulin-

sensitive brain regions have been identified, such as the hypothalamus, which controls vital bodily functions such as food intake and fluid homeostasis. Additionally, it is speculated that the insulin response in the frontal cortex has an inhibitory role on food intake by reducing the rewarding properties of food via the striato-prefrontal pathways (38). Other regions include the striatum, linked to reward-mediated behavior, and the hippocampus including the neighboring gyri in the temporal lobe, which are important for memory performance (38).

High insulin responsiveness of the brain before a lifestyle intervention was associated with less visceral fat and more weight loss and maintenance during a lifestyle intervention (39). Visceral fat is strongly linked to CVD and related risk markers, and strategies to improve insulin resistance of the brain are thus required (39). No intervention studies have assessed the effect of healthy lifestyle components on brain insulin-sensitivity (38, 39). Inorganic nitrate has already shown beneficial effects on peripheral vascular function and some studies also observed increases in regional CBF (11). It may therefore improve brain insulin-sensitivity as well, which can be quantified by measuring the gray-matter cerebral blood flow (CBF) response to intranasally administered insulin, through beneficial effects on brain vascular function, which was investigated by a study included in this thesis.

Physical exercise and vascular function

Beneficial effects on physical fitness can be attained by regular aerobic exercise and increases the capacity of the cardiovascular system to take up and transport oxygen through the body. Men aged between 18 and 79 years that performed regular aerobic training had a 17% higher CBF compared to healthy, but sedentary matched participants (40), which was associated with beneficial effects on cognitive performance (41). It is suggested that the increased blood flow during exercise induces vascular shear stress, which upregulates endothelial NOS expression, leading to an increase in nitric oxide-dependent vasodilation and enhanced basal CBF (42). This theory is derived from research in the peripheral vasculature, as the protective mechanisms of exercise on the brain vasculature are thought to be the same. Indeed, aerobic exercise training has already shown beneficial effects on some markers of peripheral vascular function (43), but further research is still needed to study if these effects are also translated to the brain. Also, aortic stiffness and brachial endothelial function improved after aerobic training in older participants (23), whereas effects on local carotid stiffness were contradictory (27). The beneficial effects on peripheral vascular function were related to a reduced oxidative stress, inhibiting inflammation (23), and a more favorable cardiometabolic risk profile (44). However, the latter explains only 50% of the effects of exercise training on CVD risk. The remaining variance may be mediated by beneficial effects on the vasculature (44).

In the randomized, controlled, cross-over trial included in this study participants performed 50 min of continuous aerobic exercise training on a cycling ergometer three times per week for eight weeks. This complies to the Dutch physical activity guidelines and is also recommended by the World Health Organization for attenuation of cognitive impairment and CVD (6, 9). Additionally, we performed a systematic review which investigated the of exercise training on CBF. In this review we also discussed the relation

between these effects with potential changes in physical fitness, which is suggested as an important determinant of CBF (45) and cognitive performance.

Dietary factors and vascular function

Dietary factors are also involved in the prevention of CVD and cognitive impairment (9). The effect of the Mediterranean diet is the most extensively studied dietary approach, which has beneficial effects on CVD and cognitive performance (46, 47). This diet is characterized by high intakes of fruits, vegetables, cereals, legumes, and unsaturated fatty acids; moderate amounts of fish and dairy products; and low amounts of (red) meat and saturated fats. Dietary consumption of these individual components, such as fruit, vegetables and unsaturated fatty acids have also been demonstrated to be associated with cognitive decline (9, 46). The neuro-protective effects may relate to the ability of dietary components to reduce oxidate stress and inflammation (46).

Consumed nutrients impact the vasculature directly through transport to the body tissues. High loads of saturated fat or carbohydrate are classified as important stressors for the endothelium, whereas other nutrients, such as flavonoids, were beneficial (48, 49). Beneficial effects on CBF after acute and longer-term supplementation of dietary components, such as flavonoid-rich foods, unsaturated fatty acids and protein on CBF were also observed (49). In this thesis we investigated the effect of sixteen-week soy nuts consumption on CBF. Soy nuts are characterized by high amounts of isoflavones, unsaturated fatty acids and proteins. Additionally, dietary factors may improve cardiometabolic risk markers, such as blood pressure, or lipid and glucose metabolism, which may indirectly play a role for maintaining CBF, and the prevention of CVD and cognitive impairment (9, 50).

Important beneficial effects of peripheral vascular function have been observed following administration of inorganic nitrate (51). Additionally, inorganic nitrate is a potential therapeutic agent in insulin resistance via multiple mechanisms including reduced oxidative stress (51). In the mouth, nitrate (NO_3^-) can be reduced to nitrite (NO_2^-) by facultative bacteria from the dorsal surface of the tongue. Once in the blood, nitrite can be further converted into nitric oxide (NO) in the vasculature [9]. Several human intervention studies have observed promising acute effects of inorganic nitrate on measures of CBF (11). Therefore, we hypothesized that inorganic nitrate may have beneficial effects on brain insulin-sensitivity via improved brain vascular function, which has never been investigated before.

Outline of the thesis

This thesis aimed to investigate the effects of components of a healthy lifestyle, consisting of a healthy diet and increased physical activity levels, on primarily cerebrovascular function, but also on peripheral vascular function. In **chapter 2**, we first introduce the utility of ASL for the non-invasive (region-specific) quantification of CBF. Additionally, the potential link between CBF and structural brain status in older individuals is discussed. Thereafter, the current literature about the effects of exercise training on CBF using different measurement methods is systematically reviewed (**chapter 3**). The next two chapters describe the results of a randomized, controlled cross-over trial with aerobic exercise training in sedentary overweight and obese older men. In **chapter 4** we first report the

training-induced effects on CBF measured with ASL and cognitive performance in the domains of executive function, memory, and psychomotor speed. Additionally, post-load glucose responses as determined with an OGTT are reported. Next, the results on different peripheral vascular function and cardiometabolic risk markers are reported in **chapter 5**. In addition, several relevant determinants were measured during daily life are reported, including monitoring of ambulatory blood pressure (ABP), glucose profiles, and physical activity levels. The effects of longer-term soy consumption in older men and post-menopausal women on CBF, cognitive performance and glucose metabolism were also investigated in a randomized controlled cross-over trial. Results of this trial are described in **chapter 6**. In **chapter 7**, we investigated the acute effect of inorganic nitrate on the insulin-induced CBF responses in middle-aged abdominally obese men, which are a measure of brain insulin-sensitivity. Finally, **chapter 8** discusses the major findings of the chapters in this thesis within a broader perspective and includes possible future research directions.

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CHAPTER 2

Cerebral blood flow measurements in elderly using arterial spin labeling at 3T

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INTRODUCTION

The world's population aged 60 years or over is predicted to grow by 56% from 901 million to 1.4 billion by the year 2030 (1). It is well known that aging is associated with impaired cognitive performance and an increased risk for developing dementia. As a result, the social challenges and the burden for healthcare systems and on society directly related to increases in the aging population will become enormous. A healthy lifestyle – consisting of a healthy diet combined with increased physical activity levels – protects against cognitive impairment (2). Underlying mechanisms still remain unclear, but it has been considered that improving brain vascular health may be a key aspect (2-5). In this respect, effects on cerebral blood flow (CBF), which is defined as a sensitive physiological marker of brain vascular health (6, 7), are of major interest because compromised vascular health in the human brain most likely precedes the development of an impaired cognitive performance (8-10).

Normal aging is associated with a progressive decline in global CBF by approximately 0.45% to 0.50% per year in middle-aged and elderly adults (3, 11, 12), and these perfusion changes are strongly associated with changes in cognitive performance and the risk to develop dementia (3, 10, 13). Therefore, many trials have focused on middle-aged and elderly adults who are also at increased vascular risk, allowing for improvement through lifestyle-based intervention strategies. Furthermore, age-related changes in cognitive performance are mainly attributed to a decrease in gray matter CBF, which results in an suboptimal removal of metabolic waste products, and supply of oxygen and nutrients to the cerebral cortex (14). In fact, the mean gray matter CBF was 15% lower in late middle-aged subjects suffering from metabolic syndrome compared to age-matched healthy controls, and was also associated with lower cognitive performance (15). In addition, CBF in specific cognitive control brain regions, such as the fusiform gyrus (attention), hippocampus (memory) and prefrontal cortex (executive function), are also of major interest (16). Furthermore, hypoperfusion in the posterior cingulate cortex and precuneus (combines attention with information from memory and perception), which are part of the default mode network that is also associated with cognitive control, has been consistently observed in subjects with decreased cognitive performance (17).

Methods for measuring CBF in humans include Positron Emission Tomography (PET) and MRI techniques, such as Dynamic Susceptibility Contract (DSC), Dynamic Contrast Enhanced (DCE) and Arterial Spin Labeling (ASL) (3). PET measures the amount of ^{15}O-labeled water radiotracer delivered to the brain tissue by blood flow and is considered the current gold standard approach to quantify CBF (18, 19). However, the invasive nature, the requirement of an onsite cyclotron and large partial volume effects, which are caused by low spatial resolution, reduce the usefulness of this technique (19). Also, DSC and DCE are invasive as they employ injections of gadolinium-based contrast agents. As a promising alternative approach, ASL enables the non-invasive assessment of CBF using magnetically-labeled water molecules in arterial blood as an endogenous tracer. The signal difference between label and control images (with and without prior labeling of the arterial blood water) can be scaled to yield highly repeatable quantitative measures of CBF (6). In a recent systematic review, PET was compared with ASL to measure CBF. The work concluded that ASL is an appropriate alternative method for accurate and reproducible quantification of gray matter CBF (19).

In our recent review, we summarized the impact of specific dietary determinants and physical exercise on CBF, and examined the relation between these effects and potential changes in cognitive performance (3). We concluded that these lifestyle factors may increase CBF, thereby improving cognitive performance. However, well-designed intervention studies investigating the potential of diet and exercise on CBF are still warranted. Especially, longitudinal studies involving middle-aged and elderly adults at increased vascular risk, who are also known to be at increased risk of cognitive impairment and dementia, would be of major interest (3). Therefore, our current research at the Department of Nutrition and Movement Sciences at Maastricht University is focused on the long-term impact of specific dietary components and physical exercise on both CBF and cognitive performance. We primarily target overweight and slightly obese elderly while using ASL to quantify changes in (regional) CBF. For example, in one of our ongoing projects, we are conducting a tightly-controlled, progressive, intervention study investigating the specific effects of aerobic-based exercise training – three times a week at 70% maximal power for a total duration of eight weeks – on brain vascular health. Besides potential beneficial effects on CBF, a healthy lifestyle may also (i) preserve the structural integrity of white matter, (ii) protect from iron accumulation and (iii) attenuate brain atrophy (20-23). Therefore, fluid-attenuated inversion recovery (T_2-FLAIR), R_2* maps calculated from T_2*-weighted gradient-echo (GRE), and T_1-weighted magnetization-prepared rapid gradient-echo (MPRAGE) images are also obtained to assess structural status of the brain. These MRI-scans are used to visualize, for example, white matter signal abnormalities (T_2-FLAIR), iron accumulation (R_2*) and brain volume (MPRAGE). In fact, these brain changes are age-dependent, and are also associated with cognitive performance and the risk of developing dementia (24-29). A detailed description of this trial has been provided on ClinicalTrials.gov (NCT03272061).

The purpose of this article is to demonstrate the utility of ASL for the non-invasive (region-specific) quantification of CBF in elderly, and to discuss the potential link between CBF and structural brain status (e.g., presence of white matter signal abnormalities, iron accumulation and brain atrophy).

MATERIAL AND METHODS

Participant

During this ongoing study, multiple study participants will be scanned. Below, we present example data from one of them. The participant was a 67-year-old male with a body mass index (BMI) of 28.9 kg/m^2 without known medical complaints. He did not use medication prior to and during the study and was sedentary as assessed by the international physical activity questionnaire (IPAQ).

Data acquisition and sequence

Measurements were performed on a 3T MAGNETOM Prisma Fit MRI-system (Siemens Healthcare, Erlangen, Germany) using a 64-channel head-neck coil (Siemens Healthcare, Erlangen, Germany) at the Scannexus research facilities in Maastricht. The participant was placed head-first-supine in the scanner. The eye centers were taken as a reference for the magnet isocenter position, which was at the level of the pons to minimize B_0 offsets in the

labeling region. Furthermore, the labeling plane was positioned perpendicular to the carotid and vertebral arteries.

Perfusion-weighted images were acquired using pseudo-continuous arterial spin labeling (PCASL) with a background-suppressed 3D-GRASE readout (TR 4000 ms, TE 13.6 ms, GRAPPA 2, labeling duration 1750 ms, post-labeling delay 2000 ms and ten label-control repetitions, duration: 9 min). Nineteen slices with a voxel resolution of 3.0mm isotropic were acquired. PCASL was used because it has a higher temporal signal-to-noise ratio (tSNR) as compared to pulsed ASL (PASL) and continuous ASL (CASL) (30). In order to allow CBF quantification, an M_0 image without magnetization preparation and with a TR of 20s was also acquired (31).

A prototype PCASL sequence from Siemens Healthcare was used which includes an optimized background suppression containing four inversion pulses, which results in a tissue signal suppression >99% (32, 33). This strong background suppression guarantees both a reduced influence of physiological noise and an increased CBF tSNR without compromising temporal resolution or efficiency. The latter is especially useful in frail elderly populations, who are prone to head motion.

One high-resolution anatomical MPRAGE scan was also performed (TR 2400ms, TE 2.18ms, TI 1040ms, 1.0mm isotropic resolution, 8 degrees flip angle and 160 sagittal slices, duration 6min). In addition, a T_2-FLAIR image was acquired (TR 9000ms, TE 89ms, TI 2500ms, voxel size 1.0mm x 1.0mm x 3.0mm, 150 degrees flip angle and 50 axial slices, duration 3min). Furthermore, a GRE sequence with four echo times was obtained (TR 31ms, TE1 2.73ms, TE2, 7.65ms, TE3 13.61ms, TE4 21.86ms, voxel size 0.9mm x 0.9mm x 1.0mm, 12 degrees flip angle and 144 axial slices, duration 5min). The field of view across the various sequences was kept constant for accurate registration and anatomical localization.

Processing

A quantitative CBF map was estimated from the ASL-data using FSL software (http://fsl.fmrib.ox.ac.uk/fsl). Brain extraction, along with tissue segmentation was performed for the MPRAGE image using Volbrain (34). First, all ASL images and the corresponding M_0 image were realigned separately employing rigid-body co-registration to the middle ASL run using the FLIRT routine to correct for motion. Next, the mean difference between label and control images was calculated. Analysis was performed following the recommendations of the ASL White Paper and using the Bayesian kinetic inference method (31, 35, 36), the BASIL tool was used and voxel-wise calibration was performed with the M_0 image. Each of the four background suppression pulse has an efficiency of 0.93, which results in a cumulative labeling efficiency of 0.64. Blood hemoglobin concentrations (ctHb) were also determined as the kinetic model inversion depends on the T_1 of blood. The T_1 of blood can be estimated based on the ctHb using the following equation: $1000/T_{1a}$ (ms) = 0.016 x ctHb (g/dL) + 0.317 (37). The used T_1 of gray matter was 1330 ms, while for bolus arrival time 1300 ms was assumed. Spatially regularized partial volume correction was performed according to the paper of Zhao et. al. (38). As an alternative method, the CBF in each voxel could also be calculated for PCASL using the formula as proposed by Alsop et. al. (31):

$$CBF = \frac{6000 * \lambda * (SI_{control} - SI_{label}) * e^{\frac{PLD}{T_{1,blood}}}}{2 * \alpha * T_{1,blood} * SI_{PD} * (1 - e^{-\frac{\tau}{T_{1,blood}}})}$$ (mL/100g tissue/min)

Where λ is the brain/blood partition coefficient in mL/g, $SI_{control}$ and SI_{label} are the time-averaged signal intensities in the control and label images respectively, $T_{1,blood}$ is the longitudinal relaxation time of blood in seconds, α is the labeling efficiency, SI_{PD} is the signal intensity of a proton density weighted image, and τ is the label duration (31).

The final ASL image containing CBF values in mL/100g tissue/min was co-registered using Affine transformation to the brain extracted MPRAGE image using the FLIRT routine. The mean gray matter CBF was calculated by taking the mean CBF over the gray matter mask (with a threshold of 0.6). The regional gray matter CBF was calculated using the Oxford-Harvard atlas, which was co-registered using the affine transformation matrix from the Montreal Neurological Institute (MNI) to MPRAGE image. T_2* maps were obtained from the multi-echo GRE data using a mono-exponential fit ($f(TE) = S_0 e^{-TE/T_2*}$). In a subsequent step, all T_2* values above 500ms were set to 0 and the R_2* map (= $1/T_2$*) was obtained. T_2-FLAIR and R_2* images were also co-registered to the MPRAGE image using Rigid Body Transformations (FLIRT routine).

RESULTS AND DISCUSSION

Mean gray matter CBF has been reported to be significantly lower in apparently healthy elderly as compared to younger subjects (n=37, mean age: 72.1 ± 8.7 years; 42.7 ± 8.8 mL/100 g/min vs. n=11, mean age: 29.3 ± 5.3 years; 52.6 ± 9.3 mL/100 g/min) (39). The case presented in this article had a mean gray matter CBF of 28.7 mL/100 g/min, which may partly be explained by his high BMI (28.9 kg/m2) that falls in the overweight range and by his sedentary behavior. **Figure 2.1** shows CBF maps representing the local brain perfusion, which has been quantified for each voxel in mL/100 g tissue/min. Since our main outcome is gray matter CBF, we masked the remaining brain tissues as illustrated in the lower two rows. Also, regional CBF in, for example, the precuneus that is strongly dependent on age (40) was found to be significantly lower in the elderly compared to the young population (43.3 ± 11.5 mL/100 g/min vs. 51.4 ± 8.7 mL/100 g/min) (39). The presented case showed a CBF in the precuneus region of 24.4 mL/100 g/min. Interestingly, the T2-FLAIR showed the presence of white matter signal abnormalities (**Figure 2.2.A**) that can be classified as periventricular and deep white matter hyperintensities (41). In addition, the high R2* showed iron accumulation in deep gray matter regions (**Figure 2.2.B**), while the MPRAGE image demonstrated ventricular enlargement and brain atrophy (**Figure 2.2.C**).

White matter signal abnormalities and iron accumulation in deep gray matter areas have been associated with aging, cognitive performance, and dementia (26, 27). Age-related patterns of brain atrophy have also been linked to cognitive performance and the risk of dementia (28). Furthermore, ventricular enlargement has been suggested to be an age-dependent risk marker for cognitive decline (42). A potential relationship between CBF and structural brain status has been suggested. Reduced gray matter CBF may be associated with subcortical brain atrophy in the presence of white matter signal abnormalities (43), and may also relate to iron accumulation (27). In fact, gray matter CBF was associated with white matter signal abnormalities independent of age. However, the decline of CBF with advancing age may also possibly exacerbate deterioration of white matter integrity (44-46).

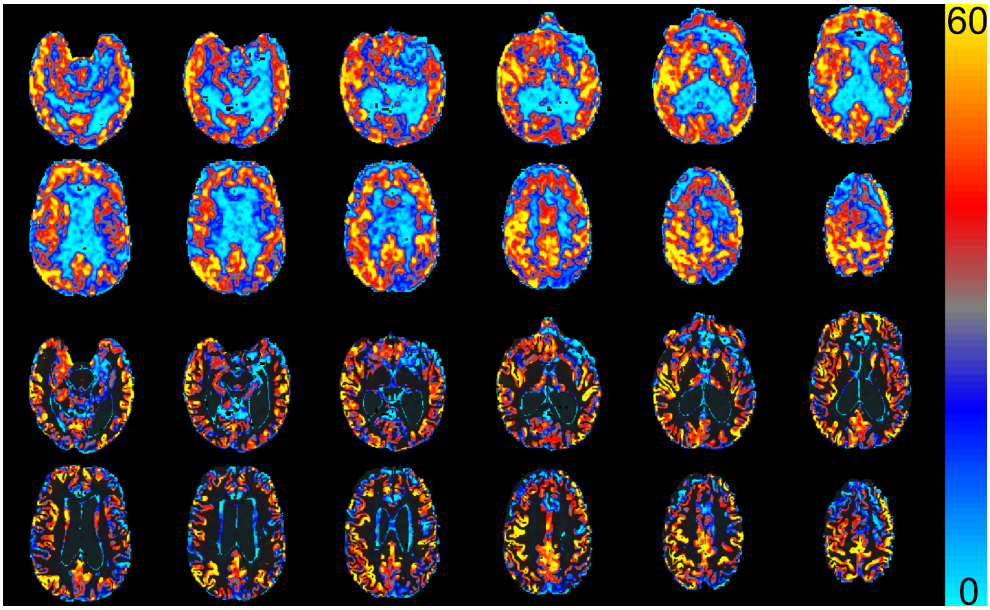

Figure 2.1. Cerebral blood flow (CBF) maps illustrating the amount of blood flow in a particular region of the brain in mL/100 g tissue/min (scale shown by color bar). The lower two rows depict gray matter CBF, while other brain tissues are masked.

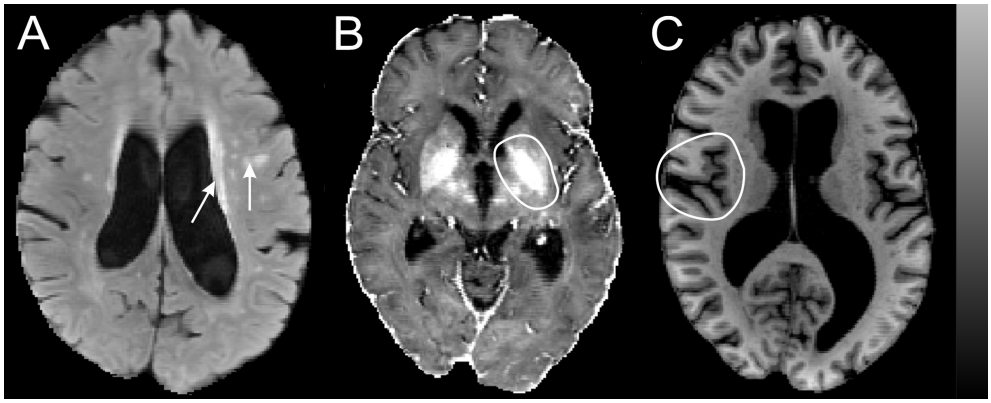

Figure 2.2. (**A**) T2-FLAIR, (**B**) R2* maps calculated from T2*weighted gradient-echo (GRE) and (**C**) MPRAGE images showing structural brain status. White matter signal abnormalities and iron accumulation appear bright (i.e., hyperintense) on the T2-FLAIR image and the R2*-weighted gradient-echo image, respectively. The MPRAGE shows a decrease in brain volume and ventricular enlargement.

Additionally, iron accumulation may plausibly reflect reduced CBF (47), due to a reduced delivery of iron binding complexes to the brain (48). In contrast, others have reported that age-associated reductions in regional CBF may be independent of concurrent age-dependent brain atrophy. Therefore, CBF can remain unaltered in regions of brain atrophy (39). These studies demonstrate a potential dissociation between brain atrophy and hypoperfusion specific to normal aging.

The development of the aforementioned structural brain changes is relatively slow and mostly without noticeable onset of symptoms linked to dementia. In fact, decreases in

cognitive performance may be determined by the severity of these structural brain changes. Cognitive impairment may remain asymptomatic until structural brain changes have affected a significant proportion of the brain (29). However, the causal relationship between CBF and structural brain changes still remains unclear. Therefore, innovative research investigating a potential causal relationship between lifestyle-induced structural brain changes and changes in CBF – using different MRI modalities – is urgently needed.

In conclusion, a healthy lifestyle may attenuate the age-related cognitive decline by improving CBF, which serves as a sensitive physiological marker of brain vascular health, maintaining sufficient CBF throughout the brain. Additionally, CBF can be quantified using ASL - an accurate and reproducible non-invasive MRI method - and may be associated with structural brain status assessed by T_2-FLAIR, R_2* maps and MPRAGE images.

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CHAPTER 3

Effects of physical exercise training on cerebral blood flow measurements: A systematic review of human intervention studies

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Submitted

ABSTRACT

Background

This systematic review aims to give an overview of the effects of physical exercise training on cerebral blood flow (CBF), which is an important physiological marker of cerebrovascular function in humans and positively associated with cognitive performance. Magnetic Resonance Imaging (MRI), transcranial doppler ultrasound and Near-Infrared Spectroscopy (NIRS) were used to measure different markers of CBF. In addition, the relation between training-induced effects on CBF with changes in physical fitness and cognitive performance will be discussed.

Methods

Potentially relevant studies were identified by a systematic search of the online-databases Medline (PubMed), EMBASE (Scopus) and CENTRAL (Cochrane Central Register of Controlled Trials) till the end of 2020. Thirty-five human intervention studies with experimental, quasi-experimental or pre-post designs were included. Thirteen studies that investigated effects of physical exercise training on CBF used MRI, fourteen studies transcranial doppler ultrasound, and eight studies NIRS.

Results

MRI studies observed consistent increases in CBF in the anterior cingulate cortex, but not in global CBF or regions within the medial temporal lobe. Effects on resting CBF as measured using transcranial doppler ultrasound and NIRS were variable, while middle cerebral artery blood flow velocity increased in response to exercise or hypercapnic stimuli. Interestingly, concomitant changes in physical fitness and regional CBF were observed, while a relation between training-induced effects on CBF and cognitive performance was also evident.

Conclusion

Physical exercise training may improve cerebrovascular function as regional CBF was changed, and these effects may underlie the beneficial effects observed on cognitive performance.

INTRODUCTION

It is well-known that human aging is associated with an increased risk to develop subjective cognitive decline (SCD), mild cognitive impairment (MCI) and ultimately (vascular) dementia (1, 2). Further, it is known that a healthy diet combined with exercise training may protect against cognitive impairment (1, 3). Underlying mechanisms are largely unknown, but lifestyle-induced beneficial effects on cerebral blood flow (CBF), which is an important physiological marker of cerebrovascular function and positively associated with cognitive performance (3-6), are thought to play an important role. In fact, several dietary approaches have already been identified that may beneficially affect CBF and cognitive performance. Exercise training may also improve cognitive performance (7, 8), but intervention studies on the effects of physical exercise training on CBF have not been reviewed so far.

Various techniques can be used to measure CBF, which makes it difficult to compare results between studies. The most frequently used imaging techniques are Positron Emission Tomography (PET), Magnetic Resonance Imaging (MRI), transcranial doppler ultrasound and Near-Infrared Spectroscopy (NIRS) (9). In brief, CBF as quantified by PET is considered to be the gold standard approach and involves intravenous injection of a radioactive contrast agent that diffuses through the blood-brain barrier. However, only a limited number of studies have used PET due to radiation exposure, while repeated scans are frequently needed. Perfusion-weighted MRI requires the intravenous injection of a non-diffusible contrast agent, such as gadolinium. Another reliable non-invasive alternative is the MRI perfusion method Arterial Spin Labeling (ASL) (10), which relies on magnetically-labeled water molecules from the blood flowing through the major arteries towards the brain. These scans result in a quantitative three-dimensional map of CBF providing information on the blood flow in a specific region or the entire brain. Transcranial doppler ultrasound is another technique that non-invasively measures blood flow velocity in the basal arteries of the brain (e.g., proximal anterior and posterior cerebral arteries). Finally, NIRS is an optical technique assessing the concentration of (de)-oxygenated hemoglobin in the cerebral cortex superficially through the scalp, which provides an indirect measure of brain activity, particularly in the pre-frontal cortex.

The aim of this systematic review is now to give an overview of the effects of physical exercise training on CBF in humans as measured by MRI, transcranial doppler ultrasound and NIRS. In addition, we will discuss the relation between these effects with potential changes in physical fitness and cognitive performance.

METHODS

Search strategy

The online-databases Medline (PubMed), EMBASE (Scopus) and CENTRAL (Cochrane Central Register of Controlled Trials) were searched until the end of 2020 to identify relevant research articles. The search terms consisted of “exercise [MeSH term]” AND “cerebrovascular circulation [MeSH term] OR cerebral oxygenation”. The complete search string for the different databases is presented in **Supplementary material**. All articles were imported into a reference manager (EndNote X9) and checked for duplicates. Remaining articles were imported into a systematic review manager (www.covidence.org).

Study selection

Only original human intervention studies, which investigated the relationship between exercise training and CBF using experimental (randomized controlled trials [RCTs]) or non-RCT's, such as quasi-experimental and pre-post designs, were included. Conference papers, posters and reviews were excluded. Articles describing the same intervention study in more than one paper were considered as one study. Only studies with an intervention period of at least one week were included. Studies combining exercise training with a dietary and/or cognitive co-intervention were also included. Articles were independently assessed for eligibility by two of the authors (J.K. and P.J.J.). When inconclusive, eligibility was discussed until consensus was reached. Finally, reference lists of the included articles and related reviews were also checked via a manual approach for relevant articles.

Data collection

Information on the study design (experimental or quasi-experimental), intervention (i.e., type and length of exercise intervention, frequency and duration of training sessions, intensity, and modality) and study population (health status, age, body mass index [BMI] and gender) was extracted and entered into a custom-made database. Data on CBF as assessed using MRI, transcranial doppler ultrasound and NIRS were also collected. Although studies were not required to assess physical fitness levels and cognitive performance (i.e., global cognition, psychomotor speed, verbal fluency, verbal and spatial memory, and executive function), information on these outcome were included in the database if reported.

RESULTS

Study characteristics

The PRISMA flow diagram is shown in **Figure 3.1**. The initial search returned 3756 articles. After removing duplicates, titles, and abstracts of 3379 articles were screened, and 409 articles were retrieved for review of the full texts. In the end, 38 articles met all the inclusion criteria. However, two intervention studies were described in five articles (11-15). Finally, 35 original intervention studies were identified, of which thirteen used MRI (including one study with PET), fourteen transcranial doppler ultrasound, and eight NIRS. Nineteen RCTs were included (MRI: n=8 (11, 16-22); transcranial doppler ultrasound: n=7 (15, 23-28); and NIRS: n=4 (29-32)) that involved a control group, which performed no exercise or activities not affecting fitness levels (e.g. yoga or stretching). The remaining sixteen studies (MRI: n=5 (33-37); transcranial doppler ultrasound: n=7 (38-44); and NIRS: n=4 (45-48)) had a quasi-experimental design, because a (suitable) control group was missing.

The type of intervention and the study population are detailed in **Table 3.1** for MRI studies, **Table 3.2** for transcranial doppler ultrasound studies and **Table 3.3** for NIRS studies. The exercise training arms included continuous exercise (MRI: n=6 (11, 18, 22, 33, 35, 36); transcranial doppler ultrasound: n=9 (15, 23, 25, 27, 28, 38, 39, 42, 43); and NIRS: n=3 (29, 31, 32)) or high-intensity interval training (HIIT; MRI: n=1 (19); transcranial doppler ultrasound: n=2 (28, 40); and NIRS: n=3 (29, 31, 45)) or a combination of different types of exercise (MRI: n=6 (16, 17, 20, 21, 34, 37); transcranial doppler ultrasound: n=3 (26, 41, 44); and NIRS: n=3 (30, 46, 47)). The exercise protocol progressed in frequency, duration and/or intensity in most studies. The progression was either standardized for the whole group or

individualized for each study participant. Studies controlled the intensity levels of aerobic exercise based on the heart rate reserve (HRR), maximum heart rate (HRmax), maximal power (Pmax) or rating of perceived exertion (RPE). The intensity of resistance training was based on the one repetition maximum (1RM) and the number of repetitions.

Magnetic Resonance Imaging

The median for the intervention duration was sixteen weeks (range: 2 – 541 weeks), and for the exercise frequency and duration three days/week (range: 2 – 7 days/week) and 60 min (range: 20 – 60 min), respectively. The median sample size was eighteen participants per study arm (range: 5 – 157). Study participants had a median age of 68 years (range: 33 – 81 years) and their median BMI was 26.7 kg/m^2 (range: 24.5 – 36.3 kg/m^2).

Transcranial doppler ultrasound

The median intervention duration was twelve weeks (range: 6 – 52 weeks) and the frequency of the exercise sessions was three days per week (range: 2 – 5 days per week). The median duration of these sessions was 50 min (range: 30 – 60 min). The median sample size per study arm was 10 (range: 4 – 42). The median age of included participants was 54 years (range: 11 – 69 years) and their median BMI was 25.3 kg/m^2 (range: 19.2 – 34.9 kg/m^2).

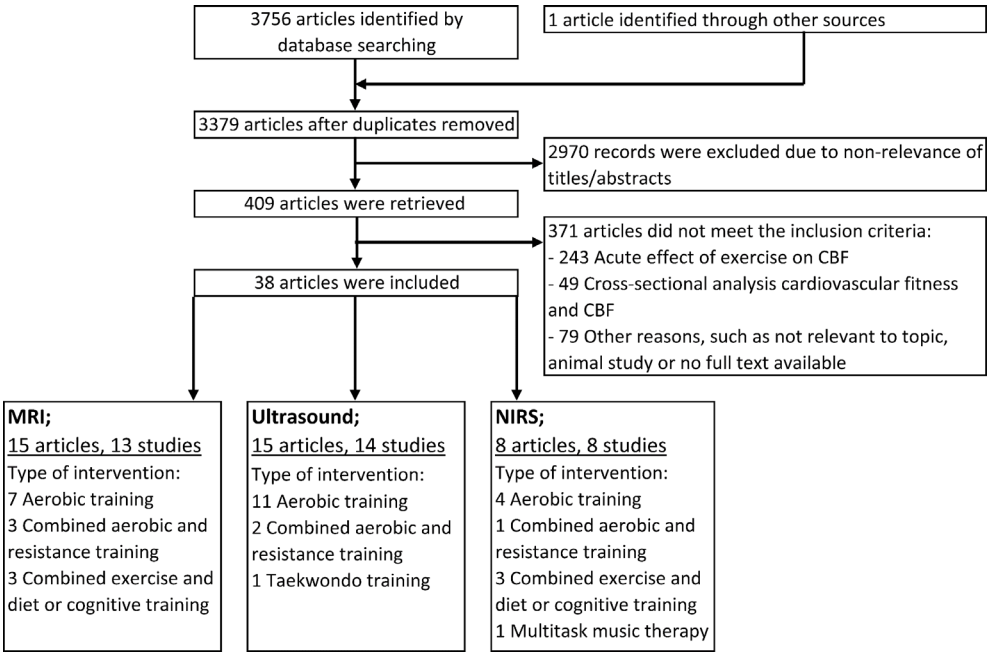

Figure 3.1. Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram of each stage of study selection. CBF: cerebral blood flow; MRI: Magnetic Resonance Imaging; NIRS: Near-Infrared Spectroscopy.

Table 3.1. Characteristics of included studies that used Magnetic Resonance Imaging.

Author, year	Health status	Study Population n	Age (y)[1]	BMI (kg/m²)[1]	F (%)	Intervention Type	wk	d/wk	Duration (min)
Aerobic exercise training									
Alfini, 2019 (33)	Healthy	18	77 ± 7	N/A	82	CE	12	4	30
	MCI	17	81 ± 6	N/A	60				
Burdette, 2010 (35)	Healthy	5	74 ± 3	28 ± 2	0	Control	16		
		6	78 ± 5	27 ± 6	50	CE		4	40
Chapman, 2013, 2016, 2017 (11-13)	Healthy, sedentary	19	64 ± 4	26 ± 3	74	Control			
		18	64 ± 4	28 ± 5	72	CE	12	3	60
		18	62 ± 3	26 ± 4	56	CT		3	60
Kleij van der, 2018 (22)	Alzheimer's disease	24	69 ± 7	N/A	41	Control	16		
		27	68 ± 7	N/A	37	CE		3	60
Kleinloog, 2019 (18)	Healthy, sedentary	17	67 ± 2	30 ± 4	0	Control and CE	8	3	50
Maass, 2015 (19)	Healthy	19	68 ± 4	25 ± 3	58	SC	12	2	45
		21	69 ± 5	25 ± 3	52	HIIT		3	30
Pareira, 2007 (36)	Healthy	11	33	N/A	82	CE	12	4	60
Combined aerobic and resistance exercise training									
Anazodo, 2016 (34)	Healthy	21	59 ± 8	25 ± 3	29	CE and RE	24	3	CE: 30, RT: N/A
	CAD	17	59 ± 6	30 ± 5	29				
Moore, 2014 (20)	6 months post stroke	20	70 ± 11	26 ± 4	20	SC	19	3	CE: 45-60, RT: N/A
		20	68 ± 8	26 ± 4	10	CE and RE			
Robertson, 2017 (37)	3 months post stroke	8	67 ± 11	27 ± 11	25	CE and RE	CE: 24, RE: 16	CE: 5, RT: 2	CE: 20-60, RT: N/A
Combination of exercise training and diet or cognitive training									
Espeland, 2018 (17)	T2DM	153	59 ± 7	36 ± 0	66	EC	541		
		157	58 ± 6	35 ± 0	73	HL		3	60
Train the Brain consortium, 2017 (16)	MCI	50	75 ± 4	N/A	45	Control	28	3	60
		53	74 ± 5	N/A	53	CT, CE and RT			
Small, 2006 (21)	Healthy	9	53 ± 10	N/A	67	Control	2		
		8	54 ± 12	N/A	63	HL		7	40-45

[1]Data are shown as mean ± SD. CAD: coronary artery disease; CE: continuous exercise; CT: cognitive training; d/wk: days per week; EC: educational control; F: female; HL: healthy lifestyle; HIIT: high-intensity interval training; MCI: mild cognitive impairment; RT: resistance training; SC: stretching control; T2DM: type 2 diabetes mellitus; wk: week.

Table 3.2. Characteristics of included studies included that used transcranial doppler ultrasound.

		Study Population				Intervention			
Author, year	**Health status**	**n**	**Age (y)[1]**	**BMI (kg/m²)[1]**	**F (%)**	**Type**	**wk**	**d/wk**	**Duration (min)**
Aerobic exercise training									
Akazawa, 2012 (23)	Healthy	10 10	51 ± 2 56 ± 3	25 [2] 25 [2]	100	Control CE	8	3-5	47 ± 4
Akazawa, 2018 (38)	Healthy, sedentary	10	62 ± 4	22 [2]	80	CE	12	5	30-45
Bailey, 2016 (2x) (14, 15)	Healthy	7 14	52 ± 6 52 ± 4	28 ± 7 29 ± 6	100	Control CE	16	3-5	30-45
Bailey, 2016 (39)	Healthy, trained	9 9	26 ± 5	24 ± 4	100	CE WI	8	3	30
Drapeau, 2019 (40)	Healthy, trained	8 9	26 ± 6 28 ± 6	23 [2] 24 [2]	0	HIIT	6	3 3	max
Hata, 1998 (25)	Healthy	10 10	23 ± 2 23 ± 1	21 ± 1 19 ± 2	100	Control CE	≥52	3-5	50
Ivey, 2011 (27)	6 months post stroke	19 19	62 ± 10 61 ± 8	26 ± 5 28 ± 6	42 42	Control CE	24	3	40
Lewis, 2019 (41)	Healthy COPD	20 23	64 ± 5 69 ± 7	26 ± 3 28 ± 3	50 43	CE and HITT	8	3	20-45
Murell, 2013 (42)	Health, sedentary	10 10	23 ± 5 53 ± 5	26 ± 3 25 ± 3	50	CE	12	3	20-50
Northey, 2019 (7)	Breast cancer survivors	6 5 6	62 ± 8 68 ± 7 60 ± 8	28 [2] 25 [2] 25 [2]	100	Control CE HIIT	12	3	20-30
Stanek, 2011 (43)	CVD	42	68 ± 9	30 ± 6	33	CE	12	3	60
Combined aerobic and resistance exercise training									
Heli, 2013 (26)	Metabolic syndrome	4 6	56 ± 6 63 ± 10	35 ± 6 31 ± 6	100	EC HIIT and RE	16	2	HIIT: 25-45, RT: 30
Tomoto, 2015 (44)	Healthy	13	N/A	21 ± 8	46	CE, HIIT and RT	16	N/A	N/A
Alternative exercise									
Cho, 2017 (24)	Healthy	15 15	11 ± 1 11 ± 1	22 ± 4 21 ± 2	40 40	Control TKD	16	5	60

[1]Data are shown as mean ± SD. [2]Calculated manually based on the length and weight. CE: continuous exercise; COPD: chronic obstructive pulmonary disease; CVD: cardiovascular disease; d/wk: days per week; EC: educational control; F: female; HIIT: high intensity interval training; RT: resistance training; TKD: taekwondo; wk: week; WI: water immersion.

Table 3.3. Characteristics of included studies that used Near Infrared Spectroscopy.

Study Population						Intervention			
Author, year	**Health status**	**n**	**Age (y)[1]**	**BMI (kg/m²)[1]**	**F (%)**	**Type**	**wk**	**d/wk**	**Duration (min)**
Aerobic exercise training									
Caen, 2019 (45)	Healthy, trained	11	22 ± 1	25.9 [2]	0	HIIT	6	3	49
Fu, 2011 (29)	CVD	13	68 ± 3	24.6 [2]	31	Control	12	3	30
		13	66 ± 2	24.5 [2]	38	CE			
		14	68 ± 2	24.5 [2]	36	HIIT			
Tsai, 2016 (31)	Healthy, sedentary	20	22 ± 1	23 ± 1	0	Control	6	5	30
		20	22 ± 1	22 ± 1		CE			
		20	22 ± 1	22 ± 1		HIIT			
Wang, 2010 (32)	Healthy	12	23 ± 1	24 ± 1	0	Normoxic control	4	5	30
		12	21 ± 0	22 ± 1		Hypoxic control			
		12	21 ± 1	23 ± 1		Normoxic CE			
		12	23 ± 1	23 ± 1		Hypoxic CE			
Combined aerobic and resistance exercise training									
Drigny, 2014 (46)	Healthy	6	49 ± 8	30 ± 1	N/A	CE, HIIT and RT	16	CE: 1, HIIT: 2, RT: 2	CE: 60, HIIT: 34-48, RT: 20
Pollock, 2019 (47)	Parkinson	12	66 ± 10	N/A	70	CE and RT	8	3	CE: 30, RT: 30
Combination of exercise training and diet or cognitive training									
Hamasaki, 2019 (30)	Healthy	18	63 ± 6	22 ± 3	N/A	Control + placebo	8	4-6	40-45
		15	66 ± 6	21 ± 3		Control + LTP			
		16	63 ± 4	22 ± 2		CE + placebo			
		15	66 ± 8	22 ± 3		CE + LTP			
Alternative exercise									
Shimizu, 2018 (48)	Healthy	9	73 ± 7	N/A	91	single-task	12	1	60
		30	75 ± 4	N/A	82	multitask			

[1]Data are shown as mean ± SD. [2]Calculated manually based on the length and weight. CE: continuous exercise; CVD: cardiovascular disease; d/wk: days per week; F: female; HIIT: high intensity interval training; LTP: lactotripeptide; RT: resistance training; wk: week.

Near-Infrared Spectroscopy
The median duration of the interventions was eight weeks (range: 4 – 16 weeks) and the frequency of exercise sessions three days per week (range: 1 – 6 days per week). The median duration of exercise sessions was 30 min (range: 30 – 60 min). The median age of the subjects was 56 years (range: 21 – 75 years) and for the BMI 22.7 kg/m^2 (range: 21.0 – 29.7 kg/m^2).

Effects on cerebral blood flow
Physical exercise training-induced effects on CBF as measured by MRI, transcranial doppler ultrasound and NIRS are summarized in **Table 3.4**.

Magnetic Resonance Imaging
Global CBF increased in two studies that used MRI (17, 37), while in six other studies no differences were observed (11-13, 18-20, 22, 34). Studies that focused on differences in blood flow in brain lobes observed an increased CBF in the limbic (17), occipital (17), medial temporal (20) and parietal lobe (37). Analyses of specific brain regions within the frontal lobe showed increases in CBF in the anterior cingulate gyrus (11, 18, 33, 34) and in the inferior frontal gyrus (33), while CBF was decreased in the left dorsolateral prefrontal cortex (21). CBF within specific regions of the medial temporal lobe also changed. Specifically, blood flow was increased in the hippocampus (35) and dentate gyrus (36), and decreased in the hippocampus CBF (19). Finally, CBF levels increased in the parahippocampal (16) and decreased in the temporal fusiform gyrus (18), which are both located in the temporal lobe.

Transcranial doppler ultrasound
Three studies that used transcranial doppler ultrasound to quantify resting CBF observed an increased middle cerebral artery (MCA) velocity (MCAv) (15, 23, 39), while ten studies observed no differences (24, 26-28, 38, 40-44). Although MCAv did not change, Stanek and colleagues reported training-induced increases in the anterior cerebral artery (ACA) velocity (ACAv) (43). The cerebrovascular conductance (CBVC), which is calculated as the mean arterial pressure divided by the MCAv, increased in one study (39), decreased in another study (23), and did not change in four studies (15, 24, 39, 40, 42). Three studies that measured cerebrovascular resistance (CVR; MCAv divided by the mean arterial pressure) did not observe differences (27, 40, 42). Finally, an increased pulsatility index (PI), calculated as the difference between peak systolic and end-diastolic flow velocities divided by the mean velocity, was observed of the ophthalmic artery (OCA) in one study (25). Five studies measured the PI of the MCA (23, 24, 38, 43, 44) and ACA (43), but no differences were observed.

Exercise training reduced the PI of the MCA 30 min after an acute exercise trigger (38). Also, dynamic cerebral autoregulation (dCA) of the MCA increased during exercise in one study (40), but no changes were observed in another study (41). Finally, two studies showed an increased CBF of the MCA in response to hypercapnia (i.e. the cerebral vasomotor reactivity [cVMR]) (27, 42), but the cVMR of the MCA did not significantly change following hypocapnia in three studies (28, 41, 42).

Near-Infrared Spectroscopy

CBF as measurement by NIRS during exercise resulted in a training-induced increase in total hemoglobin (totHb) in three study groups with HIIT training (29, 31, 45), while for two of these studies no differences in totHb were observed in the continuous exercise group (29, 31). Another study did also not observe changes in totHb after continuous exercise (32). Oxygenated hemoglobin (O_2Hb) increased in one study (45), whereas no changes were reported in four other studies (29, 31, 32, 46). Only one study observed an increased deoxygenated hemoglobin during exercise training (HHb) (46), but no significant differences were found in four studies (29, 31, 32, 45). The difference between O_2Hb and HHb that is defined as the cerebral tissue oxygenation index (cTOI) was increased in one study (45). Finally, totHb did not change during a cognitive task in one study (47), while two other studies did observe an increased O_2Hb (30, 48).

Table 3.4. Summary of exercise training-induced effects on CBF as measured with Magnetic Resonance Imaging, transcranial doppler ultrasound and Near-Infrared Spectroscopy.

	Increased	**Decreased**	**No difference**	**Not assessed**
Magnetic Resonance Imaging				
Global cerebral blood flow	2	-	6	6
Regional cerebral blood flow[1]	10	4	2	-
Transcranial doppler ultrasound				
MCA velocity	3	-	10	1
ACA velocity	1	-	-	13
CBVC	1	1	4	8
CVR	-	-	3	11
PI MCA	-	-	3	11
PI ACA	-	-	1	13
PI OCA	1	-	-	13
PI MCA 30min post-exercise	-	1	-	13
dCA		1	1	12
cVMR	2	-	2	10
Near-Infrared Spectroscopy				
During exercise				
totHb	3	-	3	1
O_2Hb	1	-	4	2
HHb	1	-	4	3
cTOI	1	-	-	7
During cognitive task				
totHb	-	-	1	7
O_2Hb	2	-	-	6

[1]Studies investigated multiple regions and could have observed a regional increase, decrease and/or no difference. Additionally, time-effects of multiple populations within one study were observed. ACA: anterior cerebral artery; CBVC: cerebrovascular conductance; cVMR: cerebral vasomotor reactivity in response to hypocapnia or hypercapnia; CVR: cerebrovascular resistance; dCA: dynamic cerebral autoregulation; MCA: middle cerebral artery; OCA: ophthalmic cerebral artery; PI: pulsatility index. cTOI: tissue oxygenation index; HHb: deoxygenated hemoglobin; O_2Hb: oxygenated hemoglobin; totHb: total hemoglobin.

DISCUSSION

The aim of this systematic review was to give a comprehensive overview of physical exercise training-induced effects on CBF in humans as measured by MRI, transcranial doppler ultrasound and NIRS. In addition, relationships between these effects with changes in physical fitness and cognitive performance were examined.

Effects on cerebral blood flow

Magnetic Resonance Imaging

Out of eight studies, global CBF was increased in one extremely long study (541 weeks) in patients with T2DM (17) and in another study with participants that had suffered from a stroke three months before the start of the intervention (37). In the latter study, however, a control group was missing, and the effect observed may have been due to the natural recovery of CBF after a stroke and not by the exercise intervention perse (49, 50). Thus, the evidence that exercise training affects global CBF is not convincing.

Regional changes in CBF were to some extent more consistent. In general, CBF was increased in the anterior cingulate cortex. However, exercise training increased - but also decreased - blood flow within regions of the medial temporal lobe. The regional CBF pattern changes during aging. This pattern may also vary between different health conditions, such as in type 2 diabetes (51), and may change during disease progression (52) that is for example observed during the development from cognitive healthy to MCI and Alzheimer's disease (53, 54). This may contribute to different CBF responses in different regions between the studies included. Alternatively, differences between studies may be directly related to MRI image acquisition or the statistical approach used. Obviously, the studies that acquired only CBF data of a predefined region may have missed potential changes in other brain regions (16, 19, 21, 33, 35, 36, 55). In contrast, studies that imaged the entire brain may be more susceptible for partial volume effects due to larger voxel-size. Another explanation for these differences may be the different statistical approaches that were used, such as region of interest (11, 16, 17, 19, 20, 22, 35, 36) or voxel-wise analyses (11, 18, 21, 33, 34). The latter approach is more sensitive as it permits statistical inferences at voxel level after normalization to a reference atlas and is not limited to pre-defined brain regions (56).

Transcranial doppler ultrasound

In healthy participants, exercise-training did not consistently change parameters related to resting CBF measured with transcranial doppler ultrasound. In fact, in some studies increases were observed (15, 23, 25, 39), while in other studies no changes were found (24, 38, 40-42, 44). In studies with patients (26-28, 41), CBF only increased in the study with CVD patients (43). All studies, but one (28), with continuous exercise training protocols observed variable changes in at least one of the CBF parameters (11, 15, 23, 25, 43, 46), while HIIT training was not effective (26, 28, 40, 44). This could be explained by the longer duration of the continuous exercise sessions, which may be needed to affect resting CBF. It should be noted, however, that results were difficult to compare because different cerebral arteries were assessed, while a great variety of outcome parameters were reported.

Despite the differences in stimuli, training increased CBF in response to exercise (38, 40, 42) and hypercapnia (27, 42) that both increase carbon dioxide levels in blood in all, but

one study (41). The study that did not find changes measured CBF during repeated squat stands after five min of supine rest (41), while two other studies did observe effects in response to ten min of standing rest (40) or a cycling trigger (38, 42). Possibly, carbon dioxide levels in the blood were not changed in the former study (41), as CBF was also not affected following a hypocapnic stimulus (28, 41, 42).

Near-Infrared Spectroscopy

Results of the various studies using NIRS were more difficult to compare. In fact, CBF changes were measured during different experimental conditions or cognitive tasks, and at different time points during exercise triggers, while different outcome parameters were reported. This may explain why changes in CBF were variable, although the only two studies measuring CBF during cognitive tasks observed an increased O_2Hb signal (30, 48). Remarkably, studies with only a continuous exercise training protocol observed no changes when exercise (29, 31, 32) or a cognitive task (47) was used as a challenge, while incorporating HIIT training was an effective approach to increase totHb during exercise (29, 31, 45). This suggests that HIIT training is more effective than continuous exercise to increase CBF during exercise, which is in contrast to findings on CBF in studies using transcranial doppler ultrasound. This may be due to the shorter duration of the continuous exercise training sessions in studies using NIRS (median: 30 min) as compared with transcranial doppler ultrasound studies (median: 45 min). HITT is well-known for its cardiovascular health benefits, which are already evident after a shorter time period compared to continuous exercise (57). The only study including older obese participants observed increased HHb during exercise (46), which suggests that deoxygenation became more efficient. However, the sample size (n=6) was probably too limited to draw any firm conclusions. Additionally, increased O_2Hb during exercise was only observed in already physically active men (45). However, whether training status affects the CBF response after training still remains to be determined.

Physical fitness and cerebral blood flow

In most studies using MRI, increases in physical fitness levels were accompanied by changes in CBF (11, 18-20, 33, 36, 37). In only one study with Alzheimer's patients, CBF did not change even though physical fitness was improved (22). This may suggest that these patients were not responsive or that a stronger intervention is needed to observe any effects on CBF. In contrast, CBF increased in one study involving coronary artery disease patients, while physical fitness levels did not significantly change (34).

Similarly, concomitant improvements in physical fitness and the transcranial doppler ultrasound parameters MCAv (15, 23, 37, 39), ACAv (43), CBVC (23, 39), or PI MCA (25) were observed. In two studies, physical fitness and CBF did both not change. (26, 44). In COPD patients, exercise did not affect CBF although oxygen uptake increased (41). Whether this indicates that a stronger intervention or a different protocol is needed to affect CBF in COPD patients warrant further study.

Physical fitness also concomitantly increased using NIRS when totHb (29, 31, 45), O_2Hb (45, 48), cTOI (45) and HHb (46) were used as read-out during exercise and O_2Hb during a cognitive task (30). In contrast, physical fitness improved without changes in totHb,

O_2Hb and HHb during exercise in one study, but this study had the shortest intervention period and lasted only for four weeks (32).

Thus, measuring physical fitness is recommended to determine the effectivity of the exercise training intervention, and has added value to understand effects of the exercise protocol on regional CBF as a marker of cerebrovascular function, which was also concluded by a recent systematic review by Chen and colleagues (58).

Cerebral blood flow and cognitive performance

Changes in CBF may underlie the well-known improvements in cognitive performance after exercise training (7, 8). In most studies (11, 16, 18-21, 33, 36), we observed concomitant changes in regional CBF measured with MRI and cognitive performance. Specifically, CBF increased in the anterior cingulate cortex, while verbal fluency (33) and memory (11, 33), and executive function (18) improved. The anterior cingulate cortex is a relevant region for these cognitive domains (59). Additionally, a positive relationship was observed between changes in hippocampal CBF, a critical region for memory function (60), and cognitive performance on a memory task (19). This suggests that regional changes in CBF, a parameter that can be measured with MRI but not with transcranial doppler ultrasound or NIRS, may be related to changes in specific cognitive domains.

An advantage of transcranial doppler and NIRS above MRI is that CBF can be measured more easily at the same time when cognitive test is performed. However, only a very limited number of the transcranial doppler ultrasound en NIRS studies also measured cognitive performance. In one study using transcranial doppler ultrasound concomitant changes in resting CBF and cognitive performance were observed (43), while no changes were observed in two studies (26, 28). In one study cognitive performance changed, but CBF did not (24). It is possible that the changes in resting CBF as measured in the basal arteries using transcranial doppler ultrasound are too low when blood flow only changes in specific regions. Studies using NIRS observed an increased CBF during exercise (46) and during a cognitive task (48) in combination with improved cognitive performance. In addition, in one study measuring totHb during exercise, both CBF and cognitive performance did not change (47).

We observed that exercise training increased CBF in the anterior cingulate cortex as measured with MRI. Changes in global CBF or within regions of the medial temporal lobe were less consistent. Effects of exercise training on markers for resting CBF measured using transcranial doppler ultrasound and NIRS were also variable. Exercise may increase MCAv in response to exercise or hypercapnic stimuli, but the number of studies is limited. Regulation of CBF is challenged during these measurements, as metabolic demands and carbon dioxide levels in blood are increased. Under these conditions, changes in CBF may become more apparent. Interestingly, concomitant changes in physical fitness and regional CBF were observed, while a relation between training-induced improvement in CBF and cognitive performance was also evident. Taken together, we conclude that exercise training may improve cerebrovascular function as regional CBF was changed, and these effects may underlie the beneficial effects observed on cognitive performance.

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SUPPLEMENTARY MATERIAL

Search string

Medline (PubMed)

((("cerebrovascular circulation"[MeSH Terms] OR ("cerebrovascular"[All Fields] AND "circulation"[All Fields]) OR "cerebrovascular circulation"[All Fields]) OR
((("cerebral"[All Fields] AND "blood"[All Fields] AND "flow"[All Fields]) OR "cerebral blood flow"[All Fields]) OR
((("cerebral"[All Fields] AND "circulation"[All Fields]) OR "cerebral circulation"[All Fields]) OR
((("cerebrum"[MeSH Terms] OR "cerebrum"[All Fields] OR "cerebral"[All Fields] OR "brain"[MeSH Terms] OR "brain"[All Fields]) AND ("cell respiration"[MeSH Terms] OR ("cell"[All Fields] AND "respiration"[All Fields]) OR "cell respiration"[All Fields] OR "oxygenation"[All Fields])))

AND

(("exercise"[MeSH Terms] OR exercise* [All Fields]) OR
((("physical"[All Fields] AND "activity"[All Fields]) OR "physical activity"[All Fields]) OR
((("physical"[All Fields] AND "activities"[All Fields]) OR "physical activities"[All Fields]) OR
((("physical"[All Fields] AND "exercise"[All Fields]) OR "physical exercise"[All Fields]) OR
((("aerobic"[All Fields] AND "exercise"[All Fields]) OR "aerobic exercise"[All Fields]) OR
((("exercise"[All Fields] AND "training"[All Fields]) OR "exercise training"[All Fields]))

Embase (Scopus)

(Exp brain circulation/ or Exp brain blood flow/ or (Exp brain/ and exp oxygenation/))

AND

(exp anaerobic exercise/ or exp aerobic exercise/ or exp exercise/ or exp physical activity/)

CENTRAL

ID	Search	Hits
#1	MeSH descriptor: [Cerebrovascular Circulation] explode all trees	
#2	MeSH descriptor: [Brain] explode all trees	
#3	MeSH descriptor: [Oxygen Consumption] explode all trees	
#4	#2 OR #3	
#5	MeSH descriptor: [Exercise] explode all trees	
#6	#4 AND #	

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CHAPTER 4

Aerobic exercise training improves cerebral blood flow and executive function: A randomized, controlled cross-over trial in sedentary older men

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ABSTRACT

Background

Physical activity may attenuate age-related cognitive decline by improving cerebrovascular function. The aim of this study was therefore to investigate effects of aerobic exercise training on cerebral blood flow (CBF), which is a sensitive physiological marker of cerebrovascular function, in sedentary older men.

Methods

Seventeen apparently healthy men, aged 60-70 years and with a BMI between 25 and 35 kg/m^2, were included in a randomized, controlled cross-over trial. Study participants were randomly allocated to a fully-supervised, progressive, aerobic exercise training or no-exercise control period for eight weeks, separated by a 12-week wash-out period. Measurements at the end of each period included aerobic fitness evaluated using peak oxygen consumption during incremental exercise (VO_2peak), CBF measured with pseudo-continuous arterial spin labeling magnetic resonance imaging, and post-load glucose responses determined using an oral glucose tolerance test (OGTT). Furthermore, cognitive performance was assessed in the domains of executive function, memory, and psychomotor speed.

Results

VO_2peak significantly increased following aerobic exercise training compared to no-exercise control by 262 ± 236 mL ($P < 0.001$). CBF was increased by 27% bilaterally in the frontal lobe, particularly the subcallosal and anterior cingulate gyrus (cluster volume: 1008 mm^3; $P < 0.05$), while CBF was reduced by 19% in the right medial temporal lobe, mainly temporal fusiform gyrus (cluster volume: 408 mm^3; $P < 0.05$). Mean post-load glucose concentrations determined using an OGTT decreased by 0.33 ± 0.63 mmol/L ($P = 0.049$). Furthermore, executive function improved as the latency of response was reduced by 5% ($P = 0.034$), but no changes were observed in memory or psychomotor speed.

Conclusion

Aerobic exercise training improves regional CBF in sedentary older men. These changes in CBF may underlie exercise-induced beneficial effects on executive function, which could be partly mediated by improvements in glucose metabolism.

This clinical trial was registered on September 5th, 2017, at clinicaltrials.org as NCT03272061.

INTRODUCTION

People over the age of 60 years represent 13% of the global population and this number is expected to increase at a rate of approximately 3% per year (1). Aging is associated with decreased cognitive performance, which is related to decreased cerebrovascular function (2, 3). As impaired cerebrovascular function may precede the decrease in cognitive performance (4-6), improving cerebrovascular function is an important target to delay cognitive impairment (7, 8). In this respect, interventions to improve cerebral blood flow (CBF), a physiological marker of cerebrovascular function (9, 10), are of major interest.

A healthy lifestyle, consisting of a healthy diet combined with increased physical activity has been proposed to protect against cognitive impairment by improving CBF (8). In fact, CBF was improved following a healthy lifestyle intervention and associated with higher cognitive performance in overweight or obese participants aged between 45 and 76 years (11). Furthermore, cross-sectional studies have observed that lower aerobic fitness in sedentary older individuals was associated with a reduced CBF and decreased cognitive performance (12). A recent meta-analysis of randomized controlled trials involving adults over the age of 50 years showed that aerobic exercise training improved cognitive performance (13). This improvement may relate to changes in CBF, since some studies suggest that CBF in the anterior cingulate and hippocampal brain regions increased following aerobic exercise training in sedentary older individuals (14-16). These exercise-induced changes in hippocampal CBF were positively related to changes in cognitive memory tasks (14, 15). Therefore, we concluded in our recent review that increases in CBF may contribute to the beneficial effects of increased physical activity levels on cognitive performance (7).

However, well-controlled trials investigating the effect of physical activity on CBF are scarce. In some studies, aerobic fitness was measured using a proxy measure which did not improve(16) or was not measured at all (17), or improvements in aerobic fitness did not sustain (14). Additionally, two studies primarily focused on the hippocampal region, potentially missing changes outside this region (16, 18). The objective of the current randomized, cross-over trial was therefore to investigate effects of a well-controlled eight-week aerobic exercise training period on CBF and cognitive performance. The study was performed in sedentary overweight or slightly obese older men, because this cohort has been shown to have a reduced CBF and cognitive performance at baseline (11, 12, 19, 20).

MATERIAL AND METHODS

Study participants

Apparently healthy overweight or slightly obese men were recruited via posters in university and hospital buildings or advertisement in local newspapers. Additionally, participants who had participated in previous studies at Maastricht University were approached if they had given written consent to contact them again for future studies. Volunteers were invited for a screening visit if they met the following inclusion criteria: aged between 60 and 70 years old, body mass index (BMI) between 25 and 35 kg/m^2; stable body weight (weight gain or loss < 3 kg in the past 3 months); non-smoker; no drug or alcohol abuse; no use of dietary supplements known to interfere with the main study outcomes; no diabetes; no use of medication known to affect blood pressure, lipid or glucose metabolism; no severe medical conditions that might interfere with the study (e.g. active cardiovascular

disease); and no participation in another biomedical study within one month prior to the screening visit.

During screening, sedentary behavior was assessed by means of the International Physical Activity Questionnaire (IPAQ) long version (21); an MRI screening list was completed; office blood pressure was measured; a 12-lead electrocardiogram (ECG) was performed; and a fasting blood sample was drawn. Based on the screening results, participants were checked against the following inclusion criteria: classified as low physically active according to the guidelines for IPAQ data processing (22); no contra-indications for MRI imaging (e.g. any metallic implants or claustrophobia); systolic (SBP) < 160 mmHg and diastolic blood pressure (DBP) < 100 mmHg, no ECG abnormalities as assessed by a cardiologist; fasting plasma glucose < 7.0 mmol/L, fasting serum total cholesterol < 8.0 mmol/L, and fasting serum triacylglycerol < 4.5 mmol/L. All participants provided written informed consent before screening. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, approved by the Medical Ethics Committee of Maastricht University Medical Center (METC173025), and registered on September 7th, 2017, at ClinicalTrials.gov (NCT03272061).

Study design

The study had a randomized, controlled cross-over design with an eight-week intervention period and an eight-week control period, separated by a twelve-week wash-out period. Participants were allocated to start either in the intervention or control period based on a computer-generated randomization scheme. Participants and investigators were unaware of the allocation prior to inclusion but could not be blinded during the intervention and measurements. However, images and blood samples were blinded prior to analysis. During the intervention period, participants followed a fully-supervised, personalized and progressive aerobic-based exercise program on a cycling ergometer for 50 min three times a week. To personalize the program, maximal exercise capacity (VO_2max) and maximal workload (Pmax) were reassessed every two weeks and training loads were adjusted accordingly. The program consisted of 10 min warm-up at 45% Pmax, 30 min at 70% Pmax and 10 min cool-down at 45% Pmax. We did not offer a program during the control and wash-out periods, and participants were requested to maintain habitual physical activity levels during the entire trial. Body weight was measured every two weeks in the intervention period and every four weeks in the control period. Participants were requested to maintain their habitual diet and use of alcoholic beverages throughout the total trial, which was checked using a food frequency questionnaire.

Measurements were performed at the start of the control and intervention periods (baseline; BL), after 4 weeks (WK4) and during two follow-up days (FU) at the end of each period. The first follow-up visit (FU-1) was performed 43 (range: 19 – 72) hours after the last training. The second follow-up visit (FU-2) was performed 117 (range: 70 – 118) hours after the maximal exercise test performed during FU-1. A schematic overview of the study design is shown in **Supplemental Figure 4.1**. On the days preceding measurements, participants were requested to have a regular meal and to refrain from alcohol. Participants arrived after an overnight fast (no food or drink after 08:00 PM, except for water) at the Scannexus research facilities in Maastricht (FU-1) or the Metabolic Research Unit Maastricht (MRUM) (FU-2). Men were asked to come by public transport or by car to standardize

measurements as much as possible. All measurements were performed in temperature-controlled rooms at 22 °C.

Maximal exercise test

Peak oxygen consumption (VO_2peak) was assessed every two weeks during the intervention period, and three times during the control period at BL, WK4 and FU-1. Heart rate was monitored simultaneously using a chest strap (Sport-tester Polar H10, Kempele, Finland). Thirty min before each maximal exercise test, the participants received a small carbohydrate rich meal including a banana and white bread with strawberry jam to optimize performance. The VO_2peak test included an incremental stepwise protocol on a calibrated bicycle ergometer (Lode Excalibur Sport 1000W/1.5V, Groningen, the Netherlands), while oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured continuously (Omnical, Maastricht University, the Netherlands).

The VO_2peak test started with a 5-minute warm-up at a load of 70W. Workload was increased by 50W every 2.5 min. When anaerobic threshold was observed, workload was increased by 25W every 2.5 min until exhaustion. The anaerobic threshold was determined when the respiratory exchange ratio (RER) was between 0.95 and 1.00. Participants had to reach an RER of at least 1.0 to fulfill the criteria of maximal exertion. Pmax was calculated as the workload completed ($P_{completed}$) plus time (t) in the last step divided by 150 and multiplied with the load increment of the final stage (ΔW): $\mathrm{Pmax} = \mathrm{P}_{\mathrm{completed}} + \frac{t}{150} \times \Delta\mathrm{W}$. Pedal frequency had to be at least 80 RPM. The exercise test was ceased when the pedal frequency remained below 60 RPM for 10 seconds. VO_2peak was determined as the maximal oxygen consumption for 5 seconds.

MRI acquisition

Scans were performed in the morning of FU-1 on a 3T MAGNETOM Prisma Fit MRI-system using a 64-channel head-neck coil (Siemens Healthcare, Erlangen, Germany). Participants were placed in the scanner with their head-first in the supine position. The eye centers were taken as a reference for the magnet isocenter position, which was at the level of the pons to minimize B_0 offsets in the labeling region. The labeling plane was positioned perpendicular to the carotid and vertebral arteries, based on an acquired angiogram (TR 21 ms, TE 7.3 ms, voxel volume 0.9 x 0.9 x 5.0 mm, 8 degrees flip angle, 26 sagittal slices, duration: 2 min).

Perfusion-weighted images were acquired after an acclimatization period of at least 20 min. During acquisition, participants were asked to look at the center of a displayed black cross to standardize measurements as much as possible and to reduce involuntary movements. Images were acquired using pseudo-continuous arterial spin labeling (PCASL) with background-suppressed segmented three-dimensional (3D) gradient and spin echo (GRASE) readouts. The sequence parameters were: TR 4000 ms, TE 13.6 ms, GRAPPA 2, labeling duration 1750 ms, post-labeling delay 2000 ms, segmentation factor 6 (the scan time per 3D volume was 24 s), and 10 label-control repetitions. The total acquisition duration, including an equilibrium magnetization scan, was approximately 9 min). Nineteen slices with a voxel resolution of 3.0 mm isotropic were acquired. In order to allow CBF quantification, a M_0 image without magnetization preparation and with a TR of 20 s was

acquired as well. **Figure 4.1.A** shows a typical perfusion-weighted image that we generated at the Scannexus research facilities in Maastricht.

One high-resolution anatomical 3D magnetization-prepared rapid acquisition with gradient echo (MPRAGE) scan was performed (TR 2400 ms, TE 2.18 ms, TI 1040 ms, 1.0 mm isotropic resolution, 8 degrees flip angle and 160 sagittal slices, duration: 6 min). The field of view across the various sequences was kept constant for accurate registration and anatomical localization.

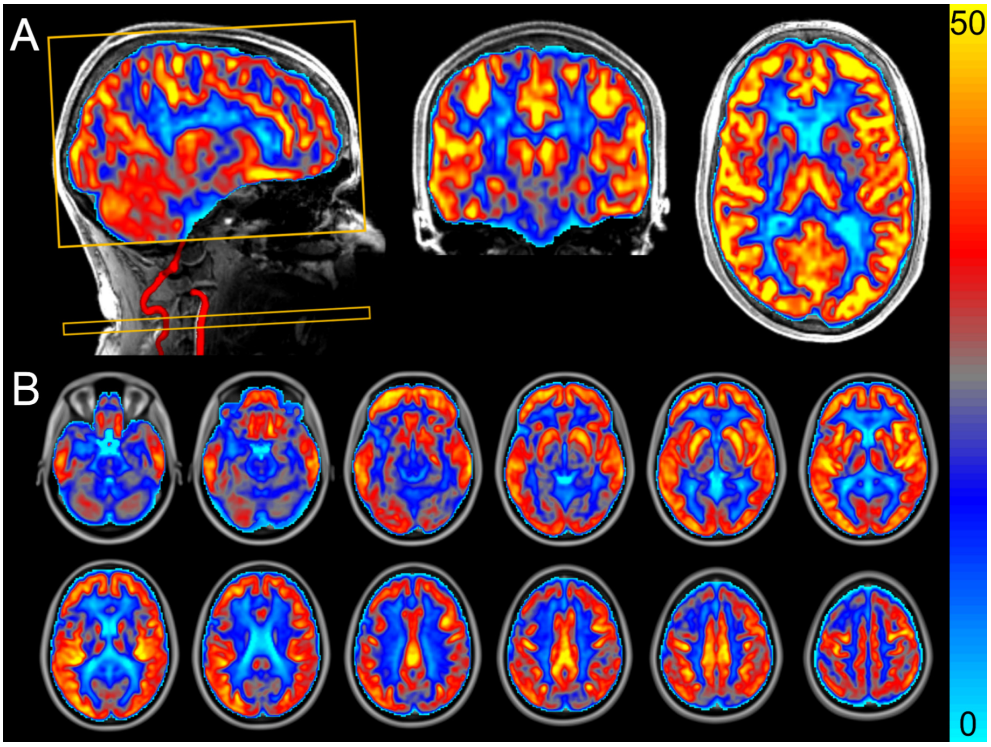

Figure 4.1. Perfusion-weighted image acquired using pseudo-continuous arterial spin labeling that we generated at the Scannexus research facilities in Maastricht. The images show the cerebral blood flow in mL/100 g tissue/min (scale shown by color bar). (**A**) Sagittal slice including angiogram from vertebral and carotid artery, coronal slice, and axial slice. The yellow rectangular boxes represent the imaging box and labeling plane perpendicular to the arteries. (**B**) Mean cerebral blood flow (CBF) map from all participants (n = 14) after the control period. Data from a randomized, controlled crossover study with sedentary older men.

MRI processing

Volbrain was used to perform brain extraction, along with tissue segmentation for the anatomical MPRAGE image (23). Motion correction was automatically performed by Siemens' scanner software. FSL software (Version 6.0) was used to estimate quantitative CBF maps from the ASL data (http://fsl.fmrib.ox.ac.uk/fsl). Pairwise subtraction of label and control images was performed on the PCASL data to generate perfusion-weighted images. Perfusion-weighted images were quantified using the BASIL tool (version 4.0.4) (24). following the recommendations of the ASL White Paper (25) The M_0 image was used for voxel-wise calibration to quantify the perfusion-weighted images. The labeling efficiency

was calculated based on the efficiency of four background suppression pulses (0.93^4), which resulted in a cumulative labeling efficiency of 0.64. The T_1 of blood depends on the blood hemoglobin concentrations (ctHb) and was estimated using the following equation: $1000/T_{1a}$ (ms) = 0.016 x ctHb (g/dL) + 0.317 (26). The used T_1 of gray matter was 1330 ms, while the bolus arrival time was set at 1300 ms.

The calibrated ASL images containing absolute CBF values in mL/100g tissue/min were co-registered using Boundary-Based Registration to the brain-extracted MPRAGE image using the FLIRT routine (27). The gray matter partial volume estimates image was thresholded at 0.6 and binarized to create a mask. The mean gray matter CBF was calculated in the anatomical space by taking the mean CBF over the gray matter mask.

The CBF images in anatomical space were registered to MNI (2 mm) space using a non-linear algorithm (FNIRT) and were used for voxel-wise statistical group comparisons. Voxel-wise analyses was performed to detect significantly changed clusters between the intervention and control periods over the whole brain without prior region of interest (28).These absolute CBF images in MNI space were spatially smoothed with a Gaussian kernel of 1 mm to account for small regional differences, which still existed across participants. Thereafter, a repeated measures mixed-effects analysis using a general linear model with a single-group paired difference (FLAME stage 1 and 2) was used to generate an image containing Z-scores for each voxel (29). Cluster information was extracted after correcting for family-wise error using a Z-threshold of 2.3 ($P<0.05$) and smoothness estimates, which were computed using the Gaussian Random Field model based on the residual error in each participant. The average probability of the location of the significant clusters was determined using the Atlasquery function in combination with the image of the cluster and the Harvard-Oxford (sub)cortical structural atlas.

Blood sampling and Oral Glucose Tolerance Test

During both periods, fasting blood samples were taken at the same time in the morning from a forearm vein by venipuncture at BL, at WK4, and at FU-1. During FU-2, a 7-point Oral Glucose Tolerance Test (OGTT) was performed as a measure of peripheral glucose metabolism. For this, blood samples were taken from an intravenous catheter at baseline (t = 0 min), and 15, 30, 45, 60, 90, and 120 min following ingestion of 75 g glucose (Novolab, Geraardsbergen, Belgium). After blood sampling, NaF-containing vacutainer tubes (Becton, Dickson and Company, Franklin Lanes, New-York, USA) were immediately placed on ice and centrifuged within 30 min at 1300 x g for 15 min at 4 °C to obtain plasma samples. Blood drawn in vacutainer SST™ II Advance tubes (Becton, Dickson and Company, Franklin Lanes, New-York, USA) were first allowed to clot for at least 30 min at 21 °C. These tubes were centrifuged at 1300 x g for 15 min at 21 °C to obtain serum samples. Plasma and serum samples were immediately portioned into aliquots, frozen in liquid nitrogen, and stored at -80 °C until analysis at the end of the study.

Plasma obtained from NaF tubes was used to determine glucose concentrations (Horiba ABX, Montpellier, France). Fasting serum samples were analyzed for insulin (RIA, Millipore, Billerica, MA, USA). The net incremental area under the curve (net iAUC) was calculated as described (30). The homeostatic model assessment index was calculated as a measure of insulin resistance (HOMA-IR) (31).

Cognitive performance

Cognitive performance was assessed in silent chambers at FU-2 using the Cambridge Neuropsychological Test Automated Battery (CANTAB). These validated, computerized assessments (32-34) have been extensively described before and measures performance in three cognitive domains: executive function, memory, and psychomotor speed. Based on the literature (35-38), the main hypothesis for the cognition parameters was that the reaction latency would improve following aerobic exercise training.

Executive function was assessed with the Multitasking Test (MTT) and Spatial Span (SSP). Focus was on four variables for the MTT: 1) Incongruency Cost (IC) was calculated by subtracting the median latency of response from the trials that were congruent from the incongruent trials; 2) Multitasking Cost (MTC) was determined as the difference between the median latency of response, in which two rules were used (respond at the side the arrow appears or the direction the arrow points) compared to when only one of the rules was used; 3) Median Latency (ML) of response for all correct trials; 4) The total number of errors (TE). For SSP, the maximal completed Span Length (SL) was used.

Memory was evaluated with the Delayed Matching to Sample (DMS) and Paired Associates Learning (PAL). The percentage of correctly answered trials for All Delays (CAD) was used for DMS, while the First Attempt Memory Score (FAMS) and TE were used for PAL.

Measurements of psychomotor speed included the Motor Screening Task (MOT) and Reaction Time (RTI). The Mean Latency (LM; from target stimulus appearance to button press) outcome variable was used for MOT. For RTI the variables Reaction Time (RT; from target stimulus appearance to release of response button) and Movement Time (MT; from release of response button to selection of target stimulus) were used.

Statistical analysis

Results are shown as mean ± standard deviation (SD), unless otherwise indicated. Before the start of the study, it was calculated that 15 participants were needed to reach a power of 80% to detect a true difference of 15% in CBF, which was the primary outcome parameter. For these calculations, a two-sided alpha of 0.05 and a within-subject variability of 19% were used (39). CBF changes of 15% may be expected and are also clinically relevant (20, 40, 41).

Intervention effects were examined using analysis of variances (ANOVA) with participant, treatment, and period as fixed factors. Linear mixed models were performed to test for differences between treatments over time, using the change from baseline as dependent variable. Time, treatment, period, and time * treatment interaction were used as fixed factors. If the interaction term was not statistically significant, it was omitted from the model. Bonferroni correction was used to correct for multiple comparisons. Statistical analyses were performed using SPSS (IBM Corp., IBM SPSS Statistics, V23, Armonk, NY, USA). Differences were considered statistically significant at $P < 0.05$ using two-tailed tests.

RESULTS

Study participants

A CONSORT flow diagram of participants throughout the study is shown in **Figure 4.2**. In total 24 men were screened for eligibility. Five participants were excluded because they were not sedentary (two men), had an abnormal ECG (two men) or a fasting plasma glucose

concentration above 7.0 mmol/L (one man). Thus, nineteen men were eligible and started the study. Two participants who started in the no-exercise control period dropped-out during the wash-out period for personal reasons and seventeen participants successfully completed the study. Two participants did not undergo the MRI measurements: one man became unexpectedly claustrophobic and another man due to remains of a metal screw in his skull following surgery which did not become apparent during the screening visit. Additionally, MRI data from one participant were excluded, because the magnetic field disturbance due to his tooth implant reduced the labeling efficiency of arterial blood. In total, fourteen participants were included in the final MRI analysis, while all seventeen participants were included in all other analyses. Baseline characteristics are shown in **Table 4.1.** Participants who completed the study had a
mean age of 67 ± 2 years and a mean BMI of 30.3 ± 2.8 kg/m^2. Body weight remained stable at the follow-up measurements between both periods (0.9 ± 3.0 kg). The median attendance of the scheduled training sessions was 100% (range: 92 – 100%).

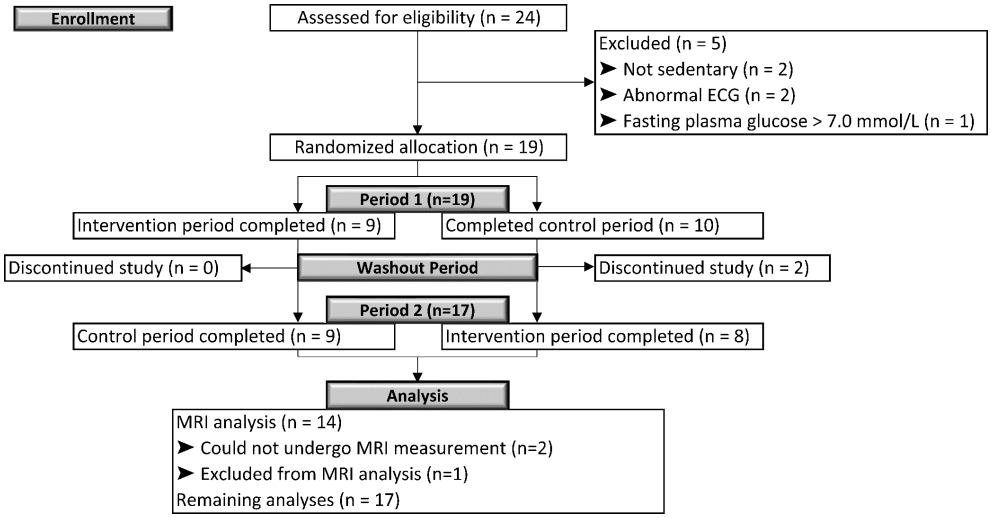

Figure 4.2. flow diagram. Diagram of the progress through the phases of this randomized, controlled crossover study with sedentary overweight or slightly obese older men.

Table 4.1. Baseline characteristics of sedentary older men who completed the study (n = 17)[1].

Participant characteristics	
Age (y)	67 ± 2
BMI (kg/m^2)	30.3 ± 2.8
Total cholesterol (mmol/L)	5.28 ± 1.10
TAG (mmol/L)	1.39 ± 0.49
Glucose (mmol/L)	5.80 ± 0.36
Systolic blood pressure (mmHg)	138 ± 13
Diastolic blood pressure (mmHg)	88 ± 6

[1]Data are shown as mean ± SD. BMI: Body Mass Index; TAG: triacylglycerol.

Maximal exercise test

All men reached a RER of at least 1.0 during all maximal exercise tests (1.12 ± 0.05), suggesting maximal exertion was reached. As expected, physical exercise training significantly increased aerobic fitness, as indicated by the significant time * treatment interaction for the VO_2peak (P = 0.018), using linear mixed models. Pairwise comparisons showed that the VO_2peak tended to increase by 99 ± 236 mL (P = 0.088) during the intervention period at week 4 and was significantly increased by 262 ± 236 mL (P < 0.001) at week 8 (**Figure 4.3.A**). Comparable results were observed for Pmax (time * treatment interaction: P < 0.001), which increased during the intervention at week 4 by 12 ± 18 W (P = 0.006) and at week 8 by 30 ± 18 W (P < 0.001; **Figure 4.3.B)**.

Cerebral Blood Flow

The mean CBF map from all participants after the control period is shown in **Figure 4.1.B**. CBF differed between intervention and control periods in three clusters with a volume of 392 mm^3 (cluster 1), 616 mm^3 (cluster 2) and 408 mm^3 (cluster 3) (**Figure 4.4; Table 4.2**). CBF was increased by 28% (6.4 ± 5.0 mL/100g tissue/min; P = 0.040) in cluster 1 and by 26% (7.0 ± 4.8 mL/100g tissue/min; P = 0.001) in cluster 2 after the intervention period. In contrast, CBF was decreased by 19% (-4.4 ± 1.9 mL/100g tissue/min; P = 0.031) in cluster 3. The average probabilities for the locations of cluster 1 were 25% in the subcallosal cortex, 11% in the anterior cingulate gyrus, 8% in the paracingulate gyrus, and 3% in the frontal medial cortex. For cluster 2, which was located contralateral to cluster 1, the average probabilities were 24% in the subcallosal cortex, 23% in the frontal medial cortex, 15% in the paracingulate gyrus, and 12% in the anterior cingulate gyrus. For cluster 3, these probabilities were 35% in the temporal fusiform cortex and 25% in the parahippocampal gyrus.

We did not observe differences between the intervention and control periods (**Table 4.2**) in global CBF (-0.5 ± 3.0 mL/100g tissue/min; P = 0.523), gray matter CBF (-0.6 ± 3.6 mL/100g tissue/min; P = 0.533), CBF in the left hemisphere (-0.5 ± 4.5 mL/100g tissue/min; P = 0.637), and CBF in the right hemisphere (-0.3 ± 3.9 mL/100g tissue/min; P = 0.723).

Glucose metabolism

A significant treatment effect was observed for the post-load glucose concentrations measured during the OGTT (P = 0.049) after the intervention period (**Figure 4.3C**). Pairwise comparisons showed a tendency towards lower glucose concentrations at 120 min (-0.86 ± 1.91 mmol/L; P = 0.083). Also, the net iAUC tended to decrease (-45 ± 96 mmol/L*2hr; P = 0.072).

Linear mixed models showed no time * treatment interactions for fasting glucose (P = 0.131) and insulin (P = 0.949) concentrations and the HOMA-IR (P = 0.772). There were also no significant treatment effects when this interaction term was omitted from the model (glucose: P = 0.146; insulin: P = 0.390; HOMA-IR: P = 0.423).

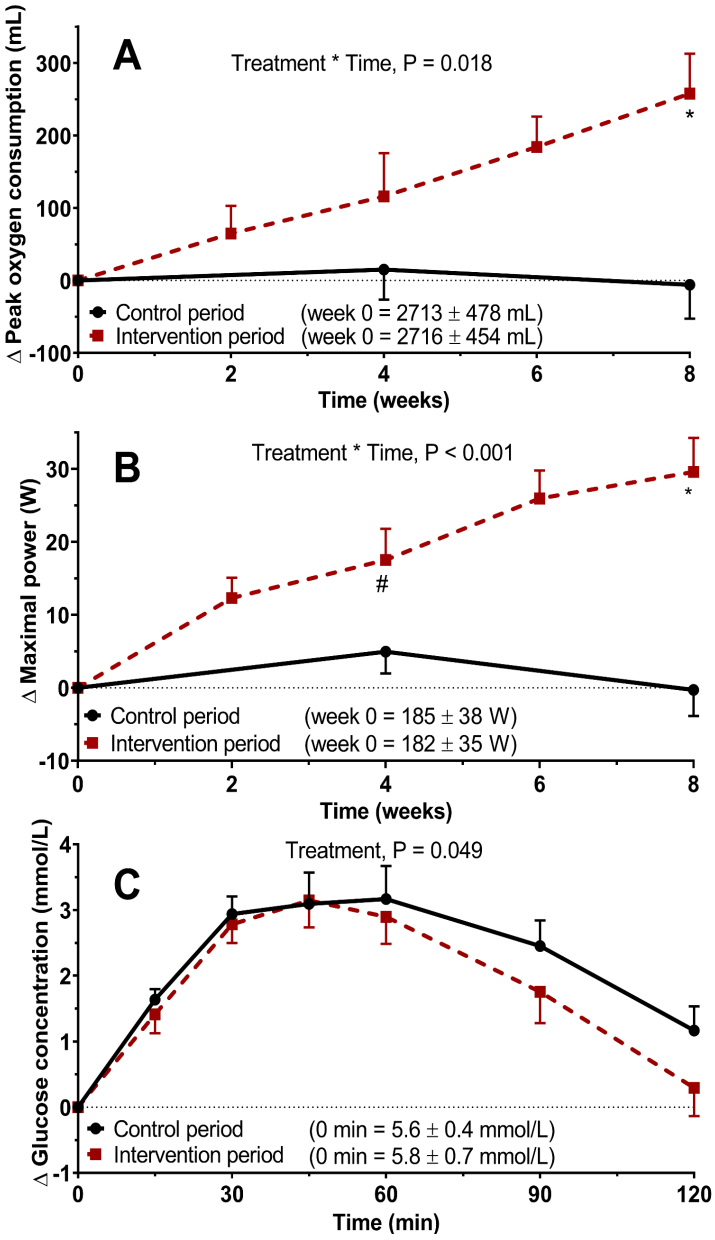

Figure 4.3. Data from a randomized, controlled crossover study with sedentary overweight or slightly obese older men (n = 17). Data were analyzed using linear mixed models on the difference between each timepoint with baseline. (**A**) Mean (± SEM) difference in peak oxygen consumption (VO_2peak) and (**B**) maximal power (Pmax) difference during the maximal exercise. Maximal exercise tests were performed every two weeks during the intervention period. During the control period, maximal exercise tests were performed at baseline, after four weeks and after eight weeks. Baseline values were not significantly different. There was a significant treatment * time interaction for VO_2peak (P = 0.018) and Pmax (P <0.001). After Bonferroni correction there was a significant difference between control and intervention period at 4 weeks (#: P = 0.006) and at 8 weeks for VO_2peak and Pmax (*: P < 0.001). (**C**) Mean (± SEM) difference in glucose concentrations during a 7-point oral glucose tolerance test (OGTT) test. There was a significant treatment effect for glucose concentration (P = 0.049).

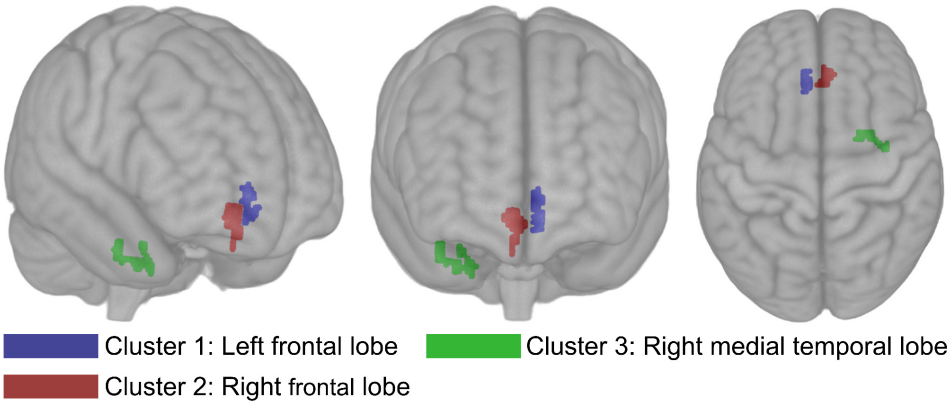

Figure 4.4. Results of voxel-wise comparisons including all acquired CBF data in three dimensional MNI template from a randomized, controlled crossover study with sedentary overweight or slightly obese older men (n = 14). Cerebral blood flow (CBF) increased bilaterally after the intervention compared to the control period P < 0.05 (Family-wise error corrected). The CBF of cluster 1 and 2 increased after the intervention period compared to the control with 6.39 mL/100g tissue/min (volume: 392mm^3) and 6.95mL/100g tissue/min (volume: 616mm^3), respectively. The CBF of cluster 3 decreased with 4.4 mL/100g tissue/min (volume: 408mm^3).

Table 4.2. Cerebral blood flow difference between intervention and control period in a randomized, controlled crossover study with sedentary older men (n = 14)[1].

	Intervention period (mL/100g/min)	Control period (mL/100g/min)	Mean difference (mL/100g/min)	P-value[2]
Gray matter CBF	27.6 ± 9.4	28.4 ± 7.9	-0.6 ± 3.6	0.533
Global CBF	23.5 ± 7.8	24.4 ± 6.4	-0.5 ± 3.0	0.523
Left hemisphere CBF	24.5 ± 8.7	25.4 ± 7.3	-0.5 ± 4.5	0.637
Right hemisphere CBF	25.7 ± 8.1	26.3 ± 6.7	-0.3 ± 3.9	0.723
Cluster 1 CBF	29.1 ± 12.2	22.7 ± 11	6.4 ± 4.8	0.040
Cluster 2 CBF	33.7 ± 17.8	27.0 ± 16.3	7.0 ± 4.9	0.001
Cluster 3 CBF	18.7 ± 5.3	23.1 ± 5.4	-4.4 ± 1.9	0.031

[1]Data are shown as mean ± SD. [2]Analysis of variances (ANOVA) with participant, treatment, and period as fixed factors. CBF: Cerebral blood flow.

Cognitive performance

Performance on the executive function MTT improved as indicated by a significant decrease in Median Latency (-37 ± 65 ms; P = 0.034), while the number of total errors remained the same (0 ± 6; P = 0.770). The other MTT variables did not change. In addition, no changes were observed for the executive function test SSP, the memory tests DMS and PAL, and the psychomotor speed tests MOT and RTI (**Table 4.3**).

Table 4.3. Cognitive outcomes in a randomized, controlled crossover study with sedentary older men (n = 17)[1].

	Intervention period	Control period	Mean difference	P-value[2]
MOT ML (ms)	846 ± 167	847 ± 177	-1 ± 157	0.980
RTI MT (ms)	281 ± 67	289 ± 56	-8 ± 56	0.574
RTI RT (ms)	399 ± 32	405 ± 45	-5 ± 33	0.510
MTT IC (ms)	117 ± 53	112 ± 48	4 ± 45	0.724
MTT MTC (ms)	281 ± 107	264 ± 109	17 ± 161	0.675
MTT RL (ms)	753 ± 87	790 ± 98	-37 ± 65	0.034
MTT IN	7 ± 9	7 ± 10	0 ± 6	0.770
SSP SL	6 ± 1	6 ± 1	0 ± 1	0.414
DMS TC (%)	80 ± 9	83 ± 7	-3 ± 12	0.336
PAL FAMS	11 ± 3	12 ± 2	-1 ± 2	0.282
PAL TE	17 ± 9	16 ± 9	2 ± 8	0.398

[1]Data are shown as mean ± SD. [2]Analysis of variances (ANOVA) with participant, treatment, and period as fixed factors. DMS: Delayed Matching to Sample; FAMS: First Attempt Memory Score; IC: Incongruency Cost; ML: Median Latency; MOT: Motor Screening Task; MT: Movement Time; MTC: Multitasking Cost; MTT: Multitasking Test; PAL: Paired Associates Leaning; RT: Reaction Time; RTI: Reaction Time; SL: Span Length; SSP: Spatial Span; TC: Total Correct; TE: Total Errors.

DISCUSSION

In this well-controlled, randomized trial in sedentary older men, aerobic exercise training affected regional CBF. It increased bilaterally in the subcallosal and anterior cingulate gyrus, which are both located in the frontal lobe. These two regions have been identified as important nodes in the limbic system and are involved in the regulation of executive cognitive functions (42, 43). Reduced CBF was observed in one cluster located in the right medial temporal lobe, mainly the temporal fusiform gyrus. In addition, latency of response was reduced for the executive function test and mean post-load glucose concentrations decreased. Recent findings suggest that unfavorable regional CBF alterations underlie reduced cognitive performance in older individuals, which may be mediated by impaired glucose metabolism (44). This underlines the potential clinical relevance of the observed concomitant improvements in regional CBF, glucose metabolism and executive function following exercise-induced increased fitness.

CBF increased by 27% bilaterally in the frontal lobe in clusters with a total volume of 1008 mm^3. The location of the cluster was comparable to the cluster identified by Chapman et al (14). However, they did not quantify the change in CBF, and the volume of the cluster was only 696 mm^3. Additionally, they did not show sustained increases in aerobic fitness. Therefore, the smaller cluster volume may be explained by the lower effectiveness of the intervention used. Reduced CBF in the frontal lobe is associated with increased age (45), and a 4-year prospective longitudinal study observed that CBF at baseline was associated with cognitive performance at follow-up (46).

Interestingly, CBF decreased by 19% in the right medial temporal lobe and this cluster had a volume of 408 mm^3. The observed decrease in CBF may attenuate progression of cognitive decline associated with human aging, as shown by a positive correlation of CBF in the temporal lobe with age (45, 47). Hays et al. have suggested that an increased temporal lobe CBF in a population with declined cognitive performance reflects neurovascular dysregulation (48). Additionally, increased CBF was observed in the medial temporal lobe early in the development of mild cognitive impairment (49, 50). In contrast

to our findings, Maass et al. observed a decrease in hippocampal CBF after exercise in older individuals (15). Pereira and colleagues also showed an exercise-induced increase in cerebral blood volume in the dentate gyrus - a subregion of the hippocampus - in young and middle-aged participants (51). The intervention periods in these studies were one to two months longer than in our study, and image acquisition techniques were particularly optimized to detect changes in the hippocampus. This may have decreased the sensitivity to detect changes outside their region of interest, while our study may have been less sensitive to detect hippocampal changes due to coarser resolution and related partial volume effects. In addition, it is possible that longer intervention periods are needed to induce CBF changes in hippocampal brain regions. Indeed, Burdette et al. have shown that hippocampal CBF was higher following exercise training (16). However, in this parallel study only post-intervention scans were performed, while the exercise training group consisted of 50% women compared to no women in the control group. Also, CBF was not corrected for hematocrit, which may have resulted in higher CBF values in women (52). Therefore, the observed CBF differences may be partly due to gender-mismatch instead of exercise training.

No changes in whole brain or gray matter CBF were observed. Gray matter CBF was based upon individually generated gray-matter masks in native space to ensure optimal overlap between structural gray matter regions and CBF. The mean gray-matter mask volume was comparable between the intervention and control periods within one participant (0.3 ± 3.0%). Gray matter CBF values were comparable with observed blood flow levels in studies that used partial volume correction (2, 44, 47, 48) as incorporated in the FSL Basil tool. In fact, the mean gray matter CBF with partial volume correction in our study was 49.8 ± 13.0 ml/100g tissue/min. However, we did not use this correction, also because the validity of proposed partial volume correction approaches has recently been questioned (53). Decreased gray matter CBF has been observed in sedentary populations (12, 20, 54), and was associated with accelerated cognitive decline (55). However, in agreement with our findings, other studies investigating the effect of aerobic exercise training on CBF also did not observe changes at the whole-brain level (14, 15).

Glucose metabolism improved, as indicated by the decreased post-load glucose concentrations. Besides the well-known effects of aerobic exercise training in (pre-)diabetics (56), Ferrara et al. also observed that exercise training increased glucose disposal in apparently healthy older men (57). Fasting plasma glucose concentrations did not change, which agrees with the result of a meta-analysis of 105 intervention studies (58). The observed beneficial effects on CBF in specific brain regions may be linked to the improved glucose metabolism. This is supported by the relation between a reduced CBF in the frontal lobe and decreased cognitive performance in type 2 diabetic patients compared to healthy controls (54). Nevertheless, a causal relationship between peripheral and brain insulin sensitivity has not yet been established (59, 60).

Several reviews concluded that aerobic exercise training improves executive function (35-38), which is in line with the current findings. The latency of response decreased when the correct answer was given, while the number of errors remained unchanged, which indicates favorable effects on cognitive performance within the domain of executive function. This decrease in latency may be associated with improved response-inhibition, and was not due to speed-accuracy trade-off (61). The favorable effects on

cognitive performance are in line with the improvements of CBF in the frontal lobe, which has been identified to be important in executive function (62). No changes in cognitive performance in (visuo-spatial) memory or psychomotor speed were observed. Similarly, visuo-spatial memory did not improve following long-term aerobic exercise as shown in a meta-analysis of 21 aerobic exercise training studies, whereas verbal-auditory memory did improve (63). Longer intervention periods may be needed to improve visuo-spatial memory, since only one trial with a twelve-month physical training intervention showed beneficial effects on visuo-spatial memory (64). Psychomotor speed only increased in studies with combined aerobic exercise- and resistance- or cognitive training (65). Multimodal combined training may thus be required to improve performance in psychomotor speed tests.

As expected, aerobic exercise training improved aerobic fitness during maximal exercise. VO_2peak increased significantly by 10% between the intervention and control group after 8 weeks, while Pmax already increased after 4 weeks. These concomitant increases were expected based on the linear relationship between VO_2peak and Pmax (66). The consistent increase in VO_2peak and Pmax of our trial emphasizes the effectiveness of the intervention. This may be attributed to a combination of several factors, including (i) the duration, frequency and tightly-controlled supervised training sessions; (ii) the individually-based progressive training intensity; and (iii) the inclusion of sedentary individuals. Maass et al. also showed an increase of 10% oxygen consumption at ventilatory anaerobic threshold after 12 weeks of 30 min interval training (15). In contrast, Chapman et al. only showed a change in VO_2peak at 6 weeks, which did not sustain after 12 weeks of aerobic exercise training (14). Burdette et al. used a proxy-measure (400m walk speed) that did not significantly differ between groups and the duration and intensity of the home-based training sessions was not controlled (16). The Train the Brain Consortium did not measure the effectiveness of the physical exercise training by means of an aerobic fitness outcome (17). Therefore, it cannot be assessed whether changes in CBF in these studies are due to exercise-induced changes in aerobic fitness.

Our tightly-controlled, progressive, aerobic exercise training showed almost perfect attendance by the participants, generating consistent improvements in aerobic fitness across the 8-week intervention period. This trial included only men to reduce gender differences as an extra source of variability, which reduces the external validity. Additionally, although we were properly powered to find changes in our primary outcome, our sample size was too limited to examine into detail relationships between changes in aerobic fitness, CBF, glucose metabolism and cognitive performance.

CONCLUSION

Our results show that aerobic exercise training improves regional CBF in sedentary older men. Also, cognitive performance in the domain of executive function improved, and beneficial effect on peripheral glucose metabolism were observed. Whether the observed exercise-induced changes in CBF underlie the beneficial effects on cognitive performance, and if they are mediated by changes in peripheral and/or brain insulin sensitivity requires further study.

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.

AUTHORS' CONTRIBUTIONS

The authors' contributed as follows; JK: designed and conducted the study, performed the statistical analyses, interpreted the data and wrote the manuscript, RM: designed the study, interpreted the data, had overall responsibility for the study, and wrote the manuscript, DI: developed the MRI sequences and analysis pipeline, interpreted the data, and reviewed the manuscript, JA: developed the cognitive performance assessment protocol, interpreted the data, and reviewed the manuscript, KU: developed the MRI sequences and analysis pipeline, interpreted the data, and reviewed the manuscript, and PJ: designed the study, interpreted the data, had overall responsibility for the study, and wrote the manuscript.

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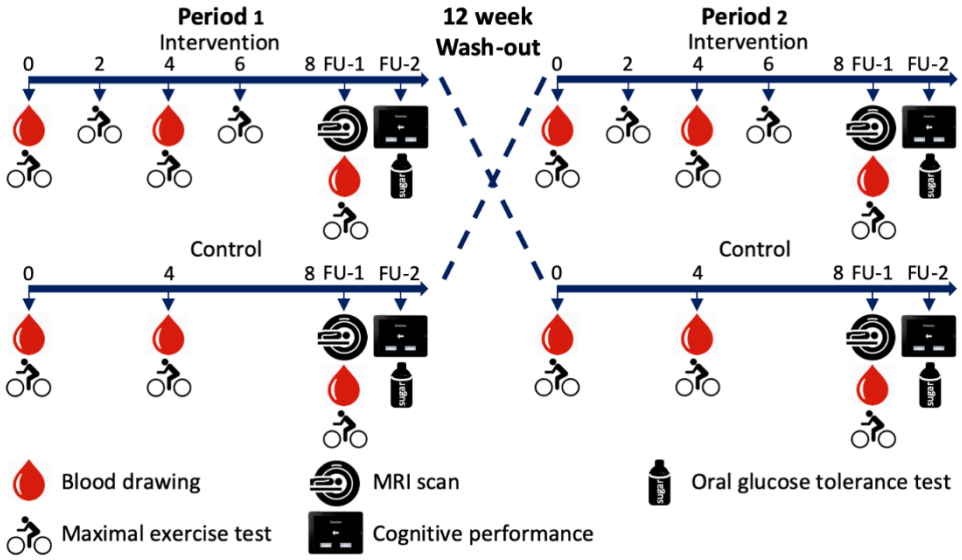
CONTRIBUTION TO THE FIELD STATEMENT

People over the age of 60 years represent 13% of the global population and this number is expected to increase at a rate of approximately 3% per year. Aging is associated with decreased cognitive performance, which is related to decreased cerebrovascular function. As impaired cerebrovascular function may precede the decrease in cognitive performance, improving cerebrovascular function is an important target to delay cognitive impairment. In this respect, interventions to improve cerebral blood flow (CBF), a physiological marker of cerebrovascular function, are of major interest. A healthy lifestyle, consisting of a healthy diet combined with increased physical activity has been proposed to protect against cognitive impairment by improving CBF. Additionally, lower CBF correlated with decreased cognitive performance. Therefore, the objective of the current randomized, cross-over trial was to investigate the effects of eight-week well-controlled aerobic exercise training on CBF and cognitive performance in sedentary overweight or slightly obese older men. Our results now show that aerobic exercise training improves regional CBF in older men. These changes in CBF may underlie exercise-induced beneficial effects on executive function, which could be partly mediated by improvements in glucose metabolism.

SUPPLEMENTAL



Supplemental Figure 4.1. Schematic overview of study design. Timeline is displayed as weeks. FU-1: Follow-up day one; FU-2: Follow-up day two.

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CHAPTER 5

Aerobic exercise training improves not only brachial artery flow-mediated vasodilatation, but also carotid artery reactivity: A randomized controlled, cross-over trial in older men

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Submitted

ABSTRACT

Background

Beneficial effects of physical exercise training on endothelial function as measured by brachial artery flow-mediated vasodilation (FMD) have been well established. This trial with older sedentary overweight and obese men examined the effects of aerobic training on other non-invasive markers of the vasculature, which have been studied into less detail.

Methods

Seventeen men aged 67 ± 2 years and with a BMI of 30.3 ± 2.8 kg/m^2 participated in this randomized, controlled cross-over study. Participants were randomly allocated to a fully supervised, progressive, aerobic exercise training (three 50-minute sessions each week at 70% maximal power) or a no-exercise control period for eight weeks, separated by a 12-week wash-out period. At the end of each period, endothelial function was assessed by the carotid artery reactivity (CAR) response to a cold pressor test and FMD, and local carotid and regional aortic stiffness by the carotid-to-femoral pulse wave velocity (PWVc-f). The retinal microvasculature and lipid profile were also determined. Finally, blood pressure and glucose concentrations during daily life were assessed.

Results

Aerobic training improved CAR by 2.23 percent point (pp; 95% CI: 0.58, 3.89 pp; P = 0.012) and FMD by 2.99 pp (95% CI: 0.58, 5.41 pp; p = 0.019). Local carotid artery stiffness increased, while PWVc-f did not change. Retinal arteriolar width improved by 3 µm (95% CI: 0, 7 µm; P = 0.041). Office blood pressure decreased, but no changes were found in ambulatory blood pressure, and serum lipid and continuous glucose concentrations.

Conclusions

Aerobic exercise training improved endothelial function and retinal arteriolar width in older sedentary overweight and obese men, which may reduce cardiovascular risk.

This clinical trial was registered on September 5th, 2017, at clinicaltrials.org as NCT03272061.

INTRODUCTION

Physical exercise training is a well-known strategy to prevent age-related health problems, such as cardiovascular disease (CVD) and cognitive decline (1, 2). A common denominator of these comorbidities is an impaired vascular function (3), which can be measured with different non-invasive techniques, each addressing a different aspect of the vasculature (1, 4). Most well-controlled trials investigating effects of exercise training on vascular function have however focused on only one specific characteristic of the vasculature (5). Thus, it has frequently been shown that vascular endothelial function of a peripheral muscular artery as measured by shear stress-induced brachial artery flow-mediated vasodilation (FMD) is improved after exercise training (6). However, endothelial function can also be examined in a major elastic conduit artery by assessing carotid artery reactivity (CAR), which involves stimulation of the sympathetic nervous system using a cold pressor test (7). CAR correlates with coronary artery responses to a cold pressor test, an independent predictor of cardiovascular events (8, 9). Effects of exercise training on CAR are not known. In addition, only a limited number of studies have examined effects of exercise on parameters reflecting local carotid stiffness, but results are inconsistent (10). Regional aortic arterial stiffness, as determined by the current non-invasive gold standard method carotid-to-femoral pulse wave velocity ($PWV_{c\text{-}f}$), is a well-established risk marker of CVD (11). However, mainly a reduced brachial-ankle pulse wave velocity (PWV) was observed after aerobic exercise training (12). Further, exercise-induced beneficial effects on the retinal microvasculature, imaged using fundus photography, are related to a reduced cardiovascular risk in obese adults (13). Finally, macrovascular complications (i.e., early signs of atherosclerotic plaque formation) were evaluated by the assessment of carotid intima-media thickness (cIMT) (14). Applying all these non-invasive techniques provides a more complete picture on the effects of exercise training among different regions of the vascular tree.

Recently, we have already reported that in sedentary overweight and obese older men a fully controlled aerobic exercise training protocol improved regional cerebral blood flow (CBF), which reflects cerebrovascular function (15). We here report aerobic-training effects on the central (i.e., carotid artery and aorta), peripheral (i.e., brachial artery), and retinal microvasculature that were assessed using different non-invasive markers for endothelial function, arterial stiffness, and vascular structure. Additionally, cardiometabolic risk markers, including blood pressure and the serum lipid profile were measured, while blood pressure levels and plasma glucose concentrations were continuously monitored during daily life.

METHODS

Study participants and design

Sedentary older overweight and obese men participated in a randomized, controlled cross-over trial with an aerobic exercise intervention and no-exercise control period of both eight weeks, separated by a twelve-week wash-out period. An overview of the study design is shown in **Supplementary Figure 5.1**. Participants were allocated based on a computer-generated randomization scheme. Participants and investigators were unaware of the allocation prior to inclusion but could not be blinded during the intervention and measurements. However, images and blood samples were blinded prior to analysis. Study details have been described before (15). In brief, men were included if they met the

following criteria: aged between 60 and 70 years, body mass index (BMI) between 25 and 35 kg/m^2, no chronic diseases, no use of medication affecting the outcome measures, systolic (SBP) < 160 mmHg and diastolic blood pressure (DBP) < 100 mmHg, and a low physical activity level as assessed with the international physical activity questionnaire (IPAQ). The intervention period consisted of a fully supervised, personalized, and progressive aerobic-based exercise program on a cycling ergometer three times a week for 50 min. The training comprised 10 min warm-up at 45% maximal workload (Pmax), 30 min at 70% Pmax, and 10 min cool-down at 45% Pmax. Maximal exercise capacity was determined before the training intervention during incremental cycling at baseline and every other week, while measuring peak oxygen consumption (VO_2peak). The training intensity was adjusted accordingly. During the control and wash-out periods, participants had to maintain or return to their habitual physical activity levels. Men were requested not to change their habitual diet and consumption of alcohol throughout the study period, which was checked using a food frequency questionnaire. Energy and nutrient intakes were calculated using the Dutch Food Composition table.

Measurements were performed at the start of the control and intervention periods (BL), after 4 weeks (WK4) and during a follow-up day (FU-1) at the end of both periods. Vascular function and blood pressure were assessed 43 hours (range: 19 – 72 hours) after the final training. Additional blood samples were taken 117 hours (range: 70 – 118 hours) after FU-1 during a second follow-up day (FU-2). Participants arrived after an overnight fast and were requested to have a regular meal the evening before, and to refrain from alcohol and exercise 24-hour prior to each visit. Between both follow-up days, ambulatory blood pressure (ABP) levels were monitored, continuous glucose measurement (CGM) was performed, and physical activity was measured using accelerometry.

The study followed the ethical guidelines of the Declaration of Helsinki and was approved by the Medical Ethics Committee of Maastricht University Medical Centre (METC-173025). All study participants gave written informed consent before the start of the intervention trial. This study was registered at ClinicalTrials.gov (registry number: NCT03272061) on September 7th, 2017.

Anthropometrics

Height was measured during the screening visit using a wall-mounted stadiometer and scale. Body weight was measured every two weeks in the intervention period and at BL, WK4 and FU-1 in the control period. BMI was calculated, and body fat distribution was assessed by measuring the waist-to-hip circumference ratio.

Vascular measurements

Vascular function measurements were performed at FU-1 after a resting period of at least 15 min in supine position in a temperature-controlled, quiet, and darkened room at the Metabolic Research Unit Maastricht (MRUM).

Central vasculature

Ultrasound echography in B-mode using a 13-MHz transducer (MyLab Gamma, Esaote, Maastricht, the Netherlands) with continuous recoding was used to visualize the left common carotid artery 2 cm proximal to the bulbous. The CAR in response to a cold pressor

test was determined. The cold pressor test consisted of a one-minute baseline period and three-minute immersion of the hand in a bucket of cold water (4.0 °C) with ice slush. The carotid artery baseline diameter was defined as the average diameter over the first minute. After immersion, the diameter was averaged for intervals of ten-seconds (16). The maximal percentage change in post-immersion arterial diameter relative to the baseline arterial diameter was calculated. The change relative to baseline was also determined for every interval of ten seconds to calculate the net incremental area under the curve (net iAUC). The echo images for were analyzed offline using a custom-written MATLAB program using automated edge-detection and wall tracking (MyFMD V15.06, AP Hoeks, Department of Biomedical Engineering, Maastricht University Medical Centre, Maastricht, Netherlands). During the baseline period five-to-six heart beats were analyzed to determine the cIMT, and systolic and diastolic diameters of the carotid artery using a custom-written MATLAB program (VidArt V13.5, AP Hoeks, Department of Biomedical Engineering, Maastricht University Medical Centre, Maastricht, Netherlands). Local arterial stiffness characteristics were calculated using radial strain, arterial distensibility, pressure-independent stiffness index β_0 (17), Young's elastic modulus (Y_e) and lumen-to-cIMT-ratio. The equations used are given in the **Supplementary Material** (18).

$PWV_{c\text{-}f}$ was determined in triplicate with a tonometer (SphygmoCor v9, AtCor Medical, West Ryde, Australia) according to the current guidelines (19). The direct distance between the left carotid and femoral artery was used. Additionally, radial artery pulse wave analyses (PWA) were performed near the wrist of the arm in triplicate using the same tonometer. Central augmentation index corrected for heart rate (CAIxHR75) was determined as described (19).

Peripheral vasculature

FMD was also assessed by ultrasound echography in B-mode using a 13-MHz transducer (MyLab Gamma, Esaote, Maastricht, the Netherlands) with continuous recoding as recommended (20). After a resting baseline period of 3 min, a pneumatic cuff placed around the forearm of the participant was inflated to 200 mmHg for 5 min. Response of the brachial artery diameter following reactive hyperemia was imaged for another 5 min. FMD was quantified as the maximal percentage change in post-occlusion arterial diameter relative to baseline diameter. The echo images were also analyzed offline with the same software as for the CAR.

Retinal microvasculature

Retinal vascular images were made to assess microvascular calibers in the eye as described previously (21). The nonmydriatic retinal camera (Topcon TRC-NW-300, Topcon Co., Tokyo, Japan) focused on the right optic disc and photographed the retina. Images were digitally analyzed to calculate mean central retinal arteriolar (CRAE) and venular equivalents (CRVE), and the arteriolar-to-venular ratio (AVR) using the Parr-Hubbard formulas (22) and appropriate software (Generalized Dual-Bootstrap Iterative Closest Point (23)). Retinal images from the intervention and control periods were analyzed simultaneously, to ensure that the same segments from at least two arteries and venules were used for a participant.

Office and ambulatory blood pressure

Office brachial SBP and DBP were measured four times using a semi-continuous blood pressure device (Omron Intellisense M7, Cemex Medische Techniek, Nieuwegein, The Netherlands). The mean of the last three measurements is reported. The mean arterial pressure (MAP) was determined using the pulse wave that was determined at the brachial artery near the antecubital fossa with a tonometer (SphygmoCor v9, AtCor Medical, West Ryde, Australia). Additionally, central systolic and diastolic blood pressure (SBP_c and DBP_c) were determined using the radial pulse wave based on the brachial DBP and MAP. ABP was also measured (Mobil-O-Graph, I.E.M. Inc., Stolberg, Germany). Brachial blood pressure levels were recorded every 15 min during daytime (07:00 h till 22:00 h) and every 30 min at night (22:00 h till 07:00 h). The first measurement was discarded, and the mean and variability (i.e., SD) of the SBP, DBP, pulse pressure (PP) and heart rate (HR) were calculated over 24-hour, and during daytime and night-time. Additionally, nocturnal SBP and nocturnal DBP dipping were calculated using the mean difference between daytime and night-time blood pressure expressed as a percentage of the daytime value.

Continuous glucose monitoring and physical activity

Glucose concentrations were measured every 15 min using the Freestyle Libre Pro (Abbott, Alameda, California, United States), which was placed precisely at the back of the upper arm. The AUC and iAUC were calculated with the trapezoid rule over a 96-hour period using GraphPad Prism 8 (24). The baseline glucose concentration for the iAUC was determined by taking the average minimal one-hour value of each measurement day. Accelerometry-based physical activity levels were measured using the activPAL3 (PAL Technologies Ltd., Glasgow, United Kingdom), a validated accelerometer for measuring physical activity and sedentary behavior (25). The accelerometer was placed on the anterior side of the left thigh 10 cm above the patella to monitor daily activity patterns and was worn uninterrupted for a median duration of 120 hours (range: 95 hours – 144 hours). Data was processed using the PAL analysis software with CREA algorithm (Version 8.11.4.61, PAL Technologies Ltd., Glasgow, United Kingdom). Physical activity score expressed as metabolic equivalent time of task per second (MET/s) and total sedentary time in seconds were extracted from the 15-second epoch file.

Serum lipid profile

Fasting blood samples were taken from a forearm vein at BL, WK4, and FU-1. At FU-2, blood samples were taken using an intravenous cannula. Blood drawn in vacutainer SST™ II Advance tubes (Becton, Dickson and Company, Franklin Lanes, New-York, USA) were allowed to clot for at least 30 min at 21 °C. Vacutainer tubes were centrifuged at 1300 x g for 15 min at 21 °C to obtain serum. Tubes containing sodium fluoride (NaF) plus Na_2EDTA (Becton, Dickson and Company, Franklin Lanes, New-York, USA) were kept on ice and centrifuged within 30 min at 1300 x g for 15 min at 4 °C to obtain plasma. Serum and plasma samples were immediately portioned, frozen in liquid nitrogen, and stored at -80 °C until analysis at the end of the study. Serum samples were analyzed for concentrations of total cholesterol (TCH: CHOD-PAP method; Roche Diagnostics, Mannheim, Germany), triacylglycerol corrected for free glycerol (TAG: GPO Trinder; Sigma-Aldrich Corporation, St. Louis, Mo, USA), HDL-cholesterol (precipitation method; Roche Diagnostics, Mannheim,

Germany) and high-sensitivity C-reactive protein (hsCRP) (immunoturbidimetric assay, Horiba ABX, Montpellier). LDL-cholesterol was calculated using the Friedewald formula. Values of FU-1 and FU-2 were averaged before statistical analyses.

Statistical analyses

When measurements were only taken at the end of both periods, effects of the exercise program were examined using analysis of variance (ANOVA) with participant number, treatment, and period as fixed factors. When repeated measurements were made during a period, differences in the effects between treatments over time were examined using linear mixed models with the change from baseline as the dependent variable. Time, treatment, period, and time * treatment interaction were used as fixed factors. The interaction term was omitted from the model if it was not statistically significant. When the time * treatment interaction was statistically significant, difference in changes at weeks 4 and 8 between the intervention and control periods were compared pairwise with Bonferroni correction. Unless otherwise indicated, normal distributed variables are shown as means ± SDs, and non-normal distributed variables are shown as medians (ranges). Effect sizes are reported as differences between intervention and control periods with 95% CIs. Concentrations of hsCRP were log-transformed, as concentrations were not normally distributed based on quantile-quantile plots. SPSS was used to perform all statistical analyses (IBM Corp., IBM SPSS Statistics, V26, Armonk, NY, USA). A $P < 0.05$ was considered to be statistically significant.

RESULTS

Study participants

A consolidated standard of reporting trials (CONSORT) flow diagram of study participants is shown in **Supplementary Figure 5.2**. A total of seventeen participants completed the study. FMD measurements for two participants and one CAR measurement could not be analyzed due to technical problems with the recording of the images. Another FMD assessment could not be analyzed due to insufficient quality of the ultrasound recordings. Accelerometer recordings of one participant were missing, because the device was lost during the recordings. Finally, CGM measurements of one study participant failed.

Baseline characteristics of the study participants, who completed the study have been described before (15). The men had a mean age of 67 ± 2 years, and their mean BMI was 30.3 ± 2.8 kg/m^2. The median attendance rate of the scheduled training sessions was 100% (range: 92 - 100%). Weight and waist-to-hip circumference ratio did not differ between treatments (treatment effects: $P = 0.830$ and $P = 0.823$, respectively) and remained stable throughout the study (time effects: $P = 0.289$ and $P = 0.373$, respectively). As anticipated, aerobic fitness improved, as indicated by a significant time * treatment interaction ($P = 0.018$) for the VO_2peak, which tended to increase by 99 mL after 4 weeks (95% CI: -15, 214 mL; $P = 0.088$) and increased significantly by 262 mL after 8 weeks (95% CI: 153, 394 mL; $P < 0.001$) (15). Energy and nutrient intakes did also not change during the study (**Supplementary Table 5.1**).

Vascular measurements

Central vasculature

CAR responses were 4.01% after exercise training and 1.78% after the control period and thus improved by 2.23 percentage points (pp) (95% CI: 0.58, 3.89 pp; P = 0.012). Additionally, the net iAUC increased by 104 %*min (95% CI: 35, 173 pp*min; P = 0.006). Baseline carotid artery diameters before the cold pressor test, but also diastolic and systolic diameters over five-to-six heart beats did not differ (**Table 5.1; Figure 5.1A and C**. Moreover, exercise training increased local arterial stiffness, as indicated by a decreased radial strain of 1.27 mm (95% CI: 0.33, 2.22 mm; P = 0.012), while the distensibility tended to decrease (Δ -4.2 MPa-1; 95% CI: -8.9, 0.3 MPa-1; P = 0.067). The stiffness index B_0 increased (Δ 1.1; 95% CI: 0.3, 1.9; P = 0.010), while the Ye did not change (Δ 0.1 MPa; 95% CI: 0.0, 0.3 MPa; P = 0.065) (Table 1). As expected, cIMT (Δ 0.02 mm; 95% CI: -0.05, 0.08 mm; P = 0.579) and the lumen-to-cIMT ratio did not change (Δ 0.02; 95% CI: -0.27, 0.34; P = 0.816). In contrast, PWVc-f (Δ 0.4 m/s; 95% CI: -0.4, 1.2 m/s; P = 0.264) and CAIxHR75 (Δ -0.8 pp; 95% CI: -2.9, 1.4 pp; P = 0.448) did not significantly change (see **Table 5.1**).

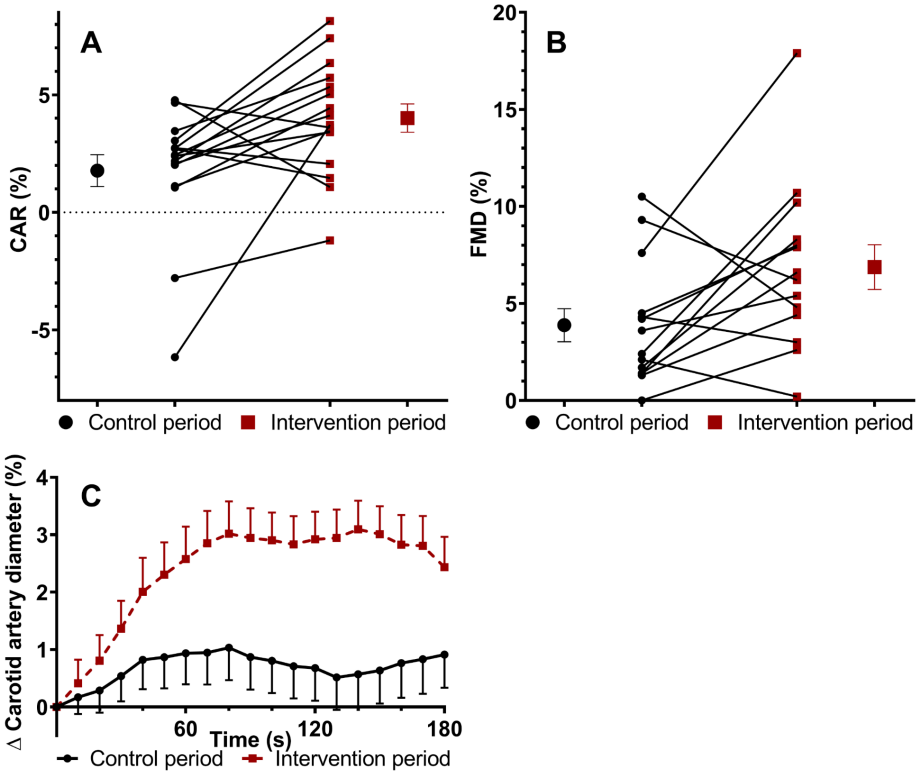

Figure 5.1. Training-induced changes of endothelial function markers of a randomized, controlled cross-over study with sedentary overweight and obese men. Mean (± SEM) and individual (**A**) carotid artery responses (CAR; n = 16) and (**B**) brachial artery flow-mediated vasodilation (FMD; n = 14) following the control and intervention period. (**C**) Mean (± SEM) carotid artery diameter changes averaged for each ten seconds during the cold pressure test (n = 16) following the control and intervention period.

Peripheral vasculature
FMD improved after the intervention as compared with the control period by 2.99 pp (95% CI: 0.58, 5.41 pp; P = 0.019), while the brachial artery diameter measured during the baseline period of the FMD decreased (Δ -0.34 mm; 95% CI: -0.17, -0.45 mm; P = 0.001) (**Table 5.1; Figure 5.1C**).

Retinal microvasculature
The CRAE significantly increased by 4 µm (95% CI: 0, 7 µm; P = 0.041), while the CRVE did not change (Δ 1 µm; 95% CI: -1, 3 µm; P = 0.153). The AVR did not change (Δ 0.02; 95% CI: 0.00, 0.03; P = 0.076).

Office and ambulatory blood pressure
Office brachial DBP decreased by 5 mmHg (95% CI: -6, -3 mmHg; P = 0.002) after the intervention as compared with the control period. Central DBP also decreased by 5 mmHg (95% CI: -6, -2 mmHg; P = 0.001). Brachial SBP did not change (Δ 3 mmHg; 95% CI: -8, 1 mmHg; P = 0.096), while central SBP significantly decreased by 5 mmHg (95% CI: 1, 9 mmHg; P = 0.015). Office (Δ 1 mmHg; 95% CI: -3, 5 mmHg; P = 0.627) and central (Δ -1 mmHg; 95% CI: -3, 1 mmHg; P = 0.340) PP did not change. Additionally, MAP decreased by 4 mmHg (95% CI: -7, -2 mmHg; P = 0.005). Heart rate was comparable after both periods (Δ -1 beats/min; 95% CI: -4, 2 beats/min; P = 0.473). There were no significant effects on mean 24-hour (24h), mean daytime, and mean night-time ABP levels. Additionally, SDs and nocturnal dipping in SBP and DBP did not differ between both periods (**Table 5.1, Figure 5.2, and Supplementary Table 5.2**).

Continuous glucose monitoring and physical activity
The AUC (Δ 18 mmol/L*h; 95% CI: -7, 44 mmol/L*h; P = 0.139) and iAUC (Δ 0 mmol/L*h; 95% CI: -0.20, 0.22 mmol/L*h; P = 0.921) for continuous glucose concentrations over a 96-hour period did not significantly change (**Table 1, Supplementary Figure 3**). No differences were observed between the intervention and control periods in physical activity score (Δ 0.01 MET/s; 95% CI: -0.02, 0.04 MET/s; P = 0.436) and sedentary time (Δ -24 seconds; 95% CI: -67, 18 seconds; P = 0.231).

Serum lipid profile
There was a significant time * treatment effect for HDL-cholesterol concentrations (P = 0.035), which increased by 0.07 mmol/L (95% CI: 0.01, 0.12 mmol/L; = 0.015) in the intervention group as compared with the control group at week 4, while the difference in changes was comparable at week 8 (Δ 0.01 mmol/L; 95% CI: -0.04, 0.07; P = 0.599). TCH, LDL-cholesterol, TAG and hsCRP concentrations did not change (**Table 2**).

Table 5.1. Vascular markers, blood pressure and continuous glucose measurement outcomes from a randomized, controlled cross-over study with sedentary older men (n = 17)[1].

	Intervention period	Control period	Mean difference (95% CI[2])	
Vascular markers				
CAR (%)[3]	4.01 ± 2.41	1.78 ± 2.71	2.23	(0.58, 3.89)*
$CAR_{net\ iAUC}$ (%*min)[3]	42 ± 116	147 ± 106	104	(35, 173)**
Carotid baseline diameter (mm)[3]	7.76 ± 1.43	7.71 ± 1.35	0.05	(-0.07, 0.18)
Brachial artery FMD (%)[4]	6.87 ± 4.32	3.88 ± 3.19	2.99	(0.58, 5.41)*
Brachial baseline diameter (mm)[4]	3.25 ± 0.34	3.59 ± 0.52	-0.34	(-0.45, -0.17)**
Carotid diastolic diameter (mm)[3]	8.22 ± 1.02	8.08 ± 0.96	0.12	(0.00, 0.25)*
Carotid systolic diameter (mm)[3]	8.54 ± 1.11	8.52 ± 1.09	0.02	(-0.09, 0.14)
Radial strain (%)[3]	3.88 ± 1.06	5.33 ± 2.18	-1.27	(-2.22, -0.33)*
Distensibility (MPa^{-1})[3]	13 ± 4.5	18 ± 9.4	-4	(-8.9, 0.3)
Stiffness index β_0[3]	7.4 ± 1.6	6.1 ± 2.1	1.1	(0.3, 1.9)*
Y_e (MPa)[3]	0.8 ± 0.3	0.6 ± 0.3	0.1	(0.0, 0.3)
$PWV_{c\text{-}f}$ (m/s)	12.6 ± 2	12.3 ± 1.8	0.4	(-0.4, 1.2)
CAIxHR75 (%)	19.5 ± 7.8	20.7 ± 7.4	-0.8	(-2.9, 1.4)
CRAE (µm)	119 ± 27	115 ± 27	4	(0, 7)*
CRVE (µm)	194 ± 32	193 ± 33	1	(-1, 3)
AVR ratio	0.61 ± 0.1	0.60 ± 0.11	0.02	(0.00, 0.03)
Office blood pressure				
Brachial SBP (mmHg)	135 ± 8	139 ± 11	-5	(-8, 1)
Brachial DBP (mmHg)	81 ± 7	85 ± 5	-4	(-6, -3)**
Brachial PP (mmHg)	54 ± 7	53 ± 8	1	(-3, 5)
Brachial MAP (mmHg)	103 ± 7	107 ± 8	-5	(-7, -2)**
Central SBP (mmHg)	118 ± 8	123 ± 11	-4	(-9, -1)*
Central DBP (mmHg)	95 ± 7	99 ± 6	-1	(-6, -2)**
Central PP (mmHg)	23 ± 4	24 ± 6	-1	(-3, 1)
HR (beats/min)	57 ± 7	58 ± 8	0	(-4, 2)
Ambulatory Blood Pressure				
24-h SBP (mmHg)	126 ± 8	127 ± 9	-2	(-5, 1)
24-h DBP (mmHg)	81 ± 5	82 ± 5	0	(-4, 1)
24-h PP (mmHg)	46 ± 6	46 ± 7	-1	(-1, 2)
24-h HR (beats/min)	69 ± 9	71 ± 11	0	(-4, 1)
CGM outcomes				
CGM_{AUC} (mmol/L*h) [a]	496 ± 103	477 ± 92	0	(-7, 44)
CGM_{iAUC} (mmol/L*h) [a]	75 ± 37	75 ± 24	0	(-20, 22)

[1]Data are shown as mean ± SD. [2]analysis of variance (ANOVA) with period as fixed factor with 95% confidence interval (95% CI): *P < 0.05, **P < 0.01. [3]n = 14, [4]n = 16. AVR: arteriolar-to-venular ratio; CAIxHR75: central augmentation index adjusted for heart rate; CAR: carotid artery reactivity; CGM: continuous glucose monitor; CRAE: central retinal arteriolar equivalent; CRVE: central retinal venular equivalent; DBP: diastolic blood pressure; FMD: brachial artery flow-mediated vasodilation; HR: heart rate; (i)AUC: (incremental) area under the curve; MAP: mean arterial pressure; net iAUC: net incremental area under the curve; PP: Pulse Pressure; $PWV_{c\text{-}f}$: carotid-to-femoral pulse wave velocity; SBP: systolic blood pressure; Ye: Young's modulus of elasticity.

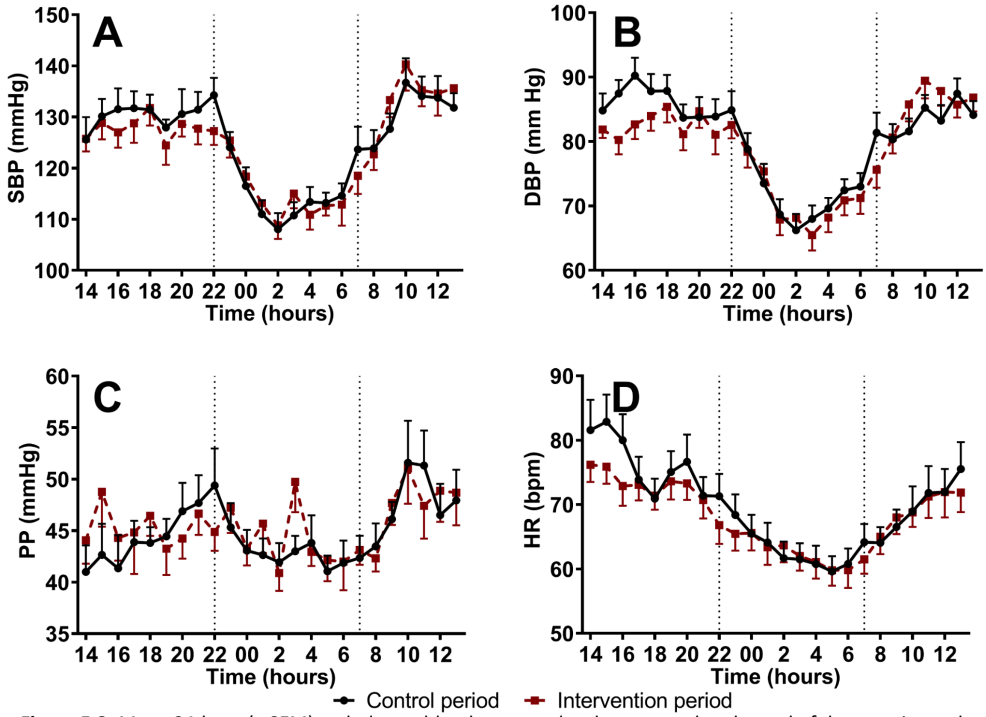

Figure 5.2. Mean 24-hour (± SEM) ambulatory blood pressure levels measured at the end of the exercise and control period in a randomized cross-over study with sedentary overweight and obese older men (n = 17). Mean **(A)** systolic blood pressure (SBP), **(B)** diastolic blood pressure (DBP), **(C)** pulse pressure (PP), and **(D)** heart rate (HR) following the control and intervention period.

Table 5.2. Metabolic measurements from a randomized, controlled cross-over study with sedentary obese older men (n = 17)[1].

	Intervention period	Control period	Mean difference		P-value[2]	
	BL	BL	Δ WK4	Δ FU	time * treatment	treatment
TCH (mmol/L)	5.77 ± 1.06	5.55 ± 1.21	0.01 ± 0.49	-0.15 ± 0.92	0.648	0.547
TAG (mmol/L)	1.35 ± 0.47	1.36 ± 0.52	0.19 ± 0.57	0.09 ± 0.57	0.411	0.497
HDL (mmol/L)	1.33 ± 0.19	1.30 ± 0.16	-0.07 ± 0.12*	0.01 ± 0.13	0.035	N/A
LDL (mmol/L)	3.82 ± 0.97	3.64 ± 1.07	-0.02 ± 0.58	-0.21 ± 0.73	0.430	0.366
CRP (mg/L) [1]	3.35 ± 5.03	2.22 ± 2.30	-1.40 ± 3.54	-1.88 ± 4.71	0.332	0.099

[1]Data are shown as mean ± SD. [2]Time * treatment and treatment effect (linear mixed model): *P < 0.05. [1]Statistics were performed after log-transformation due to non-normal distribution. BL: baseline; CRP: C-reactive protein; FU: follow-up; HDL: high density lipoprotein; LDL: low density lipoprotein; TAG: triacylglycerol; TCH: total cholesterol; WK4: week 4.

DISCUSSION

In this randomized, controlled cross-over trial with sedentary overweight and obese older men, aerobic exercise training improved endothelial function, as shown by the changes in FMD. The CAR response, which is related to effects on endothelial function and autonomic control of vascular tone, also improved. In contrast, local vascular stiffness of the carotid

artery increased. Regional aortic vascular stiffness as measured with PWVc-f did not change. Finally, retinal arteriolar diameters and office blood pressure improved, but no effects were found on ABP, serum lipids and 24-hr glucose concentrations. Participants did not change their habitual diet or regular daily physical activity pattern, underlining that the effects observed were due to the aerobic exercise training program. Effects on endothelial function and retinal arteriolar width may provide a better estimate for the decrease in CVD risk after aerobic exercise training than effects on the more conventional cardiometabolic risk markers (26).

Aerobic exercise training significantly improved the CAR response to a cold pressor test. In fact, the maximal vasodilation of the carotid artery increased to 4%, a value comparable to that of younger individuals aged 24 ± 3 years (7). The increase in CAR response may decrease cardiovascular risk and the risk for future cardiovascular events (8, 9). Catecholamines (e.g. norepinephrine) released during the cold pressor test can increase vasodilation via endothelium-dependent effects, but might at the same time cause vasoconstriction of smooth muscle cells via the sympathetic nervous system (16). Thus, we can speculate that the balance between these two processes was beneficially affected after exercise training. The observed improvement in FMD, which is associated with a decreased CVD risk (27), is in line with the results of a meta-analysis when a comparable training protocol was used (6). The baseline brachial artery diameter decreased after aerobic exercise training. However, a smaller baseline brachial artery diameter is also predictive of cardiovascular risk (28), underlining the clinical relevance of our findings. Taken together, observed changes in both major elastic conduit (CAR) and peripheral muscular arteries (FMD) supports the evidence for beneficial effects of exercise training on vascular function of central and peripheral arteries, and thereby CVD risk in overweight and obese men.

Surprisingly, we observed that local carotid arterial stiffness increased. In contrast, central arterial stiffness was lower in participants who are more physically active (29, 30). Also, daily walking for 14 weeks decreased the carotid arterial stiffness in normal-weight older men (30). However, some exercise training trials observed similar effects on vascular stiffness as we did. In fact, resistance training protocols also showed increased local carotid stiffness (10). Although underlying mechanisms are unclear, it was hypothesized that repetitive elevations in blood pressure during training sessions, as well as adaptations in vascular tone, have contributed to this observation (10). The changes in central blood pressure observed in this study may indeed change arterial compliance. However, further research on the effects of training on local artery stiffness is warranted. $PWV_{c\text{-}f}$, which is a marker for regional arterial stiffness, did not change. In contrast, a meta-analysis of 20 studies showed an improved aortic PWV, which could not be explained by changes in blood pressure and was not related to exercise intensity (12). However, this apparent discrepancy can be explained by our study duration of 8 weeks, as subgroup analyses revealed that improvements in $PWV_{c\text{-}f}$ were only observed when the training program exceeded 10 weeks (12). This also suggests that longer training duration may be needed to reduce stiffness in large central conduit arteries, as measured with the $PWV_{c\text{-}f}$. However, the positive effects on the brachial-ankle PWV, as has been observed (12), may relate to increases in shear stress during aerobic exercise training, which may in particular affect nitric oxide-producing muscular arteries (12). Retinal microvascular calibers were beneficially affected, as shown by an increased in CRAE, although CRVE did not change. These results agree with those of

other studies (13, 31) and may be related to the decrease in office blood pressure, as wider retinal arterioles are independently associated with a decreased risk to develop hypertension (32).

Office blood pressure decreased after aerobic exercise training, which was consistent with previous studies that focused on office blood pressure and is related to a reduction in CVD risk (33). It is suggested that a reduction in vascular resistance via the sympathetic nervous system and the renin-angiotensin system are involved. In contrast, ABP was not affected. A meta-analysis of 15 aerobic exercise training studies of at least 4 weeks observed a significant, albeit modest, decrease of 3 mmHg in daytime SBP and DBP (34). It is possible that the longer median study duration of 15 weeks (range: 6 – 52 weeks) and the reduction in body weight in one-third of the studies included in that meta-analysis may explain this discrepancy (34). It can also be speculated that only office BP is reduced after exercise training during a resting period and not ABP during daily activities, due to an increased sympathetic activity (35).

We have already reported improvements in post-load glucose concentration during an oral glucose tolerance test (15). However, fasting glucose and insulin concentrations and the homeostatic model assessment index as a measure of insulin resistance did not change (15). We now found that CGM and the serum lipid profile did not change following exercise training. In our study, body weight remained stable, while improvements in the serum lipid profile in other studies often coincided with weight reduction (36-39). Beneficial effects in these studies may therefore not be attributed to the exercise intervention alone. In addition, our study population may have been too healthy to improve metabolic risk markers. Indeed, Couillard and colleagues only observed beneficial effects of an exercise intervention on these serum lipids in participants with metabolic disorders (40).

This randomized, controlled, cross-over trial had a wash-out period of twelve weeks. Although VO_2peak returned to baseline, we cannot exclude that some outcome parameters had not yet returned to baseline after the wash-out period. Multiple, possibly interrelated, markers for vascular function were affected, while we also observed effects on office blood pressure, which makes it impossible to estimate the overall effect on CVD risk reduction. Also, we might have been underpowered to detect differences for some of the described outcome parameters. This trial was performed only in overweight and obese men with an age between 60 and 70 years, which reduces the external validity of our findings.

We have already reported that aerobic exercise training has an positive effects on cerebrovascular function (15). This trial provides here also evidence that aerobic exercise training in sedentary overweight and obese older men improves not only FMD, but also CAR responses and retinal arteriolar width. These effects may be important mechanisms by which aerobic exercise training reduces age-related health problems, such as CVD and cognitive decline. The results of exercise training on local carotid stiffness warrants further study.

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and K.M.R. Nijssen for their assistance during the experimental phase of the trial. Finally, we thank our participants for their cooperation and enthusiasm. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

DECLARATIONS

The project is organized by and executed under the auspices of TiFN, a public-private partnership on precompetitive research in food and nutrition. The authors have declared that no competing interests exist in the writing of this publication. Funding for this research was obtained from the Netherlands Organization for Scientific Research. The datasets analyzed during the current study are available from last author on reasonable request.

AUTHORS' CONTRIBUTIONS

The authors' contributed as follows; JK: designed and conducted the study, performed the statistical analyses, interpreted the data and wrote the manuscript, RM: designed the study, interpreted the data, had overall responsibility for the study, and wrote the manuscript, JR: set up vascular function measurements, interpreted the data, and reviewed the manuscript, DT: set up the CAR measurement, interpreted the data, and reviewed the manuscript, MH: designed the exercise protocol, and reviewed the manuscript, and PJ: designed the study, interpreted the data, had overall responsibility for the study, and wrote the manuscript.

SUPPLEMENTARY MATERIAL

Local arterial stiffness properties

Local arterial stiffness properties were calculated using the following formulas:

Equation 1

$$\text{Radial strain (\%)} = \frac{D_s - D_d}{D_d} \times 100$$

Equation 2

$$\text{Distensibility } (MPa^{-1}) = \frac{(D_s - D_d)/D_d}{SBP_c - DBP_c}$$

Equation 3

$$\beta_0 = \frac{\ln(SBP_c/DBP_c)}{(D_s/D_d) - 1} - \ln(\frac{DBP_c}{BP_{ref}})$$

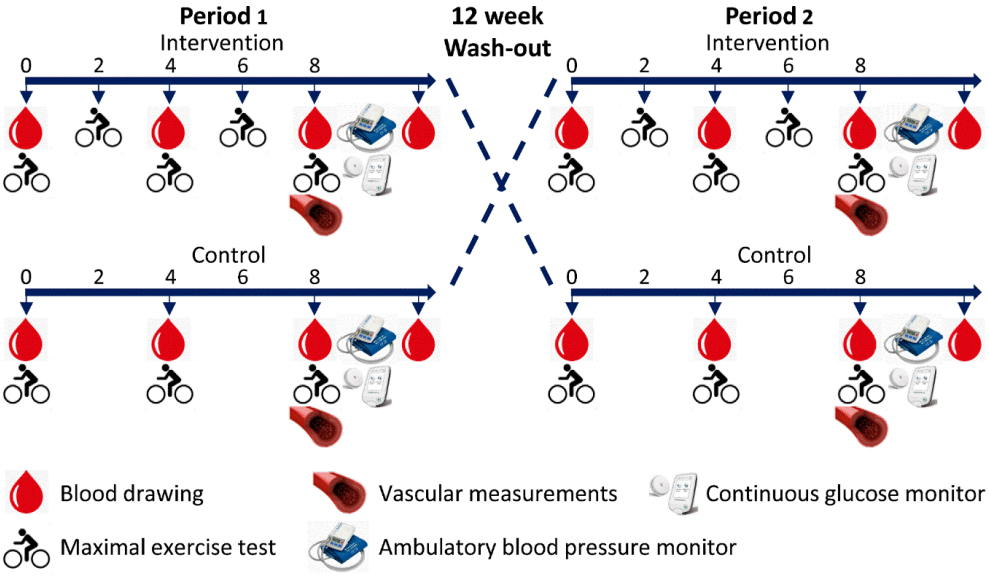
Equation 4

$$Y_e \text{ (MPa)} = \frac{SBP_c - DBP_c}{D_s - D_d} \times \frac{{D_d}^2}{cIMT}$$

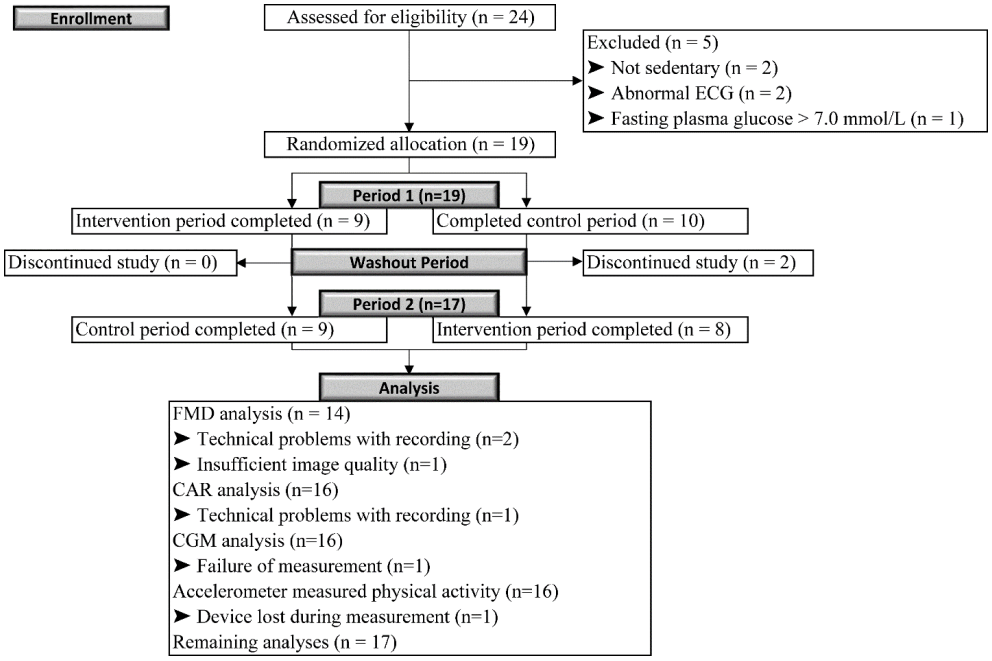
Equation 5

$$\text{lumen} - \text{to} - \text{cIMT} - \text{ratio } (mm^{-1}) = \frac{D_d}{cIMT^2}$$

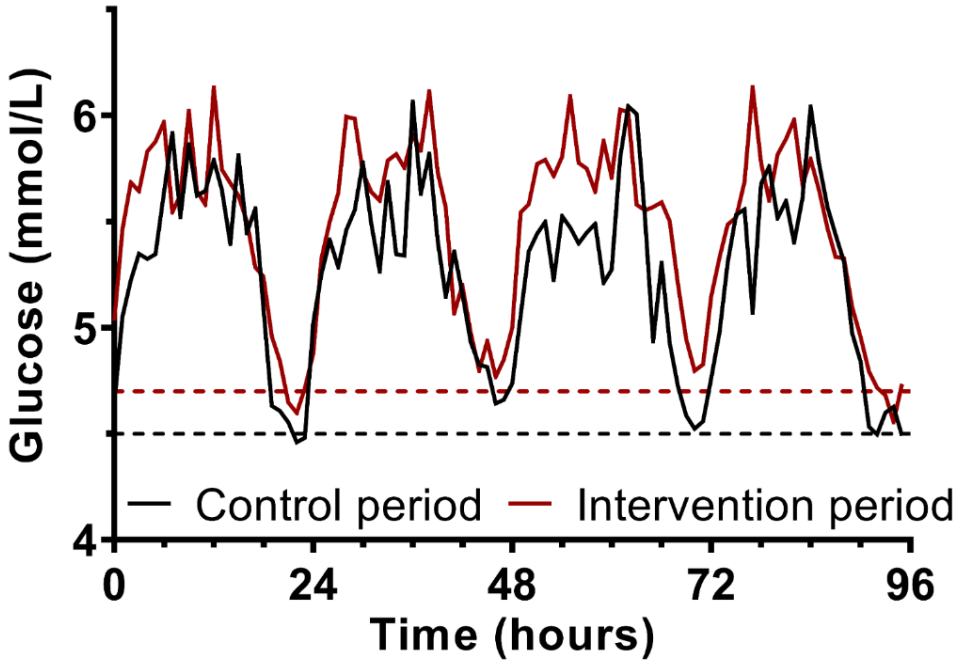
Where D_s and D_d are the carotid arterial systolic and diastolic diameter and cIMT is intima-media thickness of the carotid artery. The average DBP for both periods from all participants was used as reference blood pressure (BP_{ref}).



Supplementary Figure 5.1. Schematic overview of study design. Timeline is displayed as weeks.



Supplementary Figure 5.2. Consort flow diagram. Diagram of the progress through the phases of this randomized, controlled cross-over trial with two periods. FMD: flow-mediated vasodilation, CAR: carotid artery reactivity, CGM: continuous glucose monitor.



Supplementary Figure 5.3. Mean 96-hour continuous glucose measurements obtained from a randomized, controlled cross-over study with sedentary older men (n = 16) following the intervention and control period. The horizontal dashed lines (black = control period, red = intervention period) represent the average fasting glucose concentration that was calculated during the nights from 03:00 to 04:00 hour.

Supplementary Table 5.1. Average energy and nutrient intakes after an exercise period or no-exercise control period from a randomized, controlled cross-over study with sedentary older men (n = 17)[1].

	Intervention period	Control period	Mean difference (95% CI[1])	
Energy (KJ/day)	9320 ± 1749	9770 ± 2666	-640	(-1,648, 369)
Energy (Kcal/day)	2224 ± 418	2332 ± 638	-152	(-393, 88)
Carbohydrate (En%)	36.66 ± 7.07	36.72 ± 7.76	-0.18	(-3.52, 3.16)
Protein (En%)	16.75 ± 3.84	17.59 ± 5.65	-0.67	(-2.67, 1.33)
Total fat (En%)	39.57 ± 5.17	38.93 ± 5.26	0.57	(-1.65, 2.78)
Saturated FA (En%)	13.46 ± 2.24	13.61 ± 1.98	-0.11	(-0.77, 0.54)
Monounsaturated FA (En%)	15.01 ± 4.90	14.15 ± 4.41	0.76	(-0.95, 2.45)
Polyunsaturated FA (En%)	7.53 ± 1.45	7.43 ± 1.68	0.04	(-0.51, 0.60)
Alcohol (En%)	4.72 ± 4.80	4.68 ± 4.84	0.11	(-0.60, 0.82)
Dietary fiber (g/day)	24.53 ± 7.82	21.95 ± 8.45	1.80	(-1.22, 4.83)

[1]Data are shown as mean ± SD. [2]analysis of variance (ANOVA) with period as fixed factor with 95% confidence interval (95% CI). EN: Energy percentage; FA: fatty acids.

Supplementary Table 5.2. Ambulatory blood pressure measurements after an exercise period or no-exercise control period from a randomized, controlled cross-over study with sedentary older men (n = 17)[1].

	Intervention period	Control period	Mean difference (95% CI[2])	
Daytime SBP (mmHg)	129 ± 8	129 ± 10	0	(-5, 2)
Daytime DBP (mmHg)	83 ± 5	84 ± 6	-2	(-4, 0)
Daytime PP (mmHg)	46 ± 6	46 ± 7	1	(-2, 2)
Daytime HR (beats/min)	71 ± 9	73 ± 12	-2	(-5, 2)
Nighttime SBP (mmHg)	117 ± 8	115 ± 9	1	(-5, 3)
Nighttime DBP (mmHg)	73 ± 6	73 ± 6	0	(-4, 3)
Nighttime PP (mmHg)	44 ± 5	43 ± 7	1	(-2, 2)
Nighttime HR (beats/min)	63 ± 10	63 ± 9	0	(-3, 2)
SD 24-hour SBP (mmHg)	15 ± 3	16 ± 4	-1	(-4, 1)
SD 24-hour DBP (mmHg)	11 ± 2	11 ± 2	0	(-2, 1)
SD 24-hour PP (mmHg)	11 ± 4	12 ± 4	-1	(-2, 1)
SD 24-hour HR (beats/min)	9 ± 3	11 ± 3	-1	(-3, 1)
SD Daytime SBP (mmHg)	15 ± 4	15 ± 4	0	(-4, 2)
SD Daytime DBP (mmHg)	10 ± 2	10 ± 2	0	(-1, 1)
SD Daytime PP (mmHg)	12 ± 4	13 ± 4	-1	(-3, 2)
SD Daytime HR (beats/min)	10 ± 3	11 ± 4	-1	(-3, 2)
SD Nighttime SBP (mmHg)	12 ± 3	12 ± 4	0	(-4, 1)
SD Nighttime DBP (mmHg)	10 ± 2	10 ± 3	0	(-2, 1)
SD Nighttime PP (mmHg)	8 ± 4	7 ± 3	1	(-1, 3)
SD Nighttime HR (beats/min)	5 ± 2	6 ± 3	-1	(-2, 0)
Dipping SBP (%)	10 ± 4	10 ± 6	0	(-4, 3)
Dipping DBP (%)	13 ± 5	13 ± 6	0	(-5, 2)

[1]Data are shown as mean ± SD. [2]analysis of variance (ANOVA) with period as fixed factor with 95% confidence interval (95% CI). DBP: diastolic blood pressure; HR: heart rate; PP: pulse pressure; SD: standard deviation of individual 24-hour daytime or nighttime values (within-subject variability); SBP: systolic blood pressure.

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CHAPTER 6

Longer-term soy nut consumption improves cerebral blood flow and psychomotor speed: Results of a randomized, controlled cross-over trial in older males and females

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ABSTRACT

Background

Effects of soy foods on cerebral blood flow (CBF) - a marker of cerebrovascular function - may contribute to the beneficial effects of plant-based diets on cognitive performance. The aim of this study was to investigate longer-term effects of soy nut consumption on CBF in older adults. Changes in three different domains of cognitive performance were also studied.

Methods

Twenty-three healthy participants (age: 60-70 years; BMI: 20-30 kg/m^2) participated in a randomized, controlled, single-blinded cross-over trial with an intervention (67 g/day of soy nuts providing 25.5 g protein and 174 mg isoflavones) and control period (no nuts) of 16 weeks, separated by an 8-week wash-out period. Adults followed the Dutch food-based dietary guidelines. At the end of each period, CBF was assessed with arterial spin labeling magnetic resonance imaging. Psychomotor speed, executive function and memory were assessed using the Cambridge Neuropsychological Test Automated Battery (CANTAB).

Results

No serious adverse events were reported, and soy nut intake was well tolerated. Body weights remained stable during the study. Serum isoflavone concentrations increased (daidzein Δ 128 ± 113 ng/mL, $P < 0.001$; genistein Δ 454 ± 256 ng/mL, $P < 0.001$), indicating excellent compliance. Regional CBF increased in four brain clusters located in the left occipital and temporal lobe (Δ 11.1 ± 12.4 mL/100g/min, volume: 11,296 mm^3, $P < 0.001$), bilateral occipital lobe (Δ 12.1 ± 15.0 mL/100g/min, volume: 2,632 mm^3, $P = 0.002$), right occipital and parietal lobe (Δ 12.7 ± 14.3 mL/100g/min, volume: 2,280 mm^3, $P = 0.005$), and left frontal lobe (Δ 12.4 ± 14.5 mL/100g/min, volume: 2,120 mm^3, $P = 0.009$) that is part of the ventral network. These four regions are involved in psychomotor speed performance, which improved as the movement time reduced by -20 ± 37 ms ($P= 0.005$). Executive function and memory did not change.

Conclusions

Longer-term soy nut consumption may improve cerebrovascular function of older adults, as regional CBF increased. Effects may underlie observed improvements in psychomotor speed.

This clinical trial was registered on August 13th, 2018, at clinicaltrials.org as NCT03627637.

INTRODUCTION

Aging-related health conditions, such as impaired cognitive performance and cardiovascular diseases (CVD) are amongst the most prevalent disorders in the world (1, 2). Effective intervention strategies are therefore highly needed to prevent or reduce the burden of these conditions (1). Though less extensively studied than the potentially beneficial effects on CVD risk, consumption of plant-based diets has also been associated with improvements in cognitive performance across different cognitive domains (2, 3). Consequently, studies on the health effects of specific plant-based foods such as soy are gaining increasing attention. Soy is rich in phytoestrogens (isoflavones), *cis*-polyunsaturated fatty acids (*cis*-PUFAs) and high-quality plant proteins, which may all improve cognitive performance (4-7). Effects of soy-rich foods on cerebrovascular function are of major interest. In fact, an impaired vascular function in the brain may precede the age-related decline in cognitive performance, and several reviews already concluded that diet-induced improvements in cerebrovascular function contribute to the beneficial effects observed on cognitive performance (8-10).

The consumption of specific substances that are also present in soy may improve cerebral blood flow (CBF) (8, 11), but effects of soy products on this physiological marker of cerebrovascular function (12) have not been reported before. Moreover, glucose metabolism may play an important role as beneficial effects of interventions on CBF may be partly mediated by improvements in glucose metabolism (13-16). This randomized, controlled, cross-over trial investigated the effects of longer-term soy nut consumption on CBF, which was the primary outcome of the study, and cognitive performance in older males and females. These participants are expected to have decreased CBF and are also at increased risk of cognitive impairment (17). The non-invasive MRI perfusion method arterial spin labeling (ASL) was used as primary outcome to quantify CBF, while cognitive performance was assessed as secondary outcome using the Cambridge neuropsychological test automated battery (CANTAB). Focus was on three main domains of cognitive performance (i.e., psychomotor speed, executive function, and memory). Effects on glucose metabolism were also investigated by a 7-point oral glucose tolerance test (OGTT) and by monitoring glucose concentrations continuously during daily life.

PARTICIPANTS AND METHODS

Study participants

Healthy older males and postmenopausal females were recruited through advertisements in local newspapers, flyers in the university, the hospital, and public buildings in Maastricht, and among people who had participated in earlier studies. They were invited for a screening visit when they were aged between 60 and 70 years and had a BMI between 20 and 30 kg/m^2. During a screening visit, anthropometrics and blood pressure were measured, and a fasting blood sample was drawn. Participants were included if they met the following criteria: stable body weight (<3 kg body weight gain or loss in the past three months); systolic < 160 mmHg and diastolic blood pressure < 100 mmHg; fasting plasma glucose < 7.0 mmol/L, fasting serum total cholesterol < 8.0 mmol/L, and fasting serum triacylglycerol < 4.5 mmol/L. Participants were excluded when having an allergy or intolerance to soy; when they were smoking, or quit smoking less than 12 months before starting the study; use of dietary supplements known to interfere with the main study outcomes; no use of

medication known to affect blood pressure, lipid or glucose metabolism; and no specific contra-indications for MRI imaging (e.g. permanent make-up, surgical clips or claustrophobia). In addition, volunteers suffering from severe medical conditions, including CVD (e.g., congestive heart failure or any other CVD event in the past), diabetes mellitus, familial hypercholesterolemia, epilepsy, asthma, kidney failure, chronic obstructive pulmonary disease, inflammatory bowel diseases, autoinflammatory diseases, and rheumatoid arthritis were not allowed to participate.The study was approved by the medical ethics committee of Maastricht university medical center (METC-183017). All study participants gave written informed consent before the start of the intervention trial. This study was registered at ClinicalTrials.gov (NCT03627637) on August 13th, 2018, and performed between August 2018 and December 2019 in Maastricht, the Netherlands.

Study design

This randomized, controlled, cross-over trial consisted of a sixteen-week intervention period and a sixteen-week control period, separated by a wash-out period of 6 to 12 weeks (median: 8 weeks) (**Supplementary Figure 6.1**). During the soy nut intervention period, participants received unsalted soy nuts (Knusperkerne; Hensel, SALUS Haus, Bruckmühl, Germany), which provided about 25.5 g soy protein daily and 174 mg of isoflavones. The nutrient composition of the product is shown in **Supplementary Table 6.1**. Compliance to the intervention was checked by measuring serum daidzein and genistein concentrations as described (LGC Limited, Fordham, United Kingdom) (18). The daidzein metabolite equol was also determined to identify equol producers (19). During the intervention and control periods, participants had to adhere to the 2015 Dutch food-based dietary guidelines, for which they received instructions at baseline and throughout the study by our research assistant. Volunteers were not allowed to use other soy products or dietary supplements known to interfere with the outcomes during the whole study. Participants could consume the soy nuts at any time of the day. A validated food frequency questionnaire was completed at the end of both periods to assess energy and nutrient intakes over the past four weeks, which were calculated using the Dutch food composition table (NEVO table) (20). Participants were requested to record in diaries any protocol deviations or health problems, medication use, and alcohol intake during the whole study period. Except for the dietary changes, participants were asked not to change their habitual lifestyles during the entire study.

Allocation to treatment order was determined using a randomized block design (block size 2 or 4) with stratification for gender. The aim was to recruit an equal number of male and female participants. However, a ratio between 40 to 60% was considered to be acceptable. Except for the research assistant, all researchers were blinded to the intervention. However, due to the nature of the trial, participants could not be blinded. Measurements were performed at the start of the control and intervention periods (baseline; BL), halfway after 8 weeks (WK8) and during two follow-up days (FU1 and FU2) at the end of each period. On the days preceding measurements, participants were requested to have a regular meal and to abstain from alcohol and heavy exercise. They arrived after an overnight fast (no food or drink after 08:00 PM, except for water) by car or public transport at our Metabolic Research Unit Maastricht (MRUM).

MRI acquisition and processing

Scans were performed at FU1 during the intervention and control period at the Scannexus research facilities in Maastricht on a 3T MAGNETOM Prisma Fit MRI-system using a 64-channel head-neck coil (Siemens Healthcare, Erlangen, Germany). Details about the MRI acquisition and processing have been published before (21). In brief, one high-resolution anatomical three-dimensional magnetization-prepared rapid acquisition with gradient echo (MPRAGE) scan was acquired (TR 2400 ms, TE 2.18 ms, TI 1040 ms, 1.0 mm isotropic resolution, 8 degrees flip angle and 160 sagittal slices). Thereafter, pseudo-continuous arterial spin labeling (PCASL) was performed with background-suppressed segmented three-dimensional gradient and spin echo readouts (GRASE). The sequence parameters were: TR 4050 ms, TE 13.6 ms, GRAPPA 2, labeling duration 1750 ms, post-labeling delay 2000 ms, segmentation factor 6, 10 label-control repetitions with nineteen slices and a voxel resolution of 3.0 mm isotropic.

Prior to quantification, individual images were distortion corrected with TopUp using M_0 images with opposite phase-encoding direction and a readout time of 75 ms. Quantification followed the recommendations of the ASL White Paper (22) and was performed using FSL (Version 6.0) and the BASIL toolbox (Version 4.0.15). Images were voxel-wise calibrated using the M_0 image and with a TR of 20s (23, 24). The used labeling efficiency was 0.64 (four background suppression pulses; 0.93^4), the T_1 of gray matter was 1330 ms, and bolus arrival time was 1300 ms, images were also corrected for hemoglobin concentration (25). Volbrain was used to perform brain extraction, along with tissue segmentation for the anatomical MPRAGE image (26). CBF images were co-registered to the anatomic resolution using Boundary-Based Registration. Thereafter, mean CBF values were calculated for global brain, gray matter and both hemispheres based on the high-resolution anatomical scan. Voxel-wise comparison was performed after co-registration to the Montreal neurological institute (MNI; 2 mm) using a repeated measures mixed effects analysis with a general linear model with a single-group paired difference (FLAME stage 1 and 2), and a Z-threshold of 2.3 ($P < 0.05$). Thereafter family-wise error correction was performed based on smoothness estimates. Atlasquery was used to determine the location of significant clusters in the MNI structural and Harvard-Oxford (sub)cortical structural atlas.

Cognitive performance

Standardized cognitive performance tests were taken at FU2 using the computerized and fully-automated CANTAB cognitive research software. These tests were related to three main cognitive domains, which are known to be affected by aging: psychomotor speed, executive function, and memory (27). Participants were first familiarized with the digital tablet (iPad 5th generation; Apple, California, United States) based touchscreen test method using the motor screening task (MOT). Thereafter, psychomotor speed was assessed using the reaction time task (RTI), during which reaction time (RT) and movement time (MT) were measured. The multitasking test (MTT) was used to assess executive function. The variables used for the MTT were: incongruency cost (IC), multitasking cost (MTC), median latency (ML) and the total number of errors (TE). Cognitive tests to evaluate memory included spatial span (SSP), delayed matching to sample (DMS) and paired associates learning (PAL). Parallel tests including different patterns were used with high test-retest repeatability to increase the sensitivity to longitudinal changes by minimizing

practice effects (28, 29). For SSP, the maximal completed span length (SL) variable was used. The percentage of correctly answered trials for all delays (CAD) was used for DMS, while for PAL the first attempt memory score (FAMS) and TE were used. A summary of the cognitive tests and reported outcomes are shown in **Supplementary Table 6.2.** The cognitive tests are described in detail on the CANTAB website (27).

Blood sampling and glucose metabolism

Fasting blood samples were taken by venepuncture from a forearm vein at BL, WK8 and FU1. At FU2, blood samples were obtained using an intravenous catheter at baseline (T=0), and 15, 30, 45, 60, 90, and 120 min following ingestion of a drink containing 75 g of glucose (Novolab, Geraardsbergen, Belgium) during a 7-point OGTT. Glucose concentrations (Horiba ABX, Montpellier, France) were determined in plasma samples obtained at all time points from NaF-containing vacutainers tubes (Becton, Dickson and Company, Franklin Lanes, New-York, USA). These tubes were placed on ice immediately after sampling and centrifuged within 30 min at 1300 x g for 15 min at 4 °C. Insulin concentrations were determined in serum samples (RIA, Millipore, Billerica, MA, USA), which were obtained at all time points using vacutainer serum tubes (Becton, Dickson and Company, Franklin Lanes, New-York, USA). These tubes were first allowed to clot for at least 60 min at 21 °C and centrifuged at 1300 x g for 15 min at 21 °C. Obtained samples were immediately portioned into aliquots, frozen in liquid nitrogen, and stored at -80 °C until analysis at the end of the study.

Fasting glucose and insulin concentrations were used to calculate the homeostatic model assessment index (HOMA-IR) as a measure of insulin resistance. The post-load glucose and insulin concentrations from the OGTT were used to calculate the Matsuda index and net incremental area under the curve (net iAUC) using GraphPad (GraphPad Prism 8 Software, La Jolla California, United States). Also, muscle and liver insulin resistance indexes (MISI and HIRI) were derived from the OGTT. The MISI was calculated using the product of total area under the curve (AUC) for plasma glucose and insulin concentrations during the first 30 min of the OGTT, while the rate of decay of plasma glucose concentrations from its peak value to its nadir was divided by the mean plasma insulin concentration for the HIRI (30). Finally, continuous glucose monitoring (CGM; Freestyle Libre Pro, Abbott, Alameda, California, United States) was performed between FU1 and FU2. A sensor was placed at the back of the upper arm and measured the glucose concentration every 15 min for 96 hours. The AUC and net iAUC were calculated for the CGM using GraphPad Prism 8. For every 24 hours the minimal one-hour value was calculated and averaged, which was used as baseline to calculate the net iAUC.

Statistical analyses

Results are shown as means (± SDs), unless otherwise indicated. Based on our previous study on the effects of a lifestyle intervention on CBF (21), it was determined before the start of the study that 23 participants are needed to detect a 0.8-SD unit change in CBF with 80% power and a two-sided alpha of 0.05. A 0.8-SD unit change in CBF can be expected following dietary interventions and corresponds to a change of approximately 10 to 15% (8, 21), which is clinically relevant (31).

All variables were normally distributed based on the Shapiro-Wilk test. First, a repeated measures analysis of variance (ANOVA) with period, gender and order as between-subject factors was performed. Order effects were not observed and were therefore excluded from the final model to test for differences between treatments. Linear mixed models were performed for anthropometrics, fasting glucose and fasting insulin concentrations to test for differences between treatments over time. Time, treatment, period, gender and time * treatment interaction were used as fixed factors, and participant and intercept as random factors. The interaction term was omitted from the model if it was not significant. Best model fit was obtained with an autoregressive covariance structure based on the chi-square statistic with log-likelihood values ($P < 0.05$), and Akaike information criterion (AIC). The post-load glucose and insulin concentrations during the OGTT were analyzed using a Toeplitz covariance structure. Pearson correlations were determined between the percentage change in CBF clusters that changed significantly and changes in cognitive performance variables. SPSS was used to perform all statistical analyses (IBM SPSS Statistics V26, Armonk, New York, United States). Differences with a $P < 0.05$ using two-tailed tests were considered to be statistically significant.

RESULTS

Study participants

A consolidated standard of reporting trials (CONSORT) flow diagram is shown in **Figure 6.1**. Twenty-five older men and women were eligible and started the study. Two women dropped out during the soy nut intervention. One woman due to personal reasons and one woman due to mild gastrointestinal discomfort. A total of 23 participants (11 men and 12 women) completed the study and were included in the statistical analyses. Participants had a mean age of 64 ± 3 years, and the mean BMI was 26.8 ± 2.8 kg/m^2 for males and 25.0 ± 2.3 kg/m^2 for females. No serious adverse events or protocol deviations were reported in the diaries and the soy nut regime was well tolerated. Overall, compliance was excellent based on returned empty sachets or unused study products, and increased serum isoflavone concentrations. Specifically, serum daidzein concentrations increased by 128 ±

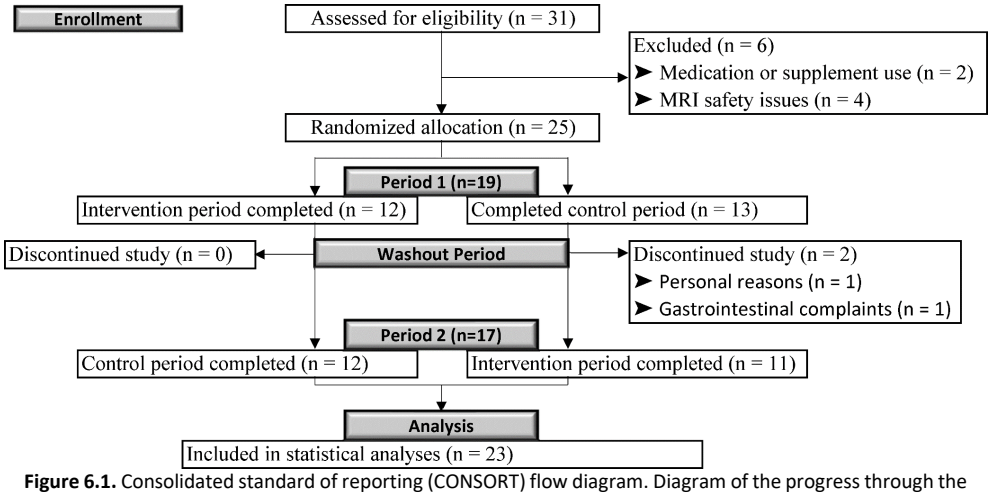

Figure 6.1. Consolidated standard of reporting (CONSORT) flow diagram. Diagram of the progress through the phases of this randomized, controlled crossover study with older males and females.

113 ng/mL (P < 0.001), and those of genistein by 454 ± 256 ng/mL (P < 0.001). Six participants (24%) could be classified as equol producers and their serum equol concentrations increased by 190 ± 79 ng/mL (P = 0.020) after the soy nut intervention (see **Table 6.1**).

As expected, food frequency data indicated a higher protein (Δ 3.1 ± 2.0 En%; P < 0.001) and a lower carbohydrate intake (Δ -2.0 ± 3.7 En%; P = 0.008) during the soy intervention period (**Supplementary Table 6.3**). Total fat intake was not changed (Δ -1.1 ± 3.4 En%; P = 0.123). However, a lower intake of saturated (Δ SFA; -1.3 ± 1.6 En%; P = 0.001) and *cis*-monounsaturated fatty acids (*cis*-MUFA; Δ -1.5 ± 1.9 En%; P = 0.001) was observed, while the consumption of *cis*-PUFAs (Δ 1.9 ± 1.4 En%; P < 0.001) was higher during the soy nut intervention. Additionally, the intake of cholesterol was reduced by 4 ± 6 mg/MJ (P = 0.002), while the intake of dietary fibers was higher (Δ 8.8 ± 3.5 g/day; P < 0.001) following soy nut intake. Although total energy intake tended to be higher during the soy nut period (Δ 111 ± 283 kcal/day; P = 0.066), body weight, BMI, and body fat percentages did not differ. However, the waist-to-hip ratio was 0.02 lower at follow-up after the soy nut intervention (time*treatment; P = 0.045) (**Table 6.2**).

Table 6.1. Serum isoflavone concentrations at the end of the 16 weeks of soy nut and control periods (n = 23)[1].

	Intervention period	Control period	Mean difference	F (1, 20)	MSE	P-value[3]
Daidzein (ng/mL)	134 ± 114	3 ± 2	128 ± 113	25.38	5482.3	< 0.001
Genistein (ng/mL)	459 ± 416	5 ± 7	454 ± 256	28.06	83310	< 0.001
Equol (ng/mL) [2]	133 ± 105	2 ± 1	190 ± 102	20.89	4316.6	0.02

[1]Data are shown as mean ± SD. [2]Equol producers (n=6). Degrees of freedom (1, 3). [3]Repeated measures analysis of variance (ANOVA) with period and gender as between-subject factors. MSE: mean square error

Cerebral blood flow

Compared with the control period, global and gray matter CBF, and the CBF in the left and right hemisphere were not different (**Figure 6.2** and **Table 6.3**). Regional blood flow, however, significantly increased in four clusters following the soy nut intervention (**Figure 6.3** and **Table 6.3)**. CBF in the largest cluster increased by 11.1 ± 12.4 mL/100 g tissue/min (Δ 36%; P < 0.001). Cluster 1 had a volume of 11,296 mm^3 and the average probability of the location based on the MNI structural atlas was in the occipital lobe (40%) and temporal lobe (16%). The specific location based on the Harvard-Oxford atlas was 13% in the left occipital pole, 7% in the temporal fusiform cortex, 5% lateral occipital cortex and 4% in the temporal occipital fusiform cortex. In cluster 2 (bilateral occipital lobe, 59%), blood flow increased by 12.1 ± 15.0 mL/100 g tissue/min (Δ 32%; P = 0.002). The volume of that cluster was 2,632 mm^3 and the specific average probability of the location was 24% in the lingual gyrus, 14% in the occipital pole, 7% in the intracalcarine cortex and 7% in the cuneal cortex. CBF increased by 12.7 ± 14.3 mL/100 g tissue/min (Δ 47%; P = 0.005) in cluster 3 (right occipital, 30%; and parietal lobe, 11%), which was 2,280 mm^3, and average probability of the location was for 22% in the right lateral occipital cortex and 6% in the right intracalcarine cortex. Finally, blood flow also increased in cluster 4 (left frontal lobe, 18%) by 12.4 ± 14.5 mL/100 g tissue/min (Δ 43%; P = 0.009). The average probability of the location of that cluster, which had a total cluster volume of 2,120 mm^3, was 10% in the left middle frontal gyrus and 9% in the left inferior frontal gyrus.

Table 2. Anthropometrics during the soy nut and control intervention throughout the intervention trial (n = 23)[1].

	Intervention period			Control period			time*treatment[2]	Treatment[2]
	Baseline	Midterm	Follow-up	Baseline	Midterm	Follow-up		
Weight (kg)	74.6 ± 10.4	74.5 ± 10.5	74.4 ± 10.5	74.4 ± 10.0	74.2 ± 10.1	74.0 ± 9.9	F(2, 83)=0.07, P=0.931	F(1, 21)=0.40, P=0.533
BMI (kg/m^2)	25.5 ± 2.7	25.5 ± 2.8	25.4 ± 2.6	25.5 ± 2.5	25.5 ± 2.5	25.4 ± 2.5	F(2, 87)=0.09, P=0.916	F(1, 21)=0.03, P=0.860
WC (cm)	86.2 ± 7.8	86.8 ± 9.1	86.0 ± 8.5	85.7 ± 9.1	85.4 ± 9.1	86.4 ± 8.5	F(2, 82)=2.20, P=0.117	F(1, 26)=0.53, P=0.474
W-H ratio	0.84 ± 0.07	0.84 ± 0.08	0.83 ± 0.08	0.84 ± 0.07	0.84 ± 0.08	0.85 ± 0.08	F(2, 89)=3.21, P=0.045	-

[1]Values are means ± SDs. [2]Linear mixed models were performed for anthropometrics to test for differences between treatment over time. Time, treatment, period, gender and time * treatment interaction were used as fixed factors, and participant and intercept as random factors. An autoregressive covariance structure was used. When the interaction term (time * treatment) did not reach statistical significance (P > 0.05), it was removed from the model to calculate P-value for treatment effects. WC: waist circumference; W-H ratio: waist-to-hip ratio.

Cognitive performance

The MT during the RTI was reduced by 20 ± 37 ms (Δ 7%; P = 0.005) from 295 ± 68 ms after the control period to 275 ± 49 ms after the soy nut intervention. This suggests that cognitive performance in the domain of psychomotor speed was improved, while the RT did not change (Δ 0 ± 24 ms; P = 0.926) (**Table 4**). After excluding one participant with extreme responses, a significant inverse correlation was observed between the percentage changes in CBF in cluster 2 (r = -0.45, P = 0.036) and cluster 4 (r = -0.46, P = 0.031), and the change in RTI MT (see **Supplementary Figure 6.2**). Correlations with changes in cluster 1 (r = -0.36, P = 0.101) and cluster 3 (r =-0.38, P = 0.084) were also negative, but did not reach statistical significance. No treatment effects were observed for the executive function tests MTT and SSP, and the memory tests DMS and PAL (see **Table 6.4**).

Glucose metabolism

The time * treatment interaction for fasting glucose (P = 0.745) and insulin (P = 0.206) concentrations, and the HOMA-IR (P = 0.425) were not statistically significant.. After the interaction term was omitted from the model, no significant treatment effects were observed (glucose: P = 0.643; insulin: P = 0.398; HOMA-IR: P = 0.150). Additionally, no differences were observed in post-load glucose (time * treatment: P = 0.952, treatment: P = 0.950) and insulin (time * treatment: P = 0.738, treatment: P = 0.737) concentrations during the OGTT (**Figures 6.4A and B**). Also, the net iAUC did not differ between treatments for glucose (Δ -8 ± 117 mmol/L*hour; P = 0.746) and insulin (Δ -147 ± 1614 µU/L*hour, P = 0.657). The MISI (Δ -0.032 ± 0.163 arbitrary units; P = 0.405) and HIRI (Δ 2.264 ± 105.303 arbitary units; P = 0.922) did also not change. Finally, continuous glucose concentrations over 96 hours did not differ as indicated by the AUC (Δ -20 ± 49 mmol/L*hour; P = 0.079) and the iAUC (Δ -4 ± 32 mmol/L*hour; P = 0.583) (see **Figure 6.4C**).

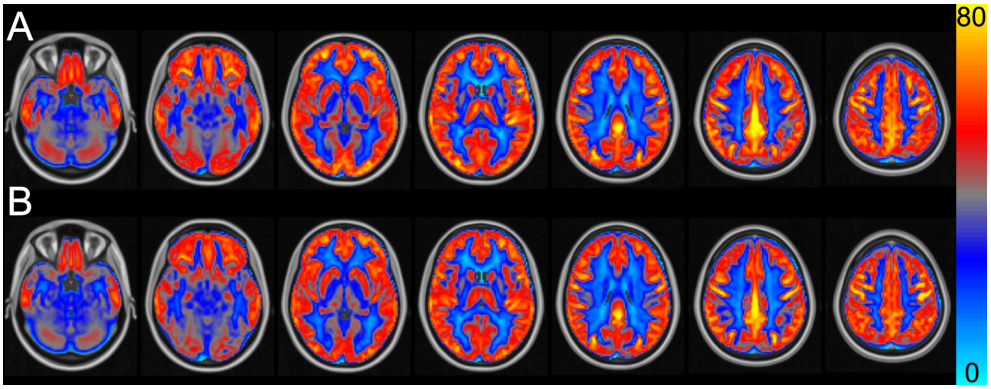

Figure 6.2. Mean cerebral blood flow (CBF) maps from a randomized, controlled crossover study in older adults (n = 23) after non-linear co-registration to the Montreal neurological institute (MNI)-template after (**A**) soy nut intake and (**B**) control period. The images show the cerebral blood flow in mL/100 g tissue/min (scale shown by color bar). No differences were observed between periods in global CBF (P = 0.567), gray matter CBF (P = 0.593) and CBF in the left (P = 0.570) and right (P = 0.542) hemisphere.

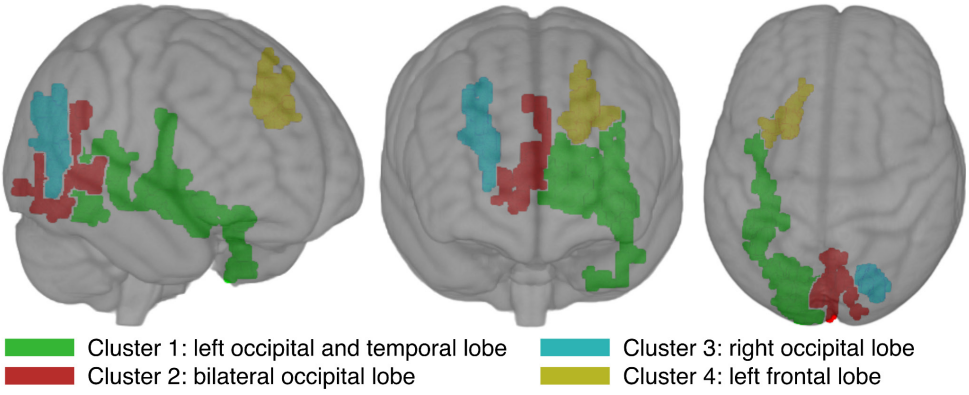

Figure 6.3. Results of voxel-wise comparisons including all acquired cerebral blood flow (CBF) data in three dimensional Montreal neurological institute (MNI)-template from a randomized, controlled crossover study in older adults (n = 23). CBF increased in four clusters after soy nut intake as compared with the control period (Family-wise error corrected). Cluster 1: left occipital and temporal lobe, Δ 11.1 ± 12.4 mL/100 g tissue/min (Δ 36%), volume 11,296 mm^3, P < 0.001; cluster 2: bilateral occipital lobe, Δ 12.1 ± 15.0 mL/100 g tissue/min (Δ 32%), volume 2,632 mm^3, P = 0.002; cluster 3: right occipital and parietal lobe, Δ 12.7 ± 14.3 mL/100 g tissue/min (Δ 47%), volume 2,280 mm^3, P = 0.005; cluster 4: left frontal lobe, Δ 12.4 ± 14.5 mL/100 g tissue/min (Δ 43%), volume 2,120 mm^3, P = 0.009.

Table 6.3. Cerebral blood flow after a soy intervention and control period in a randomized, controlled crossover study with older men and women (n = 23)[1].

Outcome	Intervention period (mL/100g/min)	Control period (mL/100g/min)	Mean difference (mL/100g/min)	F (1, 20)	MSE	P-value[2]
Global CBF	40.6 ± 8.7	41.2 ± 9.5	-0.6 ± 5.2	0.38	13.81	0.567
Gray matter CBF	48.5 ± 10.3	49.2 ± 10.9	-0.6 ± 6.0	0.33	14.11	0.593
Left hemi CBF	42.5 ± 8.9	43.1 ± 9.3	-0.6 ± 5.6	0.30	16.14	0.570
Right hemi CBF	42.2 ± 9.3	42.9 ± 10.2	-0.7 ± 5.6	0.34	12.71	0.542
Cluster 1 CBF	41.9 ± 9.1	30.8 ± 7.4	11.1 ± 12.4			< 0.001
Cluster 2 CBF	49.6 ± 12.4	37.6 ± 6.8	12.1 ± 15.0			0.002
Cluster 3 CBF	39.6 ± 11.3	26.9 ± 6.8	12.7 ± 14.3			0.005
Cluster 4 CBF	41.0 ± 10.1	28.6 ± 8.3	12.4 ± 14.5			0.009

[1]Data are shown as mean ± SD. [2]Repeated measures analysis of variance (ANOVA) with period and gender as between-subject factors and participant and treatment as fixed factors. Clusters were the result of a voxel-wise analysis within FSL applying a repeated measures mixed effects analysis using a general linear model with a single-group paired difference (FLAME stage 1 and 2), and a Z-threshold of 2.3 (P < 0.05). Family-wise error correction was performed based on smoothness estimates. CBF: cerebral blood flow; hemi: hemisphere; MSE: mean square error

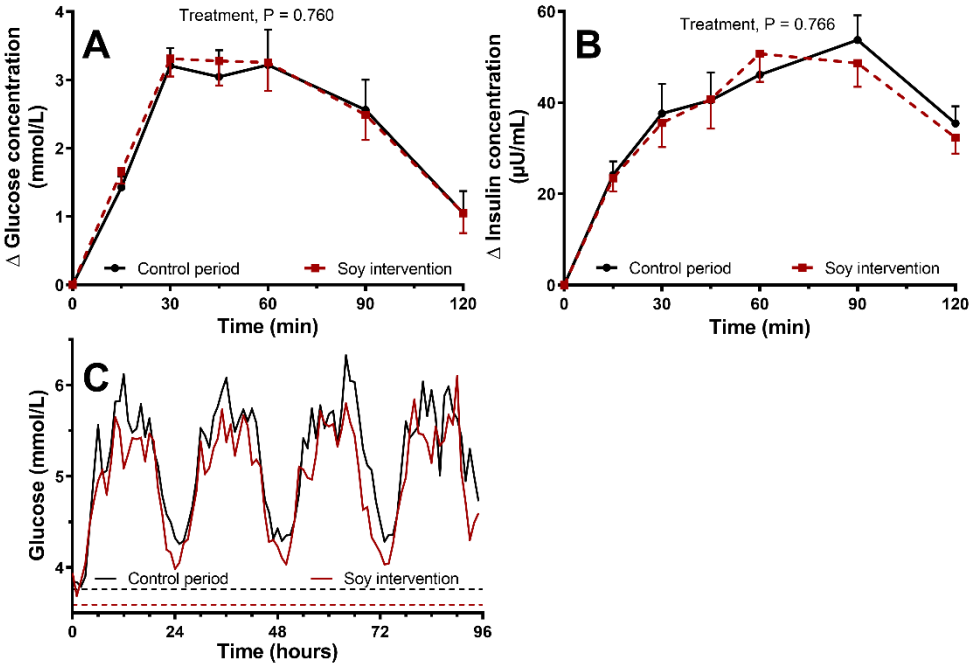

Figure 6.4. Mean ± SEM differences in glucose (**A**) and insulin concentrations (**B**) during a 7-point oral glucose tolerance test (OGTT) test (n = 23). Data were analyzed using linear mixed models between the difference at each timepoint with baseline after the soy nut intervention and control periods. No treatment effect was observed for glucose (P = 0.760) and insulin concentrations (P = 0.766). Mean 96-hour continuous glucose measurements (**C**). The horizontal dashed lines (red = soy nut intervention period, black = control period) represent the mean minimal fasting glucose concentration of each day.

DISCUSSION

In this randomized, controlled cross-over trial with older males and females, longer-term soy nut consumption increased regional CBF in four brain clusters. Three clusters were located in the bilateral occipital and parietal lobes, while the largest cluster extended to the left temporal lobe. The fourth brain cluster was located in the left frontal lobe. Further, cognitive performance within the domain of psychomotor speed improved, but no changes were observed in executive function or memory. Finally, fasting and post-load glucose and insulin concentrations did not change, and glucose concentrations - measured during daily life with a CGM - were also not affected.

Effects of soy products on CBF have not been studied before in humans. However, consumption of specific substances, that are also present in soy, such as phytoestrogens (isoflavones), *cis*-PUFAs and plant proteins, may improve CBF (14-16). Specifically, isoflavones in soy are distinct from those of other plant products, and mainly consists of genistein and daidzein. Effects of these isoflavones on CBF are not known. Yet, supplementation for 12 weeks with a blueberry concentrate rich in the flavonoid anthocyanin increased in older adults CBF in the occipital and parietal lobes, which agrees with our findings (32). Further, flavanol-rich cocoa acutely increased CBF in two clusters located in the frontal lobe and parietal lobe in older adults (33). Although the different classes of flavonoids may have different effects, such as antioxidant and anti-inflammatory activities (34), they may all increase nitric oxide bioavailability thereby improving CBF (35).

Other soy components that may account for the observed effects on CBF include *cis*-PUFAs and high-quality plant proteins (11). Soy mainly contains linoleic acid (C18:2, *n-6*), but effects of linoleic acid on CBF have not been studied. However, it has been suggested that the majority of linoleic acid entering the brain is converted into relatively polar compounds (36), including linoleic acid-derived oxylipins that may increase CBF (37). The soy nuts also provided daily about 0.8 g of α-linolenic acid (ALA, C18:3n-3) that can be converted into the long-chain *n-3* PUFAs eicosapentaenoic (EPA; C20:5, *n-3*) and docosahexaenoic acid (DHA; C22:6, *n-3*), though in limited amounts (38). Daily supplementation for twelve weeks with fish (39), krill or sardine oil (40), which are rich in EPA and DHA, increased CBF in the prefrontal cortex. These studies measured CBF using near-infrared spectroscopy (NIRS) during a cognitive task in healthy young (39) or older adults (40). Circulating DHA gets incorporated into human brain lipids (41), thereby possibly affecting CBF responses. Finally, isolated soy proteins (8 g) acutely increased CBF in the prefrontal cortex in young healthy adults as measured with NIRS in superficial cortical regions, which may be due through their beneficial effects on neurotransmission (42) and nitric oxide metabolism (43).

Soy nut consumption significantly improved cognitive performance within the domain of psychomotor speed. Effects of soy foods on cognitive performance have hardly been studied. In contrast to our findings, consumption of a soy drink did not affect psychomotor speed in post-menopausal females (44). This may relate to the shorter study duration (12 weeks vs. 16 weeks) and the lower daily intake isoflavones (10-60 mg vs. 174 mg in our study). Interestingly, several reviews have reported beneficial effects of soy isoflavones on cognitive performance (5, 45-47). A recent meta-analysis of sixteen randomized controlled trials (RCTs) in mainly postmenopausal women indeed concluded that isolated soy isoflavones with intakes ranging from 60 to 160 mg per day improved

overall cognitive performance (5). However, we only observed effects on psychomotor speed. Of note, the only study in the meta-analysis involving a similar study population of healthy older males and females also observed an improved psychomotor speed (48). Whether effects on cognitive performance depends on study population warrants further study. Effects on cognitive performance may also relate to the increased intake of *cis*-PUFAs and plant proteins. Although positive associations have been observed (7), no randomized controlled trials have addressed the effects of linoleic acid on cognitive performance. However, some evidence exists that in healthy older adults EPA and DHA have beneficial effects on psychomotor speed (49). Finally, daily consumption of 50 g isolated soy protein for eight weeks improved results of a multi-choice reaction time task, which agrees with our findings, while memory was also not affected (50).

A relationship was found between regional CBF increase and the favorable effects observed on psychomotor speed. Brain clusters were located in cortical regions that are known to be affected by aging (17) and may thus be more sensitive to the effects of diet. Specifically, clusters 1 and 3 were partly located in the lateral occipital cortex that is involved in object recognition (51), while the occipital pole (clusters 1, 2 and 3) and temporal fusiform cortex (cluster 1) have been linked to visual information processing (52, 53). Furthermore, cluster 4 was located in the frontal gyrus, which is part of the ventral attention network that is involved when performing the five-choice reaction time psychomotor speed tasks (51). The faster movement time during the psychomotor speed test may thus be due to faster recognition and processing of the target in combination with improved reorientation to the stimuli. Interestingly, concomitant changes in regional CBF and performance during a psychomotor speed test were already reported in older adults after twelve-week supplementation with an anthocyanin-rich blueberry concentrate (32).

Effects on CBF or cognitive performance in our study were not related to changes in glucose metabolism, as suggested by other studies (13-16). In fact, markers of glucose metabolism did not change at all. This is in line with results of a meta-analysis including 24 RCTs (54). In nine studies soy foods were used, in ten studies soy isoflavone extracts, and in five studies soy proteins. No effects on fasting or post-load glucose and insulin concentrations were observed (54). A more recent meta-analysis reported that plant-derived *cis*-PUFAs also did not affect fasting glucose concentrations. However, a dose-dependent decrease in fasting insulin concentrations was observed (55). Based on the results of that meta-analysis, however, the additional intake of *cis*-PUFAs in our study was probably too low to affect fasting insulin concentrations (55). Intervention studies with dietary ALA (56) in healthy individuals did also not show beneficial effects on glucose metabolism or plasma insulin concentrations.

We used the MRI perfusion method ASL, which is considered the non-invasive gold standard (12), to quantify changes in CBF, and CANTAB as a standardized, validated and sensitive method to detect changes in cognitive performance following dietary interventions (57). Focus was on both older males and females that had to adhere to food-based dietary guidelines (58), meaning that soy nut effects were evaluated as part of a recommended diet. Compliance based on serum isoflavone concentrations was excellent. An inherent limitation of our study was that participants could not be blinded. Except for the research assistant, however, researchers were blinded. Even though body weight remained stable, it should be considered that participants only partly replaced the extra

energy from the intake of the nuts, as energy intake tended to increase during the soy nut intervention. Additionally, soy-nut effects cannot be disentangled from those due to the replacement of food products by the intake of the soy nuts. Some studies have suggested that people who can convert daidzein into equol benefit more from the potential health benefits of soy. However, only six participants (24%) were equol-producers, which is in line with other studies (59). Unfortunately, this number is too low to compare with sufficient statistical power effects between equol producers and non-producers.

In conclusion, longer-term soy nut intervention increased regional CBF. These effects may underlie the observed beneficial effects on cognitive performance in the psychomotor speed domain, suggesting a potential mechanism by which an increased intake of soy-rich foods beneficially affects cognitive performance in older males and females.

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AUTHORS' CONTRIBUTIONS

JK: analyzed data and performed statistical analysis and wrote paper; LT: conducted research and reviewed the manuscript; RM: designed research, had primary responsibility for final content and wrote paper; TA: reviewed the manuscript; PJ: designed research, had primary responsibility for final content and wrote paper.

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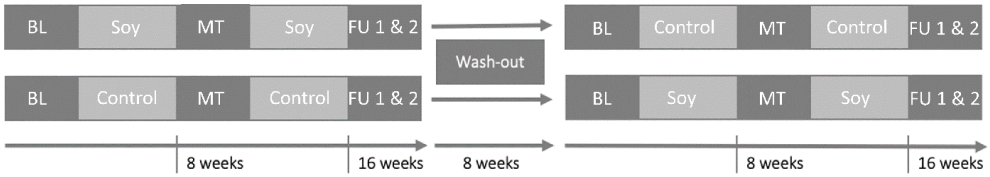
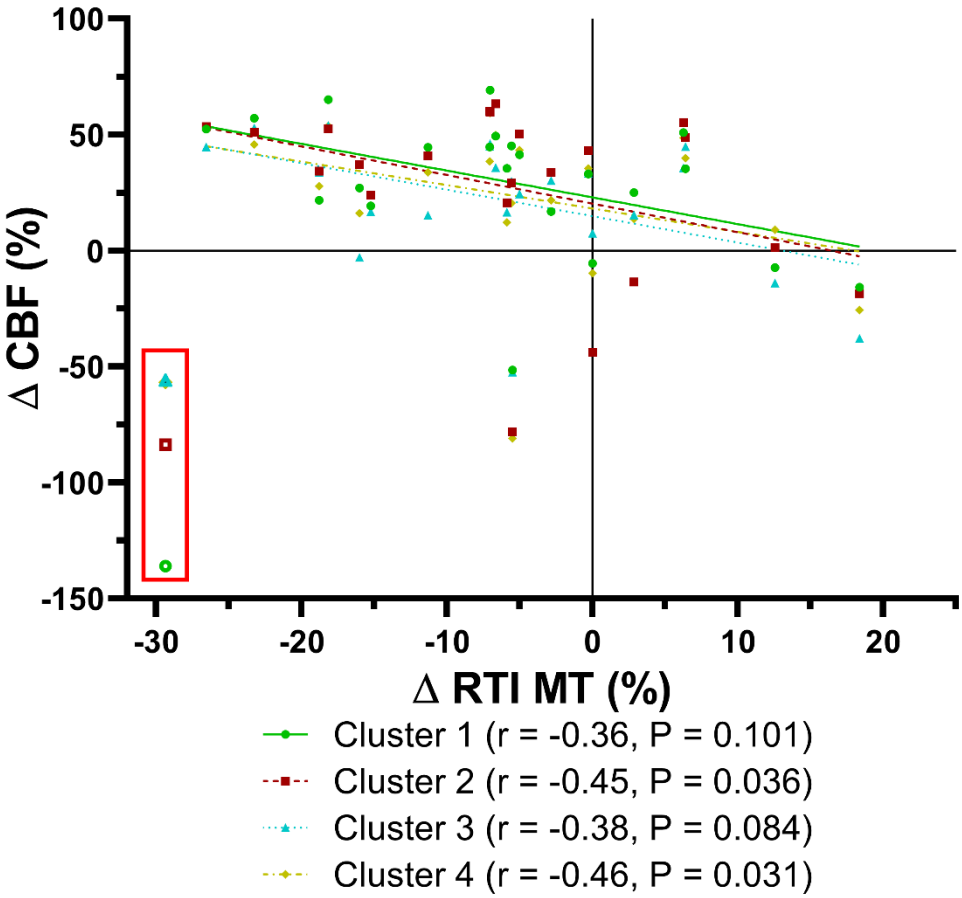
CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL



Supplementary Figure 6.1. Study design. BL = baseline measurements; MT = midterm measurements; FU = follow-up measurements. BL and MT: anthropometrics, glucose metabolism; FU: anthropometrics, glucose metabolism, cerebral blood flow, cognitive performance.



Supplementary Figure 6.2. Correlation between changes in cerebral blood flow (CBF) in clusters 1 till 4 and the movement time during the reaction time task (RTI MT). One participant shown with open symbols and framed in red was excluded from the analyses.

Supplementary Table 6.1. Nutrition facts of unsalted soy nuts[1].

	Per 100 g	Per portion (67 g)
Energy (kJ / kcal)	1568 / 374	1051 / 251
Protein (g)	38	25.5
Carbohydrates (g)	17	11.4
Sucrose (g)	8	5.4
Total fat (g)	17	11.4
Saturated fatty acids (g)	2.5	1.7
Monounsaturated fatty acids (g)	3.5	2.4
Polyunsaturated fatty acids (g)	11	7.4
Dietary fibers (g)	15	10.1
Sodium (g)	0.02	0.01
Isoflavones (mg)	259	174

[1]Knusperkerne (Hensel, SALUS Haus, Bruckmühl, Germany).

Supplementary Table 6.2. Summary of the cognitive tests and reported variables.

Cognitive tests	**Outcome**
Psychomotor speed	
Five choice reaction time test (RTI)	Movement time (MT) from button release until the target
	Reaction time (RT) until button release
Executive function	
Multitasking test (MTT)	Incongruency cost (IC); median latency of congruent minus incongruent trials
	Multitasking cost (MTC); median latency of block in which both rules were used minus blocks which in which a single rule was used
	Reaction latency (RL) of all correct trials
	Total errors (TE)
Memory	
Spatial Span (SSP)	Span length (SL); longest sequence of successfully recalled boxes
Delayed Matching to Sample (DMS)	Total correct (TC) of assessments containing a delay
Paired Associated Learning (PAL)	First attempt memory score (FAMS)
	Total errors (TE)

Supplementary Table 6.3. Average food intake over four weeks as assessed by food frequency questionnaires after 16 weeks of soy nut and control intervention (n=23)[1].

	Intervention period	Control period	Mean difference	F (1, 20)	MSE	P-value[2]
Energy intake (kcal/day)	2235 ± 361	2119 ± 440	111 ± 283	3.77	37334	0.066
Protein (En%)	18.8 ± 1.4	15.7 ± 1.4	3.1 ± 2.1	53.76	1.98	< 0.001
Carbohydrates (En%)	40.9 ± 5.0	42.0 ± 4.6	-2.0 ± 3.7	8.81	5.04	0.008
Total fat (En%)	35.4 ± 4.9	36.5 ± 4.3	-1.1 ± 3.4	2.60	5.36	0.123
Saturated fatty acids (En%)	11.1 ± 2.0	12.4 ± 2.0	-1.3 ± 1.6	16.07	1.30	0.001
Monounsaturated fatty acids (En%)	12.5 ± 2.5	14.0 ± 2.5	-1.5 ± 1.9	14.86	1.69	0.001
Polyunsaturated fatty acids (En%)	8.6 ± 1.7	6.8 ± 1.9	1.9 ± 1.4	42.49	1.00	< 0.001
Cholesterol (mg/MJ)	20 ± 6	24 ± 6	-4 ± 6	12.91	15.24	0.002
Dietary fibers (g)	33.6 ± 5.6	24.8 ± 5.6	8.8 ± 3.5	144.95	6.11	< 0.001

[1]Values are means ± SD. MSE: mean square error. [2]Repeated measures analysis of variance (ANOVA) with period and gender as between-subject factors ANOVA with participant number, treatment and period as fixed factors.

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CHAPTER 7

Acute inorganic nitrate intake increases regional insulin action in the brain: Results of a double-blind, randomized, controlled cross-over trial with abdominally obese men

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To be submitted

ABSTRACT

Background

Improving brain insulin sensitivity by increasing the action of insulin in the brain may be a promising approach in the prevention and treatment of age-related non-communicable diseases. The aim of this study was to investigate acute effects of inorganic nitrate on the regional brain insulin responsiveness in abdominally obese men.

Methods

Eighteen apparently healthy men, aged 18-60 years and with a waist circumference ≥ 102cm, participated in a randomized, double-blind, placebo-controlled cross-over trial. The study consisted of two test days that were separated by at least one week. Participants received in random order a drink providing 10mmol (i.e., 625mg nitrate) potassium nitrate or an isomolar placebo drink with potassium chloride. Brain insulin action was assessed 120min after the drinks by quantifying acute effects of nasal insulin on regional CBF using arterial spin labeling MRI. Plasma glucose and serum insulin concentrations were measured at regular intervals, while blood pressure was determined fasted and at 240min.

Results

Inorganic nitrate increased regional insulin action in five brain clusters. The two largest clusters were located in the right temporal lobe (ΔCBF: 7.0±3.8mL/100g/min, volume: 5296mm3, P<0.001; and ΔCBF: 6.5±4.3mL/100g/min, volume: 3592mm3, P<0.001), while two other cortical clusters were part of the right frontal (ΔCBF: 9.0±6.0mL/100g/min, volume: 1096mm3, P=0.007) and the left parietal lobe (ΔCBF: 6.1±4.3mL/100g/min, volume: 1024mm3, P=0.012). One subcortical cluster was located in the striatum (ΔCBF: 5.9±3.2mL/100g/min, volume: 1792mm3, P<0.001). No differences were observed before administration of the spray. Finally, inorganic nitrate did not affect cardiometabolic risk markers. Reduced glucose concentrations were observed over time (P<0.001), but insulin concentrations did not change. Furthermore, blood pressure increased (P<0.05), while heart rate decreased (P<0.001).

Conclusion

Acute inorganic nitrate intake increases the regional insulin action in brain regions that are involved in the regulation of various metabolic and cognitive processes, as well as in processes underlying food intake in abdominally obese men.

This clinical trial was registered on January 7th, 2021, at clinicaltrials.org as NCT04700241.

INTRODUCTION

Brain insulin resistance, which can be defined as the failure of brain cells to adequately respond to insulin, is a characteristic of several diseases such as dementia and type 2 diabetes mellitus (T2D) (1-3). Therefore, improving brain insulin sensitivity by increasing the action of insulin in the brain may be a promising approach in the prevention and treatment of these age-related non-communicable diseases. Insulin may modulate cerebral blood flow (CBF) responses via direct vasodilatory effects (4), while reduced brain insulin action was associated with cerebrovascular disturbances possibly via an impaired endothelium-dependent vasodilation (5). Additionally, insulin regulates various metabolic and cognitive processes in the brain, and may also control food intake (3). It has been shown that high brain insulin responsiveness prior to a two-year healthy lifestyle intervention resulted in more weight loss that was attributed to less visceral fat and less regain of fat mass during a nine-year follow-up (6). However, trials investigating strategies that affect brain insulin action are still missing.

Inorganic nitrate, which is primarily found in beetroot and green leafy vegetables, may play a role in the prevention of insulin resistance and T2D by improving nitric oxide (NO) homeostasis through the enterosalivary nitrate-nitrite-NO pathway (7, 8). Although inorganic nitrate is well-known for its beneficial effects on the peripheral vasculature (9), a limited number of studies have also already demonstrated an improved cerebrovascular function following the acute intake of nitrate (10). In fact, CBF, a sensitive marker for cerebrovascular function, improved after a diet providing 773 mg of dietary nitrate over a 24-hour feeding period in older participants (11). In addition, CBF acutely increased in young adults measured with transcranial doppler during exercise (12) and near-infrared spectroscopy during cognitive tasks (13) after beetroot juice providing 750 mg and 342 mg of nitrate, respectively.

Effects of increased NO bioavailability after inorganic nitrate intake on regional CBF responses to intranasal insulin are unknown. Therefore, the aim of the present randomized, controlled, double-blind cross-over trial was to examine the acute effects of inorganic nitrate on brain insulin action. Focus was on abdominally obese men as they have been shown to have a reduced brain insulin responsiveness (6). The action of insulin was assessed using a whole-brain approach by quantifying the acute effects of insulin as nasal spray on regional CBF (14, 15) using the non-invasive perfusion method pseudo-continuous arterial spin labeling (ASL) magnetic resonance imaging (MRI) (16).

METHODS

Study participants

Abdominally obese men were recruited by approaching participants from previous studies at Maastricht University, via online advertisements, and via local advertisements in university and hospital buildings. Men were invited for a screening visit if they were aged between 18 and 60 years and were right-handed. Additionally, participants had to meet the following criteria: minimal waist circumference 102 cm; no contra-indications for MRI imaging (e.g. any metallic implants or claustrophobia); stable body weight (weight gain or loss < 3 kg in the past 3 months); non-smoker; no drug or alcohol abuse; no use of dietary supplements known to interfere with the main study outcomes; no diabetes; no use of medication known to affect blood pressure, lipid or glucose metabolism; no medical

conditions that might interfere with the study (e.g. active cardiovascular disease); and no participation in another biomedical study within one month prior to the screening. During screening, blood pressure was measured in seated position according to the latest recommendations (17). Systolic (SBP) and diastolic blood pressure (DBP) had to be lower than 160 mmHg and 100 mmHg, respectively. Additionally, a venous blood sample was drawn to determine if fasting plasma glucose was < 7.0 mmol/L and fasting serum total cholesterol < 8.0 mmol/L. Written informed consent was provided by all participants before screening. The study was conducted according to the guidelines described in the Declaration of Helsinki, approved by the Medical Ethics Committee of Maastricht University Medical Center (METC 20-078), and executed between January 2021 and May 2021. The trial was registered on January 6th 2021 at ClinicalTrials.gov as NCT04700241.

Study design

This study was a randomized, double-blind, placebo-controlled, cross-over trial with a wash-out period of at least one week (median: 9 days, range: 7 – 18 days). The study design is shown in **Supplementary Figure 7.1**. Participants received a drink (30 g of tap water) in which 10 mmol (i.e., 625mg nitrate) potassium nitrate (KNO_3; Merck KGaA, Darmstadt, Germany) was dissolved or an isomolar placebo drink with potassium chloride (KCl; Merck KGaA, Darmstadt, Germany). This dose of potassium nitrate was chosen based on the maximal reference dose for chronic oral exposure set by the United States Environmental Protection Agency (EPA; 7.0 mg per kg body weight per day, which equals 630 mg for a 90 kg individual) (18) and because it has beneficial effects on the peripheral vasculature (19, 20). For logistic reasons, participants started both test days consistently at either 08:00 or 09:00 in the morning. MRI measurements were performed 120 min or 150 min after supplementation depending on the start time at the Scannexus research facilities in Maastricht. In fact, earlier studies have clearly shown that plasma nitrate and nitrite concentrations (21) and the NO pool (22) reached peak levels and plateaued 120 min after consuming inorganic nitrate. The insulin spray was administered between two MRI sessions and 30 min before CBF was measured for the second time, which is a common approach (23). Additionally, office blood pressure was measured at baseline and 240 min after the drink in supine position using an intermittent blood pressure device (Omron M7 Intelli IT, Cemex Medische Techniek, Nieuwegein, The Netherlands) (17). Blood samples were taken from an intravenous catheter before the drink was provided (T=0) and 60, 120, 180, 210, 240 and 330 min after the drink.

One week prior to the test day and throughout the study, participants were not allowed to use antibacterial mouth wash or antibacterial toothpaste, chewing-gum or tongue-scraping. Additionally, participants had to avoid nitrate-rich food products for which a list was provided, have a regular dinner, and were not allowed to drink alcohol on the day preceding the test day. Participants arrived by car or public transport after an overnight fast (no food or drink after 08:00 PM, except for water) at the Metabolic Research Unit Maastricht (MRUM), which is temperature controlled at 22 °C. A wheelchair was used for the transport of the participants to the Scannexus research facilities for the brain measurements. During the whole study period, study participants were kindly requested to maintain their habitual diet and use of alcoholic beverages.

Magnetic resonance imaging

Acquisition

Brain perfusion measurements were performed in supine-position prior to and 30 min after intranasal insulin administration using a 3T MAGNETOM Prisma Fit MRI-system and a 64-channel head-neck coil (Siemens Healthcare, Erlangen, Germany). Insulin was administered intranasally by four puffs of 0.4 mL (two per nostril) at 30-second intervals, amounting to a total dose of 1.6 mL insulin (160 U Insulin Novorapid; Novo Nordisk, Mainz, Germany). CBF was measured using pseudo-continuous arterial spin labeling (PCASL) after 15 min of rest inside the MRI-scanner. The acquisition and processing has been described in detail before (24). In brief, the scan was performed with background-suppressed segmented three-dimensional (3D) gradient and spin echo (GRASE) readouts. The default repetition time (TR) was 4050 ms but required prolongation up to 5470 ms for some of the participants depending on the Specific absorption rate (SAR) estimation. The TR was kept constant for each participant across measurements. The other sequence parameters were: TE 13.6 ms, GRAPPA 2, labeling duration 1750 ms, post-labeling delay 2000 ms, segmentation factor 6, 10 label-control repetitions with nineteen slices and a voxel resolution of 3.0 mm isotropic. Preceding each PCASL measurement one high-resolution anatomical 3D magnetization-prepared rapid acquisition with gradient echo (MPRAGE) scan (TR 2400 ms, TE 2.18 ms, TI 1040 ms, 1.0 mm isotropic resolution, 8 degrees flip angle and 160 sagittal slices) was performed.

Pre-processing

PCASL-images were analyzed using FSL (Version 6.0) and the BASIL toolbox (Version 4.0.15) (25-27) following the recommendations of the ASL White Paper (22). First, individual PCASL-images were distortion corrected with TopUp using M0 images with opposite phase-encoding direction and a TR of 20 s. The used labeling efficiency was 0.64 (four background suppression pulses; 0.934), the T1 of gray matter was 1330 ms, the T1 of blood was calculated using the hemoglobin concentration of the participant measured on the test day (26), while the bolus arrival time was 1300 ms. Mean CBF was determined in the following pre-defined regions: global, gray matter, cortical and subcortical (i.e., caudate, putamen, thalamus, globus pallidus, hippocampus, amygdala and nucleus accumbens) after Boundary-Based co-registration to the anatomical MPRAGE image, which was segmented using Volbrain (23).

Voxel-wise analysis

Voxel-wise comparison was performed after non-linear followed by linear co-registration to the Montreal Neurological Institute (MNI; 2 mm) using a repeated measures mixed effects analysis with a general linear model with a single-group paired difference. The effect of inorganic nitrate on brain insulin action was assessed using the difference between the post-insulin and pre-insulin CBF-maps during the nitrate and placebo test day. For evaluation of the effect of inorganic nitrate on CBF the scans after the nitrate and placebo pre-insulin administration were compared, while the effect of insulin on CBF was determined using the post-insulin and pre-insulin scans during the placebo test day. FLAME stage 1 and 2 was run. Cluster-wise interference was performed on the whole-brain excluding the cerebellum, because of issues with co-registration to the common space. We

used a Z-threshold of 2.1, a connectivity of 26 ($P < 0.05$) and included family-wise error correction based on smoothness estimates. Atlasquery was used to determine the location of significant clusters in the Harvard-Oxford (sub)cortical structural atlas.

Cardiometabolic risk markers

Blood pressure was measured on the left (non-dominant) arm in supine position after at least 15 min of rest in a quiet and darkened temperature-controlled room following the latest recommendations (17). Blood pressure was measured four times, while the average of the last three measurement was used for statistical analyses.

Serum was obtained from vacutainer SST™ II Advance tubes (Becton, Dickson and Company, Franklin Lanes, New-York, USA), which were allowed to clot for at least 30 min and centrifuged at 1300 x g for 10 min at 21 °C. Fasted serum samples were analyzed for concentrations of total cholesterol (TCH: CHOD-PAP method; Roche Diagnostics, Mannheim, Germany), high-density lipoprotein (HDL)-cholesterol (precipitation method; Roche Diagnostics, Mannheim, Germany), triacylglycerol corrected for free glycerol (TAG: GPO Trinder; Sigma-Aldrich Corporation, St. Louis, Mo, USA), and high-sensitivity C-reactive protein (hsCRP) (immunoturbidimetric assay, Horiba ABX, Montpellier). Low-density lipoprotein (LDL)-cholesterol concentrations were also calculated using the Friedewald formula (28). Insulin concentrations were determined in serum samples from all timepoints (ELISA, Christal Chem, Elk Grove Village, IL, USA). Plasma glucose concentrations were also determined at all timepoints using NaF-EDTA-containing vacutainers tubes (Becton, Dickson and Company, Franklin Lanes, New-York, USA), which were placed on ice immediately after sampling and centrifuged within 30 min at 1300 x g for 15 min at 4 °C. All samples were immediately portioned into aliquots, frozen in liquid nitrogen, and stored at -80 °C until analysis at the end of the study.

Statistical analyses

Based on our previous studies (24), it was estimated before the start of the study, that eighteen participants would be needed to detect a 1-SD unit change in brain insulin action between treatments with 80% power and a two-sided alpha of 0.05. This change in CBF corresponds to a change of approximately 10 to 15%, which can be expected following dietary interventions (8, 20) and is clinically relevant (27).

The statistical approach used for the voxel-wise analyses has been described under the MRI-section. All other analyses were performed using SPSS (IBM Corp., IBM SPSS Statistics, V26, Armonk, NY, USA). A two-tailed p-value < 0.05 was considered to be statistically significant. Results were first checked for normality using the Shapiro-Wilk test and are shown as means ± SDs. Only hsCRP was analyzed using the Wilcoxon signed rank test because of a non-normal distribution and results are presented as medians (interquartile range). To test for differences in anthropometrics and the fasting lipid profile before administration of the drink, analysis of variance (ANOVA) was performed with treatment and test day as fixed factors, and participant as random factor. Linear mixed models were performed to test for differences in brain insulin action between treatments using test day, insulin spray, treatment, and insulin spray * treatment as fixed factors. The interaction term was omitted from the model, if it was not statistically significant, which made it possible to investigate the effects of treatment and the insulin spray. Participant

was included as random factor and a random intercept was used. The best model fit was obtained with a Toeplitz covariance structure based on the chi-square statistic with log-likelihood values ($P < 0.05$), and Akaike information criterion (AIC).

Differences in insulin and glucose concentrations relative to the nasal insulin spray were also investigated using a similar linear mixed model. The three blood samples prior and two blood samples after the insulin spray were used, because the interval between blood samples was similar. The change from the first blood sample was used as the dependent variable. Test day, time, treatment, and time * treatment were used as fixed factors and participant and intercept were included as random factor. The interaction term was omitted from the model if it was not statistically significant to investigate the effect of treatment and time. Differences in time were compared pairwise with Bonferroni correction when statistically significant. Finally, linear mixed models were also used to test for differences in blood pressure using the change in blood pressure from T=0 as dependent variable. Test day and treatment were used as fixed factor and participant and intercept were included as random factor. Baseline differences in insulin and glucose concentrations, and blood pressure were investigated using a repeated measures ANOVA with treatment as a fixed factor.

RESULTS

Study participants

A CONSORT flow diagram of participants throughout the study is shown in **Supplementary Figure 7.2.** In total, 23 men were assessed for eligibility. One man was excluded because his fasting total cholesterol exceeded 8.0 mmol/L and another man because of MRI safety issues (i.e., metal implant in the jaw). Therefore, 21 men started the study. Two participants dropped out due to personal reasons before the first test day and one participant discontinued the study after the first test day because he suffered from vasovagal responses during the blood draws. A total of eighteen participants thus completed the study and were included in the statistical analyses. Participants who completed the study had a median age of 50 years (range 23 – 60 years). Their body weight (inorganic nitrate: 111.0 ± 14.8 kg vs. placebo: 111.0 ± 15.4 kg), BMI (inorganic nitrate: 33.5 ± 5.0 kg/m^2 vs. placebo: 33.4 ± 5.0 kg/m^2), waist-circumference (inorganic nitrate: 118.7 ± 10.3 cm vs. placebo: 118.5 ± 10.1 cm) and waist-to-hip ratio (inorganic nitrate: 1.00 ± 0.04 vs. placebo: 1.00 ± 0.04) were comparable between both test days (see **Table 1**).

Brain insulin action and cerebral blood flow responses

Inorganic nitrate did not affect the CBF in response to nasal insulin administration as compared with placebo in pre-defined brain regions (i.e., global, gray matter, cortical and subcortical). Also, inorganic nitrate did not have an effect on CBF before administration of the spray (**Table 7.2**). The nasal insulin spray, however, increased CBF in the mean subcortical CBF by 1.4 ± 1.4 mL/100g/min ($P = 0.001$), but not in the other brain regions (see **Table 7.2**).

As compared with placebo, inorganic nitrate significantly increased brain insulin action in five brain clusters based on voxel-wise analyses (**Figure 7.1A** and **Table 7.3A**). The CBF increases in response to insulin in clusters 1N and 2N were 7.0 ± 3.8 mL/100g/min ($P <$

Table 7.2. Brain insulin action and cerebral blood flow responses after administration of a nitrate and placebo drink in a randomized, double-blind, controlled crossover study with abdominally obese men (n=18)[1].

	Inorganic nitrate		Placebo		P-value[2]		
	Pre-insulin (mL/100g/min)	Post-insulin (mL/100g/min)	Pre-insulin (mL/100g/min)	Post-insulin (mL/100g/min)	Treatment * insulin[3]	Treatment[4]	Insulin[5]
Global CBF	41.4 ± 8.1	42.2 ± 8.1	42.1 ± 8.6	42.2 ± 8.4	0.228	0.722	0.154
GM CBF	50.1 ± 10.1	50.9 ± 10.1	51 ± 11	51 ± 10.8	0.278	0.695	0.374
Cortical CBF	54.7 ± 11.2	55.2 ± 11.2	55.5 ± 11.9	55.3 ± 11.6	0.703	0.76	0.649
Subcortical CBF	34.3 ± 7.5	36.2 ± 7.1	35.4 ± 8.2	36.2 ± 8.1	0.146	0.563	< 0.001

[1]Data are shown as mean ± SD. [2]Linear mixed models with Toeplitz covariance structure were performed using test day, insulin spray, treatment, and insulin spray * treatment as fixed factors. Participant was included as random factor and a random intercept was used. The interaction term was omitted from the model if it was not statistically significant. [3]Effect of inorganic nitrate on brain insulin action. [4]Difference in cerebral blood flow between the nitrate and placebo period adjusted for insulin effect. [5]Difference in brain insulin action adjusted for the nitrate drink. CBF: cerebral blood flow; GM: gray matter.

0.001) and 6.5 ± 4.3 mL/100g/min (P < 0.001), respectively. Both clusters were located in the right temporal lobe (36% and 26%, respectively) based on the MNI structural atlas.

According to the Harvard-Oxford atlas, the specific location for cluster 1N, which had a total volume of 5296 mm^3, was in the temporo-occipital part of the inferior (13%) and middle (7%) temporal gyrus, inferior lateral occipital cortex (6%), and temporal occipital fusiform cortex (4%). The volume of cluster 2N was 3592 mm^3 and the specific average probability of the location was 12% in the posterior temporal fusiform cortex, 3% in the parahippocampal gyrus, 3% in the inferior and 2% in the middle temporal gyrus, 3% in the planum temporale, 3% in the parietal operculum cortex, 3% in the temporal occipital fusiform cortex, and 1% in the Heschl's gyrus. CBF in cluster 3N increased by 5.9 ± 3.2 mL/100g/min (volume: 1792 mm^3, P < 0.001) and this cluster was located for 23% subcortical in the left putamen (15%), amygdala (5%), accumbens (2%) and pallidum (2%), and partly in the frontal orbital cortex of the frontal lobe (3%). CBF responses to intranasal insulin following inorganic nitrate significantly increased by 9.0 ± 6.0 mL/100g/min (P = 0.007) in cluster 4N (right frontal lobe, 61%), which had a volume of 1096 mm^3. The specific average probability of the location was 53% in the frontal pole, 12% in superior frontal gyrus and 2% in paracingulate gyrus. Finally, CBF also increased in cluster 5N (left parietal lobe, 58%) by 6.1 ± 4.3 mL/100g/min (volume: 1024 mm^3, P = 0.012), which was located in both the precuneus cortex (36%) and posterior cingulate gyrus (22%). The location probability of these brain clusters is also shown in **Supplementary Table 7.1**. No significant differences were observed before administration of the spray between inorganic nitrate and placebo following voxel-wise analyses.

Effects on nasal insulin spray on CBF were evaluated during the placebo test day. Insulin increased CBF in four clusters (cluster 1I – 4I), while a decreased CBF was found in four other brain clusters (cluster 5I – 8I). These results are shown in **Figure 7.1B** and **Table 7.3B**). CBF increased in cluster 1I (3.5 ± 2.4 mL/100g/min, volume: 1976 mm^3, P < 0.001) and 2I (3.0 ± 1.9 mL, volume: 1064 mm^3, P = 0.011), which were both located in the bilateral occipital (31% and 30%, respectively) and parietal lobe (20% and 18%, respectively). CBF in cluster 3I, which was located in the left thalamus (79%), increased by 4.0 ± 3.3 mL/100g/min

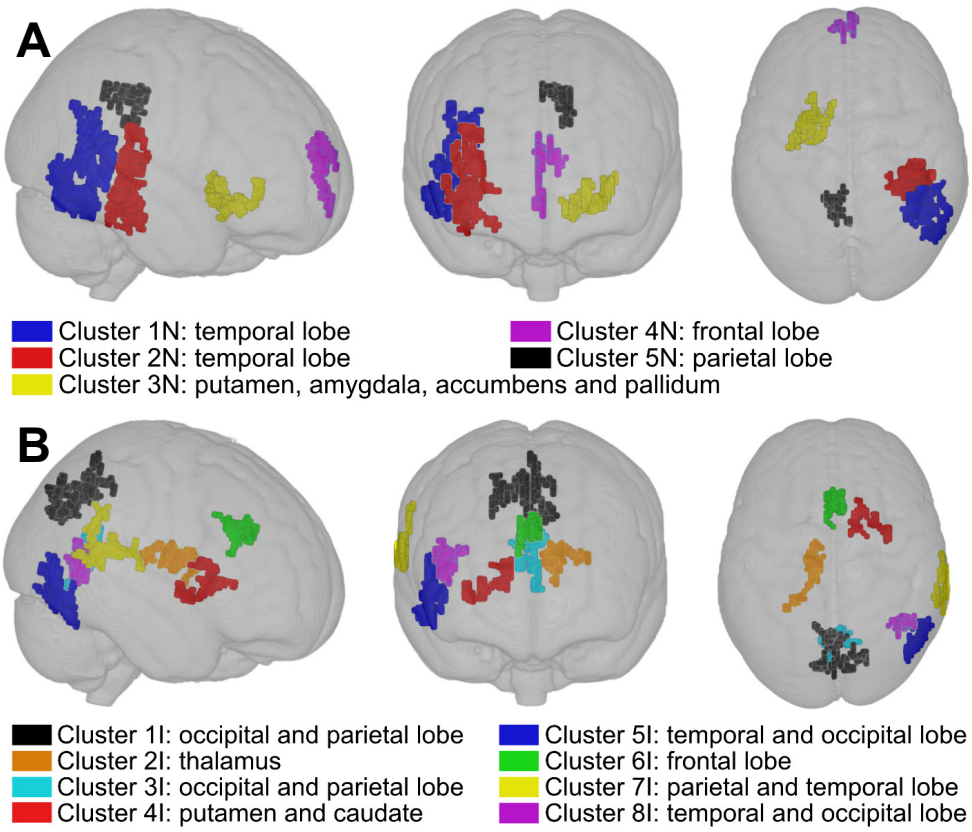

Figure 7.1. Results of voxel-wise comparisons (family-wise corrected) of the whole-brain excluding the cerebellum showing the effect of (**A**) inorganic nitrate on brain insulin action (treatment * insulin) and (**B**) brain insulin action during placebo (insulin) in three dimensional Montreal Neurological Institute (MNI)-template from a randomized, controlled, crossover study in abdominally obese adults (n = 18). Mean changes and cluster volumes are shown in **Table 7.3**, and cluster locations in **Supplementary Table 7.1** and **7.2**.

(cluster volume: 1336 mm^3, P = 0.002). Finally, an increased CBF by 3.6 ± 2.8 mL/100g/min (P = 0.018) was also observed in cluster 4I (volume: 992 mm^3) that was located in the right putamen (26%) and caudate (17%). In contrast, CBF decreased by 6.5 ± 3.4 mL/100g/min (P = 0.010) in cluster 5I (1368 mm^3) that was located in the right temporal (60%) and occipital lobe (14%). Also, a decreased CBF following spray (5.4 ± 4.3 mL/100g/min, 952 mm^3, P = 0.023) was observed in cluster 6I (bilateral frontal lobe, 65%). In addition, CBF in cluster 7I decreased by 6.6 ± 5.9 mL/100g/min (P = 0.025). The volume of that cluster was 944 mm^3 and the average probability of location was in the right parietal (37%) and temporal lobe (27%). Finally, in cluster 8I (right temporal lobe, 29%; and occipital lobe, 10%), CBF decreased by 5.6 ± 5.1 mL/100g/min (P = 0.042, volume: 872 mm^3). The specific location probability of these brain clusters is also presented in **Supplementary Table 7.2.**

Table 7.3. The effect of inorganic nitrate on brain insulin action (**A**) and CBF response to nasal insulin during the placebo test day (**B**) in a randomized, double-blind, controlled crossover study with abdominally obese men (n=18)[1].

A		**Inorganic nitrate (mL/100g/min)**	**Placebo (mL/100g/min)**	**Mean difference (mL/100g/min)**	**Volume (mm^3)**	**P-value[2]**
Treatment * insulin	Cluster 1N	2.7 ± 3.2	-4.2 ± 4.9	7.0 ± 3.8	5296	< 0.001
	Cluster 2N	3.2 ± 4.0	-3.3 ± 4.2	6.5 ± 4.3	3592	< 0.001
	Cluster 3N	4.7 ± 2.8	-1.2 ± 2.6	5.9 ± 3.2	1792	< 0.001
	Cluster 4N	4.3 ± 3.5	-4.7 ± 5.0	9.0 ± 6.0	1096	0.007
	Cluster 5N	4.5 ± 3.6	-1.6 ± 3.4	6.1 ± 4.3	1024	0.012
B		**Pre-insulin (mL/100g/min)**	**Post-insulin (mL/100g/min)**	**Mean difference (mL/100g/min)**	**Volume (mm^3)**	**P-value[2]**
Insulin	Cluster 1I	49.7 ± 10.5	53.2 ± 11.1	3.5 ± 2.4	1976	< 0.001
	Cluster 2I	47.1 ± 9.0	51.1 ± 9.0	4.0 ± 3.3	1336	0.002
	Cluster 3I	50.3 ± 10.9	53.3 ± 11.4	3.0 ± 1.9	1064	0.011
	Cluster 4I	41.8 ± 6.9	45.3 ± 7.0	3.6 ± 2.8	992	0.018
	Cluster 5I	50.6 ± 13.8	44.0 ± 12.4	-6.5 ± 7.4	1368	0.001
	Cluster 6I	56.5 ± 14.1	51.1 ± 14.6	-5.4 ± 4.3	952	0.023
	Cluster 7I	52.5 ± 16.8	45.9 ± 15.9	-6.6 ± 5.9	944	0.025
	Cluster 8I	40.7 ± 9.4	35.1 ± 7.6	-5.6 ± 5.1	872	0.042

[1]Data are shown as mean ± SD. [2]Clusters were the result of a voxel-wise analysis applying a repeated measures mixed effects analysis using a general linear model with a single-group paired difference (FLAME stage 1 and 2), and a Z-threshold of 2.3 (P < 0.05). Family-wise error correction was performed based on smoothness estimates. The effect of inorganic nitrate on brain insulin action (treatment * insulin) was assessed using the difference between the post-insulin and pre-insulin CBF-maps during the nitrate and placebo test day (cluster N). For evaluation of brain insulin action, the post-insulin and pre-insulin scans during the placebo test day were compared (Cluster I). Inorganic nitrate did not affect CBF when the scans after the nitrate and placebo pre-insulin administration were compared.

Cardiometabolic risk markers

No differences were observed in fasting TCH, HDL, LDL, TAG and hsCRP concentrations between both test days (**Table 7.1**). No effects were observed of inorganic nitrate on serum insulin concentrations over time (time * treatment: P = 0.478). Also, no treatment (P = 0.388) or time (P = 0.100) effects were found as shown in **Figure 7.2**. There was no significant time * treatment interaction (P = 0.845) or treatment effect (P = 0.916) for glucose concentration, but the effect of time was significant (P < 0.001, **Figure 7.2**). Inorganic nitrate did not affect SBP (P = 0.764) and DBP (P = 0.538), and heart rate (HR: P = 0.346). However, SBP and DBP increased over time by 4 ± 5 mmHg (P = 0.009) and 3 ± 3 mmHg (P = 0.001), respectively, while HR decreased by 5 beats per min (P < 0.001; **Figure 3**). Baseline differences were not observed for these risk markers (insulin: P = 0.404, glucose: P = 0.680, SBP: P = 0.929, DBP: P = 0.881, and HR: P = 0.683).

DISCUSSION

In this double-blind, randomized, controlled, cross-over trial with abdominally obese men, inorganic nitrate acutely increased the CBF response to nasal insulin in five brain clusters, which reflects an improved regional insulin action in the brain. The two largest clusters were located in the right temporal lobe (i.e., temporal gyrus and fusiform cortex), while two other cortical clusters were part of the right frontal (i.e., prefrontal) and the left parietal lobe (i.e.,

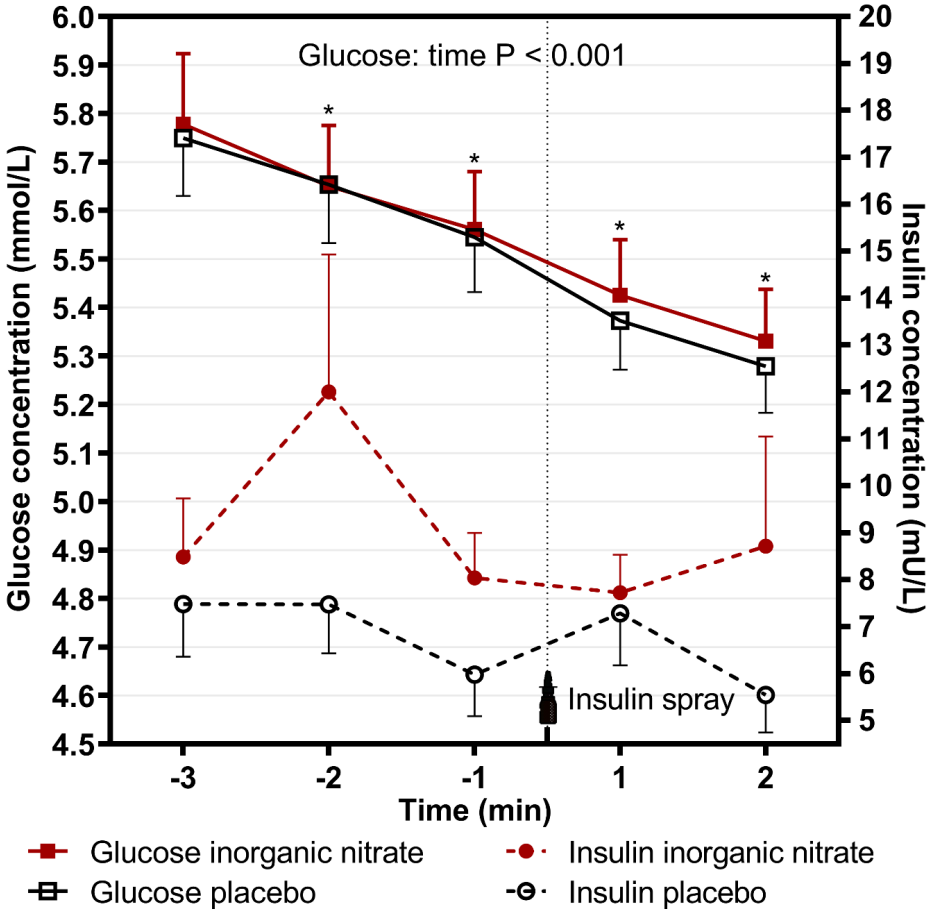

Figure 7.2. Mean (± SEM) glucose and insulin concentration during the inorganic nitrate and placebo period in abdominally obese men (n = 18). The x-axis shows blood sample relative to the nasal-insulin spray. Linear mixed models with Toeplitz covariance structure was performed with time, treatment, period, and time * treatment as fixed factors. Participant was included as random factor and a random intercept was used. The P-value for the time effect was calculated after the statistically non-significant interaction term was omitted from the model. Concentrations between treatments did not differ. * After Bonferroni's correction significantly different from each other P < 0.002.

precuneus cortex and posterior cingulate gyrus). One subcortical cluster was located in the striatum (i.e., putamen, amygdala, accumbens and pallidum).

All cortical brain regions that showed increased brain insulin responsiveness following inorganic nitrate intake belonged to the default mode network (DMN), which comprises the lateral regions in the temporal lobe, the prefrontal and precuneus cortex, and the posterior cingulate gyrus. The DMN is a network of interacting brain regions that accounts for 90% of the energy consumed by the brain (29), which is mainly active at rest and essential for main cognitive functions such as memory and executive function (30). The increased CBF response to intranasal insulin spray may increase the delivery of energy substrates to the DMN. This can be relevant as (glucose) hypometabolism in the DMN has been reported in patients with neurodegenerative diseases using fluorodeoxyglucose

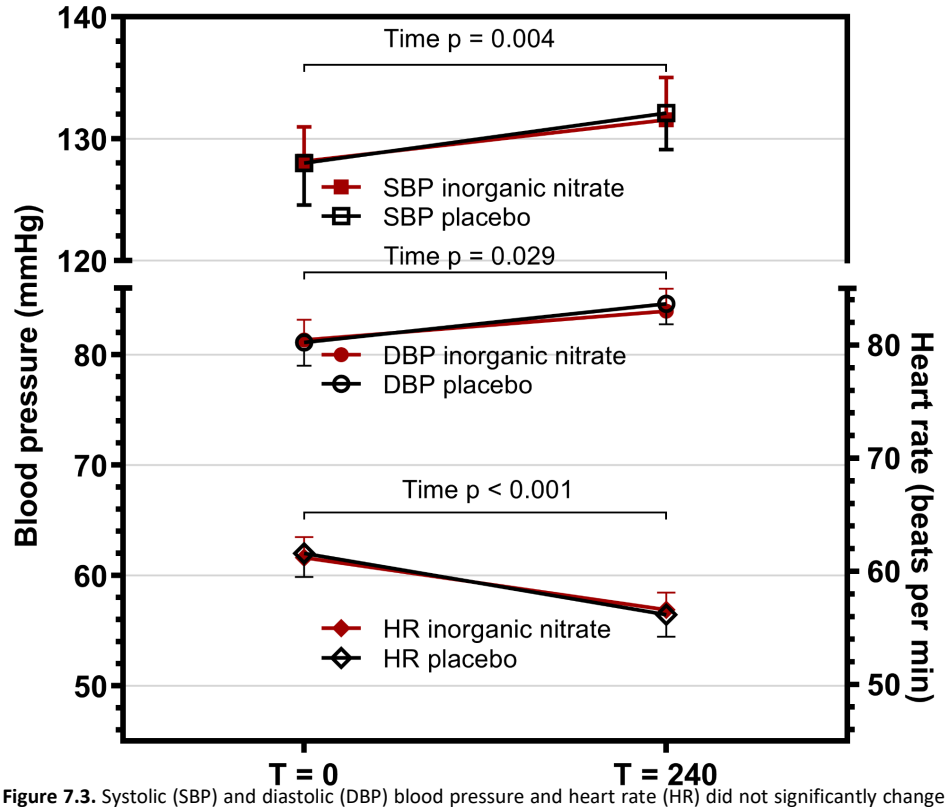

Figure 7.3. Systolic (SBP) and diastolic (DBP) blood pressure and heart rate (HR) did not significantly change 240 min after consumption of inorganic nitrate as compared with placebo in abdominally obese men (n = 18). Data are shown as Mean (± SEM). Linear mixed models were performed using the change from baseline as dependent variable. Test day and treatment were used as fixed factors, and participant was included as a random factor.

amyloid positron emission tomography (FDG-PET) (30, 31). Further, brain insulin resistance is associated with impaired cognitive performance, possibly due to changes in the connectivity of brain networks (32). Therefore, the increase in brain insulin action may not only be beneficial for our population consisting of abdominally obese individuals, but also for patients suffering from T2D or neurodegenerative diseases like dementia who have a decreased insulin responsiveness and functional connectivity of the DMN (2, 30, 33). However, long-term studies in different patient groups, using different doses of inorganic nitrate, and with functional outcomes are still needed to prove or disapprove this hypothesis.

The cortical ventral and dorsal striatal circuit, which consists of prefrontal, temporal and striatal clusters, are involved in the regulation of food intake by modifying specific brains reward processes (2). These circuits also showed an increased brain insulin responsiveness after the intake of inorganic nitrate. Both brain circuits are activated by the neurotransmitter dopamine, while insulin inhibits the action of dopamine and has anorexigenic properties by reducing the activation of these reward circuits (34, 35). Interestingly, increased CBF responses to intranasal insulin in the striatum have already

been observed in healthy participants (35, 36). In our study, similar effects were only found following nitrate intake, which may suggest that in abdominally obese participants an increased NO bioavailability is required to observe these findings. Glucose metabolism measured with FDG-PET increased both in the prefrontal cortex and in the striatum during a hyperinsulinemic-euglycemic clamp in insulin-sensitive men, while a less pronounced response was observed in insulin-resistant counterparts (37). Similar findings were observed in response to glucose intake (38) and intranasal insulin (15) in normal-weight as opposed to obese individuals. It is therefore of interest if these acute effects of inorganic nitrate are also evident at the long-term, thereby possibly counteracting the reduced inhibitory control contributing to overeating behavior as has been observed in some insulin-resistant and obese individuals (2, 3).

Inorganic nitrate did not affect CBF before administration of the spray. In contrast, a study involving older adults observed an increased CBF in the frontal lobe as measured with ASL after the consumption of a high-nitrate diet, including 500 mL of beetroot juice providing 773 mg of dietary nitrate, over a 24-hour period (11). The higher dose of nitrate supplied by wholefoods over a longer period of time may explain these apparent inconsistent results. An acute increase in CBF was also observed in young adults after the consumption of beetroot juice as measured with transcranial doppler during exercise (12) or near-infrared spectroscopy during cognitive tasks (13). These results indicate that the intake of inorganic nitrate may affect CBF following stimuli challenging the regulation of blood flow in the brain.

Abdominally obese men are known to have an impaired brain insulin responsiveness (6), which is in line with the observed regional CBF responses in our population during the placebo test day. We observed an increased thalamic CBF response to insulin in abdominally obese men, which is opposite to responses following the intake of foods in normal-weight adults (39). Increased thalamic insulin responses were positively associated with the amount of visceral adipose tissue (6, 15). Additionally, an increased CBF was observed in substructures of the striatum (i.e., putamen and caudate), while CBF decreased in the frontal lobe (i.e., anterior cingulate gyrus). Comparable responses in these brain regions, which are involved in energy homeostasis, attention, reward, sensory perception and motivation, have previously been associated with impaired insulin responses after an oral glucose load (2, 40). In our study population, as well as in normal-weight participants (41), CBF was reduced in response to nasal insulin in the fusiform and temporal gyrus, and the medial part of the frontal lobe. It would therefore be of interest if the magnitude of these responses may play a role in the termination of food intake following a meal (41) as a more pronounced reduction in CBF in these brain regions was associated with less visceral adipose tissue (6, 15). Finally, CBF was also affected by the insulin spray in brain clusters located in the occipital lobe that are involved in the modulation of food preferences, which provides further evidence that insulin plays an important role in the regulation of processes in the brain underlying food intake and appetite (42, 43).

Serum insulin concentrations were not affected by the intake of inorganic nitrate and did also not change following the application of the spray. In contrast, a transient increase in serum insulin concentrations was observed 15 min after the intranasal application of 160 U of insulin (human insulin, Actrapid) in a dose-response study (40, 80

and 160 U), which was due to spillover of the spray into the peripheral circulation without affecting plasma glucose concentrations (23). We did not observe an increase in insulin concentrations following the application of a similar dose of insulin aspart (Novorapid). This may be due to differences between the two types of insulin in kinetics following intranasal administration of the sprays. Although we might have missed a moderate increase in serum insulin concentrations, it was however still concluded that a transient increase in peripheral insulin concentrations did not significantly affect CBF responses (23, 44). Finally, we did observe reduced plasma glucose concentrations over time that were probably due to the long fasting period (45), and not related to the insulin spray as glucose concentrations were also not affected in the dose-response study (23).

Compared with the placebo drink, the acute intake of inorganic nitrate did not affect blood pressure after 240 min, which could therefore not explain the observed differences in CBF responses in our study. In contrast, decreased blood pressure levels were observed in a recent meta-analysis following the acute intake of (dietary) nitrate (46). A possible explanation might be that inorganic nitrate has more pronounced blood pressure effects when part of wholefoods (46). In general, effects on blood pressure were only observed two-to-three hours following nitrate administration, indicating that blood pressure levels may already have been restored after four hours. An interesting observation is that blood pressure levels increased over time, while HR decreased. This could be due to the natural circadian blood pressure and HR rhythm (47). Alternatively, the insulin spray may induce sympathoexcitation of blood pressure regulatory centers in the brain, which was suggested by Benedict et al. (48).

Our primary aim was to investigate the acute effects of inorganic nitrate on regional insulin action in the brain. Effects were tested using a whole-brain approach except for the cerebellum, which involved family-wise corrections for multiple comparisons. A possible limitation is that we did not use a placebo spray (23). In addition, only abdominally obese men were included, because they are thought to have an impaired brain insulin responsiveness and to exclude any possible sex effects (2, 6). Finally, it remains to be determined in longer-term studies whether the observed increases in brain insulin action also translate into beneficial functional outcomes, such as improved cognitive performance and food intake regulation.

In conclusion, this study involving abdominally obese men showed that acute inorganic nitrate intake affects regional insulin action in the brain. Specifically, an increased brain insulin responsiveness was observed in regions that are involved in the regulation of various metabolic and cognitive processes in the brain, as well as in processes underlying the intake of food.

AUTHOR CONTRIBUTIONS

JK: designed and conducted the study, analyzed data, performed statistical analysis, and wrote the manuscript; RM: designed the study, wrote the manuscript and had primary responsibility for final content; ES: conducted the study and reviewed the manuscript; DI: developed the MRI sequences and assisted with the statistical analysis, and reviewed the manuscript; PJ: designed the study, wrote the manuscript and had primary responsibility for final content.

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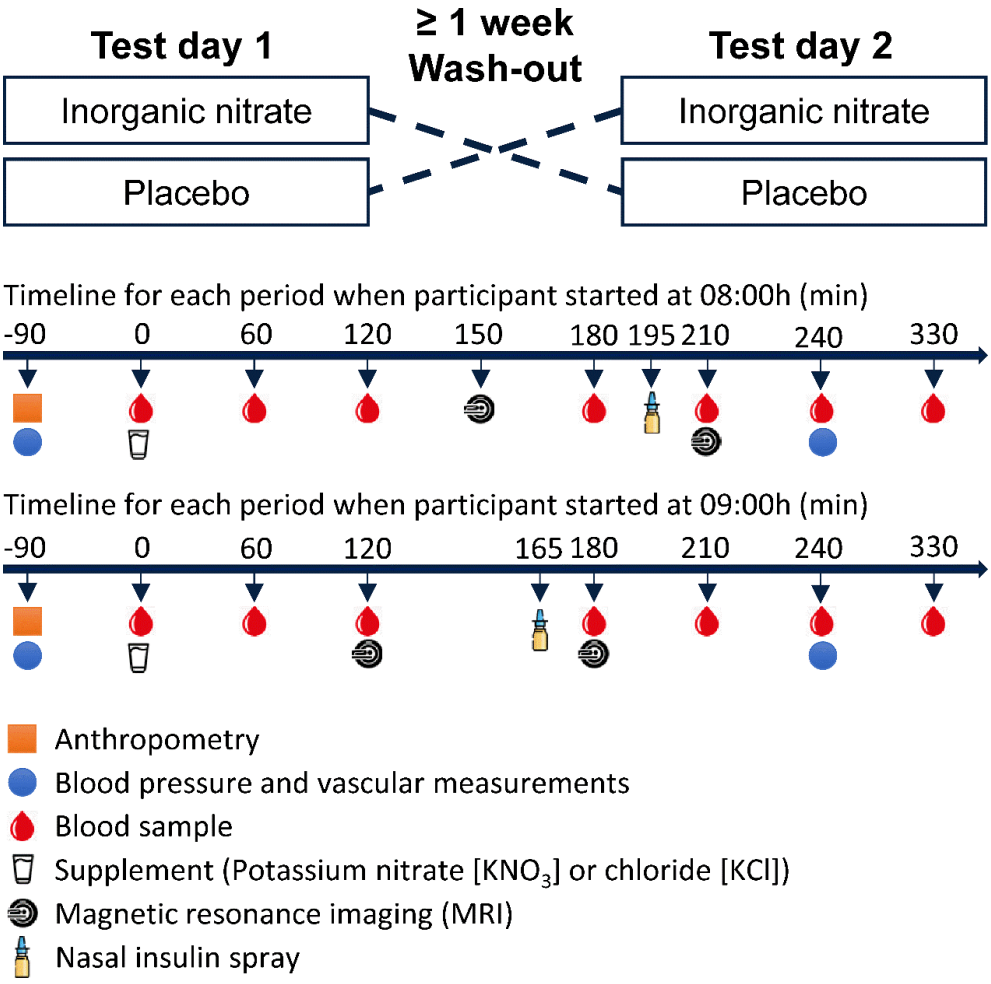
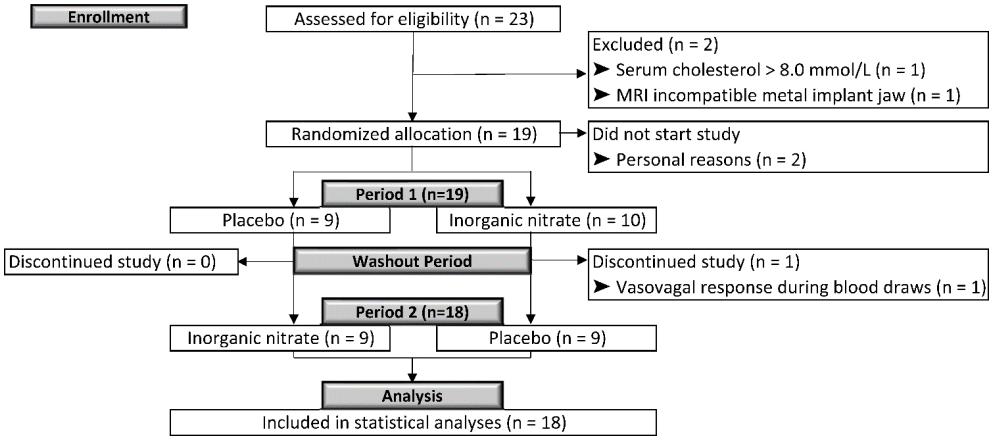
CONFILICT OF INTEREST

The project is organized by and executed under the auspices of TiFN, a public-private partnership on precompetitive research in food and nutrition. The authors have declared that no competing interests exist in the writing of this publication. Funding for this research was obtained from the Netherlands Organization for Scientific Research (grant number: ALWTF.2016.012).

SUPPLEMENTAL MATERIAL



Supplementary Figure 7.1. Schematic overview of study design.



Supplementary Figure 7.2. CONSORT flow diagram of the progress through the phases of this randomized, cc crossover study with abdominally obese men.

Supplementary Table 7.1. Location probability of the significantly changed clusters (treatment * insulin, cluster N) determined using the Atlasquery function of FSL using the MNI structural atlas and Harvard-Oxford atlas. The effect of inorganic nitrate was investigated during a randomized, double-blind, placebo-controlled crossover study in abdominally obese men (n = 18).

	Location			
	MNI structural atlas	Probability	Harvard-Oxford atlas	Probability
Cluster 1N	Right temporal lobe	36%	Temporo-occipital part of the inferior temporal gyrus	13%
			Temporo-occipital part of the middle temporal gyrus	7%
			Inferior lateral occipital cortex	6%
			Temporal occipital fusiform cortex	4%
Cluster 2N	Right temporal lobe	26%	Posterior temporal fusiform cortex	12%
			Parahippocampal gyrus	3%
			Inferior temporal gyrus	3%
			Middle temporal gyrus	2%
			Planum temporale	3%
			Parietal operculum cortex	3%
			Temporal occipital fusiform cortex	3%
			Heschl's gyrus	1%
Cluster 3N	Left subcortical		Putamen	15%
			Amygdala	5%
			Accumbens	2%
			Pallidum	2%
Cluster 4N	Right frontal lobe	61%	Frontal pole	53%
			Superior frontal gyrus	12%
			Paracingulate gyrus	2%
Cluster 5N	Left parietal lobe	58%	Precuneus cortex	36%
			Posterior cingulate gyrus	22%

Supplementary Table 7.2. Location probability of the significantly changed clusters (insulin, cluster I) determined using the Atlasquery function of FSL using the MNI structural atlas and Harvard-Oxford atlas. The effect of inorganic nitrate was investigated during a randomized, double-blind, placebo controlled, crossover study in abdominally obese men (n = 18).

	Location			
	MNI structural atlas	Probability	Harvard-Oxford atlas	Probability
Cluster 1I	Bilateral occipital lobe	31%	Cuneal cortex	23%
			Superior occipital cortex	13%
			Intracalcarine cortex	16%
			Supracalcarine cortex	6%
	Bilateral parietal lobe	20%	Precuneus cortex	30%
Cluster 2I	Left subcortical		Thalamus	79%
Cluster 3I	Bilateral occipital lobe	30%	Lingual gyrus	31%
			Intracalcarine cortex	16%
			Supracalcarine cortex	6%
	Bilateral parietal lobe	18%	Precuneus cortex	13%
Cluster 4I	Right subcortical		Putamen	26%
			Caudate	17%
Cluster 5I	Right temporal lobe	60%	Inferior lateral occipital cortex	33%
			Temporo-occipital part of the inferior temporal gyrus	27%
			Temporo-occipital part of the middle temporal gyrus	2%
	Right occipital lobe	14%	Occipital fusiform gyrus	2%
			Temporal occipital fusiform cortex	2%
Cluster 6I	Bilateral frontal lobe	65%	Anterior cingulate gyrus	64%
			Paracingulate gyrus	11%
Cluster 7I	Right parietal lobe	37%	Posterior supramarginal gyrus	20%
			Angular gyrus	6%
	Right temporal lobe	27%	Posterior superior temporal gyrus	16%
			Posterior middle temporal gyrus	2%
			Middle temporal gyrus	7%
			Planum temporale	3%
Cluster 8I	Right temporal lobe	29%	Temporo-occipital part of the middle temporal gyrus	16%
			Temporo-occipital part of the inferior temporal gyrus	5%
			Angular gyrus	2%
	Right occipital lobe	10%	Inferior lateral occipital cortex	4%

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CHAPTER 8

General discussion

A healthy lifestyle, consisting of increased physical activity levels and a healthy diet, is instrumental to attenuate or prevent the risk to develop age-related conditions, such as cardiovascular disease (CVD), cognitive impairment and dementia (1, 2). CVD has already been the leading cause of morbidity and mortality worldwide for decades, while the incidence of cognitive impairment and dementia is growing rapidly (3). Addressing lifestyle-related behavioral risk factors is therefore important, as this approach has the greatest potential of all known strategies for reducing the risk for developing or progression of these age-related conditions (4, 5). Lifestyle-induced changes in the more traditional cardiometabolic risk markers (e.g., serum lipid profile and blood pressure) could only partly explain the risk reduction of these age-related conditions following specific dietary and exercise training interventions, and therefore current intervention and prevention strategies also focus on effects on the vasculature, as vascular function may also play an important role (6). An innovative approach is to focus on cerebral blood flow (CBF), which is a sensitive marker for cerebrovascular function, that is estimated to decrease with 0.45% to 0.50% each year in middle-aged and older adults (7). A representative example of a younger and older participant is shown in **Figure 8.1**. Regional changes in CBF have also been observed during aging (8) and these changes are associated with age-related cognitive decline (9). Beneficial effects of a healthy lifestyle on regional CBF may therefore be important, as they may underlie the improvements on cognitive performance (5, 10). In addition to cerebrovascular function, effects on peripheral vascular function markers can be assessed. These markers are used to address vascular endothelial function and arterial stiffness, which may contribute the decreases in CVD risk after healthy dietary and exercise training interventions (6). However, well-controlled trials investigating effects of a healthy diet and exercise training on cerebrovascular and peripheral vascular function are lacking (11). Further, the role of peripheral glucose metabolism and insulin actions in the brain on cerebrovascular function has been suggested to play an important role in the development of these diseases that warrants further research (12). The research described in the present thesis therefore first described measurement of CBF using arterial spin labeling (ASL) magnetic resonance imaging (MRI) and presented a case-study (**Chapter 2**). Thereafter, the effects of physical exercise training interventions on cerebral blood flow were reviewed (**Chapter 3**). Effects of a trial with an aerobic exercise training (**Chapter 4**) and longer-term soy nut intervention (**Chapter 6**) on CBF, cognitive performance and glucose metabolism were described in the next chapter. Effects of aerobic exercise training on different regions of the vascular tree were also investigated (**Chapter 5**). Moreover, the effects of inorganic nitrate, which has already been shown to have beneficial effects on peripheral vascular function and possibly regional CBF (7), on brain insulin action was determined (**Chapter 7**).

Measurement of vascular function

Human aging is related to both decreased cerebrovascular and peripheral vascular function, which lead to cognitive impairment and CVD, respectively. Whether these age-related conditions have common underlying mechanisms is unclear. It has been proposed that age-related changes in vascular function and structure affect CBF (13). Arterial stiffening and wall thickening of central elastic arteries elevate systolic blood and pulse pressure, subsequently augmenting CBF pulsatility (peak systolic minus end diastolic flow) (13). Indeed, CBF was decreased, while the brain pulsatility index was increased, which was

associated with an increased CVD risk quantified by the Framingham General Cardiovascular Risk Profiles score (14). Additionally, these changes in CBF have been related to changes in brain structure (i.e., white matter signal abnormalities, iron accumulation and brain atrophy), which are also associated with cognitive performance (**Chapter 2**).

Cerebrovascular function

Adequate CBF is necessary for supply of oxygen and energy substrates, while removing waste products, and is required to support normal brain function during healthy aging. Aging is associated with a decreased CBF in specific cortical brain regions (8) and these regions may be more sensitive to the effects of a healthy lifestyle. Various techniques can be used to measure CBF (15). Depending on the technique, CBF can be defined as the blood volume that flows per unit mass per unit time in brain tissue or in terms of flow velocity per unit volume of brain tissue. Positron Emission Tomography (PET) is the gold standard method. However, applicability in human intervention studies is limited due to intravenous injection of a radioactive contrast agent that diffuses through the blood-brain barrier. The novel method MRI ASL assess CBF non-invasively (16), which relies on magnetically-labeled water molecules from the blood flowing through the major arteries towards the brain. These images are compared with control imaged in which no inversion pulse is applied. The mean difference between these images can be calibrated using an M0 image without magnetization preparation to obtain a quantitative three-dimensional CBF map in milliliters of blood per 100 g of tissue per minute; an example is given in **Figure 8.1**. When studies using MRI ASL were compared with PET CBF imaging, it was concluded that the scan-rescan reproducibility is better, while PET CBF imaging was an accurate method (17). The coefficient of variation of the scan-rescan reproducibility however varies per region, which should be taken into account in the study design. The between-session coefficient of variation was 13% for global CBF in the performed studies, which was used for power calculations to determine the sample size, as we focused on whole-brain changes. Within session coefficient of variation was only 5%, as observed in the acute study on the effects of inorganic nitrate on brain insulin action. MRI ASL also has significant advantages over other MRI techniques such as dynamic susceptibility contrast imaging, because these techniques require injection of a non-diffusible contrast agent (e.g., gadolinium) and do not provide quantitative CBF values. Blood oxygenation level-dependent (BOLD) MRI is a susceptibility-based method that relies on relative changes in oxygenated and deoxygenated hemoglobin accompanying brain activation and is appropriate for event-related study designs, whereas ASL has better spatial localization, less susceptibility effects and is more suitable to determine changes over longer-term (18). Other non-invasive methods include transcranial doppler ultrasound and near-infrared spectroscopy (NIRS), but these methods are not region specific, which is an important limitation as regional perfusion has been associated with for example aging. Transcranial doppler ultrasound measures flow velocity in the basal arteries of the brain (e.g., proximal anterior arteries and posterior cerebral arteries), but does not provide information on the flow of arterial blood into the capillary beds. NIRS is an optical technique that measures the concentration of (de)-oxygenated hemoglobin in the cerebral cortex superficially through the scalp, which provides an indirect measure of brain activity. The latter method depends on relative changes in blood flow and oxygen metabolism that have taken place during a stimulus, such

as cognitive tasks of exercise, which should be considered when interpreting results. Therefore, the intervention studies described in the present thesis used MRI ASL to quantify (regional) CBF (**Chapter 4, 6 and 7**).

We have used the pseudo-continuous (PC)ASL approach, which was also recommended by the consensus paper by Alsop et al. (19). For PCASL, labeling occurs over a longer period (1750 ms), while 1000 or more shaped radiofrequency pulses are applied at a rate of approximately one per second. In contrast, pulsed (P)ASL uses a single short pulse or a limited number of pulses (10-20 ms) to invert a thick slab of arterial water spins. However, the signal-to-noise ratio is higher for PCASL due to the temporal duration and higher magnetization of the labeled bolus. The scan duration was approximately 10 min and contained 10 tag and control images with a resolution of 3 mm isotropic to reduce variability, while four background suppression pulses were used with a total label efficiency of 0.64 (0.93^4) to improve signal to noise ratio. Due to the large field of view, we were able to image the entire brain excluding a part of the cerebellum, while other studies often only images a specific region. It is important to consider the arterial transit time, as post labeling delay is ideally just longer than the longest value of arterial transit time in the participants. We used a post labeling delay of 2000 ms, which is recommended for older participants and patients to account for expected longer arterial transit time. The main feeding arteries of the brain (i.e., carotid and vertebral arteries) were labeled perpendicular in a relatively straight part based on an angiogram, which was kept similar between repeated scans. Additionally, the relaxation time (T_1) of the label in blood should be considered, which depends largely on the blood water content. The T_1 of blood was therefore estimated using the measured hemoglobin concentration at each measurement day, as it may be affected by interventions and can change over time. This may affect CBF values up to 35% (20), thereby inducing extra variation in studies that did not correct for hemoglobin concentration.

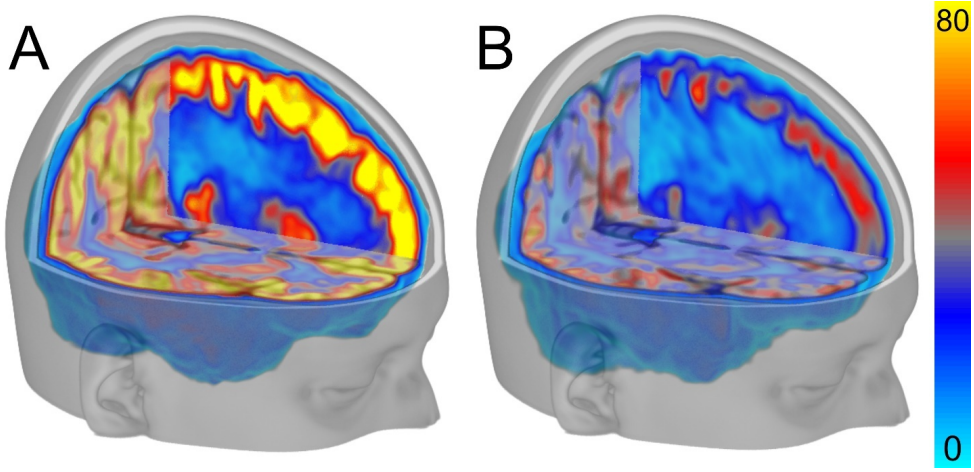

Figure 8.1. Representative example of a quantitative three-dimensional CBF map in ml/100g/min of a young (**A**) and old participant (**B**), which was acquired during one of our studies.

There are different approaches to analyze the CBF data based on the study design and the objective of the study. Considerations about the region of interest and voxel-size need to be taken into account. As a specific region of interest may benefit from a smaller voxel-size, while partial volume effects may increase as voxel size increases. The images in the studies described in **Chapters 4, 6 and 7** acquired CBF of the entire brain with a voxel-size of only 3 mm isometric, which can be possible nowadays to the recent technological advancements. In comparison, Maass et al. (21) could only image a part of the brain with specific focus on the hippocampus with a comparable voxel size, while Burdette et al. (22) had a slice thickness of 8 mm. Still, partial volume effects arise as the spatial resolution of the ASL image is larger than the thickness of the adult cortex, which is approximately 2.5 mm. As such, voxels around the cortex will contain a CBF mixture of cortical and non-cortical tissues. Correction of these partial volume effects uses voxel-wise estimates of partial volumes to separate out the signals arising from each tissue. However, the efficacy is limited by the accuracy of the volumetric segmentation of complex geometries and transformation from the structural to the functional voxel grid. This approach was therefore questioned by the group of Chappell et al. (23) and we decided not to implement this step in the reported CBF maps.

The mean CBF of all voxels within a pre-defined region of interest could be calculated, which restricts the analysis to specific boundaries. In line with commonly observed values, global CBF was on average 42 mL/100 g of tissue/min, while gray matter CBF was 50 mL/100 g of tissue/min. Within all the studies described in this thesis, the CBF values were also calculated in native space using the Harvard-Oxford atlases and Volbrain subcortical segmentations. In neither of the studies, regional changes were observed. Therefore, the more sensitive voxel-wise analysis method was used to investigate differences between treatments, which is not restricted to the size and boundaries of the pre-specified region (24). Family-wise error correction, which is a commonly used approach for MRI images, was used to deal with the multiple-testing problem. In the studies described in **Chapters 4, 6 and 7** voxel-wise analysis was performed after non-linear registration to a common template, the Montreal Neurological Institute (MNI)-atlas with a 2 mm voxel-size. This improves the transformation of local deformation, which is present within the populations and increases resolution. The analysis could also be performed within subjects, which is a big advantage of the cross-over design used in the studies performed. To standardize measurements as much as possible, they were performed at the same time of the day for a particular study participant (25). Many other factors may also affect CBF, such as dietary intake (7), alcohol consumption (26), or physical activity (27). Therefore, individuals were requested to have a regular dinner, and not to consume alcoholic beverages, or to perform any strenuous physical exercise on the day preceding the measurements. Further, on the morning of the measurements, participants arrived after an overnight fast (no foods or drinks after 08.00 PM, except for water) at the research facilities by public transport or by car to standardize CBF measurements as much as possible.

Peripheral vascular function

Well-controlled trials focused in general on only one specific characteristic of the vasculature, while an integrated overview of the effects on different regions of the vascular tree, such as central (i.e., carotid artery and aorta), peripheral (i.e., brachial artery), and

retinal microvasculature arteries is currently lacking. Brachial artery flow-mediated vasodilation (FMD) measurement is considered an important non-invasive method for assessing endothelial function, which is an early atherosclerotic marker that precedes asymptomatic structural vascular alterations as well as clinical symptoms of CVD (28, 29). Reactive hyperemia-induced shear stress following the release of a pneumatic blood pressure cuff placed around the brachial artery is the stimulus for the largely nitric oxide (NO) dependent FMD (29). Meta-analyses of prospective epidemiological studies have suggested that for every 1 percentage point (pp) increase in FMD, the risk for future cardiovascular events decreases by 8-13% (30, 31) Highly reliable FMD measurements can be achieved when standardized protocols are followed (29). FMD was measured in a quiet and darkened room after at least 15 min in supine rest, while images were analyzed using custom continuous edge-detection software (**Chapter 5**). The baseline diameter should be considered when interpreting the FMD, which did decrease after exercise. However, a smaller baseline brachial artery diameter is also predictive of cardiovascular risk (32), underlining the clinical relevance of our findings. The FMD relates to coronary artery endothelial function (33), which can also be assessed with the carotid artery reactivity (CAR) in response to a cold pressor test (34). The CAR is an easier method to assess endothelial function in a major elastic conduit artery compared to the FMD in a peripheral muscular artery. The CAR involves stimulation of the sympathetic nervous system. Catecholamines (e.g., norepinephrine) released during the cold pressor test can increase vasodilation via endothelium-dependent effects, but might at the same time cause vasoconstriction of smooth muscle cells via the sympathetic nervous system (35). The balance between these two processes may determine the vasomotor response and is an independent predictor of cardiovascular events (36). Although the FMD and CAR are both measures of vascular endothelial function, we did not observe a correlation between both outcomes. This may be explained by the difference in vascular structure and function of the peripheral muscular brachial artery versus the central elastic conduit carotid artery. The CAR was also not correlated to any of the other vascular measures, but the sample size was probably too limited to examine relationships into detail.

Pulse wave velocity between the carotid and femoral artery ($PWV_{c\text{-}f}$) measured using tonometry is the gold standard to assess regional arterial stiffness non-invasively and is used for risk stratification of CVD (37). Many clinical studies and meta-analysis observed an association between PWV and coronary, cerebral and carotid atherosclerosis (37). In fact, a meta-analysis has suggested that the risk of CVD events decreases by 14% when $PWV_{c\text{-}f}$ improves by 1.0 m/s (38). Nowadays, results from different studies can be more easily compared due to a greater uniformity in the measured vascular territory and methodology used, based upon consensus and reference standards (39). We adhered to these recommendations in the study performed in **Chapter 5** and used strict quality control with at least three repeated measures. The individual $PWV_{c\text{-}f}$ values should not differ more than 1.0 m/s from the average value; otherwise, a fourth measurement was performed. The reported values were the mean of all $PWV_{c\text{-}f}$ measurements. Alternatively, brachial-ankle pulse wave velocity ($PWV_{b\text{-}a}$) can be measured, which includes a greater territory of the arterial tree, but show similar associations with CVD risk factors (40). However, the sensitivity to interventions may differ, as also NO producing muscular peripheral arteries are included.

Office blood pressure was measured according the recent guidelines (41), while ambulatory blood pressure was also determined, which more closely relates to preclinical target organ damage (42). The mean arterial pressure (MAP) and central blood pressure can be determined accurately using the pulse wave assessed with a tonometer, that was determined at the brachial artery near the antecubital fossa, based on the brachial systolic and diastolic blood pressure (41). Also, pulse wave analysis of the radial artery was performed in triplicate to assess the central augmentation index corrected for heart rate (CAIxHR75), as a measure of pulse wave reflections. PWV and CAIxHR75 provide information about the properties of the arterial tree, where CAIxHR75 may be modifiable to a higher degree than PWV (43). However, the association between CVD risk factors and CAIxHR75 reduced in people over 60 years, due to the curvilinear association between CAIxHR75 and age (43). In addition, local carotid arterial stiffness (44-46) can be determined using a radial cross-section of the carotid artery. For this purpose, the systolic and diastolic diameters need to be assessed during the peak and end phase of each heart cycle (45). The diameter is determined using custom-written software with automated detection of the anterior and posterior walls based on the pixel intensity of the lumen relative to the adventitia. Reduction in the difference between the systolic and diastolic blood pressure (i.e., radial strain) is an indicator for increased artery wall stiffness. However, exercise affected blood pressure. Therefore, correcting for central blood pressure is necessary for interpretation (i.e., arterial distensibility, pressure-independent stiffness index β_0) (44). Additionally, an estimate of the elasticity of the arterial wall can be determined using the Young's modulus of elasticity (45). Both structural and functional elements have been implicated in the vascular stiffening processes with advancing age, which may be modifiable by lifestyle factors (46).

Finally, fundus photography focusing on the retinal vasculature was used to quantify diameters of the microvasculature in **Chapter 5** (47). The retinal vasculature may provide a window into the cerebrovascular brain health, as they share similar embryological origin, and structural and physiological features (48). A non-mydriatic retinal camera (Topcon TRC-NW-300) focused on the optic disc and photographed the retina of the right eye. Although the choice of eye was arbitrary, no differences are expected between eyes as high levels of retinal symmetry have been reported (49). Images were digitally analyzed with semiautomated software (Generalized Dual-Bootstrap Iterative Closest Point) (50) to identify at least two arteriolar and two venular segments. Preferably up to six vessels are identified to calculate the central retinal vessels, but this is difficult with repeated measurements as exactly the same segments need to be identified for each individual. The central retinal arteriolar (CRAE) and venular equivalents (CRVE), and the arteriolar-to-venular ratio (AVR) were derived using the Parr-Hubbard formulas (51). These equivalents are projected calibers for the central retinal vessels, which enter and leave the eye through the center of the optic nerve and originates from the internal carotid artery, that also supplies the brain. Reducing the number of vessels reduces CRAE and CRVE values, but the AVR remains similar (52). However, for the intervention studies mainly the change in diameter is important. Thus, by using the same vessels for each measurement for an participant, the internal validity increases, but the external validity of the calculated diameters may decrease (53). Other formulas exist to calculate equivalents of the central

retinal vessels using weighted means with lower weights for the largest and the smallest calibers measured. However, outcomes of the various formulas are highly correlated (53).

Cerebrovascular and peripheral vascular function

Cross-sectional and cohort studies already demonstrated associations between retinal vasculature and cerebral vascular diseases (54, 55). Recent cross-sectional study in older individuals related retinal microvasculature and CBF in the frontal lobe (56). We now observed a significant inverse Spearman correlation (r = -0.62) between the change in CBF in a cluster of the frontal lobe and CRVE after exercise-training (**Figure 8.2.A**). This underlines the potential of the retinal vasculature as surrogate marker for cerebrovascular function. The retinal vessel diameters were also associated with a reduced risk to develop hypertension (57) stroke incidents (58), and coronary heart disease (59). The significant inverse correlation between the exercise-induced decrease in systolic blood pressure (SBP) and an increase in CRAE is in line with previous literature (**Figure 8.2.B**).

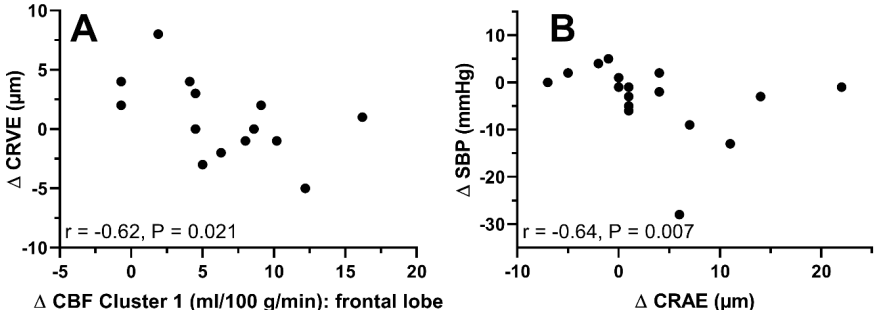

Figure 8.2. Spearman correlation between the changes in retinal microvascular calibers (central retinal arteriolar, CRAE; and venular equivalents, CRVE) and cerebral blood flow (CBF; **A**) and systolic blood pressure (SBP; **B**) measured at the end of both periods during the exercise study (**Chapter 4**).

Cognitive performance

Assessment of cognitive performance requires a battery of valid, reliable, and sensitive measures that are related to specific cognitive domains. Common cognitive domains include attention, information processing speed, executive function, and memory. Development of tests to measure various cognitive functions can be traced back over a century, and some tests have been developed or adapted to measure the different cognitive domains in relation to physical activity or food intake (60, 61). The methodology should be accurate, standardized, and robust, and assessments should have good sensitivity and specificity for the outcomes being assessed. Additionally, construct validity and retest reliability are required for cognitive measures. Measures should also have biological plausibility, even if the detailed mechanism of action of the marker is not fully understood.

The studies described in **Chapters 4 and 6** used the fully automated Cambridge Neuropsychological Test Automated Battery (CANTAB), which is a touchscreen based, standardized, validated and sensitive method to detect changes in cognitive performance in the domains of attention and psychomotor speed, executive function and memory following lifestyle interventions (60). The CANTAB system was initially developed in the 1980s to assess cognitive function in older people and in populations with impaired cognitive performance (62). The tests can be used in populations with a wide range of

cognitive abilities as they have been developed to avoiding floor and ceiling effects. Psychomotor speed was assessed using the five-choice reaction time test to determine reaction time until button release and movement time from button release until the target. For executive function, the multitasking test was used, while incongruency cost (median latency of congruent minus incongruent trials), multitasking cost (median latency of block in which both rules were used minus blocks in which a single rule was used), reaction latency of all correct trials were assessed. Three different tests were performed to assess different aspects of spatial memory. First, spatial span determined the longest sequence of successfully recalled boxes, which are placed in random locations on the screen. During the second test, participants had to select the pattern matching the sample after a delay (delayed matching to sample), while the total correct assessments was used. Finally, the location of several patterns needed to be remembered and paired with the matching sample (paired associated learning). Parallel versions with a high test-retest reliability of the memory tests were used (63, 64) to increases sensitivity to longitudinal changes by minimizing practice effects. The test duration was restricted to 60 min, as longer test batteries have significant effects of fatigue (65).

Interestingly, after excluding one participant with extreme responses, a significant inverse correlation was observed between the changes in CBF and cognitive performance in the psychomotor speed domain after soy supplementation (**Chapter 6**). This suggests a relationship between soy-induced changes in CBF and cognitive performance. Most studies, as well as the results from the studies in **Chapters 4 and 6**, observed only significant effects on some variables within a domain (60). However, most likely not only those variables or domains were affected as sensitivity to the intervention-induced changes may differ (61, 66). Nonsignificant results may thus be due to a lack of sensitivity of the selected test or a lack of statistical power (i.e., a type 2 error or insufficient sample size, rather than a true absence of an effect) (67, 68). Different tests may claim to measure the same cognitive domain, but subtle differences in the level of difficulty or the cognitive processes required may influence the sensitivity to a certain intervention. Moreover, the type and length of intervention, or cognitive status and physical fitness or nutrient status at baseline could also affect intervention outcomes, and thus explain discrepant results of efficacy (60). CANTAB has been widely used in clinical research, with over 1300 peer-reviewed papers supporting its use, resulting in a large base of knowledge about the sensitivity of cognitive tests to specific interventions in certain populations.

Peripheral glucose metabolism

Impaired glucose metabolism is an important contributor to the pathophysiology of diabetes type 2 and the age-related conditions (69, 70). Additionally, impaired glucose metabolism was related with lower CBF and cognitive performance (12, 71-74). Insulin is an essential peptide hormone that regulates glucose metabolism in various organs and tissues such as skeletal muscle, liver and adipose tissue (75), but has also important physiological effects in the brain (76, 77) and on the vascular endothelium for vascular homeostasis (78). Decreased sensitivity or responsiveness to metabolic actions of insulin, such as the insulin-mediated uptake of glucose, is defined as insulin resistance (69). Impaired glucose metabolism is reversible, even at initial stages of β-cell dysfunction (79), but requires intensive healthy lifestyle interventions and frequently substantial weight loss (80). It is

therefore important to identify and treat people with impaired glucose metabolism at an early stage.

Many methods and indices are available for the estimation of glucose metabolism. The hyperinsulinemic euglycemic clamp is the gold standard to measure insulin sensitivity. However, it is time consuming and expensive (69, 70). Therefore, simplified approaches have been developed. A reliable method is the oral glucose tolerance test (OGTT), which involves a standardized amount of glucose dissolved in a drink, while plasma and insulin concentration are sampled during 120 min. Also, muscle and liver insulin resistance indexes (MISI and HIRI) can be derived from the OGTT, as interventions may affect insulin sensitivity tissue specific (81). For example, exercise is known to specifically improve muscle insulin sensitivity (82). The HIRI is calculated by the product of the total area under the curve (AUC) of plasma glucose and insulin concentrations during the first 30 min of the OGTT, while the rate of decay of plasma glucose concentrations from its peak value to its nadir is divided by the mean plasma insulin concentration to calculate the MISI (83). Exercise training was effective in improving post-load glucose concentrations, as measured with the OGTT (**Chapter 4**). Although insulin concentrations during the OGTT were not determined in this study, we can speculate that the MISI improved based on the increased steepness of the slope (**Figure 8.3**). In contrast, soy did not change glucose metabolism (**Chapter 5**), also not the MISI and HIRI. Alternatively, the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) can be calculated by using fasting plasma concentrations of insulin and glucose. The HOMA-IR is a robust clinical and epidemiological tool for the assessment of steady state whole body insulin-resistance, with a high correlation with the clamp method (84).

Due to technological advances, we were also able to assess glucose concentrations continuously (CGM), allowing for monitoring of glycemic control during daily live. Glucose is measured in the interstitial fluid with CGM, which causes a delay of approximately 15 min compared to glucose measured in plasma. To assess reliability of the CGM we compared the glucose concentrations measured with CGM and blood plasma during the OGTT of the exercise study (**Figure 8.3**). Although there is some slight variation in baseline values (**Figure 8.3.A**) and the CGM tends to overshoot glucose values, the pattern of response is similar. During both the exercise and soy study, CGM did not change. Meal composition, size and timing were however not standardized between participants and measurements, which induced large variation in the measurements. We tried to define one variable to describe the continuous measurement using the incremental area under the curve (iAUC). The area below baseline was excluded to increase susceptibility to changes, which was determined by the minimum value averaged over each day.

Brain insulin action

Insulin acts as an important signaling hormone in the brain that regulates eating behavior and homeostatic, reward-related, and higher cognitive brain functions, as reflected by multiple behavioral and metabolic effects (85). In contrast to the periphery, glucose can be utilized independently of insulin-mediated processes. It can enter the brain by diffusing across the blood-brain barrier and is absorbed by brain cells via a range of insulin-insensitive glucose transporters. The responsiveness of the brain to insulin gets disturbed during aging, and brain insulin resistance is a shared pathological feature of dementia, impaired glucose metabolism and obesity (76). Insulin-sensitivity in the brain can be assessed using various

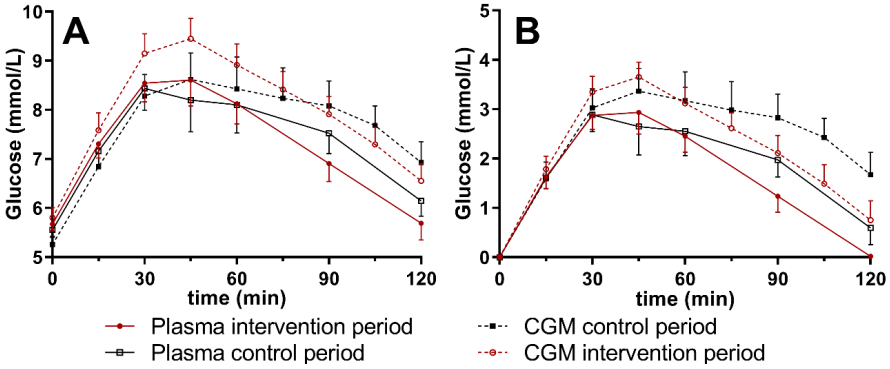

Figure 8.3. Absolute (**A**) and change from baseline (**B**) in glucose concentrations measured in venous plasma samples and continuous glucose measurement during the OGTT at the end of the control and intervention period of the exercise study.

methods, while insulin release can be stimulated endogenously via glucose administration. Exogenous insulin can be administered via an intravenous catheter or intranasally (76). An advantage of intranasally administered insulin is that it bypasses the blood-brain barrier, resulting in minimized systemic exposure compared with other administration protocols, disentangling peripheral from central insulin effects. Nasally administered insulin is transported by peptide transporters near the olfactory bulb into the cerebrospinal fluid (CSF), where they also reach brain cells (86).

In our study, brain insulin action was measured by the CBF response 30 min after administration of 160 U intranasal insulin. Insulin concentration in the CSF increased within 10 min after administration of 40 U insulin nasal spray (87); functional changes in brain activity occurred within a comparable time frame (88). However, measurement of the CBF response was optimal after 30 min (89). Nasal insulin administration dose-dependently modulated regional brain activity, with the strongest effect effects of 160 U (89). This dose, however, was accompanied by a transient increase in circulating insulin concentrations due to spillover into circulation (~0.1U), which was not related to the effects on the brain. Additionally, no major effects on blood glucose were observed, while the small rise in circulating insulin may induce effects in peripheral tissues (90). Hence, intranasal insulin-suppressed endogenous glucose production was observed between 180 and 360 min after administration of intranasal insulin (91), which stimulated glucose uptake (90). Abdominally obese men were included in the study, as they were expected to have a reduced brain insulin action. Participants were aged between 18 and 60 years. Older participants were excluded, because receptor density in the brain may be reduced in older individuals (92). Women were excluded as estrogen signaling may modulates the brain's sensitivity to the impact of insulin (76), which induces an extra source of variation. Several insulin sensitive regions have already been identified (e.g., the hypothalamus, fusiform gyrus, striatum, insular cortex, frontal cortex) that were confirmed by our study (**Chapter 7**). Additionally, we observed an increased CBF in response to insulin in clusters in the occipital lobe, which have not previously been reported. No placebo nasal spray was used in this study, because of insurmountable practical complexity. A placebo is necessary to ensure that responses can specifically be attributed to insulin.

Healthy lifestyle factors and vascular function

The results presented in this thesis show that healthy lifestyle factors, specifically aerobic exercise training and soy nuts, can beneficially affect cerebrovascular and peripheral vascular function, which may underlie the beneficial effects on cognitive performance. An overview of the main outcomes and the clinical relevance of these findings is presented in **Table 8.1** and presented schematically in **Figure 8.4**.

The case study presented in **Chapter 2** suggested that a healthy lifestyle may have beneficial effects for cognitive performance by improving CBF, which can be quantified accurately and reproducibly using MRI ASL and is associated with structural brain status. The systematic review of **Chapter 3** investigating the effect of physical exercise training showed increased regional, but not global, CBF in studies with MRI, while resting CBF variables measured using transcranial doppler ultrasound and NIRS did not change. Furthermore, aerobic exercise training improved CBF and cognitive performance in the executive function domain in sedentary older men (**Chapter 4**), while also endothelial function of a major elastic conduit artery and peripheral muscular artery improved (**Chapter 5**). Additionally, retinal arteriolar width and office blood pressure improved, which may reduce CVD risk. In older men and women, longer-term soy nut consumption also improved CBF. These effects may underlie the observed improvements in cognitive performance in

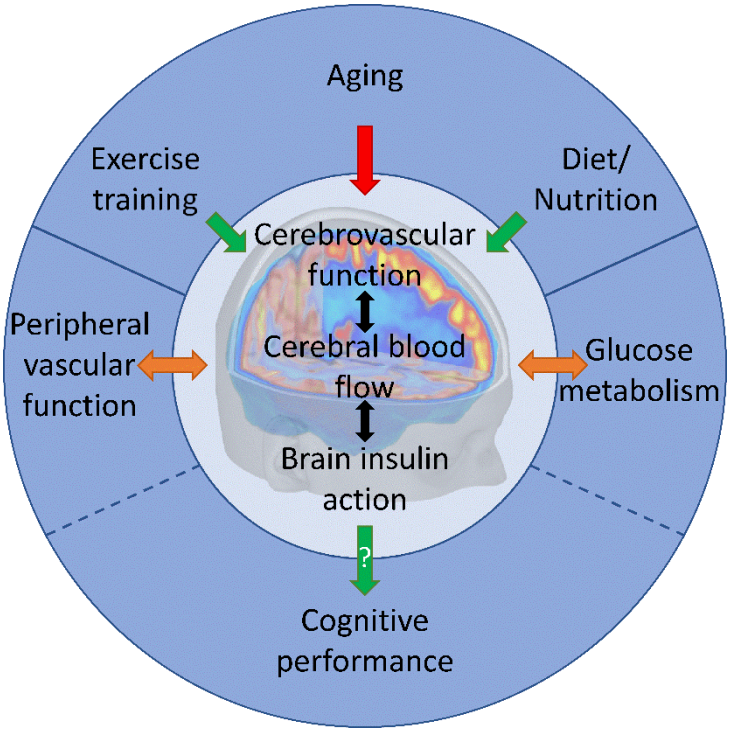

Figure 8.4. Overview of the central role of cerebral blood flow. Whereas aging decreases CBF, exercise training and diet/nutrition had a beneficial effect on cerebral blood flow, which may underlie the beneficial effects observed on cognitive performance. Although exercise training improved vascular function and post-load glucose metabolism, the relation with cerebral blood flow is still largely unknown.

the domain of psychomotor speed (**Chapter 6**). Finally, inorganic nitrate increased regional brain insulin action in regions relevant for eating behavior and homeostatic regions (**Chapter 7**).

Interventions should primarily target the population at risk for developing CVD and cognitive impairment. Recently, it was concluded that sedentary lifestyle and obesity negatively impacts CBF causing loss of brain volume and reducing its contribution to cognitive performance (93). We therefore included sedentary overweight and obese older men in the longer-term exercise study, which are expected to have a reduced CBF at baseline. In the soy study, older men and post-menopausal women were included to minimize hormonal fluctuations as an extra source of variability. The acute effects of a dietary supplement on brain insulin action included young and middle-aged abdominally obese men, as they are prone to brain insulin resistance and are at risk for developing CVD and cognitive impairment. The section below discusses the healthy lifestyle factors investigated in this thesis on the various non-invasive cerebrovascular and peripheral vascular function markers in an adult population.

Physical exercise training

Several types of physical exercise training were used in studies investigating its effects on CBF, as described in **Chapter 3**. Due to large heterogeneity between studies, we could not identify physical exercise protocols that were superior compared to others. We did observe concomitant improvements in aerobic fitness and regional CBF, suggesting that increasing aerobic fitness is an important determinant of CBF irrespective of the type of training. In the aerobic exercise study described in **Chapters 4 and 5**, the peak oxygen uptake consistently increased over 8 weeks, with a difference of 10% during maximal exercise testing at the end of the intervention period compared to the control period. The consistent increase in VO_2peak and Pmax of this trial emphasizes the effectiveness of the intervention, which may be attributed to a combination of several factors, including the duration, frequency and tightly-controlled supervised training sessions, the individually-based progressive training intensity, and the inclusion of sedentary individuals. A comparable increase in aerobic fitness was observed after 12 weeks of 30 min interval training, which correlated with the change in hippocampal CBF (94). In contrast, Chapman et al. only showed a change in VO_2peak at 6 weeks, which did not sustain after 12 weeks of aerobic exercise training, while CBF in the anterior cingulate increased (95). The size of the cluster in this study was only 696 mm^3 and one-sided compared to the bilateral change with a size of 1008 mm^3 in our study, while the absolute change in CBF could not be compared as Chapman et al. did not report the changes. Burdette et al. also observed an increased CBF in the hippocampus but used a proxy-measure (400 m walk speed) that did not significantly differ between groups. Also, the duration and intensity of the home-based training sessions were not controlled (22). The Train the Brain Consortium did not measure the effectiveness of the physical exercise training by means of an aerobic fitness outcome (96). Therefore, it cannot be assessed whether changes in CBF in these studies are truly due to exercise-induced changes in aerobic fitness.

Exercise effects on CBF may be mediated by increased circulating growth factors on arteriole or capillary density, as circulating growth factors VEGF, BDNF and IGF increased. However, it remains unclear whether exercise-induced angiogenesis is a reflection of

increased metabolic activity in the brain during exercise, or an effect of increases in circulating growth factors and hormones produced elsewhere, such as the muscles (97).

The aerobic exercise study described in **Chapter 5** was the first randomized controlled trial to assess CAR in response to a cold pressor test. The CAR increased with 2.23 pp to 4.01%, a value comparable to that of younger individuals aged 24 ± 3 years (34). The increase in CAR response may decrease cardiovascular risk and the risk for future cardiovascular events (36, 98). Further research should confirm these findings. Also, FMD increased, which is in line with a meta-analysis when a comparable training protocol was used (99). The aerobic fitness at baseline before starting the intervention did not affect the FMD response, but aerobic training with a high intensity and volume of at least 150 min per week had the largest effect on FMD (99). Additionally, the FMD response was dependent on the disease status, as it was more effective in diseased compared to asymptomatic healthy individuals, independent of age (99). The observed changes in vascular function of central and peripheral arteries supports evidence for beneficial on CVD risk in overweight and obese men. Local carotid stiffness increased in our study, while daily walking decreased arterial stiffness (100). Resistance exercise studies also observed increased local carotid stiffness (46). The change in local carotid stiffness may thus be dependent on the intensity of the exercise training (46). However, further research on the effects of training on local artery stiffness is warranted. $PWV_{c\text{-}f}$, did not change after eight weeks of aerobic exercise training, while a meta-analysis of 20 studies showed an improved $PWV_{c\text{-}f}$ (101). However, subgroup analyses revealed that improvements in $PWV_{c\text{-}f}$ were only observed when the training program exceeded 10 weeks (101). This suggests that longer training duration may be needed to reduce stiffness in large central conduit arteries, as measured with the $PWV_{c\text{-}f}$. Additionally, a higher effect-size was observed in the $PWV_{b\text{-}a}$ after exercise training (101), which may relate to increases in shear stress during aerobic exercise training that affect NO-producing muscular arteries in particular (101). Retinal microvascular calibers were also beneficially affected, as shown by an increased in CRAE, although CRVE did not change. These results agree with those of other studies (102, 103) and have been shown to decrease the risk to develop hypertension (57).

Exercise only improved post-load glucose concentrations in older men with below impaired fasting glucose concentrations. This may explain why only changes were observed after a challenge test. In populations with (pre-)diabetes, aerobic exercise training also improved fasting glucose concentrations (104). In contrast, Ferrara et al. observed only improved glucose disposal in apparently older men, whereas fasting glucose did not change in a meta-analysis of 105 intervention studies (105). Additionally, intervention-induced changes in glucose metabolism often coincided with weight loss, which may account for the observed effects (106). Beneficial effects on CBF in specific brain regions may be related to the improved glucose metabolism, which is supported by the relation between a reduced CBF in the frontal lobe in type 2 diabetic patients compared to healthy controls (73).

Cross-over studies are not common with physical exercise training, as there is a concern that physical activity levels during daily life remain higher when starting with the exercise protocol. Participants were asked to maintain their habitual physical activity level throughout the wash-out and no-exercise control period. The physical activity and sedentary time were measured with accelerometry, which did not differ between periods. Additionally, VO_2peak at baseline was similar between periods. This indicates that physical

activity returned to habitual levels during the wash-out period when participants started with the exercise period.

Although office blood pressure improved, no changes were observed in ambulatory blood pressure (ABP) at the end of the exercise period compared to the control period. Measurement of ABP was performed every 15 min for 24 hours, while activity was monitored continuously. These measurements were therefore aligned to investigate the influence of activity during the measurement of ABP. Measurements during the night were excluded to reduce skewness of data distribution due to long periods of low physical activity. The continuous metabolic equivalent of task scores (METs) were categorized into three classes, corresponding to a specific type of physical (in)activity: activity class 1 (1.0 – 1.4 METs; sedentary behavior), activity class 2 (1.4 - 1.8 METs; standing) and activity class 3 (1.8 – 5.3 METs; walking with a speed of 3-7 km/h) (107). Interestingly, a significant interaction between treatment and activity class ($P = 0.004$) was observed for SBP after performing linear mixed models. After Bonferroni correction, SBP decreased by 4 ± 1 mmHg ($P \leq 0.001$) for activity class 1, but not for higher activity classes. This indicates that the effect of the intervention on blood pressure becomes less with higher activity classes. Sympathetic activity during daily activities may thus affect ambulatory blood pressure (108), thereby reducing the intervention effects.

Soy supplementation

Diets higher in plant foods and lower in animal foods were associated with a lower risk of CVD morbidity and mortality in large community-based cohort of middle-aged adults (109). Additionally, plant-based diets have been associated with improvements across different cognitive domains (10, 110), although less extensively studied than the effects on CVD risk markers. Several reviews concluded that diet-induced improvements in cerebrovascular function contribute to the beneficial effects observed on cognitive performance (7, 111, 112). However, the effects of soy nuts on CBF, which is rich in phytoestrogens (isoflavones), *cis*-polyunsaturated fatty acids (*cis*-PUFAs) and high-quality plant proteins, which may all contribute to the effects on cognitive performance (113-116), have not been investigated before. The randomized, controlled, cross-over trial described in **Chapter 6** showed that longer-term soy nut consumption improved regional CBF and cognitive performance in the psychomotor speed domain. Glucose metabolism, a suggested mediator of the beneficial effect on CBF and cognitive performance, did not change. The specific substances that are also present in soy may improve CBF via various mechanisms (7, 117). Soy is distinct from other plant products due to its high concentration of isoflavones (i.e., genistein and daidzein). It has been suggested that people who have the ability to produce the daidzein metabolite equol by gut microbiota exhibit a more pronounced effect on cognitive performance (118), potentially via CBF (119), but this has not yet been investigated. Within the soy study only six subjects (24%) were equol-producers, which is in line with other studies in Western populations (120), but this is not sufficient to make a comparison between equol- and non-equol-producers. Supplementation of other flavonoids, such as anthocyanin in blueberry concentrate (121) and flavanol-rich cacao (122) also increased CBF. Although these flavonoids vary in structure and may have different mechanisms of action, they have been linked to antioxidant and anti-inflammatory effects (123) that all increase NO bioavailability thereby improving CBF (124). Effects on CBF of the most

abundant *cis*-PUFA (linoleic acid [C18:2, *n-6*]) in soy have never been studied. However, soy also provides some α-linolenic acid (ALA, C18:3, *n-3*), which can be converted in limited amounts into long-chain *n-3* PUFAs eicosapentaenoic (EPA; C20:5, *n-3*) and docosahexaenoic acid (DHA; C22:6, *n-3*). These *cis*-PUFAs supplemented as fish (125), krill or sardine oil (126) increased CBF during cognitive testing in related cerebral regions. Circulating DHA gets directly incorporated into human brain lipids, mainly membrane phospholipids (127), thereby possibly affecting CBF responses. In contrast, CBF may be affected by linoleic acid via conversion into relatively polar compounds, such as acid-derived oxylipins (128, 129). Finally, soy proteins acutely increased CBF after consumption of isolated soy proteins (8 g), which may be due to beneficial effects on neurotransmission (130) and NO metabolism (131). Neurotransmitter systems are involved in brain functions, such as learning and memory processes (132), while NO is an important intercellular messenger in cerebral and peripheral hemodynamics (133). In **Chapter 6**, we concluded that the function of the regions with increased CBF may be related to the psychomotor speed domain, suggesting a potential mechanism by which an increased intake of soy rich foods beneficially affects cognitive performance in older males and females.

Inorganic nitrate supplementation

Inorganic nitrate has beneficial effects on CVD (134) and increases NO bioavailability via bioconversion to nitrite. Increased bioavailability of NO is known for its beneficial effects on vascular function an glucose metabolism (135). Additionally, a high nitrate diet for 24-hours providing 773 mg (136) of inorganic nitrate and a single dose of beetroot juice providing 750 mg (137) and 342 mg (138) increased CBF. We proposed that inorganic nitrate may also improve (regional) brain insulin action (76), via improved regulation of CBF, which was investigated in **Chapter 7**. This is highly relevant because improving brain-insulin sensitivity may be important for the prevention of cognitive decline and the onset of dementia. The study described in **Chapter 7** showed that inorganic nitrate, a potent vasodilator, induced an increase in regional brain insulin action measured with the CBF response to intranasally administered insulin. Improving brain insulin action may have a beneficial effect on weight loss, as suggested a lifestyle intervention study, with a shift towards a more favorable body fat distribution (76, 139). Additionally, the weight regain after the intervention was less in participants with improved brain insulin action. Therefore, improved brain insulin action may on the long-term both support body mass reduction and weight maintenance. Improved brain insulin action has also been suggested to attenuate the development of obesity, peripheral insulin resistance and cognitive impairment (76).

Conclusion and future directions

The research described in this thesis focused on the effects of different healthy lifestyle factors on CBF, cognitive performance and glucose metabolism. Additionally, the effect of aerobic exercise training on a variety of vascular function markers was investigated. We provide evidence that increased physical activity levels and soy nuts which can be part of a healthy dietary pattern affect regional CBF, which may underlie the observed beneficial effects on cognitive performance. Improved endothelial function and retinal arteriolar width may be important mechanisms by which aerobic exercise training reduces age-related health problems, such as CVD and cognitive decline. Besides, inorganic nitrate

increased regional brain insulin action in regions of the default mode network and the striatum, which are important for metabolism and eating behavior. An overview of the main results of the studies presented in the present thesis and the potential clinical relevance of these findings is shown in **Table 8.1** and is presented schematically in **Figure 8.4.** The described and applied MRI ASL method with voxel-wise analysis was sensitive to detect regional CBF changes after healthy lifestyle interventions.

In **Chapter 2** we suggested a potential link between CBF, which serves as a sensitive physiological marker of brain vascular health, and structural brain status (140, 141). Development of structural brain changes is relatively slow and mostly without noticeable onset of symptoms linked to cognitive impairment. Cognitive impairment may remain asymptomatic until structural brain changes have affected a significant proportion of the brain (142). However, a causal relationship between CBF and structural brain changes remains unclear. Large longer-term trials should investigate the potential causal relationship between healthy lifestyle-induced, including exercise training and a healthy diet, changes in CBF and structural brain changes.

The effects found in the aerobic exercise trial (**Chapter 4**) were suggested that physical activity may attenuate age-related cognitive decline by improving CBF. Soy nuts also improved CBF and cognitive performance in the psychomotor speed domain (**Chapter 6**). Peripheral glucose metabolism only improved after exercise training, but not after the soy nuts intervention. Although impaired glucose metabolism may be related to cognitive impairment (12, 74), its contribution is still unclear. Large intervention studies should investigate the causal relationship between peripheral and brain insulin action (12, 88). Additionally, determinants of intervention effects such as health status, sex and age should be examined, because these characteristic may lead to different CBF patterns (143).

Aerobic exercise training also improved central and peripheral endothelial function and improved retinal arteriolar width (**Chapter 4**). Surprisingly, carotid arterial stiffness increased, while previous research showed that central arterial stiffness was lower in participants who are more physically active (100, 144). Daily walking also decreased (100), but resistance training protocols (46) increased local carotid stiffness. Although underlying mechanisms are unclear, it was hypothesized that adaptations in vascular tone or composition of the arterial wall may have contributed to this observation (46). Further research on the effects of different types of training on local artery stiffness is warranted to clarify these contradictory findings.

The population in the longer-term soy study (**Chapter 6**) consisted of older, apparently healthy adult. The effects of different study populations in relation to the effects of soy on cognitive performance needs to be investigated. Recent meta-analysis of sixteen randomized controlled trials (RCTs) concluded that soy isoflavones improved overall cognitive performance, which was mainly attributed to the improvement in memory (114). However, most research focused on postmenopausal women, because of the postulated association between reduced memory performance and decline in estrogen concentrations during menopause and after the cessation of the menstrual cycle, while isoflavones are known for their estrogen-like effects (145). The only study that also included older men and women also observed improvements in psychomotor speed (146). Therefore, more research on the different effects in men and women on cognitive performance after soy supplementation is warranted.

We showed in **Chapter 7** that brain insulin action is acutely modifiable by the nutritional component inorganic nitrate. Brain insulin resistance may be a novel target for the reduction in CVD and cognitive impairment (76, 88). However, it not known how brain insulin resistance arises, although it is conceivable that brain insulin resistance is a cause rather than a consequence of these age-related conditions. Whether brain insulin action can be modified by longer-term healthy-lifestyle intervention and the effects on CBF, cognitive performance and peripheral glucose metabolism is currently unclear. Longer-term improvements in brain insulin action may also support body weight management or loss (147).

Overall, components of a healthy lifestyle interventions had beneficial effect on regional CBF, while global CBF remained similar. This suggests that regulation of blood in the brain was improved as also beneficial effects on cognitive performance in related domains was observed. Thus, a healthy lifestyle may attenuate or prevent the decrease in cognitive performance by improved regulation of CBF. Additionally, inorganic nitrate acutely increased brain insulin action in regions associated with glucose metabolism and eating behavior. Whether inorganic nitrate also has longer-term effects on brain insulin action and is related to health benefits remains to be determined.

Table 8.1. Overview of the main results of the studies presented in this thesis. Including three intervention studies, investigating the effects of aerobic exercise training on cerebrovascular and peripheral vascular function, effects of longer-term soy nut intervention on cerebrovascular function, and the effects of acute inorganic nitrate supplementation on brain insulin action.

Ch	Study design	Exposure	Main results	Conclusion
2	**Case-study** sedentary men, age: 66 y, BMI: 28.9 kg/m^2		↓ Gray matter CBF ↑ brain atrophy, white matter signal abnormalities and iron accumulation	Potential relationship between lifestyle, CBF and structural brain status
3	**Systematic review**	Effects of physical exercise training on CBF	MRI: = global CBF, ↑ anterior cingulate gyrus, ↕ regions with medial temporal lobe Transcranial doppler ultrasound: = resting CBF, ↑ exercise or hypercapnia CBF, = hypocapnia CBF NIRS: = resting CBF, = exercise CBF, ↑ cognitive CBF	Exercise training may affect regional CBF measured with MRI, which may underlie the beneficial effects observed on cognitive performance.
4	**Aerobic exercise study**[1] Randomized, cross-over trial, 17 sedentary men, age: 67 ± 2 y, BMI: 30.3 ± 2.8 kg/m^2	Three 50-minute sessions each week at 70% maximal power) or no-exercise control separated 8 weeks intervention, 12 weeks wash-out	↑ bilateral subcallosal and anterior cingulate gyrus CBF, executive function ↓ temporal fusiform gyrus CBF, post-load glucose concentration	Regional CBF improved, while cognitive performance in a related domain of executive function also improved. Whether these effects are mediated by the post-load glucose metabolism warrants further study.
5			↑ FMD, CAR, CRAE, local carotid stiffness ↓ brachial DBP, MAP, central BP = $PWV_{c\text{-}f,}$ CAIxHR75, CRVE, AVR-ratio, brachial SBP, 24-h BP, CGM	The beneficial effects on endothelial function may be and important mechanism by which aerobic exercise training reduces age-related health problems, such as CVD and cognitive decline. The results of exercise training on local carotid stiffness warrants further study.
6	**Soy nut study**[1] Randomized, cross-over trial, 23 adults, age: 64 ± 3 y, BMI: 25.5 ± 2.7 kg/m^2	67 g soy nuts daily or no nuts on top of a healthy diet (Dutch nutritional guidelines, wheel of five) 16 weeks intervention, 8 weeks wash-out	↑ regional CBF in four clusters, psychomotor speed = glucose metabolism	The beneficial effects on CBF may underlie beneficial effects on cognitive performance in the psychomotor speed domain, suggesting a potential mechanism by which an increased intake of soy rich foods beneficially affects cognitive performance in older males and females.
7	**NO-BRAINS**[1] Randomized, double-blind, cross-over trial, 18 abdominally obese men, age: 48 ± 10 y, BMI: 33.4 ± 4.9 kg/m^2	10 mmol (625 mg) potassium nitrate or isomolar placebo drink Acute effects, 1 week wash-out	↑ brain insulin action after inorganic nitrate compared to placebo = nitrate effect on CBF or insulin on CBF during the placebo	Inorganic nitrate increases brain insulin action in regions related to impaired glucose metabolism in neurodegenerative diseases and regulation of food intake.

[1]Values are means ± SD. AVR-ratio: arteriolar-to-venular ratio; BMI: body mass index; CAIxHR75: central augmentation index adjusted for heart rate; CAR: carotid artery reactivity; CBF: cerebral blood flow; CGM: continuous glucose measurements; Ch: chapter; CRAE: central retinal arteriolar equivalent; CRVE: central retinal venular equivalent; DBP: diastolic blood pressure; FMD: brachial artery flow-mediated vasodilation; MAP: mean arterial pressure; $PWV_{c\text{-}f}$: carotid-to-femoral pulse wave velocity; SBP: systolic blood pressure; 24-h BP: 24-hour blood pressure

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Summary

A healthy lifestyle, consisting of sufficient physical activity and a healthy diet, is a cornerstone to prevent or attenuate the development of cognitive impairment and cardiovascular disease (CVD), which are important causes of morbidity and mortality worldwide. Lifestyle-induced improvements in cerebrovascular function may account for the beneficial effects on cognitive performance. Furthermore, improvements in peripheral vascular function have also been shown following a healthy lifestyle and these changes are related to a reduced CVD risk. Beneficial effects on vascular function may be mediated by improvements in glucose metabolism. Also, brain insulin action is thought to play an important role in the progression of these age-related conditions. However, most research only focused on cross-sectional relationships between physical fitness, cerebrovascular function and cognitive performance, and trials investigating longer-term effects of exercise training are limited. In addition, these trials collected region specific brain data or only focused on a specific part or aspect of the peripheral vasculature, thereby possibly missing important intervention effects. Except for physical fitness, plant-based diets also have beneficial effects on age-related conditions. However, less is known about the vascular health effects of specific foods that can be part of these healthy diets, such as soy. Finally, inorganic nitrate is known to have beneficial effects on peripheral vascular function. However, whether these effects also translate to the human brain is not fully understood, while effects on brain insulin action are unknown. This thesis therefore primarily focused on the effects of aerobic exercise training and longer-term soy nut consumption on cerebrovascular function, cognitive performance and glucose metabolism. Effects of aerobic exercise training on different regions of the peripheral vascular tree were investigated. Moreover, acute inorganic nitrate effects on regional insulin action in the brain were determined.

In **chapter 2**, it is discussed that cerebral blood flow (CBF) is a sensitive marker for cerebrovascular function and related to cognitive performance. Additionally, different methods to measure CBF are discussed, including the non-invasive gold standard method Magnetic Resonance Imaging (MRI) Arterial Spin Labeling (ASL), as used in the intervention studies described within this thesis. The current literature on the effects of exercise training interventions on CBF using MRI, ultrasound or near infrared spectroscopy (NIRS) is reviewed in **chapter 3**. It was shown that exercise training in MRI studies consistently increased CBF in the anterior cingulate cortex, but not in global CBF or within medial temporal lobe regions. Effects on resting CBF measured using transcranial doppler ultrasound and NIRS were variable, while middle cerebral artery blood flow velocity increased following exercise or hypercapnic stimuli. Interestingly, concomitant changes in physical fitness and regional CBF were observed, while a relation between training-induced effects on CBF and cognitive performance was evident.

The results of a randomized, controlled, cross-over trial investigating the effects of fully-supervised, progressive aerobic exercise training have been described in the next two chapters. In **chapter 4** the outcomes on CBF, cognitive performance and glucose metabolism are discussed, while **chapter 5** describes the effects on the peripheral vasculature and the more conventional cardiometabolic risk markers. Also, effects on relevant determinants of the peripheral vasculature during daily life were examined. Seventeen sedentary men, aged 67 ± 2 years and with a BMI of 30.3 ± 2.8 kg/m^2, were randomly allocated to start either with an eight-week aerobic exercise training or a no-

exercise control period for eight weeks, separated by a 12-week wash-out period. It was found that the peak oxygen consumption, as a measure for aerobic fitness, increased by 262 ± 236 mL ($P < 0.001$) following training as compared with no-exercise control. Further, CBF was increased by 27% bilaterally in the frontal lobe, specifically the subcallosal and anterior cingulate gyrus (cluster volume: 1008 mm3; $P < 0.05$), while CBF was reduced by 19% in the right medial temporal lobe and in particular the temporal fusiform gyrus (cluster volume: 408 mm3; $P < 0.05$). Mean post-load glucose concentrations determined using an OGTT decreased by 0.33 ± 0.63 mmol/L ($P = 0.049$). Furthermore, executive function improved as the latency of response was reduced by 5% ($P = 0.034$), but no changes were observed in memory or psychomotor speed. From this study, we concluded in **chapter 4** that regional CBF improved after aerobic exercise training in sedentary older men. Observed changes in CBF may underlie the exercise-induced beneficial effects on executive function, which could be partly mediated by improvements in glucose metabolism. As described in **chapter 5**, peripheral vascular function also improved following exercise training. In fact, vascular endothelial function improved, as carotid artery reactivity response to a cold pressor test increased by 2.23 ± 3.13 percent point (pp; $P = 0.012$) and brachial artery FMD increased by 2.99 ± 4.73 pp ($P = 0.019$). Local carotid artery stiffness increased, including stiffness index B_0 (Δ 1.1 ± 1.4; $P = 0.010$), while regional aortic stiffness did not change. Retinal arteriolar width improved by 3 ± 7 µm ($P = 0.041$), and office blood pressure decreased, but no changes were found in ambulatory blood pressure levels and metabolic risk markers, including continuous glucose concentrations. It was concluded that an improved peripheral vascular function can be an important mechanism by which aerobic exercise training reduces CVD risk in sedentary older men.

The effects of a longer-term study on the effects of soy nut consumption as part of a healthy diet on CBF, cognitive performance and glucose metabolism have been described in **chapter 6**. A single-blinded, randomized, controlled, cross-over trial was performed with a sixteen-week intervention and control period, separated by a wash-out period of eight weeks. In this study, a total of twenty-three men and women, aged 64 ± 3 years and with a BMI of 25.9 ± 2.7 kg/m^2, were allocated to start either with the soy nut intervention (67 g of soy nuts daily providing 25.5 g soy protein) or control period (no nuts). It was reported that serum isoflavone concentrations, which were assessed as a measure of compliance to the treatment, increased after the soy nut intervention (daidzein Δ 133 ± 113 ng/mL, $P < 0.001$; genistein Δ 456 ± 256 ng/mL, $P < 0.001$). Interestingly, regional increases in CBF were observed in four brain regions that are involved in object recognition, visual information processing and target reorientation. These brain clusters were located in the (1) left occipital and temporal lobe (Δ 36%, volume 11296 mm^3, $P < 0.001$), (2) bilateral occipital lobe (Δ 32%, volume 2632 mm^3, $P = 0.002$), (3) right occipital and parietal lobe (Δ 47%, volume 2280 mm^3, $P = 0.005$). The fourth cluster was located in the left frontal lobe (Δ 43%, volume 2120 mm^3, $P = 0.009$), and is part of the ventral network, which is involved in task-relevant stimuli. Movement time during the psychomotor speed test was beneficially reduced by 7% ($P = 0.005$), while no changes were observed in executive function or memory. From these results, we concluded in **chapter 6** that longer-term soy nut intervention may improve cerebrovascular function in older adults, which may underlie observed beneficial effects on cognitive performance in the psychomotor speed domain.

Finally, acute effects of inorganic nitrate on the action of insulin in the brain were investigated in a double-blind, randomized, controlled, cross-over trial involving eighteen healthy men with a median age of 50 (range 23 – 60 years) and mean waist-circumference of 118.5 ± 10.1 cm. The study consisted of two test days that were separated by at least one week. Participants received in random order a drink containing 10 mmol (625 mg) inorganic nitrate or an isomolar placebo drink with potassium chloride. Brain insulin action was assessed 120 min after the drinks by quantifying acute effects of insulin as nasal spray (160 U) on regional CBF. Brain insulin responsiveness increased in five brain clusters following the acute intake of inorganic nitrate. The two largest brain clusters were located in the right temporal lobe (Δ 7.0 ± 3.8mL/100g/min, volume 5296 mm^3, $P < 0.001$; and Δ 6.5 ± 4.3 mL/100g/min, volume 3592 mm^3, $P < 0.001$), while two other cortical clusters were part of the right frontal (Δ 9.0 ± 6.0 mL/100g/min, volume 1096 mm^3, $P = 0.007$) and the left parietal lobe (Δ 6.1 ± 4.3 mL/100g/min, volume 1024 mm^3, $P = 0.012$). One subcortical cluster was located in the striatum (Δ 5.9 ± 3.2 mL/100g/min, volume 1792 mm^3, $P < 0.001$). No significant differences were observed before administration of the spray between inorganic nitrate and placebo. Finally, glucose and insulin concentrations were measured at regular intervals throughout the test day, but no effects of inorganic nitrate were observed. We concluded that acute inorganic nitrate intake increases regional insulin action in brain regions that are involved in the regulation of various metabolic and cognitive processes, as well as in processes underlying food intake.

In conclusion, this dissertation provides further evidence that lifestyle factors, specifically aerobic exercise training and soy nuts, improve cerebrovascular function in adults. The observed changes in CBF following exercise training and longer-term consumption of soy nuts may underlie the beneficial effects on cognitive performance. In the studies described, the non-invasive perfusion method pseudo-continuous ASL MRI was used, which a good method to quantify acute and longer-term changes in regional CBF following lifestyle interventions. Exercise training also improved several markers in the peripheral vasculature, which may be related to a reduced CVD risk. Furthermore, exercise training improved post-load glucose uptake, whereas soy nuts did affect glucose metabolism. Finally, we showed for the first time that brain insulin action was increased by the acute intake of inorganic nitrate.

Samenvatting

Een gezonde leefstijl, waar voldoende bewegen en een gezond voedingspatroon deel van uitmaken, is de basis voor het voorkomen of vertragen van leeftijdsgerelateerde aandoeningen, zoals cognitieve stoornissen en hart- en vaatziekten. Met cognitieve stoornissen wordt bedoeld dat de hersenen minder goed in staat zijn om informatie te verwerken. Deze cognitieve stoornissen kunnen een voorloper zijn van het ontwikkelen van de ziekte van Alzheimer en andere vormen van dementie, hetgeen wereldwijd een erg belangrijke oorzaak van ziekte en sterfte is. Gunstige effecten van een gezonde leefstijl op de vaatfunctie in de hersenen zouden de positieve effecten op cognitieve prestaties kunnen verklaren. Bovendien kan de verlaging voor het risico op hart- en vaatziekten mogelijk verklaard worden door een verbetering van andere aspecten van de vaatfunctie in het lichaam. Verder wordt verondersteld dat gunstige effecten op de suikerhuishouding een rol spelen bij de verklaring van de positieve effecten van bewegen en een gezonde voeding op cognitieve stoornissen en hart- en vaatziekten. Onderzoek heeft inmiddels aangetoond dat fysieke fitheid, de vaatfunctie in de hersenen en cognitieve prestaties met elkaar geassocieerd zijn. Onderzoek naar de langetermijneffecten van bewegen is beperkt, terwijl vaak slechts in een beperkt deel van de hersenen de bloedtoevoer was gemeten. Ook waren de gebruikte onderzoeksmethoden vaak niet optimaal. Verder was het effect van bewegen op de vaatfunctie slechts in een beperkt deel van het lichaam onderzocht, waardoor een goed en volledig overzicht van mogelijke effecten ontbreekt. Ook is niet veel bekend over de effecten van plantaardige voedingsmiddelen. Wel is bekend dat nitraat, een stofje dat in rode bieten en bladgroente voorkomt, gunstige effecten heeft op de vaatfunctie in het lichaam en mogelijk ook op de suikerhuishouding. De invloed van nitraat op de gevoeligheid van de hersenen voor het hormoon insuline, hetgeen niet enkel een rol speelt bij de regulatie van de suikerspiegel maar ook van de voedselinname en cognitieve functies, is echter onbekend.

Het belangrijkste doel van dit proefschrift was om langetermijneffecten van duurtraining en een plantaardig voedingsproduct op de vaatfunctie in de hersenen te onderzoeken. Bovendien zijn effecten op cognitieve prestaties en suikerhuishouding onderzocht, evenals de effecten van duurtraining op andere aspecten van de vaatfunctie in het lichaam. Het acute effect van nitraat op de insulinegevoeligheid en vaatfunctie van de hersenen is tenslotte onderzocht in een laatste studie.

In **hoofdstuk 2** wordt de bloedtoevoer naar de hersenen besproken als een gevoelige marker voor de vaatfunctie in de hersenen. Veroudering gaat samen met een verlaging van de bloedtoevoer naar de hersenen. Daarnaast zijn afwijkende patronen in de bloedtoevoer in specifieke hersendelen gerelateerd aan cognitieve stoornissen en diabetes type 2. Bovendien worden verschillende methoden besproken om deze bloedtoevoer te meten. Onze focus lag op de non-invasieve gouden standaardmethode, genaamd “arterial spin labeling (ASL)”, gemeten met magnetische resonantie beeldvorming (MRI). In dit hoofdstuk is een casus besproken van een persoon met een verlaagde bloedtoevoer naar de hersenen. Dit heeft mogelijk geleid tot ongunstige structurele veranderingen in de hersenen, hetgeen het risico op cognitieve achteruitgang vergroot.

Vervolgens is in **hoofdstuk 3** de huidige literatuur over de effecten van fysieke training op de bloedtoevoer naar de hersenen besproken. De bloedtoevoer kan met drie verschillende

methoden worden gemeten: MRI, transcraniële doppler-echografie en nabij-infraroodspectroscopie (NIRS). De conclusie van dit overzicht was dat training een effect heeft op de bloedtoevoer naar bepaalde gebieden van de hersenen. Deze zogenaamde regionale effecten kunnen alleen met MRI worden gemeten. Effecten op de bloedtoevoer naar de hersenen in rust gemeten met transcraniële doppler-echografie en NIRS waren echter variabel. De bloedtoevoer in een belangrijke slagader van de hersenen was echter verhoogd na een stimulus. Dit is een indicatie dat na training de regulatie van de bloedtoevoer was verbeterd, waardoor de aanvoer van zuurstof en voedingsstoffen kan toenemen. De fysieke fitheid en regionale bloedtoevoer naar de hersenen nam in bijna alle studies toe. Bovendien was er een duidelijke relatie tussen de effecten van fysieke training op de bloedtoevoer naar de hersenen en de cognitieve prestaties.

In de volgende twee hoofdstukken worden de resultaten van een gerandomiseerde cross-over studie beschreven, die de effecten van een volledig begeleide progressieve duurtraining heeft onderzocht. In **hoofdstuk 4** zijn de uitkomsten op de bloedtoevoer naar de hersenen, cognitieve prestaties en suikerhuishouding besproken. In **hoofdstuk 5** zijn de uitkomsten op andere aspecten van de vaatfunctie in het lichaam besproken, zoals endotheelfunctie, lokale en regionale vaatstijfheid, en structuur van de kleine bloedvaten in het oog. Voor deze studie startten zeventien lichamelijk inactieve mannen met een leeftijd van 67 ± 2 jaar en een BMI van 30,3 ± 2,8 kg/m^2 in willekeurige volgorde met duurtraining of een controleperiode (zonder training) gedurende acht weken. Deze twee perioden werden gescheiden door een periode van twaalf weken. De maximale zuurstofopname tijdens een inspanningstest was na de trainingsperiode verhoogd in vergelijking met de controleperiode. De maximale zuurstofopname is een maat voor de fysieke fitheid. De bloedtoevoer naar de hersenen verbeterde in regio's die ook een rol spelen tijdens het uitvoeren van executieve functies die ook verbeterden. Er werden echter geen veranderingen in geheugenprestaties en de psychomotorische snelheid gevonden. Daarnaast werden suikers sneller uit de bloedbaan opgenomen. Uit deze studie blijkt dan ook dat de regionale bloedtoevoer naar de hersenen verbetert na duurtraining in inactieve oudere mannen. Deze veranderingen in de bloedtoevoer naar de verschillende regio's in de hersenen hebben mogelijk een gunstig effect of cognitieve prestaties in het executieve functie domein. In hoeverre de verbeteringen in de suikerhuishouding hierbij een rol spelen is onduidelijk. Andere aspecten van de vaatfunctie in het lichaam, met name de endotheelfunctie, verbeterden ook aangezien de diameter van de halsslagader toenam, wanneer een pijntest werd uitgevoerd door de hand in ijskoud water te houden. De diameter van de armslagader nam ook toe bij een test die de bloedstroom naar de hand stimuleert. De plaatselijke stijfheid van de halsslagader nam toe, maar er werd geen verandering gevonden in de stijfheid van de centrale lichaamsslagader, gemeten tussen de hals- en liesslagader. Bovendien nam de diameter van een kleine slagader in het oog toe en was de bloeddruk verlaagd. Deze resultaten tonen aan dat een verbeterde vaatfunctie een belangrijk mechanisme is waarlangs duurtraining het risico op hart- en vaatziekten vermindert.

De resultaten van een gerandomiseerde cross-over studie naar de langetermijneffecten van sojanoten op de bloedtoevoer naar de hersenen en suikerhuishouding is beschreven in

hoofdstuk 6. Gedurende 16 weken moesten drieëntwintig volwassenen met een leeftijd van 64 ± 3 jaar en een BMI van 25.9 ± 2.7 kg/m^2 een voedingspatroon volgen, gebaseerd op de Nederlandse richtlijnen Goede Voeding 2015. Dit voedingspatroon werd, in willekeurige volgorde, al dan niet aangevuld met dagelijks 67 g sojanoten. De interventie- en controleperiode waren gescheiden door een periode van twaalf weken. Sojanoten zijn rijk in isoflavonen en deze werden in het bloed teruggevonden, hetgeen betekende dat de deelnemers zich goed aan de voorgeschreven studierichtlijnen hadden gehouden. De bloedtoevoer naar de hersenen verbeterde in vier regio's, die betrokken zijn bij het herkennen van - en oriëntatie naar - een doel. Bovendien was de cognitieve prestatie verbeterd hetgeen bleek uit een psychomotorische test, waarbij de bewegingstijd naar een doel verbeterde. Executieve functie en geheugen veranderden niet. Ook de suikerhuishouding bleef ongewijzigd. Op basis van deze resultaten concludeerden we dat dagelijkse consumptie van sojanoten de vaatfunctie in de hersenen kan verbeteren in ouderen. Deze effecten zorgen mogelijk voor de verbetering in de cognitieve prestatie.

De acute effecten van nitraat op de insulinegevoeligheid van de hersenen worden beschreven in **hoofdstuk 7.** Mannen met een leeftijd van 18 tot 60 jaar en een gemiddelde buikomtrek van 118,5 ± 10,1 cm dronken in willekeurige volgorde een drankje met 625 mg nitraat of een controledrankje op twee testdagen. Deze testdagen werden gescheiden door een periode van minimaal één week. De insulinegevoeligheid van de hersenen werd bepaald door het kwantificeren van veranderingen in de regionale bloedtoevoer naar de hersenen na het toedienen van insuline neusspray. Acute nitraatinname verhoogde de gevoeligheid van de hersenen voor het hormoon insuline in vijf hersenregio's. Deze regio's zijn betrokken bij de regulatie van verschillende metabole en cognitieve processen in de hersenen, en hebben een invloed op de voedselinname. Er werden echter geen effecten gevonden op de bloedtoevoer voordat de spray werd toegediend. Daarnaast werden insuline- en glucoseconcentraties gemeten in het bloed, maar deze werden niet beïnvloed door nitraat. Deze resultaten tonen aan dat nitraat acuut de regionale insulinegevoeligheid van de hersenen verhoogd in mannen met abdominale obesitas.

Samenvattend kan gesteld worden, dat de gebruikte meetmethode om de bloedtoevoer naar de hersenen te meten (MRI ASL) gevoelig is om regionale verschillen aan te tonen na leefstijlinterventies. De studies beschreven in dit proefschrift tonen dan ook aan dat onderdelen van een gezonde leefstijl, namelijk duurtraining en voedingsfactoren zoals soja en nitraat, een rol spelen in het verbeteren van de vaatfunctie in de hersenen. De gevonden veranderingen in de bloedtoevoer naar de hersenen dragen mogelijk bij aan gunstige effecten op cognitieve prestaties. Duurtraining heeft bovendien ook een gunstig effect op andere markers voor de vaatfunctie in het lichaam, hetgeen samengaat met een verlaging op het risico van hart- en vaatziekten. Duurtraining had daarnaast een gunstig effect op de suikerhuishouding, terwijl dit effect niet werk teruggevonden voor soja. Daarnaast hebben we voor het eerst aangetoond dat de insulinegevoeligheid van de hersenen beïnvloed kan worden door een acute inname van nitraat.

Impact

The findings of this dissertation provide evidence that improved vascular function in the brain and in the periphery are important mechanisms by which exercise training and soy nuts reduce the risk for cardiovascular disease (CVD) and cognitive impairment. Additionally, inorganic nitrate may acutely increase brain insulin action, which may be important as brain insulin resistance is considered a characteristic of both dementia and type 2 diabetes mellitus (T2D). Our findings are important as the worlds' population, the proportion of older people (1), and the incidence of CVD (2), cognitive impairment (3, 4) and (T2D) are estimated to further increase the coming years. Therefore, it is crucial to develop strategies to lower the risk for developing age-related non-communicable diseases (5). The potential scientific, societal, environmental and economic relevance, and the implications for the translation into practice of the findings described in this thesis will be discussed in the following paragraphs.

Scientific relevance

Exercise training and soy nuts improved regional cerebral blood flow (CBF), which may underlie the observed beneficial effects on cognitive performance. Therefore, further evidence was provided that CBF as measured with arterial spin labeling (ASL) magnetic resonance imaging (MRI) is a sensitive non-invasive marker, which can be used to assess lifestyle-induced changes in cerebrovascular function. Also, exercise training improved endothelial function in a major elastic conduit (i.e., carotid) and peripheral muscular (i.e., brachial) artery, thereby lowering CVD risk (6-8). Due to the proven sensitivity of these markers, they may be used in the future for identification of people at risk of disease and, for example, to track the progression of diseases. Moreover, inorganic nitrate acutely increased brain insulin action, and this finding should now be further investigated in future longer-term studies.

Societal relevance

An unhealthy lifestyle, consisting of a sedentary lifestyle and an unhealthy diet, is a major cause of CVD and cognitive impairment (9). CVD is the leading cause of death worldwide accounting for 17.9 million deaths in 2016 (2), while an impaired cognitive performance is the fastest growing condition reaching 82 million people suffering from this condition globally in 2030 (3, 4). Worldwide, on average 25% of the adult population does not meet the global recommendations for physical activity set by the World Health Organization (10). Depending on the economic development of a country, this can be as high as 70%, due to changing patterns of transportation, increased use of technology and urbanization (10). Approximately 5.3 million deaths every year are attributable to physical inactivity (11), which doubles the risk of CVD, type 2 diabetes, and obesity (12), and is also associated with decreased cognitive performance (13). An unhealthy diet is another leading cause of disability and mortality. Eleven million disability-adjusted life-years could be attributed to dietary risk factors in 2017 (14). Improvement of diet could potentially prevent one out of five deaths globally, which is more than any other risk globally. Specifically, consumption of plant-based diets has been associated with improvements in cognitive performance across

different cognitive domains (3, 15). This highlights the importance of a healthy diet in preventing or attenuating the development of these age-related conditions.

The observed effects of exercise training and soy nuts on top of a recommended diet contribute to the reduced risk of CVD and increased cognitive performance. Results from prospective epidemiological studies suggested that the observed exercise-induced improvement in brachial endothelial function reduces CVD risk by about 24% (23). Besides, vasodilation of the carotid artery increased after exercise training to a level previously observed in healthy young individuals. In addition, the beneficial effects of exercise training and soy nuts on regional CBF and cognitive performance may therefore have a great societal impact. Inorganic nitrate acutely increased brain insulin action in regions that were shown to be impaired in populations with neurodegenerative diseases and T2D, and were related to eating behavior (16-18).

Human aging is another important risk factor for the development of the non-communicable conditions mentioned above. The population aged over 65 years will almost double by the year 2050 accounting for 1.5 billion people (1). Physical activity and a healthy diet are effective modifiable lifestyle factors that can be implemented at any age to reduce the duration and exposure to risk factors for age-related conditions, thereby attenuating or preventing those disease outcomes (19, 20). Therefore, it is important to understand how these lifestyle factors affect healthy aging. In this thesis we showed that not only cerebrovascular and peripheral vascular function improved, but also perceivable benefits, including cognitive performance and physical fitness. Moreover, reducing the risk for cognitive impairment also beneficially impacts the psychosocial consequences of this condition for both the individuals and their relatives.

Economic relevance

The total numbers of deaths due to CVD increased with 14.5% to 17.6 million people globally between 2006 and 2016 (21). The CVD-related economic costs were estimated to be 169 billion dollar translating to 230 dollar per person in 2004, of which 68% were direct costs (e.g., hospitalization) and the remaining indirect costs were mainly due to losses of productivity and short- or long-term disability (22). Exercise training may reduce these costs by decreasing the risk for CVD via its effects on peripheral vascular function. Additionally, reduced risk for cognitive impairment may be realized by exercise and soy nuts via beneficial effects on cerebrovascular function. The rapidly growing incidence of cognitive impairment is also a global public health problem. Around 50 million people suffer from cognitive impairment worldwide, which is projected to reach 82 million in 2030 and 152 million in 2050 (3). Not only governments are faced with increased costs due to cognitive impairment, but also whole communities, families and individuals are affected. The global societal cost of cognitive impairment was estimated to be 818 billion dollar (3). Stimulating a healthy lifestyle, consisting of increased physical activity levels and a healthy diet, is a cost-effective intervention and prevention strategy. Costs for health care systems attributable to physical inactivity were estimated to be 54 billion dollar worldwide (23), which already yielded cost savings when a reduction of inactivity rates by about 20% was realized (24). Besides, it was

estimated that 45% of the cost associated with cardiometabolic diseases is related to an unhealthy diet, accounting for more than 50 billion dollar in the United States (25). Approximately 1.93 million CVD events could be prevented and 39.7 billion dollars in healthcare costs would be saved in the United States, if a 30% subsidy would be provided on healthful foods, including fruit and vegetables and plant-based foods (26). This clearly highlights the economic relevance of a healthy lifestyle by lowering the health care costs of non-communicable diseases in the aging population.

Target groups

The two longer-term intervention studies were performed in older adults aged 60 to 70 years. The study investigating the effect of aerobic exercise training included sedentary overweight and obese men, while normal-to-overweight men and women participated in the soy study. These populations were expected to have a reduced cerebrovascular and peripheral vascular function at baseline, thereby allowing for improvement by lifestyle interventions. Multimodal interventions in aging overweight and obese participants may effectively attenuate the societal, economic, and psychosocial consequences of the age-related conditions. Whether the observed effects of exercise training also translate to a broader population consisting of young adults and CVD patients requires further study. The study investigating the acute effects of inorganic nitrate on brain insulin action focused on apparently healthy men that were aged 18 – 60 years and may benefit most from lifestyle interventions. However, this research did not include women to eliminate possible gender differences, which obviously reduces the generalizability of the outcomes. Future research should now also investigate whether the findings also translate to populations with subjective cognitive decline and reduce the progression to mild cognitive impairment and eventually dementia.

Translation into practice

Intervention and prevention trials in humans are essential for the development of knowledge about processes that lead to health problems, and how these problems can be prevented. The translational research performed in this thesis showed that aerobic exercise training can already have beneficial effects on vascular function, cognitive performance, and glucose metabolism after eight weeks in older men. The compliance to the intervention was excellent, although feasibility over a prolonged period with a larger population may be more challenging. However, other physical activity protocols may also be beneficial, which should be confirmed in future studies. Additionally, soy nuts on top of a healthy diet showed beneficial effects on cerebrovascular function and cognitive performance, which emphasizes the benefits of shifting to a more plant-based diet. The soy nuts were well tolerated, while body weight remained stable and no (serious) adverse events were observed. Participants were requested to consume soy nuts. However, similar beneficial effects may also be observed with other soy products which requires further study. Finally, inorganic nitrate acutely increased brain insulin action. These results may also apply to whole foods rich in nitrate, such as beetroot and leafy green vegetables (16), but this needs

to be confirmed by follow-up research. Additionally, improvements in perceivable benefits (e.g., physical fitness and cognitive performance) may also assist in adherence to the intervention in practice. Finally, the outcomes of this study may be relevant for the development of dietary and physical activity recommendations that will aid in the reduction in prevalence of CVD and cognitive impairment. The observed results may also be interesting for the food industry to develop, for example, healthy evidence-based food formulations and concepts.

The findings described in one of the chapters are published in Siemens FLASH, which is primarily read by clinicians. The first study was already published open-access, to increase visibility and reuse of knowledge. The other studies described in this dissertation will also be published in peer-reviewed scientific journals and have already been presented at scientific conferences to increase awareness and share knowledge, which functions as a foundation for future research. The findings may also be used to create more awareness for, and to promote, a healthy lifestyle.

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Curriculum Vitae

Jordi Peter Dick Kleinloog was born on March 11th, 1994 in Eindhoven, the Netherlands. He completed secondary school at the van Maerlant Lyceum, Eindhoven in 2013. From 2013 till 2016, he studied Biomedical Sciences, specialization Human Movement Sciences at Maastricht University for which he graduated. At the free university of Amsterdam, he completed the minor Sports- and Performance Psychology. His bachelor internship was spent at the department of Human Biology and Movement Sciences at Maastricht University, which was supervised by Dr. Guy Plasqui. He was responsible for a recently published study that assessed the validity and reproducibility of maximal exercise testing in a respiration chamber. Thereafter, he continued with his master Human Movement Sciences, for which he graduated in 2017. His master internship was conducted at the Maxima Medical Centre in Veldhoven. Under supervision of medical doctor sports medicine Dr. Goof Schep, he investigated pedal power measurement as diagnostic tool for functional vascular problems, which he concluded with a published article. Since the end of 2017, he worked on his PhD project "Cerebrovascular and peripheral vascular function in adults: Effects of exercise training, soy nuts and inorganic nitrate" at the department of Nutrition and Movement Sciences at Maastricht University under the supervision of Prof. dr. ir. Ronald P. Mensink and Dr. Peter J. Joris. This project was part of a project by the Top Institute for Food and Nutrition (TIFN) and was funded by the Dutch Research Council (NWO). During his PhD project, he investigated the effects of a healthy lifestyle, consisting of physical activity and dietary interventions, on cerebrovascular and peripheral vascular function. His main focus was on the application of a novel non-invasive technique to measure cerebrovascular function, which is called arterial spin labeling (ASL) magnetic resonance imaging (MRI). He developed the acquisition and analysis protocols, and applied these brain scans within human intervention studies performed within the Physiology of Human Nutrition (PHuN) Research Group. Some of the study results are already published in international, peer-reviewed journals. As part of his PhD, he was also involved in teaching and supervising students during their thesis.

List of publications

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Kleinloog JPD, Mensink RP, Roodt op't J, Thijssen DHJ, Hesselink MKC, Joris PJ. Aerobic exercise training improves not only brachial artery flow-mediated vasodilatation, but also carotid artery reactivity: A randomized controlled, cross-over trial in older men.

Manuscripts in preparation

Kleinloog JPD, Mensink RP, Smeets ETHC, Dimo Ivanov, Joris PJ. Acute inorganic nitrate intake increases regional insulin action in the brain: Results of a double-blind, randomized, controlled cross-over trial with abdominally obese men

Smeets ETHC, Mensink RP, **Kleinloog JPD**, Joris PJ. Acute effects of inorganic nitrate on brachial and femoral flow-mediated vasodilation, and on carotid artery reactivity: Results of a randomized double-blinded, placebo-controlled trial with abdominally obese men