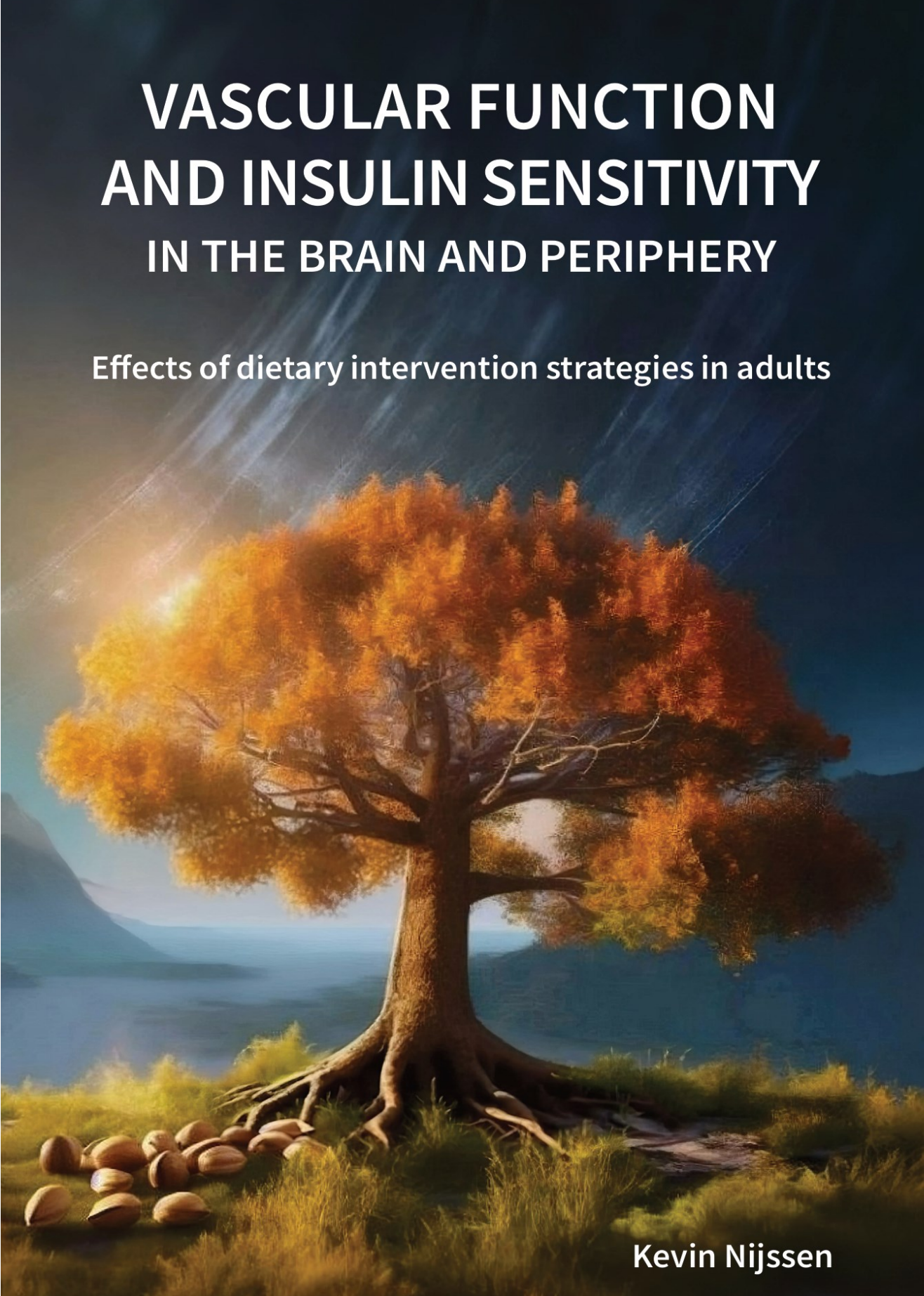
Vascular function and insulin sensitivity in the brain and periphery

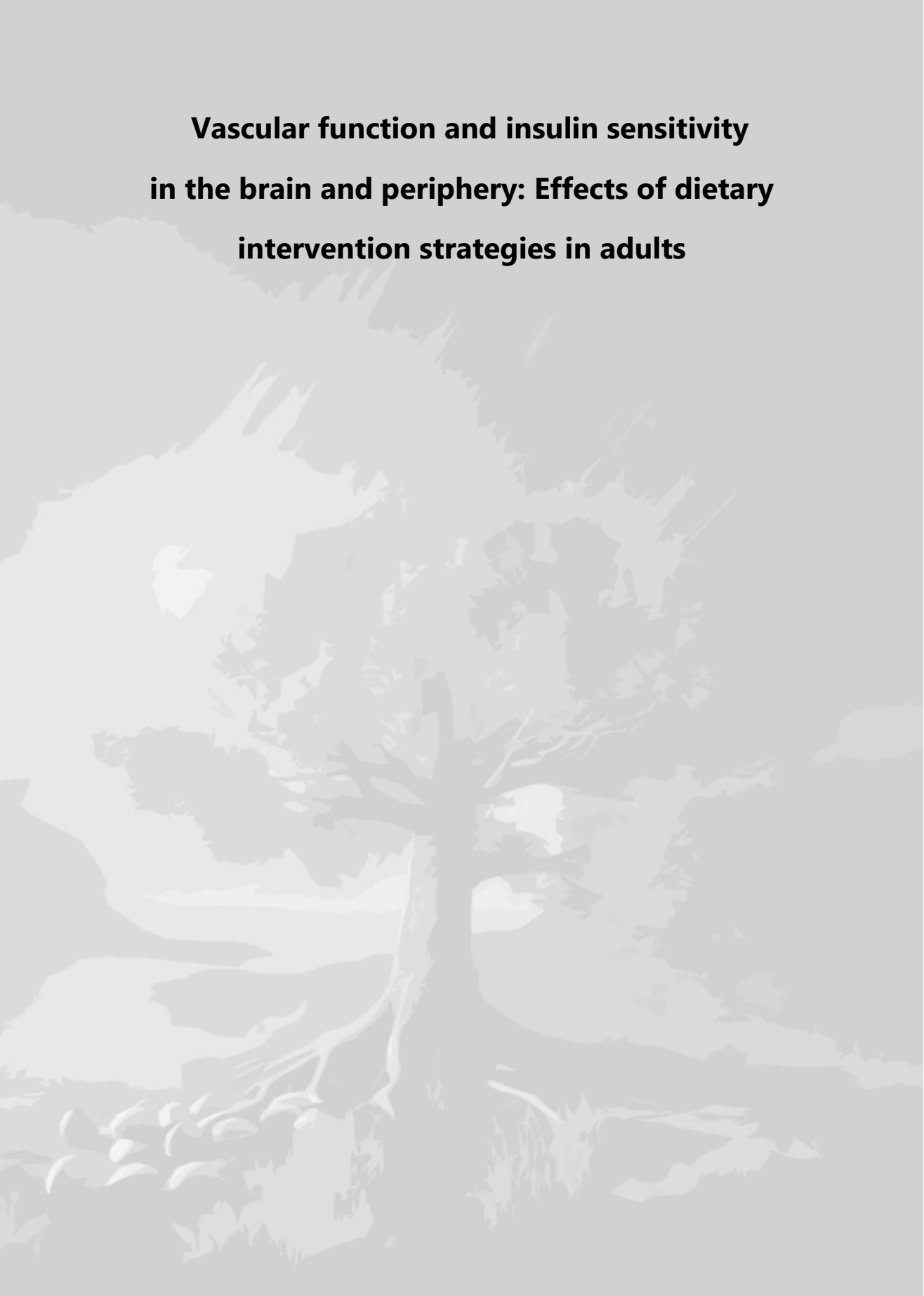
VASCULAR FUNCTION AND INSULIN SENSITIVITY IN THE BRAIN AND PERIPHERY

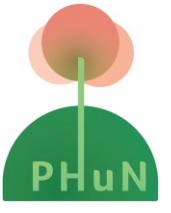
Effects of dietary intervention strategies in adults



Kevin Nijssen

Vascular function and insulin sensitivity in the brain and periphery: Effects of dietary intervention strategies in adults





This research described in this dissertation was performed within the Physiology of Human Nutrition (PHuN) research group at the Department of Nutrition and Movement Sciences, which is embedded within NUTRIM Research Institute of Nutrition and Translational Research in Metabolism. The research was partly funded by a grant obtained from the International Nut and Dried Fruit Council (INC), and the study in Chapter 5 was financially supported by Newtricious R&D BV.

Cover: Kevin M.R. Nijssen
Layout: Kevin M.R. Nijssen
Printed by: Gildeprint – The Netherlands
ISBN: 978-94-6496-011-2

Vascular function and insulin sensitivity in the brain and periphery: Effects of dietary intervention strategies in adults

DISSERTATION

to obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus, Prof. dr. Rianne M. Letschert
in accordance with the decision of the Boards of Deans,
to be defended in public on Thursday 25 January 2024, at 13:00 hours

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CHAPTER 1

General introduction

THE AGING POPULATION

The number of adults aged 60 years and older is expected to double from 1.2 billion in 2022 to 2.1 billion by 2050, comprising 22% of the world's population [1]. This demographic shift will have significant socio-economic implications and is expected to increase the prevalence of age-related non-communicable disorders, such as cardiovascular disease (CVD), type 2 diabetes (T2D), and dementia [2]. CVDs, including cerebrovascular and coronary heart disease, remain the leading causes of mortality, accounting for about 19 million deaths in 2020 [3]. Atherosclerosis is the primary underlying cause for CVD development, characterized by lipids and cholesterol deposition in arterial walls, leading to plaque formation that narrows and stiffens blood vessels. Modifiable risk factors contribute to approximately 90% of CVD risk, including traditional risk factors like high blood pressure (BP) or serum cholesterol concentrations [4, 5]. Furthermore, the global prevalence of T2D was 537 million adults in 2021 that will rise to 783 million by 2045 [6]. T2D is characterized by progressive pancreatic β-cell failure and impaired insulin secretion. This leads to impaired glucose homeostasis and insulin resistance, which refers to the reduced ability of insulin to act on insulin sensitive organs, including the skeletal muscle, liver, and adipose tissue [7, 8].

Studies have consistently shown that these age-related disorders are associated with cognitive decline [9, 10]. Cognitive processes encompass a variety of domains such as attention and psychomotor speed, memory, and executive function, which are essential for decision-making, problem-solving, and daily functioning [11]. Cognitive decline is evident at different stages, with some decline observed in middle age for processing speed and working memory, and acceleration occurring in adults older than 60 years for memory and executive function [12]. Mild cognitive impairment (MCI) is a syndrome characterized by measurable cognitive impairments and subtle functional impairments, while dementia is a progressive form of decline that affects multiple cognitive domains and interferes with being able to perform daily activities. The prevalence of dementia is also increasing globally with an estimated 50 million patients in 2020 that is expected to triple by 2050 [13]. Lifestyle interventions, including dietary modifications, have been shown to reduce the prevalence of age-related non-communicable disorders by as much as 70% [14]. Diet also plays a crucial role in the management of traditional CVD markers and insulin resistance, particularly in earlier stages like obesity or prediabetes [8], but also in adults with metabolic syndrome that present a cluster of CVD and T2D risk markers (e.g., central obesity, dyslipidemia, hypertension, and insulin resistance) [15]. Moreover, it is important to note that cognitive decline can be also be delayed by dietary interventions that promote healthy cognitive aging.

VASCULAR HEALTH

Peripheral vascular function

Changes in traditional CVD risk markers, such as BP or serum total cholesterol concentrations, may not fully explain the mechanisms underlying CVD risk reduction with lifestyle interventions [16]. In contrast, non-invasive vascular markers have been emerged to address key mechanisms in the pathophysiology of CVD, such as vascular endothelial function, arterial stiffness, and retinal vascular structure [17]. While many studies assessed vascular function in specific regions or aspects of the vasculature, a comprehensive approach that includes multiple markers is necessary to fully understand the impact of lifestyle across the vascular tree.

Endothelial dysfunction plays an important role in CVD pathogenesis, and refers to the impaired ability of the endothelium to maintain vascular tone and blood flow by regulating the production of vasoactive molecules such as nitric oxide (NO) [18]. A variety of non-invasive methods have been developed to assess endothelial function, including flow-mediated dilation (FMD) and carotid artery reactivity (CAR). FMD is a well-established non-invasive measure of NO-dependent vasodilation of muscular arteries, such as the brachial artery, in response to increased blood flow after temporary occlusion of the artery [19]. CAR assesses the ability of the endothelium to modulate vascular tone by measuring the change in carotid artery diameter in response to cold stress, which triggers the sympathetic nervous system and causes vasoconstriction of the carotid artery. This causes an increase in BP that results in shear stress on the endothelium leading to vasodilation and an increase in blood flow to compensate for the vasoconstriction [20]. Both FMD and CAR have been used as prognostic markers for CVD. For example, each 1% increase in FMD has been associated with a 13% reduction in risk of cardiovascular events [21]. Arterial stiffness refers to the elasticity of the arterial walls and is important for maintaining BP and reducing CVD risk. Two non-invasive methods commonly used to assess arterial stiffness are pulse wave velocity (PWV) and carotid stiffness. PWV measures the speed at which pressure waves travel along the arteries and requires a tonometer to be placed at two different arterial sites [22]. In addition, local artery stiffness is typically measured by ultrasound of the carotid artery and quantified as the carotid β_0-stiffness index [23]. Both PWV and carotid artery stiffness are strong independent predictors of cardiovascular events [24]. Furthermore, retinal vascular calibers refer to the diameter of the arterioles and venules, which can be assessed non-invasively using fundus photography. The retinal microvasculature shares anatomical and physiological similarities with the microvasculature in other organs, including the heart and brain. Therefore, abnormalities in retinal vascular caliber have been associated with increased risk of CVD and age-related cognitive decline [25].

Brain vascular function

The human brain requires a constant blood flow through cerebral arteries and veins to ensure delivery of adequate oxygen and essential nutrients to the brain, but also to remove carbon dioxide and other metabolic products. Regional cerebral blood flow (CBF) values are tightly regulated to meet metabolic demands. However, individual variation exists due to factors such as sex and age, and there are no established cutoff values for defining abnormal CBF. Nonetheless, in adults aged 60 years or older, CBF tends to decline with a decrease of approximately 0.5-1.0% per year, particularly in frontal, temporal, and parietal lobes, and subcortical regions [26, 27]. Declines in global and regional CBF, mainly in cingulate, precuneus, parietal lobes and inferior frontal regions are observed in dementia patients that are related to cognitive performance [26]. Moreover, the presence of peripheral vascular risk factors may contribute to the age-related reduction in CBF [28].

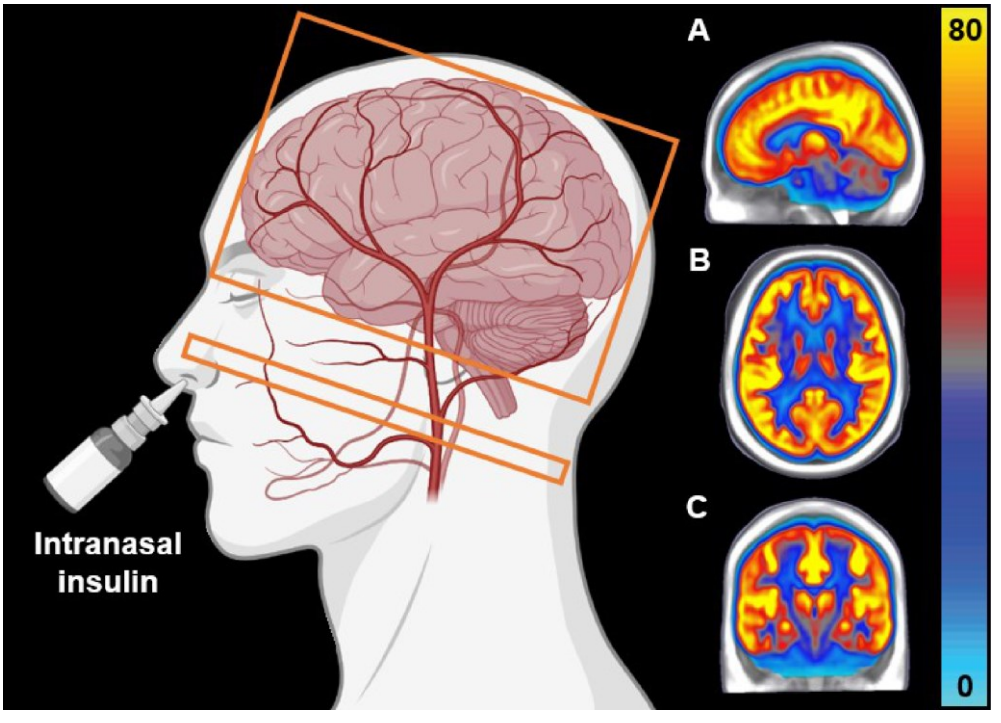

Figure 1 – Example of a perfusion-weighted image using pseudo-continuous arterial spin labeling magnetic resonance imaging (pCASL-MRI). The orange rectangular boxes represent the imaging box and labeling plane perpendicular to the vertebral and carotid artery. Three-dimensional cerebral blood flow maps (CBF in ml/100 g brain tissue/min, scale shown by color bar) were obtained: (**A**) sagittal view, (**B**) axial view and (**C**) coronal view. These CBF maps were obtained in the fasted state to assess brain vascular function. Moreover, brain insulin responsiveness was quantified by the change in CBF before and 30 minutes after intranasal insulin application.

Several methods are available for assessing CBF in humans, including Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET), and Near-Infrared Spectrometry (NIRS) [29]. The focus of this dissertation is on pseudo-continuous arterial spin labeling (pCASL)-MRI, which is a non-invasive technique that utilizes magnetically labelled arterial blood as an endogenous tracer to assess CBF. This method uses radiofrequency pulses to perpendicularly label blood protons in carotid and vertebral arteries. Labeled images are acquired when the blood has reached the brain, while separate control images are obtained without prior labeling. The difference between control and labeled images provides a measurement of labelled blood delivered to the tissue by perfusion. After calibration, this technique generates a three-dimensional CBF map of the brain (**Figure 1**). Studies have shown the quantitative nature of pCASL-MRI to assess CBF in both healthy and diseased populations, making it a promising tool to study brain vascular function [29]. Furthermore, pCASL-MRI exerts good reproducibility and can be used to explore the effects of dietary interventions aimed at improving CBF [30]. The use of pCASL-MRI holds clinical relevance in the diagnosis of disorders associated with CBF [31].

INSULIN SENSITIVITY

Peripheral insulin sensitivity

For the assessment of peripheral insulin sensitivity, the focus should be on traditional metabolic risk factors like fasting glucose and insulin or impaired glucose tolerance as well as methods for quantifying peripheral insulin sensitivity. The gold standard technique for evaluating peripheral insulin sensitivity is the hyperinsulinemic-euglycemic clamp, which - despite its accuracy - is invasive, labor-intensive, and expensive. As a result, peripheral insulin sensitivity is often quantified using data obtained from a fasting blood sample and/or an oral glucose tolerance test (OGTT). Commonly used indices for peripheral insulin sensitivity include the Homeostatic Model Assessment of Insulin Resistance ($HOMA_{IR}$) and the Matsuda index [32, 33]. However, to comprehensively assess the impact of lifestyle modifications and treatment strategies on these increasingly prevalent metabolic diseases, there is a growing interest in novel tissue-specific markers that capture the complex dynamics of glucose metabolism and its regulation. Addressing this need, the hepatic insulin resistance index (HIRI) and the muscle insulin sensitivity index (MISI) have been developed to assess liver and skeletal muscle insulin sensitivity, respectively. These tissue-specific methods have been validated against the gold standard two-step hyperinsulinemic-euglycemic clamp technique, thereby enhancing our understanding of metabolic disorders and facilitating targeted interventions [34].

Brain insulin sensitivity

The conventional belief that the brain is an insulin-insensitive organ has been challenged by emerging evidence, revealing the important role of insulin in the central nervous system (CNS). While the brain does not require insulin for glucose transport, insulin signaling in the brain exerts region-specific effects on neural circuits involved in cognitive performance, but also systemic energy metabolism and eating behavior [35, 36]. The wide expression of insulin receptors in brain regions, such as the hippocampus, hypothalamus, and cortex, further supports its potential role in multiple cognitive and metabolic processes [37]. Brain insulin resistance, characterized by reduced brain insulin responsiveness, has emerged as an important pathological feature in age-related health conditions, including T2D and dementia. Peripheral insulin resistance often coincides with impaired brain insulin signaling, suggesting a systemic dysregulation of insulin sensitivity. However, impaired brain insulin signaling in dementia and various neurodegenerative diseases may also develop independently of peripheral effects [35, 38]. By understanding this complex interplay between insulin and the brain, innovative therapeutic or lifestyle approaches can be designed for preserving brain health and preventing the risk of age-related cognitive decline.

Several methods have been utilized to evaluate insulin sensitivity in the human brain [36]. Within this dissertation, brain insulin responsiveness is assessed using the pCASL-MRI technique to quantify the change in CBF following intranasal insulin application (**Figure 1**) [39]. Whereas circulating insulin enters the cerebrospinal fluid (CSF) via a saturable transport mechanism, nasal insulin directly bypasses the blood-brain barrier. The majority of nasally dispensed insulin effectively reaches distinct brain areas within 30 to 60 min via paracellular transport into the CSF of the subarachnoid space, or via bulk flow along olfactory nerves and trigeminal perivascular channels [36, 40]. However, a minor proportion of nasal insulin may enter the systemic circulation through the rich vasculature of the nasal epithelium and can be distributed throughout the body [39]. This approach offers a non-invasive and targeted mean to investigate regional brain insulin sensitivity in both healthy and diseased populations [35, 37]. It remains however unclear whether brain insulin resistance is reversible. Recent trials have provided initial evidence that pharmacological and lifestyle interventions (i.e., weight loss and maintenance or aerobic exercise) may improve regional brain insulin responsiveness [41, 42]. However, no studies to date have focused on the role of a healthy diet in this context.

COGNITIVE PERFORMANCE

Traditional pencil and paper methods for assessing cognitive performance, such as neuropsychological tests, have limitations in terms of sensitivity and precision. However, computerized tools such as the Cambridge Neuropsychological Test Automated Battery (CANTAB) have been emerged as a more valid assessment tool to assess cognitive performance across different cognitive domains, including attention and psychomotor speed, memory, and executive function [43]. CANTAB has been validated for use in various clinical populations and research settings with high sensitivity and specificity to provide information on the progression of cognitive impairment. CANTAB may be particularly useful to identify early stages of cognitive decline when interventions are most effective and to monitor the progression of cognitive impairment [44, 45]. Moreover, CANTAB is a sensitive method to detect relevant changes in cognitive performance in response to dietary interventions [46]. However, the underlying mechanisms for dietary effects on different domains of cognitive performance still need to be explored. Therefore, this dissertation also focused on the potential effects of dietary interventions on brain vascular function and brain insulin sensitivity as potential underlying mechanisms.

DIETARY INTERVENTION STRATEGIES

To prevent or reverse the global burden of age-related conditions, promoting healthy dietary patterns for the management of risk factors is important. We focused on the physiological and functional effects of dietary components on metabolic aberrations, particularly those related to CVD and brain health. To achieve this, well-controlled dietary longer-term intervention studies are conducted with well-defined study populations. In this dissertation, we focused on older adults who are overweight or obese, or have metabolic syndrome, as they are at increased risk for cognitive decline. While using non-invasive techniques, a strong focus is on components that affect vascular function, as well as lipid and glucose metabolism in both peripheral tissues and the brain. For this, the Metabolic Research Unit Maastricht (MRUM) infrastructure together with the MRI scanning facilities at Scannexus, are used within our facilities in Maastricht.

Among various dietary approaches, the Mediterranean diet has been extensively studied and shown to have beneficial effects on both cardiometabolic parameters and cognitive performance [47]. This diet is characterized by high intakes of fruits, vegetables, cereals, legumes, and unsaturated fatty acids, moderate consumption of fish and dairy products, and low amounts of (red) meat and saturated fats. The individual components of this diet have also been shown to be associated with cognitive performance, potentially attributed to their ability to reduce oxidative stress and inflammation [48]. Notably, the Mediterranean diet has been consistently associated with a reduced risk of cognitive decline and dementia, providing strong evidence for its protective effects on brain health [49]. Nuts are nutrient-dense foods that are part of the

Mediterranean diet and rich in bioactive components, including unsaturated fatty acids, polyphenols, fibers, phytosterols, tocopherols and proteins. Nut consumption has been extensively studied for their potential effects on traditional CVD risk factors. In this context, there is already evidence that the inclusion of tree nuts in the diet has beneficial effects on overall cardiometabolic health, including cholesterol-lowering and antihypertensive properties, and effects on peripheral vascular function [50-52]. However, it is highly relevant to study whether the peripheral effects of nut consumption can be extended to the brain. Increasing evidence suggests that nut consumption protects against cognitive impairments [53]. In fact, studies that incorporated mixtures of nuts into the Mediterranean diet have shown beneficial effects on cognitive performance in older adults [54, 55], but underlying mechanisms remain to be elucidated. In this thesis we therefore investigated the effect of a sixteen-week mixed nut intervention on vascular function and insulin sensitivity in the brain of older adults with overweight or obesity.

Foods containing functional ingredients, such as protein hydrolysates, may also be of potential interest in the prevention of age-related metabolic and vascular impairments. These hydrolysates possess antihypertensive properties due to angiotensin-converting enzyme (ACE)-inhibition and improve glycemic control due to dipeptidyl peptidase 4 (DPP-IV) inhibition [56-59]. For example, NWT-03 is a dietary egg-protein hydrolysate, derived from the digestion of lysozyme with alcalase [60, 61]. Following the acute or short-term intake of NWT-03, recent studies already show BP-lowering effects in mild-hypertensive adults [62], and improved carotid-to-radial PWV ($PWV_{c\text{-}r}$) and cardiometabolic markers (i.e., glucose and lipid metabolism) in adults with impaired glucose tolerance or T2D [63]. While egg-protein hydrolysates are promising in CVD and T2D risk reduction, longer-term trials are required [64]. Therefore, we conducted a four-week randomized, controlled crossover trial to investigate the effects of NWT-03 consumption on arterial stiffness and cardiometabolic markers in adults with metabolic syndrome, a condition with a clustering of risk markers that promote arterial stiffening, and increase the risk to develop CVD and T2D [65].

OUTLINE OF THE THESIS

The overall aim of the current dissertation was to investigate the effects of dietary intervention strategies on vascular function and insulin sensitivity of the brain and periphery in adults, which is schematically presented in **Figure 2**. In **chapter 2**, we first systematically reviewed the current literature on CBF responses to intranasal insulin to assess regional brain insulin sensitivity in healthy and diseased populations. Moreover, relationships between changes in brain insulin sensitivity and cognitive performance were explored. The next two chapters describe the results of a randomized, controlled cross-over trial with longer-term mixed nut consumption in older adults with overweight or obesity. In **chapter 3**, we first report the effects of longer-term mixed

nuts consumption on regional brain insulin sensitivity as measured with pCASL-MRI. Moreover, effects on peripheral insulin sensitivity assessed with an OGTT, and cardiometabolic risk markers are reported. **Chapter 4** describes the effects of longer-term mixed nut consumption on brain vascular function assessed by pCASL-MRI, and cognitive performance in domains of memory, psychomotor speed, and executive function. Furthermore, peripheral vascular function was also assessed using different non-invasive markers for endothelial function, arterial stiffness, and microvascular function. In **chapter 5**, the results are described of a long-term human intervention study in which the effects of NWT-03 supplementation, a protein hydrolysate, on arterial stiffness and cardiometabolic markers are investigated in adults with the metabolic syndrome. Finally, **chapter 6** summarizes the major findings of the different studies and reviews described in the dissertation. The main results from the chapters are discussed in a broader perspective and directions for future research are provided.

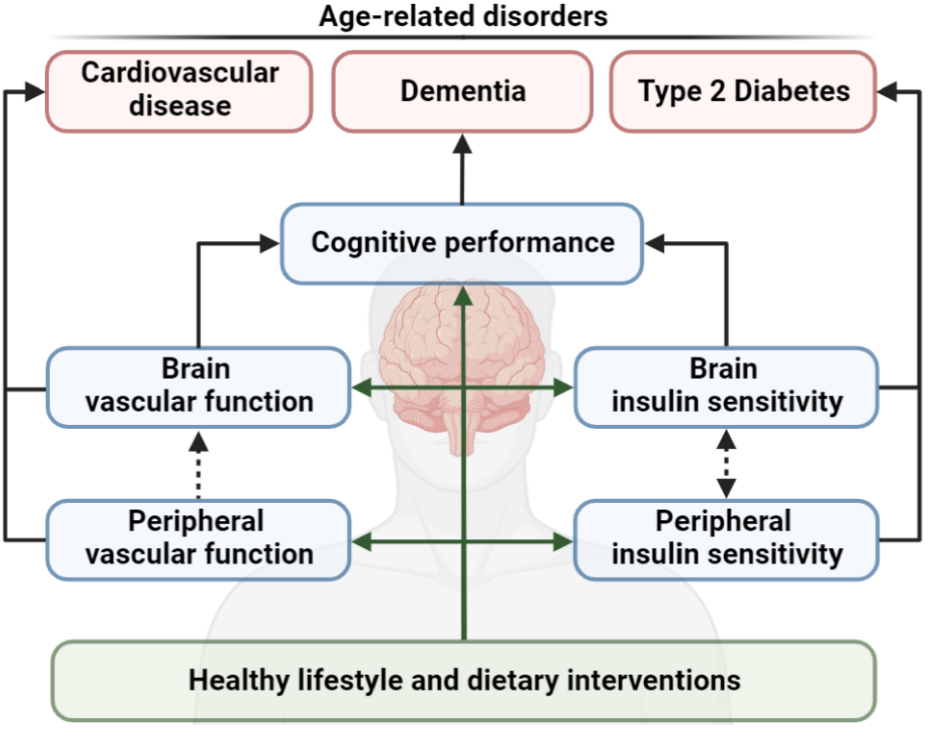

Figure 2 – Schematic overview of hypothesized mechanisms in which healthy dietary interventions may contribute to the prevention of age-related disorders, including cardiovascular disease, dementia, and type 2 diabetes, through improvements in vascular function and insulin sensitivity of the periphery and brain (black and green solid lines). Moreover, the potential of brain vascular function and brain insulin sensitivity as underlying mechanisms for cognitive performance will be explored (orange dotted lines).

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CHAPTER 2

Effects of intranasal insulin administration on cerebral blood flow and cognitive performance in adults: a systematic review of randomized, placebo-controlled intervention studies

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Neuroendocrinology, August 2022

ABSTRACT

Introduction

Brain insulin resistance is an important hallmark of age-related conditions, including type 2 diabetes (T2D) and dementia. This systematic review summarized effects of cerebral blood flow (CBF) responses to intranasal insulin to assess brain insulin sensitivity in healthy and diseased populations. We also explored relationships between changes in brain insulin sensitivity and cognitive performance.

Methods

A systemic literature search (PROSPERO: CRD42022309770) identified 58 randomized, placebo-controlled trials (RCTs) that investigated effects of intranasal insulin on (regional) CBF, cognitive performance, and systemic spill-over in adults.

Results

Acute intranasal insulin did not affect whole-brain CBF in healthy adults, but increased regional CBF of the inferior frontal gyrus, dorsal striatum and insular cortex, and reduced CBF around the middle frontal gyrus and hypothalamus. Obese adults showed increased CBF responses following internasal insulin for the middle frontal gyrus, but decreased CBF for hypothalamic and cortico-limbic regions. Furthermore, increased CBF responses were reported for the insular cortex in T2D patients, and for occipital and thalamic regions in older adults. The spray also improved memory and executive function, but a causal relation with regional CBF still needs to be established. Finally, intranasal insulin resulted in only a small amount of systemic spill-over, which is unlikely to have an impact on the observed findings.

Conclusions

Region-specific changes in CBF after intranasal insulin administration were affected by obesity, T2D, and normal aging, indicating altered brain insulin sensitivity. Future RCTs should investigate longer-term effects of intranasal insulin and explore potential associations between effects on CBF and cognitive performance.

INTRODUCTION

Age-related health conditions, such as type 2 diabetes (T2D) and dementia, are among the most prevalent disorders in the world [1]. These comorbidities share common pathophysiological characteristics such as peripheral insulin resistance [2, 3] and impaired cerebrovascular function [4, 5]. Additionally, brain insulin resistance, defined as the failure of brain cells to respond adequately to insulin, can be part of these conditions. Brain insulin resistance may result from downregulation of brain insulin receptors or impaired downstream signaling [1]. Furthermore, insulin receptors are unevenly distributed throughout the brain [6, 7], suggesting that the degree of brain insulin resistance may differ between specific brain regions. As a consequence, regional brain insulin resistance may lead to cognitive impairment and neurodegeneration [8, 9].

Non-invasive neuroimaging techniques can be used to assess insulin sensitivity in the human brain following intranasal insulin administration. Different arterial spin labeling (ASL) methods are commonly employed to quantify changes in cerebral blood flow (CBF), a well-known physiological marker for cerebrovascular function, and they offer the possibility to assess regional brain insulin sensitivity [6, 10, 11]. The ASL white paper [12] recommends pseudo-continuous ASL (pCASL) for clinical applications due to the higher signal-to-noise ratio (SNR) and lower magnetization effects compared to pulsed ASL (PASL), and higher labeling efficiency compared to continuous ASL (CASL) [13]. Further improvement of the SNR can be obtained by modular features, including background suppression that reduces noise from fluctuations in the static tissue signal [13].

The majority of nasally dispensed insulin effectively reaches distinct brain areas within 30-to-60 minutes via paracellular transport into the cerebrospinal fluid (CSF) of the subarachnoid space, or via bulk flow along olfactory nerves and trigeminal perivascular channels [14-17]. Whereas circulating insulin enters the CSF via a saturable transport mechanism, nasal insulin directly bypasses the blood-brain barrier. This approach can therefore be used to directly assess brain insulin responsiveness in different brain regions [1, 18]. However, a minor proportion of nasal insulin may enter the systemic circulation through the rich vasculature of the nasal epithelium and can be distributed throughout the body, but no adverse effects have been reported so far [16].

Currently, the effects of intranasal insulin on cerebrovascular function are unclear. Therefore, we systematically reviewed randomized, placebo-controlled intervention trials (RCTs) that assessed effects of nasal insulin on (regional) CBF to study brain insulin sensitivity in healthy and diseased target populations. Furthermore, a potential association between changes in CBF and cognitive performance was explored, as multiple studies using the spray have already reported cognitive benefits [19-22]. Finally, we quantified the amount of systemic insulin spill-over and effects on blood pressure, which both might interfere with the functional impact of nasal insulin on the human brain [23, 24].

METHODS

The protocol for this systematic review was registered in the International Prospective Register of Systemic Reviews (PROSPERO: CRD42022309770) [25].

Search strategy

The preferred reporting items for systematic review and meta-analyses (PRISMA) checklist was used to structure the systematic review [26]. Potentially relevant studies published before March 2022 were identified through a systemic literature search in Ovid Medline, Embase and the Cochrane Central Register of Clinical Trials. The following search terms were used: “brain insulin” or “brain insulin-sensitivity” or “intranasal insulin” (All fields), combined with “human” or “humans” (Publication type) and “trial” or “clinical study” or “RCT” or intervention” (Publication type). Reference lists from the selected articles were also screened via a manual approach to retrieve potentially relevant studies that were not identified by the literature search.

Selection procedure

Only studies investigating the effects of intranasal insulin administration were selected. Publication language had to be English. The selection procedure was divided into two stages: a title and abstract selection, followed by a full-text selection of the selected articles. Papers were included when they met the following criteria: (i) intervention study involving adults; (ii) study using intranasal insulin as experimental variable and placebo spray as control; and (iii) co-intervention that made it impossible to estimate intranasal insulin effects. All articles were independently reviewed by two authors (KN and PJ) for inclusion. Any discrepancies were resolved by discussion until consensus was reached.

Data collection

For each included article, the following data were extracted into a custom-made spreadsheet: study information (first author, publication year), study characteristics (study design, study product, and dose, duration, time between measurements), and participant characteristics (sample size, age, gender, body mass index (BMI), health status). Furthermore, the main findings on CBF, cognitive performance, blood pressure (BP), and circulating plasma biomarkers (glucose, C-peptide, insulin, free fatty acids (FFA), and triacylglycerol (TAG)) were collected. If mean concentrations were not provided in a table, these were estimated from graphs using a pixel ruler. For parallel studies, the effect of intranasal administration was defined as the difference between the change before and after intranasal insulin administration versus the change before and after placebo application. The effect in crossover trials was defined as the difference between post-intervention values after insulin and placebo application. Cognitive tasks were categorized into domains of executive function (working memory, attention, and information processing), and memory (declarative, spatial and non-declarative memory). The Cochrane

revised risk-of-bias tool for RCTs was used to assess the risk of bias for the included studies [27].

RESULTS

Search results

A total of 465 records were identified from the selected databases (**Figure 1**). Titles and abstracts were screened, and 373 papers were excluded based on the predefined selection criteria. The full texts of 92 articles were reviewed and 35 papers were excluded. One study was added through searching reference lists of selected articles. In total, 58 articles met all the inclusion criteria but some articles were focused on different measurements of the same intervention study. Therefore, the review included a total of 34 acute studies and 14 studies on the longer-term effects of intranasal insulin (**Supplemental Table 1**). The risk of bias assessment showed some concerns for four studies and a low risk of bias (89%) for the remaining 44 studies, indicating an overall high quality of evidence provided by the included studies (**Supplemental Table 2**).

Cerebral blood flow

Four studies reported the acute effects of nasal insulin on whole-brain CBF. Doses of 40 or 160 IU did not evoke changes in whole-brain CBF in healthy normal-weight and overweight adults, and T2D patients [28-31]. Eight studies reported the acute impact of nasal insulin on regional CBF [6, 28-35], while no longer-term studies were performed (**Table 1**). Methodological information on both the data acquisition (manufacturer, magnetic field and ASL technique) and analysis (software, ROI or voxel-wise analysis, and P-value adjustments) is presented in **Supplemental Table 3**. Four studies used pCASL [29-31, 34], two studies used PASL [6, 32], and two studies used CASL [28, 33].

Cerebral cortex

Three studies reported the effects on CBF of the frontal cortex [6, 29, 33]. In young and older men, no changes were found after 40 IU [29]. A significant increase was found in a cluster involving the opercular part of the inferior frontal gyrus in healthy young adults [33]. Another study in normal-weight subjects showed a decrease in prefrontal CBF after 160 IU. However, an increase was observed in overweight or obese adults [6].

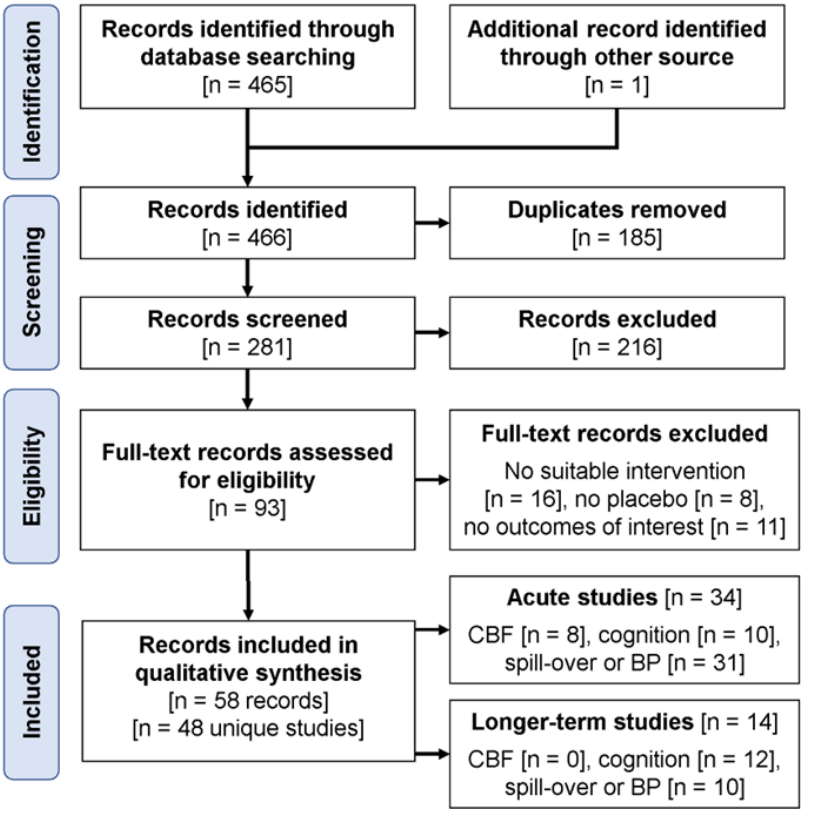

Figure 1 – PRISMA flow diagram of the study selection procedure of human intervention studies for included in the qualitative synthesis of the systematic review.

Five studies investigated the effects on CBF within other major brain regions [6, 28, 29, 31, 33]. In normal-weight or overweight and obese adults, no changes in CBF of the parietal, temporal or occipital lobe were reported [6, 29, 33]. After 40 IU, CBF increased in the occipital lobe of older adults [29]. One study showed an increase in insular CBF after 40 IU in healthy adults [33], while another study showed an increase in T2D patients but not in a healthy population [28].

Subcortical structures

Three studies focused on effects on CBF of the diencephalon [6, 29, 32]. Thalamic CBF did not change after 40 IU in healthy young adults, but increased in older subjects [29]. After a dose of 160 IU, however, the spray caused a decrease in hypothalamic CBF in normal-weight and overweight adults [6]. Effects on hypothalamic CBF were dose-dependent and the most pronounced decreases were found after 80 IU or 160 IU [34]. Insulin-induced changes in hypothalamic CBF were positively associated with peripheral insulin-sensitivity and insulin secretion [32, 35].

Three studies described effects on CBF of the limbic system [30, 31, 33]. In healthy adults, 40 IU did not change hippocampal CBF [33]. Wingrove and colleagues also found no changes in hippocampal CBF after 160 IU in overweight or obese adults, but CBF decreased in the left and right amygdala [30]. Another study found in overweight men decreases in regional CBF of the left hippocampus, left and right parahippocampal gyrus and left fusiform gyrus, while no changes were observed in a normal-weight group [31]. Increases in the putamen and a cluster of the left caudate nucleus and putamen were reported after 40 IU in healthy adults [33]. After 160 IU, no effects on CBF of the putamen were found in normal-weight men, but a decrease was found in overweight men [31].

Cognitive performance

Twenty-two studies examined the effects of nasal insulin on cognitive performance (Supplemental table 1): ten acute [28, 36-45] and twelve longer-term studies [46-60]. The main outcomes of these studies have been summarized in **Table 2**.

Executive function

Seven studies examined the effects on working memory [40-42, 44, 48, 53, 60]. In overweight adults, working memory was not changed by a low-dose (10 to 60 IU) of insulin [41, 42]. Improvements however were observed 75 minutes after a dose of 160 IU in normal-weight adults [40, 60]. Benedict and colleagues showed improvements in women only [60]. In patients with Mild Cognitive Impairment (MCI) or Alzheimer's Disease (AD), no effects were observed after acute application [41, 42, 44], while a daily dose of 40 IU for 3 weeks improved working memory [48]. Finally, working memory in schizophrenic patients was not changed following a daily dose of 160 IU for 8 weeks [53].

Ten studies studied the effects on cognitive tasks assessing attention [41, 42, 44, 46, 48, 52-55, 57]. In overweight adults and MCI/AD patients no effects were observed after acute doses of 10 to 60 IU [41, 42], but one study showed an improvement in visual attention after 20 IU in MCI/AD patients [44]. Daily application of 160 IU for 8 weeks did not affect attention or inhibition in overweight adults [46, 54]. In MCI/AD patients, a daily dose of 40 IU for 3 weeks either improved response inhibition [42] or had no effects [48]. No changes were observed in schizophrenic or bipolar patients [52, 53, 55].

Six studies examined the effects on cognitive tasks assessing information processing [28, 42, 44, 53, 55, 56]. Acute doses of 10 to 60 IU did not affect verbal fluency or psychomotor speed in normal-weight, T2D or MCI/AD patients [28, 42, 44]. Four-weeks of daily 40 IU improved verbal fluency in Parkinson's patients [56], while no changes were observed in schizophrenic or bipolar patients [53, 55].

Table 1 – Summary of intervention studies on the effects on intranasal insulin of regional CBF.

Author	Study design	Study product	N (%male)	Age (years)	BMI (kg/m^2)	Health status	Effects on CBF (ml/100g/min) compared to placebo
Schilling, 2014 [33]	Parallel	Actrapid (40 IU)	13 (100)	24	N/A	Healthy	↑ Cluster of inferior frontal gyrus and left caudate nucleus (Left) putamen (6.3 – 6.8) Insular cortex (4.3 – 7.0) = Hippocampus
Heni, 2014 [32]	Crossover	Actrapid (160 IU)	10 (100) 5 (100)	26 28	22.8 33.2	Healthy Obese	Positive correlation of hypothalamic CBF with peripheral insulin sensitivity
Novak, 2014 [28]	Crossover	Novolin (40 IU)	29 (41) 15 (53)	62 62	N/A N/A	Healthy T2D	= Whole-brain ↑ Insular cortex (7.2)
Kullmann, 2015, 2017 [6, 35]	Crossover	Actrapid (160 IU)	25 (60) 23 (52)	26 27	22.7 27.8	Healthy Overweight	↓ Middle frontal gyrus, hypothalamus ↑ Middle frontal gyrus ↓ Hypothalamus, positive correlation of hypothalamic CBF with peripheral insulin sensitivity and insulin secretion
Akintola, 2017 [29]	Crossover	Actrapid (40 IU)	8 (100) 11 (100)	22 65	23.6 24.1	Healthy (young) Healthy (older)	= Whole-brain ↑ Thalamus (5.0), Occipital lobe (4.0)
Kullmann, 2018 [34]	Crossover	Actrapid (40/80160 IU)	9 (100)	27	23.3	Healthy	↓ Hypothalamus (dose-dependent)
Wingrove, 2019 [30]	Crossover	Actrapid (160 IU)	16 (100)	25	27.8	Overweight	↓ Amygdala (4.0 – 4.1)
Wingrove, 2021 [31]	Crossover	Humulin (160 IU)	12 (100) 14 (100)	27 25	22.4 27.5	Healthy Overweight	= Whole-brain ↓ Cluster of (left) hippocampus, parahippocampal gyrus, (left) insula, (left) putamen and (right) fusiform gyrus[a]

CBF, cerebral blood flow; BMI, body mass index; Effects include significant increases (↑), significant decreases (↓) or no effects (=).

[a] Regional CBF data was statistically corrected for mean whole-brain gray matter CBF values.

Memory

Seventeen studies investigated the effects on declarative memory [28, 39, 41-44, 48-55, 57, 58]. Acute doses of 10 to 160 IU did not improve memory in normal-weight or overweight adults [39, 41, 42]. In MCI/AD patients, individuals carrying the APOE-ε4 allele showed no or even worsened memory scores, while in those without the allele intranasal insulin improved memory recall [41, 42, 44]. A dose of 20 IU improved immediate recall in adults at increased risk of dementia [43]. The 8-week application of a daily 160 IU improved delayed recall in normal-weight and overweight adults [46, 47, 54, 58]. In MCI/AD patients, application of 10 to 60 IU for 3 to 16 weeks reported beneficial effects on memory [48-51, 57], although one study found decreased memory scores in patients carrying the APOE-ε4 allele [48]. No effects on declarative memory were observed in schizophrenic [52, 53] or bipolar patients [55].

Table 2 – Summary of 10 acute and 12 longer-term studies that investigated the effects of intranasal insulin application of cognitive performance.

	Executive function			**Memory**		
	Working memory	***Attention/ Inhibition***	***Information processing***	***Declarative memory***	***Spatial memory***	***Non-declarative***
Acute studies (n = 10)						
BMI 18-25 kg/m^2	↑ (2)		= (1)	= (1)	↑ (3)/= (2)	↑ (1)/= (2)
BMI > 25 kg/m^2	= (2)	= (2)		= (2)		
T2D patients			= (1)		↑ (1)	
MCI / AD	= (3)	↑ (1)/= (2)	= (1)	↑ (2)[a]/ = (1)/↓(2)[a]	↑ (1)	
Other				↑ (1)		
Longer-term studies (n = 12)						
BMI 18-25 kg/m^2		= (1)		↑ (3)		= (2)
BMI > 25 kg/m^2		= (1)		↑ (1)		= (1)
T2D patients						
MCI / AD	↑ (1)	↑ (1)/= (1)		↑ (4)[a]/↓ (1)[a]		
Other	= (1)	= (3)	↑ (1)/= (2)	= (3)		

BMI, Body Mass Index; T2D, Type 2 Diabetes; MCI, Mild Cognitive Impairment; AD, Alzheimer's Disease; Other: Bipolar disorder, schizophrenia and Down's syndrome. Effects include the number of studies that report significant increases (↑), significant decreases (↓) or no effects (=).

[a] In two acute studies and one longer-term study, decreased declarative memory performance in MCI/AD patients carrying the APOE-ε4 allele was found, but increased performance in non-carriers.

Seven acute studies investigated effects on cognitive tasks assessing spatial memory [28, 36, 37, 40, 44, 45, 60]. In normal-weight adults, acute doses of 40 or 160 IU improved spatial memory [28, 36, 45, 60], while other studies showed no effects [37, 40]. Spatial memory scores in T2D patients were also improved after 40 IU insulin [28, 45] and after 20 IU in MCI/AD patients [44].

Six studies investigated the effects on non-declarative memory [38, 39, 46, 54, 58, 60]. In normal-weight adults, no effects on procedural memory were found [39, 60], while one study reported a facilitated fear extinction response in after a dose of 160 IU [38]. The 8-week application of daily 160 IU did not affect non-declarative memory in normal-weight or overweight individuals [46, 54, 58].

Systemic spill-over, blood pressure and heart rate

The main outcomes of 41 studies that examined the effects of intranasal insulin administration on systemic spill-over or blood pressure and heart rate are summarized in **Table 3**.

Systemic spill-over

Thirty-five studies reported the effects on serum insulin concentrations. For normal-weight adults, mean intervention effects over time of 40 IU [29, 34, 36, 41, 61-67], 80 IU [34], and 160 IU [24, 31, 34, 40, 68-74] were summarized in **Figure 2A**. Circulating insulin concentrations did not appear to change after a dose of 40 IU. However, concentrations immediately increased following a dose of 80 IU or 160 IU, reaching maximal increases of respectively 26 and 44 pmol/L after 15 minutes. In overweight and obese subjects, two studies showed increased insulin concentrations after 100 IU [75] or 160 IU [31, 75], while other studies did not show effects [24, 73, 76, 77]. In patients with T2D or MCI/AD, no changes were observed [41, 52, 69]. Longer-term application of nasal insulin (20 to 160 IU daily) up to 8 weeks did not affect fasting insulin concentrations [46, 47, 54, 57, 58, 77-79].

Thirty-seven studies investigated effects on plasma glucose concentrations. For normal-weight adults, mean intervention effects over time of 40 IU [28, 29, 34, 36, 41, 61-63, 65-67, 80], 80 IU [34], and 160 IU [24, 31, 34, 38, 40, 64, 68-71, 73, 74, 81] were summarized in **Figure 2B**. Overall, glucose concentrations did not change after 40 IU, but higher doses of 80 and 160 IU reduced glucose concentrations up to 30 to 40 minutes after use, with a maximal reduction of about 0.4 mmol/L. After both doses, glucose concentrations returned to baseline values after approximately 60 minutes. In overweight or obese adults, one study found a decrease after 160 IU [75], while no changes were observed in other studies [6, 30, 31, 73, 76, 77], or in patients with T2D or MCI/AD patients [28, 41, 69, 80]. Longer-term application (20 to 240 IU daily) up to 24 weeks did not affect fasting glucose levels [46-48, 57, 58, 77-79, 82].

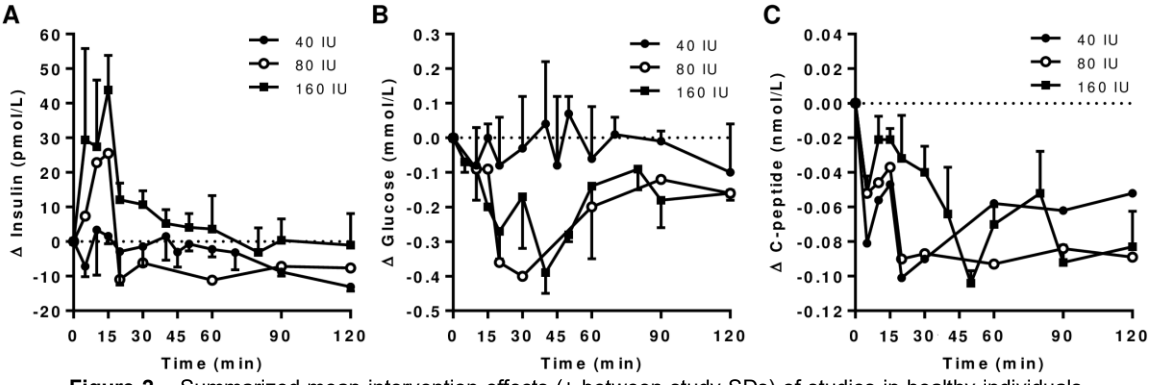

Figure 2 – Summarized mean intervention effects (± between-study SDs) of studies in healthy individuals showing effects of acute intranasal insulin (40 IU, 80 IU, or 160 IU) over time on (**A**) serum insulin concentrations; (**B**) plasma glucose concentrations; and (**C**) serum C-peptide concentrations. Standard deviations were missing for those timepoints representing data from only one study.

Table 3 – Summary of 31 acute and 10 longer-term that investigated the effects of intranasal insulin application on systemic spill-over or blood pressure.

	Systemic spill-over				**Blood pressure**		
	Insulin	**Glucose**	**C-peptide**	**TAG / FFA**	**SBP**	**DBP**	**HR**
Acute studies (n = 31)							
BMI 18-25 kg/m^2	↑ (7) / = (13)	= (15) / ↓ (7)	= (5) / ↓ (4)	= (1)/ ↓ (2)	= (5)	= (5)	= (7)
BMI > 25 kg/m^2	↑ (2) / = (2)	= (3) / ↓ (1)	= (1) / ↓ (1)				
T2D patients	= (3)	= (3)	= (1)	= (1)	= (1)	= (1)	= (1)
MCI / AD	= (1)	= (2)					
Other					↑ (1)	= (1)	= (1)
Longer-term studies (n = 10)							
BMI 18-25 kg/m^2	= (3)	= (6)			= (1)	= (1)	= (1)
BMI > 25 kg/m^2	= (2)	= (2)			= (1)	= (1)	↑ (1)
T2D patients		= (1)					
MCI / AD	= (2)	= (1)					
Other	= (3)	= (3)					

TAG, triacylglycerides; FFA, free fatty acids; BMI, Body Mass Index; T2D, Type 2 Diabetes; MCI, Mild Cognitive Impairment; AD, Alzheimer's Disease; Other: Bipolar disorder, schizophrenia and Down's syndrome. Effects include the number of studies that report significant increases (↑), significant decreases (↓) or no effects (=).

Fifteen studies reported effects on C-peptide concentrations. Mean intervention effects over time were summarized for doses of 40 IU [34], 80 IU [34], and 160 IU [24, 31, 34, 40, 63, 68-71, 73, 81] administered to healthy normal-weight adults (**Figure 2C**). C-peptide concentrations decreased over time independent of the insulin dose. After 15 to 45 minutes, C-peptide concentrations were lowest with a reduction of about 0.10 nmol/L and had not returned to baseline after 120 minutes. In overweight or obese adults, two studies showed decreased concentrations after 40 or 160 IU [31, 75], while other studies did not report any changes [73, 76]. No longer-term effects were found [77, 82].

Five studies, all using a high dose of 160 IU intranasal insulin, reported effects on circulating lipid concentrations. Two studies found a significant reduction in FFA concentrations in healthy adults 60 minutes after nasal application [69, 83], while no effects were observed in other studies involving healthy adults [68] or T2D patients [69]. The longer-term daily administration of daily 160 IU insulin for four weeks did not result in differences in FFA concentrations [77]. TAG concentrations were not changed by the acute [83] or longer-term application [77, 79].

Blood pressure and heart rate

Ten studies reported effects of intranasal insulin on blood pressure or heart rate (**Table 3**). Acutely, no changes were found for SBP, DBP and heart rate in healthy or obese adults or T2D patients [28, 34, 61, 71, 72, 74]. In adults with increased risk for dementia, SBP was increased after 20 IU insulin [43]. Longer-term use of nasal insulin for up to eight weeks did not change SBP or DBP [46, 54, 82]. One study in overweight or obese adults showed a decreased heart rate after 8 weeks of daily 160 IU [54].

DISCUSSION

In this systematic review, randomized, placebo-controlled intervention studies examining effects of intranasal insulin on regional CBF were evaluated. Also, relations between changes in regional CBF with cognitive performance were explored. Furthermore, systemic insulin spill-over, and effects on blood pressure and heart rate were quantified. An overview of brain regions that responded to intranasal insulin application in different target populations is shown in **Figure 3**. These findings and related effects on cognitive performance will be discussed in the following paragraphs.

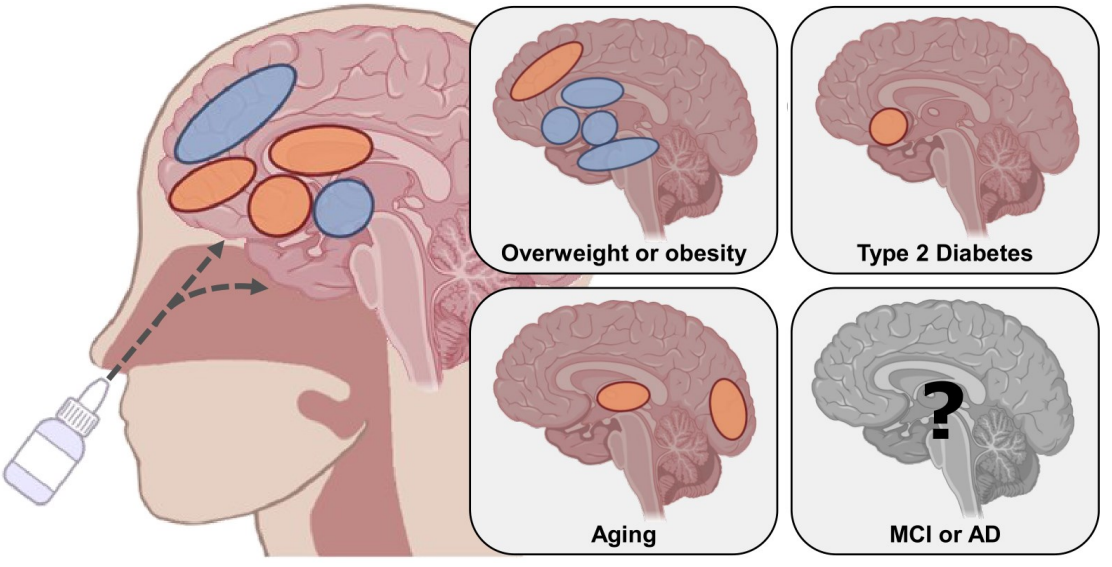

Figure 3 – An overview of the effects of intranasal insulin on regional increases (orange shapes) or decreases (blue shapes) in cerebral blood flow (CBF). In healthy adults, nasal insulin increased CBF of the inferior frontal gyrus, dorsal striatum and insular cortex and decreased CBF of the middle frontal gyrus and hypothalamus. CBF responses to the spray were different in overweight or obese adults with increased CBF of the middle frontal gyrus, an attenuated decrease in hypothalamic CBF and a decreased CBF within cortico-limbic regions. In Type 2 Diabetes patients, nasal insulin increased insular CBF compared to healthy controls. Increased CBF of the thalamus and occipital lobe were reported in elderly compared to younger adults. No studies have been conducted in patients with Mild Cognitive Impairment (MCI) or Alzheimer's Disease (AD).

Intranasal insulin and (regional) cerebral blood flow responses

Intranasal insulin administration did not affect whole-brain CBF in healthy adults, but changed regional CBF. More specifically, CBF in brain regions with a lower density of insulin receptors was hardly affected [1, 15], while CBF did change in insulin-sensitive brain regions [9]. Previous studies have already indicated that intranasal insulin does not exert direct vasoactive effects, but that changes in CBF reflect changes in regional neuronal activity. The neurovascular unit, which is comprised of different cell types, such as endothelial and neuronal cells, and astrocytes, plays a fundamental role in coupling the energy demand of activated brain regions with regional CBF. This phenomenon, also known as neurovascular coupling, links increases or decreases in neuronal activity to subsequent changes in local CBF via the concept of functional hyperemia [84]. We found that CBF in the inferior frontal gyrus, dorsal striatum and insular cortex was increased after the spray, which may reflect increases in neuronal activity in a cortical network important for appetite suppression and the processing of gustatory information [33]. In contrast, regional CBF was reduced around the middle frontal gyrus and hypothalamus [6, 34] that may

respectively relate to an inhibitory control towards food cues or an increase in satiety [64, 85]. Insulin-induced changes in CBF have therefore mostly been associated with feeding or reward behaviors [86]. Thus, regional CBF can either be increased or decreased following nasal insulin, which could explain the absence of an effect at the whole-brain level.

Acute administration of intranasal insulin beneficially affected spatial memory and executive function in healthy normal-weight adults, while the longer-term application also improved declarative memory in these adults. Recently, Hallschmid reviewed possible mechanisms underlying the beneficial effects of insulin on cognitive performance [8]. In short, brain insulin signaling contributes to various neuronal mechanisms essential for proper cognitive functioning, including catecholamine release and uptake, ion channel trafficking, and activation of neurotransmitter receptors [8]. Also, brain insulin signaling may rapidly induce GLUT-4 translocation in brain regions with high cognitive demands, thereby increasing regional glucose uptake and affecting cognitive functions [87]. Furthermore, insulin may contribute to activity-dependent processes of synaptic plasticity, thereby improving the establishment of memory traces [88]. An association between acute intranasal insulin-induced cognitive benefits and changes in regional CBF has already been established in one study. In that study, Novak and colleagues observed that acute insulin-related improvements in visuospatial memory and verbal fluency were associated with greater vasodilation in anterior cortical areas, which are important areas involved in attention and memory outcomes [28]. However, the longer-term impact of intranasal insulin on brain vascular function and related cognitive effects are not yet investigated.

Intranasal insulin responses in populations at risk for brain insulin resistance

Obesity

Obesity is associated with impaired brain insulin action and related to a decreased CBF in most brain regions [73, 89]. We found that CBF responses to intranasal insulin were different between overweight or obese adults and normal-weight individuals. Intranasal insulin decreased CBF in the middle frontal gyrus in normal-weight adults, but opposite effects were observed in overweight and obese adults. Moreover, the decrease in hypothalamic CBF after the spray was attenuated in obesity, and a decreased regional CBF within a large cluster involving cortico-limbic regions was observed. These latter effects were not found in normal-weight adults [6, 30, 31, 34], which may imply that changes in brain insulin sensitivity result in a decreased satiety and inhibition towards food cues as described by Kullmann and colleagues [6, 15]. Moreover, stronger insulin-induced CBF responses of the hypothalamus were associated with the amount of weight reduction and a more favorable body fat distribution [90], while chronic insulin administration reduced adiposity [78]. This suggests that lifestyle interventions can improve brain insulin action and regional brain insulin resistance may be beneficially affected. Moreover, longer-term intranasal insulin administration improved declarative memory in obese adults [54].

Insulin signaling within the hippocampus, which is a well-established brain region that is vital for long-term declarative memory formation and consolidation [91], has been shown to hyperpolarize neurons, increase GABA-related activity and reduce neuronal firing rates [92, 93]. Previous studies have already identified an inverse relationship between hippocampal CBF and memory performance [94-96]. Therefore, decreased hippocampal CBF following spray application in obese adults, which has been reported by Wingrove and colleagues [31], may underlie the beneficial effects of intranasal insulin observed on cognitive performance.

Type 2 Diabetes

Peripheral insulin resistance, which is a key feature of T2D, has been associated with reduced cerebral glucose metabolism and brain insulin resistance [1]. We observed that spray-induced reductions in CBF of the hypothalamus and amygdala were positively associated with peripheral insulin resistance in non-T2D patients [30, 32, 35]. However, effects of nasal insulin on regional CBF in T2D were examined in only one study that showed increased insular CBF in T2D patients, but no effects in healthy adults [28]. Interestingly, the change in insular CBF was positively associated with improved spatial memory, which provides further evidence for the relevance of cerebrovascular reactivity to the spray in relation with cognitive performance. Previous studies have already shown impairments in functional connectivity between the insula and other brain regions in T2D patients that may contribute to cognitive decline [97].

Mild cognitive impairment and dementia

A reduced brain insulin sensitivity may also manifest independently from peripheral insulin resistance, which has already been observed in patients with MCI and dementia [22]. Moreover, lower cortical insulin concentrations and receptor binding have already been reported in elderly without dementia as compared with younger adults [98, 99], indicating that impaired brain insulin signaling already develops during normal human aging. To date, only one study has compared side-by-side the effect of aging on changes in regional CBF by nasal insulin. In that study, no changes CBF in the thalamic and occipital regions in young adults were found, while CBF within these regions increased in older individuals [29]. In MCI or dementia patients, major abnormalities are present in brain insulin signaling molecules, which are related to disease progression and cognitive decline [1]. Beneficial effects on memory and executive function have been reported in patients with MCI or dementia following both acute and longer-term application of the spray. Unfortunately, no studies have been conducted on the effects of nasal insulin on regional CBF in these patients, while improvements in cerebrovascular function might act as an important underlying mechanism for the observed effects on cognitive performance. Increased amyloid clearance, and supporting synapse formation and neuronal plasticity may also be involved leading to cognitive benefits [8]. Moreover, we found differential effects of intranasal insulin on memory based on the APOE-ε4 genotype, with no effects or worsened scores in ε4-

carriers and improvements in non-carriers. Greater hippocampal CBF was associated with worsened memory performance in ε4-carriers, but better memory scores were observed in non-carriers [100]. APOE-ε4 status should therefore be considered as a modifying factor in the development of brain insulin resistance and cognitive decline.

Considerations for intranasal insulin responses

Multiple factors may influence the impact of intranasal insulin on regional CBF responses. First, systemic insulin spill-over to the periphery might play a role [16]. We found that plasma insulin concentrations increased dose-dependently up to 15 minutes following the spray with consequent minor reductions in glucose, C-peptide and FFA concentrations. However, systemic spill-over is unlikely to contribute to the responses observed. In fact, changes in peripheral insulin concentrations were small compared with subcutaneous insulin infusion or after meal intake [101, 102], while insulin transport across the blood-brain barrier is a saturable process [103, 104]. Second, we did address the effects of nasal insulin on blood pressure, as cerebral hemodynamics have profound effects on CBF responses [105]. However, systemic blood pressure was not influenced following spray administration and probably does not affect brain insulin actions on CBF. Third, Kullmann *et al.* reported that doses of at least 80 IU are needed to detect changes in brain activity [34], but other studies already showed specific effects after 40 IU, which may be due to the specific threshold activations of regions [33]. Doses exceeding 160 IU may however cause potential side effects, including increases in cortisol and growth hormone that could also affect the functional impact of the spray [106].

CONCLUSION

In this systematic review, the effects of intranasal insulin administration on CBF were summarized with the purpose to study brain insulin sensitivity in healthy and diseased target populations. In healthy adults, CBF changes upon the spray in regions that have typically been related to feeding and reward behaviors. Important determinants of the CBF response to the intranasal spray were obesity, T2D, and normal human aging, which indicates altered brain insulin sensitivity. Intranasal insulin has been shown to improve memory and executive function, but a causal association between regional brain insulin sensitivity and cognitive performance still needs to be established. Future studies should consider the insulin dose on the functional impact of the spray, while the amount of systemic spill-over is small and no effects on blood pressure were observed. Future well-designed RCTs are warranted to investigate the longer-term effects of intranasal insulin and focus on potential associations between effects on CBF and cognitive performance. Furthermore, effects of (longer-term) lifestyle interventions on regional brain insulin sensitivity should be investigated, because improving brain insulin resistance may be important for the prevention of cognitive decline.

STATEMENT OF ETHICS

An ethics statement is not applicable because this systematic review is based exclusively on published literature. All included studies complied with the guidelines for human studies according to the World Medical Association Declaration of Helsinki.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

FUNDING SOURCES

The authors did not receive support from any organization for the submitted work.

AUTHOR CONTRIBUTIONS

Kevin M.R. Nijssen was involved in the conceptualization, study selection, data acquisition, data interpretation, and writing the manuscript. Ronald P. Mensink was involved in the conceptualization, data interpretation, and writing the manuscript. Peter J. Joris was involved in the conceptualization, study selection, data acquisition, data interpretation, and writing manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

All data generated during this study are included in this article. Further enquiries can be directed to the corresponding author.

SUPPLEMENTAL MATERIAL

Supplemental table 1 – Study characteristics of 58 included studies and their main outcomes.

Author	Study design	Study product	Dose (IU)	N	Age (years)	BMI (kg/m^2)	Health status	Main effects							
								CBF	MEM	EXE	INS	GLU	C-PEP	LIP	BP
Acute studies															
Reger, 2006	Crossover	Novolin	20, 40	35	75	25.6	Healthy		=	=	=	=			
				14	77	24.9	MCI/AD, ε4+		↓	=	=	=			
				12	77	24.5	MCI/AD, ε4-		↑	=	=	=			
Reger, 2008	Crossover	Novolin	10, 20, 40, 60	59	74	26.0	Healthy		=	=	=	=			
				22	77	26.5	MCI/AD, ε4+		↓	=	=	=			
				11	76	25.6	MCI/AD, ε4-		↑	=	=	=			
Bohringer, 2008	Parallel	Actrapid	40	13	24	21.7	Healthy				=	=			=
		Placebo		13	25	22.5									
Guthoff, 2010	Crossover	Actrapid	160	9	25	21.4	Healthy				↑	=			
Krug, 2010	Crossover	Actrapid	160	14	24	57.6	Healthy		↑	=	=	=	=		
Stingl, 2010;	Crossover	Actrapid	160	10	26	20.9	Healthy				=	=	=		
Guthoff, 2011				10	27	28.8	Overweight				=	=	=		
Benedict, 2011	Crossover	Actrapid	160	19	23	23.5	Healthy				↑	↓	=	=	
Hallschmid, 2012	Crossover	Actrapid	160	13	23	22.9	Healthy				=	↓	↓		
Jauch-Chara, 2012	Crossover	Actrapid	40	15	25	22.2	Healthy				=	=	=		
Heni, 2012	Crossover	Actrapid	160	103	28	22.6	Healthy				↑	↓	↓		
Brünner, 2013	Crossover	Actrapid	40	17	25	22.2	Healthy				=	↓			
Kullmann, 2013	Crossover	Actrapid	160	17	25	21.2	Healthy				↑	=			
Schilling, 2014	Parallel	Actrapid	40	13	24	N/A	Healthy	↑							
Iwen, 2014	Crossover	Actrapid	160	14	25	24.4	Healthy							↓	

Supplemental table 1 – Study characteristics of 58 included studies and their main outcomes (continued).

Author	Study design	Study product	Dose (IU)	N	Age (years)	BMI (kg/m^2)	Health status	Main effects							
								CBF	MEM	EXE	INS	GLU	C-PEP	LIP	BP
Novak, 2014;	Crossover	Novolin	40	14	60	N/A	Healthy	=	↑	=		=			
Zhang, 2015				15	62	N/A	T2D	↑	↑	=		=			
Rosenbloom, 2014	Crossover	Glulisine	20	12	72	N/A	AD		↑/=	↑/=					
Heni, 2014	Crossover	Actrapid	160	10	26	21.8	Healthy	/			=	=	=		
				5	28	33.2	Obese	/			=	=	=		
Kullmann 2015,	Crossover	Actrapid	160	25	26	22.7	Healthy	↓				=			
Kullmann 2017				23	27	31.3	Obese	↓				=			
Brünner, 2015	Crossover	Actrapid	40	18	24	22.6	Healthy		↑		=	↓			
Gancheva, 2015	Crossover	Actrapid	160	10	26	23.1	Healthy				↑	↓	↓	↓	
				10	61	29.0	T2D				=	=	=	=	
Brünner, 2016	Crossover	Actrapid	40	16	25	23.1	Healthy		=		=	=			
Feld, 2016	Crossover	Actrapid	160	32	24	22.8	Young		=		↑	↓			=
Thienel, 2017 Santiago, 2017				19	71	25.3	Older				=	=			=
Akintola, 2017	Crossover	Actrapid	40	8	22	23.6	Young	=			=	=			
Opstal, 2017				11	65	24.1	Older	=/↑			=	=			
Rodiguez-Raecke, 2017	Crossover	Actrapid	40	24	25	23.5	Healthy				=	=			
Krug, 2018	2x2 design	Actrapid	160	16	24	22.6	Healthy				=	↓	=		=
		Placebo	160	16	24	23.0									
Kullmann, 2018	Crossover	Actrapid	80, 160	9	27	23.4	Healthy				↑	=	=		=
Rodiguez-Raecke, 2018	Crossover	Actrapid	40	30	25	21.9	Healthy				↑	=			

Supplemental table 1 – Study characteristics of 58 included studies and their main outcomes (continued).

Author	Study design	Study product	Dose (IU)	N	Age (years)	BMI (kg/m²)	Health status	Main effects							
								CBF	MEM	EXE	INS	GLU	C-PEP	LIP	BP
Reger, 2008	Parallel	Novolog	40/d, 3 weeks	13	77	26.9	MCI/AD		↑	↑	=	=			
		Placebo		12	79	26.0									
Fan, 2011	Parallel	Humulin	40/d, 8 weeks	30	50	N/A	Schrizophrenic outpatients		=	=	=	=			
		Placebo													
McIntyre, 2012	Parallel	Novolin	40/d, 8 weeks	34	41	27.9	Bipolar patients		=	=					
		Placebo		28	39	29.6									
Craft, 2012; Claxton, 2013	Parallel	Novolin	20/d or 40/d, 4 months	36	73	26.7	MCI/AD		↑/↓						
				38	70	26.9									
		Placebo		30	75	27.4									
Fan, 2013; Li, 2013	Parallel	Humulin	160/d, 8 weeks	21	49	28.5	Schrizophrenic outpatients		=	=					
		Placebo		24	44	32.2									
Claxton, 2015	Parallel	Levemir	20/d or 40/d, 3 weeks	21	72	27.3	MCI/AD		=	↑		=			
				19	73	25.6			↑/↓			=			
		Placebo		20	71	26.9									
Scherer, 2017	Parallel	Actrapid	160/d, 4 weeks	10	29	25.4	Healthy				=	=		=	
		Placebo		10	36	24.6									
Craft, 2017	Parallel	Levemir	20/d, 4 months	12	67	29.4	MCI/AD		↑						
		Humulin		12	71	28.8									
Ritze, 2018	Parallel	Actrapid	160/d, 8 weeks	12	27	23.5	Healthy		↑		=	=			
		Placebo													
Novak, 2019	Parallel	Novolin	40/d, 4 weeks	8	63	23.9	Parkinson's patients			↑					
		Placebo	40/d, 8 weeks	6	62	28.8	Schrizophrenic outpatients								

CBF, cerebral blood flow; MEM, memory; EXE, executive function, INS, blood insulin concentrations, GLU, blood glucose concentrations; C-PEP, blood C-peptide concentrations; LIP, blood lipid concentrations; BP, blood pressure; T2D, Type 2 Diabetes; MCI, mild cognitive impairment; AD, Alzheimer's disease. Main effects include significant increases (↑), significant decreases (↓) or no effects (=).

Supplemental Table 2 – Cochrane risk of bias assessment for studies investigating acute or longer-term internasal insulin application.

Unique study ID: Author(s) included articles	D1	DS	D2	D3	D4	D5	Overall
1: Reger, 2006	+	+	+	+	+	+	+
2: Reger, 2008a	+	+	+	+	+	+	+
3: Bohringer, 2008	+	n/a	+	+	+	+	+
4: Guthoff 2010	+	+	+	+	+	+	+
5: Krug, 2010	+	+	+	+	+	+	+
6: Stingl, 2010; Guthoff, 2011	+	+	+	+	+	+	+
7: Benedict, 2011	+	+	+	+	+	+	+
8: Hallschmid, 2012	+	+	+	+	+	+	+
9: Jauch-Chara, 2012	+	+	+	+	+	+	+
10: Heni, 2012	+	+	+	+	+	+	+
11: Brünner, 2013	+	+	+	+	+	+	+
12: Kullmann, 2013	+	+	+	+	+	+	+
13: Schilling, 2014	+	n/a	+	+	+	+	+
14: Iwen, 2014	!	+	+	+	!	+	!
15: Novak, 2014; Zhang, 2015	+	+	+	+	+	+	+
16: Rosenbloom, 2014	+	+	+	+	+	!	!
17: Heni, 2014	+	+	+	+	+	+	+
18: Kullmann, 2015; Kullmann 2017a	+	+	+	+	+	+	+
19: Brünner, 2015	+	+	+	+	+	+	+
20: Gancheva, 2015	+	+	+	+	+	+	+
21: Brünner, 2016	+	+	+	+	+	+	+
22: Feld, 2016; Thienel, 2017; Santiago, 2017	+	+	+	+	+	+	+
23: Akintola, 2017; Opstal, 2017	+	+	+	+	+	+	+
24: Rodriguez-Raecke, 2017	+	!	+	+	+	+	!

Legend: + *Low risk*; ! *Some concerns*; - *High risk*

D1, randomization process; DS, bias arising from period and carryover effects (only for crossover designs); D2, deviations from the intended interventions; D3, missing outcome data; D4, measurement of the outcome; D5, selection of the reported result.

Supplemental Table 2 – Cochrane risk of bias assessment for studies investigating acute or longer-term internasal insulin application (continued).

Unique study ID: Author(s) included articles	D1	DS	D2	D3	D4	D5	Overall
25: Krug, 2018	+	+	+	+	+	+	+
26: Kullmann, 2018	+	+	+	+	+	+	+
27: Rodriguez-Raecke, 2018	+	+	+	+	+	+	+
28: Dhindsa, 2018	+	+	+	+	+	+	+
29: Wingrove, 2019	+	+	+	+	+	+	+
30: Thanarajah, 2019	+	+	+	+	+	+	+
31: Rodriguez-Raecke, 2020	+	+	+	+	+	+	+
32: Ferreira de Sá, 2020	+	n/a	+	+	+	+	+
33: Rosenbloom, 2020	+	+	+	+	+	!	!
34: Wingrove, 2020	+	+	+	+	+	+	+
35: Kupila, 2003	+	+	+	+	+	+	+
36: Benedict, 2004, 2005; Hallschmidt, 2004	+	n/a	+	+	+	+	+
37: Benedict, 2007	+	n/a	+	+	+	+	+
38: Hallschmidt, 2008	+	n/a	+	+	+	+	+
39: Reger, 2008b	+	n/a	+	+	+	+	+
40: Fan, 2011	+	n/a	+	+	+	+	+
41: McIntyre, 2012	+	n/a	+	+	+	+	+
42: Craft, 2012; Claxton, 2013	+	n/a	+	+	+	+	+
43: Fan, 2013; Li, 2013	+	n/a	+	+	+	+	+
44: Claxton, 2015	+	n/a	+	+	+	+	+
45: Scherer, 2017	+	n/a	+	+	+	+	+
46: Craft, 2017	+	n/a	+	+	+	+	+
47: Ritze, 2018	+	n/a	+	+	+	+	+
48: Novak, 2019	+	n/a	+	+	+	+	+

Legend

+ *Low risk*

! *Some concerns*

- *High risk*

D1, randomization process; DS, bias arising from period and carryover effects (only for crossover designs); D2, deviations from the intended interventions; D3, missing outcome data; D4, measurement of the outcome; D5, selection of the reported result.

Supplemental Table 3 – Methodological information on MRI data acquisition and analysis of the eight included studies that included MRI measurements.

Author	Data acquisition	Data analysis
Schilling, 2014 [10]	**Manufacturer**: Philips. **Magnetic field**: 1.5 Tesla. **ASL technique:** 2D amplitude-modulated CASL.	**Software**: SPM8 & MATLAB. **ROI analyses**: Left/right insula, hippocampus and putamen. **Voxel-wise analyses**: Whole-brain gray matter **P-value adjustment**: Pre-insulin values and family-wise error correction.
Heni, 2014 [30]	**Manufacturer**: Siemens. **Magnetic field**: 3 Tesla. **Sequence/Tagging scheme:** 2D PASL PICORE-Q2TIPS.	**Software**: SPM8 & ASL-toolbox **Voxel-wise analyses**: Whole-brain gray and white matter and hypothalamus. **P-value adjustment**: Pre-insulin values.
Novak, 2014 [33]	**Manufacturer**: GE. **Magnetic field**: 3 Tesla. **Sequence/Tagging scheme:** 3D CASL.	**Software**: Interactive data language. **Voxel-wise analyses**: Whole-brain gray and white matter. **P-value adjustment**: None.
Kullmann, 2015 & 2017 [31, 32]	**Manufacturer**: GE. **Magnetic field**: 3 Tesla. **Sequence/Tagging scheme:** 2D PASL PICORE-Q2TIPS.	**Software**: SPM8 & ASL-toolbox. **Voxel-wise analyses**: Whole-brain gray and white matter and hypothalamus. **P-value adjustment**: Pre-insulin values, gender and family-wise error correction.
Akintola, 2017 [23]	**Manufacturer**: Philips. **Magnetic field**: 3 Tesla. **Sequence/Tagging scheme:** 3D PCASL.	**Software**: FMRIB Software Library v5.0.6. **ROI analyses**: Whole-brain gray matter, frontal lobe, temporal lobe, parietal lobe, occipital lobe, thalamus, and hypothalamus. **P-value adjustment**: None.
Kullmann, 2018 [16]	**Manufacturer**: Siemens. **Magnetic field**: 3 Tesla. **Sequence/Tagging scheme:** 2D PCASL.	**Software**: FMRIB Software Library v5.0.9. **Voxel-wise analyses**: Hypothalamus (ROI). **P-value adjustment**: Pre-insulin values, HOMA-IR and family-wise error correction.
Wingrove, 2019 [25]	**Manufacturer**: GE. **Magnetic field**: 3 Tesla. **Sequence/Tagging scheme:** 3D PCASL.	**Software**: FMRIB Software Library v3.20. **ROI analyses**: Whole-brain gray matter, left/right hippocampus, left/right amygdala. **P-value adjustment**: None.
Wingrove, 2021 [27]	**Manufacturer**: GE. **Magnetic field**: 3 Tesla. **Sequence/Tagging scheme:** 3D PCASL.	**Software**: FMRIB Software Library v3.20. **Voxel-wise analyses**: Whole-brain gray-matter and ROI for hypothalamus, amygdala, insula, and putamen. **P-value adjustment**: Individual whole-brain gray-matter cerebral blood flow, HOMA-IR and family-wise error correction

CASL, continuous arterial spin labeling; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; PASL, pulsed arterial spin labeling; pCASL, pseudo-continuous arterial spin labeling; PICORE, proximal inversion with control for off-resonance effects; Q2TIPS, Quantitative Imaging of Perfusion using a Single Subtraction II with Thin-slice TI1 Periodic Saturation; ROI, region of interest.

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CHAPTER 3

Mixed nut consumption improves brain insulin sensitivity: a randomized, single-blinded, controlled, crossover trial in older adults with overweight or obesity

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The American Journal of Clinical Nutrition, December 2023

ABSTRACT

Introduction

Improving brain insulin sensitivity, which can be assessed by measuring regional cerebral blood flow (CBF) responses to intranasal insulin, may prevent age-related metabolic and cognitive diseases. This study aimed to investigate longer-term effects of mixed nuts on brain insulin sensitivity in older individuals with overweight/obesity.

Methods

In a randomized, single-blinded, controlled, crossover trial, twenty-eight healthy adults (mean ± SD; 65 ± 3 years; BMI: 27.9 ± 2.3 kg/m^2) received either daily 60 g mixed nuts (15 g of walnuts, pistachio, cashew, and hazelnuts) or no nuts (control) for 16 weeks, separated by an 8-week washout period. Throughout the study, participants were instructed to adhere to the Dutch food-based dietary guidelines. During follow-up, brain insulin action was assessed by quantifying acute effects of intranasal insulin on regional CBF using arterial spin labeling magnetic resonance imaging. Furthermore, effects on peripheral insulin sensitivity (oral glucose tolerance test), intrahepatic lipids, and cardiometabolic risk markers were assessed.

Results

Body weight and composition did not change. Compared with control, mixed nut consumption improved regional brain insulin action in five clusters located in the left (difference in CBF responses to intranasal insulin: -4.5 ± 4.7 mL/100g/min; P<0.001; -4.6 ± 4.8 mL/100g/min; P<0.001; and -4.3 ± 3.6 mL/100g/min; P=0.007) and right occipital lobe (-4.3 ± 5.6 mL/100g/min; and -3.9 ± 4.9 mL/100g/min; P=0.028). A fifth cluster was part of the left frontal lobe (-5.0 ± 4.6 mL/100g/min; P<0.001). Peripheral insulin sensitivity was not affected. Intrahepatic lipid content (-0.7 %-point; 95%CI: -1.3 to -0.1; P=0.027), serum LDL cholesterol (-0.24 mmol/L; 95%CI: -0.44 to -0.04; P=0.019), and systolic blood pressure (-5 mmHg; 95%CI: -8 to -1; P=0.006) were lower after the mixed nut intervention.

Conclusions

Longer-term mixed nut consumption affected insulin action in brain regions involved in the modulation of metabolic and cognitive processes in older adults with overweight/obesity. Intrahepatic lipid content and different cardiometabolic risk markers also improved, but peripheral insulin sensitivity was not affected.

Trial registration number: NCT04210869 (ClinicalTrials.gov).

INTRODUCTION

Brain insulin resistance is an important pathological feature of type 2 diabetes (T2D) and other age-related diseases, such as dementia [1-3]. Within the central nervous system, insulin acts on neural circuits in a region-specific way, which may affect the regulation of peripheral energy metabolism, eating behavior, and cognitive performance [4, 5]. Various studies have combined non-invasive neuroimaging techniques with intranasal insulin administration to assess region-specific brain insulin sensitivity, which is reflected as increases or decreases in cerebral blood flow (CBF) due to changes in neuronal activity upon spray application [6, 7]. In our recent review, we have concluded that changes in brain insulin responsiveness were age-dependent, and related to obesity and T2D [7]. However, it remains largely unknown whether brain insulin resistance is reversible and can be beneficially affected by intervention strategies. Recent human trials have already provided some evidence that pharmacological and lifestyle interventions involving weight reduction or aerobic exercise training improved brain insulin responsiveness [8, 9], which may be important for the prevention of age-related metabolic and cognitive diseases [1]. The effects of dietary intervention studies on region-specific brain insulin sensitivity have however not yet been investigated before.

Ground and tree nuts are nutrient-dense foods with complex matrices rich in unsaturated fats and other bioactive compounds [10]. Evidence already indicated that the inclusion of nuts in the diet reduced the risk to develop metabolic and cognitive diseases [10, 11], but underlying mechanisms remain unknown. Recently, we have reported that in older adults with overweight or obesity longer-term mixed nut intake (60 g/day: 15 g of walnuts, pistachio, cashew, and hazelnuts) beneficially affected not only peripheral vascular function, but also vascular function in the brain [12]. The more traditional cardiometabolic risk markers, including serum lipoprotein concentrations and blood pressure [10, 13] may also improve, and previous meta-analyses have already provided evidence for beneficial effects of nut consumption on peripheral insulin sensitivity [14]. However, it remains to be investigated if effects on insulin sensitivity can also be observed specifically in the brain. We here report effects of our randomized, controlled, single-blinded, crossover trial in older adults with overweight or obesity on brain insulin sensitivity, as these individuals are at increased risk to develop brain insulin resistance [7]. Brain insulin responsiveness was non-invasively quantified by the change in regional CBF assessed using pseudo-continuous arterial spin labeling (pCASL) magnetic resonance imaging (MRI) following intranasal insulin application [15, 16], while effects on peripheral insulin sensitivity and more traditional cardiometabolic risk markers were also reported.

METHODS

The study was approved by the Medical Ethics Committee of the University Hospital Maastricht and Maastricht University and registered on ClinicalTrials.gov on December 26th, 2019 as NCT04210869, and followed the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all volunteers. This trial was performed between January 2020 and December 2022.

Study population

Healthy males and postmenopausal females were recruited via online advertisements and flyers throughout university and hospital buildings. In addition, participants of our previous intervention studies were contacted if they had given written consent. Adults were invited for a screening visit when they were aged between 60 and 70 years and had a BMI between 25 and 35 kg/m^2. During the screening visit, anthropometrics and office blood pressure (BP) were measured, and a fasting blood sample was collected. Inclusion criteria were: stable body weight (< 3 kg body weight loss or gain in the past three months); office BP below 160/100 mmHg; fasting plasma glucose < 7.0 mmol/L; fasting serum total cholesterol (TC) < 8.0 mmol/L; fasting serum triacylglycerol (TAG) < 4.5 mmol/L; and no blood donation eight weeks prior to screening and during the trial. The exclusion criteria were: allergy or intolerance to nuts; left-handedness; T2D diagnosis; familial hypercholesterolemia; active cardiovascular disease; use of medication to treat BP, lipid metabolism or glucose metabolism; severe medical conditions that interfere with the study outcomes (e.g. epilepsy or inflammatory bowel disease); use of dietary supplements known to interfere with the study outcomes; current smoker or smoking cessation less than a year ago; alcohol or drug abuse; MRI contra-indications; and participation in another biomedical intervention within one month prior to the screening visit.

Study design

A randomized controlled, single-blinded, cross-over design with a 16-week intervention and control period was conducted, separated by an 8-week washout. Except for the research assistant who provided the study products, the researchers conducting the study and analyzing the outcomes were blinded to the interventions. Study participants were allocated to start either in the intervention or control period (no nuts) based on randomization schedules in blocks of two or four stratified by sex. During the intervention period, subjects consumed sachets of 60 g (359 kcal) unsalted and unroasted mixed nuts (15 g of walnuts, cashew, hazelnuts, and pistachio; BasBoerNoten, Ridderkerk, the Netherlands), as described previously [12]. This mixture of nuts was deliberately chosen to provide a diverse mixture of bioactive components. Also, dietary guidelines do not prefer one type of nuts over the other. The empty and unused sachets were returned during follow-up to assess compliance. Throughout both periods, subjects were not

allowed to consume any products from a predefined list of foods with high amounts of n-3 PUFAs (e.g., other nuts, seeds, or fish oil capsules), and participants were requested and instructed to adhere to the Dutch food-based dietary guidelines [17]. Protocol deviations or abnormalities regarding health status, medication use, and alcohol intake were recorded in diaries. Visits included short measurements at baseline and after 8 weeks, and two follow-up visits after 16 weeks. All measurements were performed in quiet and temperature-controlled rooms (20°C). Participants fasted overnight for 12 h and abstained from alcohol and heavy exercise 48 h preceding the visits and came to the Metabolic Research Unit Maastricht by car or public transport. During all visits, body weight was measured, and BMI was calculated, and fasting blood samples were collected. Measurements of brain and peripheral insulin sensitivity, and intrahepatic lipid content were performed only during follow-up (week 16 of each period). A validated food frequency questionnaire (FFQ) was filled out after 8 weeks and at follow up, and results were averaged to assess energy and nutrient intakes based on the Dutch Food Composition Database [18].

Brain insulin sensitivity

MRI measurements were performed on a 3T MAGNETOM Prisma Fit MRI-system using a 64-channel head-neck coil (Siemens Medical Solution, Erlangen, Germany) at the Scannexus research facilities in Maastricht. CBF was measured using pCASL after 15 min of rest in the supine position. The scans were performed before and 30 min after intranasal insulin administration. Insulin was applied intranasally in the seated position by four puffs of 0.4 mL (two per nostril) at 30-s intervals, accounting for a total of 1.6 mL (160 IU Actrapid, Novo Nordisk, Mainz, Germany). The acquisition and processing have been described in detail before [12]. In short, scans were performed with background-suppressed segmented three-dimensional gradient and spin echo (GRASE) readouts. Sequence parameters included: TR 4300 ms, TE 13.9 ms, GRAPPA 2, labeling duration 1750 ms, post-labeling delay 2000 ms, segmentation factor 6, 10 label-control repetitions with 19 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 2.19 ms, TI 1040 ms, 1.0 mm isotropic resolution, 8 degrees flip angle and 160 sagittal slices) was performed. pCASL-images were analyzed using FSL (Version 6.0) and the BASIL toolbox (Version 4.0.15) [19-21], following the recommendations of the ASL White Paper [22]. Individual pCASL-images were distortion corrected with TopUp using M0 images with opposite phase-encoding directions (TR 2000 ms, labeling efficacy 0.64, T1 gray matter 1330 ms, T1 blood was based on blood hemoglobin). Mean CBF was determined after boundary-based co-registration to the MPRAGE image, which was segmented using Volbrain [20].

Blood collection and analyses

Fasting blood samples were drawn from the antecubital vein by venipuncture at baseline (week 0), during the mid-term visit (week 8) and at the end of the intervention period (week 16). Blood in serum STT-II advance tubes (Becton Dickinson, Erembodegem, Belgium) clotted for at least 30 min at room temperature before centrifugation (10 min at 1300g at 21 °C). NaF plus Na_2EDTA-containing tubes were directly placed on ice after withdrawal and immediately centrifuged (10 min at 1300g at 4 °C). After centrifugation, plasma and serum samples were distributed in aliquots, snap frozen, and stored at -80°C until analysis at the end of the study.

Peripheral insulin sensitivity

Samples from baseline, after 8 weeks, and follow-up were used for the analyses of fasting serum insulin (Millipore Corporation, Billerica, USA) and fasting plasma glucose concentrations (Horiba, ABX, Montpellier, France). During the second follow-up visit, a 7-point oral glucose tolerance test (OGTT) was performed. Blood samples were taken from an intravenous catheter before (t = 0) and 15, 30, 45, 60, 90, and 120 min after ingestion of 75 g glucose (Novolab, Geraadsbergen, Belgium) to assess serum insulin and plasma glucose concentrations. During the OGTT, participants remained seated and were not allowed to walk, eat or drink. Incremental area under curves (iAUCs) for glucose and insulin were calculated by the trapezoidal rule. The homeostasis model assessment of insulin resistance (HOMAIR) was calculated as (fasting glucose [mmol/L] × fasting insulin [mIU/L])/22.5 [23]. HOMA of β-cell function (HOMA-β) was defined as (20 × fasting insulin [mIU/L])/(fasting glucose [mmol/L] – 3.5) [23]. Matsuda index was calculated as: 10,000 / √(fasting plasma glucose [mmol/L] x fasting insulin [pmol/L]) x (mean glucose [mmol/L] x mean insulin [pmol/L]), using glucose and insulin values at time points 0, 30, 60, 90, and 120 min [24] . Disposition index was defined as: Matsuda index * (Insulin AUC0-30min / Glucose AUC0-30min). Hepatic insulin resistance index (HIRI) was calculated as: Glucose AUC0-30min * Insulin AUC0-30min [25]. The cubic spline method was used for the estimation of muscle insulin sensitivity index (MISI), defined as: dG/dt / mean insulin (pmol/L). In this formula, dG/dt is the rate of decay of plasma glucose concentration (mmol/L) during the OGTT, calculated as the slope of the least square fit to the decline in plasma glucose concentration from peak to nadir [26].

Lipid and lipoprotein metabolism, and low-grade systemic inflammation

Serum from baseline, after 8 weeks, and follow-up were used for the analyses of TC (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany), high-density lipoprotein cholesterol (HDL cholesterol; precipitation method, Roche Diagnostics, Mannheim, Germany), triacylglycerol corrected for free glycerol (GPO-Trinder, Sigma Diagnostics, St Louis, USA), and high-sensitivity C-reactive protein (hsCRP; immunoturbidimetric assay, Horiba ABX, Montpeillier, France). Low-

density lipoprotein (LDL) cholesterol concentrations were calculated using the Friedewald formula [27].

Brain-derived neurotrophic factor (BDNF)

For the analysis of brain-derived neurotrophic factor (BDNF), fasting plasma and serum samples were used from the first day of follow-up (week 16). BDNF was analyzed by an enzyme-linked immunosorbent assay (Duo Kit ELISA, R&D Systems, Minneapolis, USA) according to instructions by manufacturer. The ELISA did not distinguish between precursor BDNF (proBDNF) and mature BDNF (mBDNF), and we thus report the sum of proBDNF and mBDNF.

Office and ambulatory blood pressure

Office brachial SBP, DBP and heart rate (HR) were monitored using a semi-continuous BP monitoring device four times (Omron Intellisense M7, Nieuwegein, The Netherlands) after at least 15 min in supine position. The first measurement was discarded and the last three were averaged. Pulse pressure (PP) was defined as SBP minus DBP. Mean arterial pressure (MAP) was determined using pulse wave analyses at the brachial artery near the antecubital fossa with a tonometer (SphygmoCor v9, AtCor Medical, Australia). Central SBP and DBP were determined using the radial pulse wave based on the brachial DBP and MAP. Ambulatory BP was measured for at least 24 h using a Mobil-O-Graph (I.E.M. Inc., Stolberg, Germany). Brachial BP was recorded every 15 min during daytime and every 30 min at night to assess BP over 24 h, during daytime and nighttime, BP variability, and dipping.

Intrahepatic lipid content

Whole-body transversal scans were performed on a 3T MAGNETOM Prisma Fit MRI-system (Siemens Medical Solution, Erlangen, Germany) using a 4-channel Flex coil and 24-channel Spine Matrix coil. After initial localizer scans, abdominal MRI was performed using a 3D CAIPIRINHA-VIBE 6-point Dixon sequence (LiverLab; TR 9.0 ms, TE (1.1, 2.5, 3.7, 4.9, 6.2, 7.4) ms, flip angle 4°, slice thickness 3.5 mm). This acquired 64 axial slices at vertebral level T12 for the quantification of intrahepatic lipid content. Four sets of MR images (T1-weighted in- and opposed-phase, fat and water images) were generated. A multi-peak fat model with a complex-based water-fat separation algorithm was used [28]. Using the water-only and fat-only images extracted from the 6-point Dixon data, regions-of interest (ROIs) were manually placed throughout the hepatic lobes, in which vascular and biliary structures were avoided. Four ROIs (total surface area ≥5 cm^2) were selected for each participant and these were matched between visits on the same axial slice. Liver fat fractions for each ROI were calculated as the signal intensity from fat divided by the sum of fat and water signal intensities, and the average liver fat fraction of the ROIs was used.

Statistical analyses

Data were presented as means ± standard deviations (SDs) unless otherwise indicated. For non-normally distributed variables, as assessed with the Kolmogorov-Smirnov test, results are reported as the median changes with interquartile ranges (IQR). As described previously [12], at least 27 participants were needed to detect a 7.5% change in fasting CBF, which was the primary outcome. Based on our previous study [29], this sample size is also sufficient to detect differential changes in regional brain insulin responsiveness, which was a secondary outcome. The effects of mixed nuts on brain insulin responsiveness were assessed using voxel-wise comparison of differences in CBF before and after intranasal insulin. This analysis was performed after non-linear and linear co-registration to the Montreal Neurological Institute (MNI; 2 mm). A gray matter mask was applied, as insulin receptors throughout the brain are limited to the gray matter [1]. Repeated measure mixed effects analyses were conducted using a general linear model, which included a single-group paired difference for treatment and subject as random factor. FLAME stage 1 and 2 were run and cluster-wise interference was performed on the whole-brain excluding the cerebellum, due to issues with co-registration to the common space. A Z-threshold of 2.1 and a connectivity of 26 ($P < 0.05$) were used, and a family-wise error correction was included based on smoothness estimates. Atlasquery was used to determine the location of significant clusters in the Harvard-Oxford cortical and subcortical structural atlases.

For outcomes with repeated time measurements, linear mixed models were used including treatment, time, time*treatment, period, sex as fixed factors, participant as random factor, and baseline values as covariate. The time*treatment interaction provided information if the treatment effect was comparable at all timepoints. However, the interaction was omitted from all statistical models as it never reached statistical significance. Residual covariance structures were selected based on maximum likelihood estimation using Akaike's Information Criteria. For parameters only assessed at the end of each period, random-intercept model analyses were performed including treatment, period, and sex as fixed factors. Carry-over effects were examined by including treatment order as fixed factor, but no significant effects were found and the factor was therefore omitted from all models. Pearson's correlations were used to explore potential relationships between changes in brain insulin sensitivity and other significantly changed outcome parameters. All analyses were completed using SPSS (IBM Corp., IBM SPSS Statistics, V26, Armonk, NY, USA). P-values ≤ 0.05 were considered statistically significant.

RESULTS

Study participants

The CONSORT flow diagram is shown in **Supplemental Figure 1**. pCASL MRI data were unavailable for one participant. Intrahepatic lipid quantification could not be performed for two participants and one participant was not analyzed due to insufficient quality of the images obtained. One participant was unwilling to perform the ambulatory BP measurement. Fourteen males and fourteen females (65 ± 3 years; BMI: 27.9 ± 2.3 kg/m^2) completed the study (**Supplemental Table 1**), of which baseline characteristics have been described before (12). BMI did not change throughout the study (P=0.251), no serious adverse events or protocol deviations were reported, and compliance based on the returned sachets was excellent (median 98% [IQR: 93-100%]). Total energy and protein intakes did not differ between interventions (**Supplemental Table 2**). However, mixed nut consumption lowered carbohydrate intake compared with control (-4.9 En%; 95%CI: -7.1 to -2.5; P<0.001). Total fat intake was 5.6 En% higher (95%CI: 3.2 to 8.1; P<0.001), with lower intakes of saturated fatty acids, but higher intakes of cis-monounsaturated fatty acids, cis-polyunsaturated fatty acids, linoleic acid and alpha-linolenic acid (all, P<0.01).

Brain insulin sensitivity

Whole-brain analysis revealed six significant clusters that showed lower CBF responses to intranasal insulin following the mixed nut intervention compared to the control period (**Figure 1A-B**; **Table 1)**. Five of these clusters were mainly located in the occipital lobe. The largest cluster (1081 voxels) was located in the left lateral occipital cortex, occipital pole, and superior parietal lobule (-4.5 ± 4.6 mL/100 g/min; P<0.001). The other clusters were located in the left lateral occipital cortex (209 voxels; -4.6 ± 4.8 mL/100 g/min; P<0.001), left lingual gyrus and precuneus cortex (143 voxels; -4.3 ± 3.6 mL/100 g/min; P=0.007), right cuneal cortex, occipital pole and supracalcarine cortex (119 voxels; -4.3 ± 5.6 mL/100 g/min; P=0.028), and right lateral occipital cortex (119 voxels; -3.9 ± 4.9 mL/100 g/min; P=0.028). Another large cluster (364 voxels) within the frontal lobe was located in the left precentral, postcentral and middle frontal gyrus (-5.0 ± 4.6 mL/100 g/min; P<0.001). No significant differences between treatments were observed for CBF responses to the spray of global CBF, gray-matter CBF, cortical CBF, or subcortical CBF (**Supplemental Table 3**).

Peripheral insulin sensitivity

No significant differences between the mixed nut intervention and control period were observed for fasting plasma glucose and serum insulin concentrations, and post-load glucose iAUC and insulin iAUC during the OGTT (**Figure 2A-F**). In addition, no differences were observed for HOMAIR, HOMA-β, Matsuda Index, Disposition Index, HIRI, or MISI (**Supplemental Table 4**).

Cardiometabolic risk markers

Effects on fasting metabolic markers are summarized in **Table 2**. The mixed nut intervention lowered TC by 0.27 mmol/L (95%CI: -0.50 to -0.04; P=0.024), LDL-C by 0.24 mmol/L (95%CI: -0.44 to -0.04; P=0.019), and TC:HDL-C ratio by 0.26 (95%CI: -0.42 to -0.09; P=0.003), as compared to the control period. No significant differences between treatments were observed for HDL-C or TAG. Changes in median hsCRP concentrations were comparable between the mixed nut and control intervention at weeks 8 and 16. Effects on office and ambulatory BP are summarized in Table 3. Mixed nut consumption lowered office brachial SBP (-5 mmHg, 95%CI: -8 to -1; P = 0.006), central SBP (-7 mmHg, 95%CI: -10 to -3; P = 0.002), and MAP by 3 mmHg (95%CI: -6 to -1; P = 0.020). Moreover, lower office brachial (-3 mmHg 95%CI: -6 to -1; P = 0.009) and central PP (-5 mmHg, 95%CI: -8 to -2; P = 0.002), as well as ambulatory 24h PP (-2 mmHg, 95%CI: -4 to 0; P = 0.025), and daytime PP (-2 mmHg, 95%CI: -5 to -1; P = 0.012). The mixed nut intervention did not affect DBP, HR, BP variability or dipping (**Supplemental Figure 2 & Supplemental Table 5**). No significant correlations were observed between changes in brain insulin responsiveness and cardiometabolic risk markers.

Brain-derived neurotrophic factor

No significant differences between treatments were observed for serum and plasma BDNF concentrations (**Table 2**).

Cardiometabolic risk markers

The mixed nut intervention lowered intrahepatic lipid content by 0.7 %-point (95%CI: -1.3 to -0.1; P = 0.027) as compared to the control period (**Figure 1C**). Furthermore, the change in intrahepatic lipid content was positively correlated with the relative change in the CBF response to intranasal insulin of the left lateral occipital cortex (cluster 1: r = 0.45, P = 0.026, **Figure 1D**). However, no correlation was found for the other significant brain clusters.

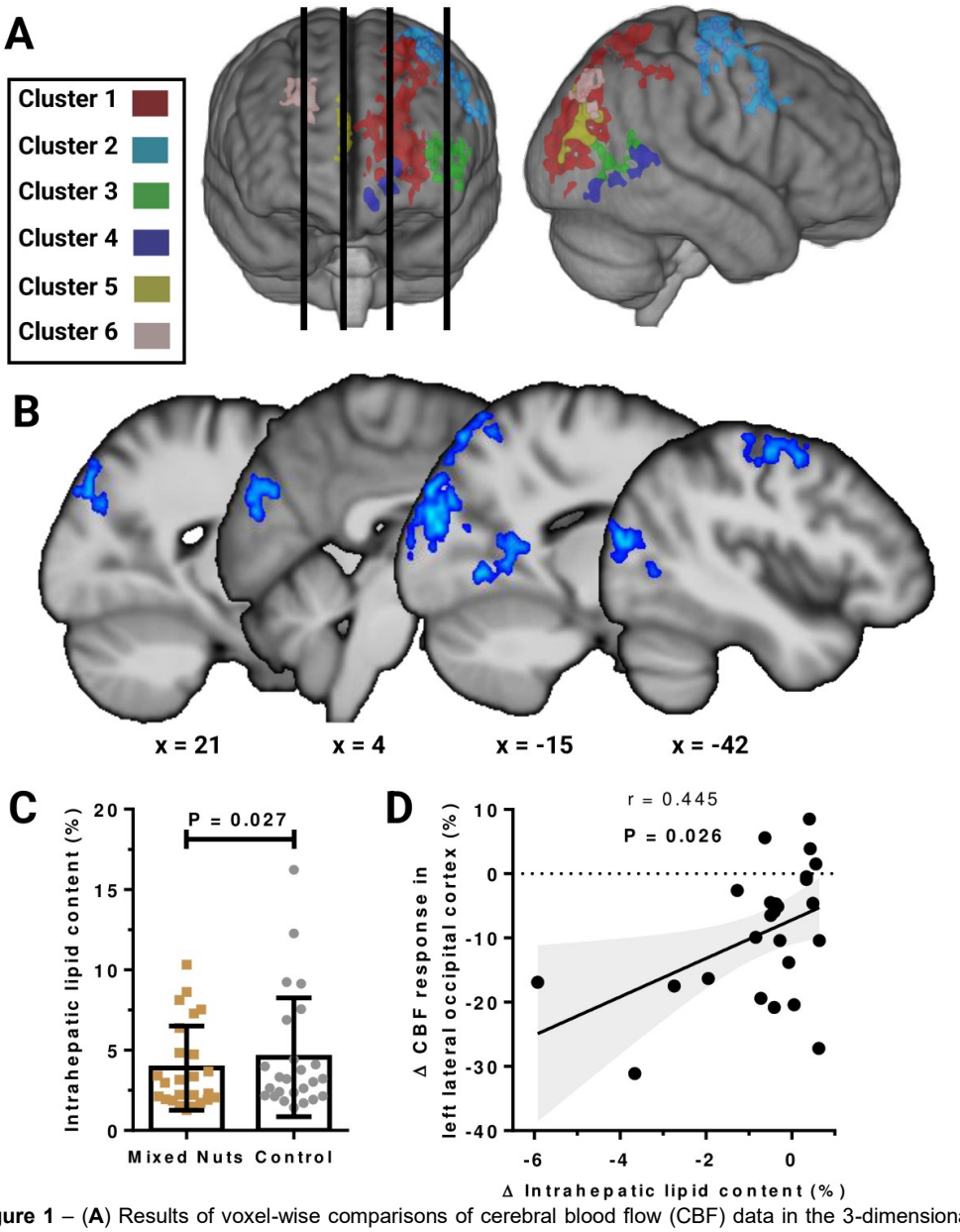


Figure 1 – (**A**) Results of voxel-wise comparisons of cerebral blood flow (CBF) data in the 3-dimensional Montreal Neurological Institute template. Six significant clusters showed lower CBF responses to intranasal insulin following mixed nut intake as compared with the control period (family-wise error corrected, n = 27). (**B**) Clusters overlaid on sagittal (posterior to anterior) slices of the structural MNI template with the color bar from dark blue to light blue indicates lower CBF responses to the nasal spray. (**C**) Intrahepatic lipid content and (**D**) correlation between changes in CBF responses to intranasal insulin of the left lateral occipital cortex (cluster 1) and those in intrahepatic lipid content following mixed nut intake. P-values for treatment effects were analyzed with linear mixed model analysis with a random-intercept, period, sex, and treatment as fixed factors and participant as random factor, and Pearson's correlation analyses were used.

Table 1 – Changes in cerebral blood flow responses to intranasal insulin in six significant clusters based on voxel-wise analyses following the mixed nut and control period[1].

Anatomical region	Mixed nuts < Control		Absolute CBF responses (mL/100 g/min)		Treatment effect[2]
	Cluster size, mm3 (voxels)	Peak MNI coordinates (x, y, z)	Mixed nut intervention	Control intervention	
Occipital lobe					
Left lateral occipital cortex, occipital pole & superior parietal lobule	8648 (1081)	-22, -86, 12	-2.7 ± 5.5	1.8 ± 4.2	-4.5 ± 4.6, **p < 0.001**
Left lateral occipital cortex	1672 (209)	-40, -64, 2	-2.6 ± 6.0	2.0 ± 5.0	-4.6 ± 4.8, **p < 0.001**
Left lingual gyrus & precuneus cortex	1144 (143)	-8, -78, -10	-2.9 ± 3.3	1.4 ± 3.6	-4.3 ± 3.6, **p = 0.007**
Right cuneal cortex, occipital pole & supracalcarine cortex	952 (119)	4, -82, 30	-3.0 ± 5.1	1.2 ± 4.4	-4.3 ± 5.6, **p = 0.028**
Right lateral occipital cortex	952 (119)	22, -82, 42	-2.5 ± 5.2	1.4 ± 4.7	-3.9 ± 4.9, **p = 0.028**
Frontal lobe					
Left precentral, postcentral & middle frontal gyrus	2912 (364)	-28, -18, 72	-2.0 ± 5.3	3.0 ± 4.7	-5.0 ± 4.6, **p < 0.001**

[1] Values are means ± SDs; n = 27. Location probability of the anatomical regions was determined using Atlasquery of FSL using the Harvard-Oxford atlas. MNI, Montreal Neurological Institute.

[2] Cluster-wise comparison; repeated measured effects analysis using a general linear model with a single group paired difference; FLAME stage 1 and 2. Mean differences (± SD) between the mixed nut and control intervention and P-values (family-wise error [FWE]-corrected) were reported.

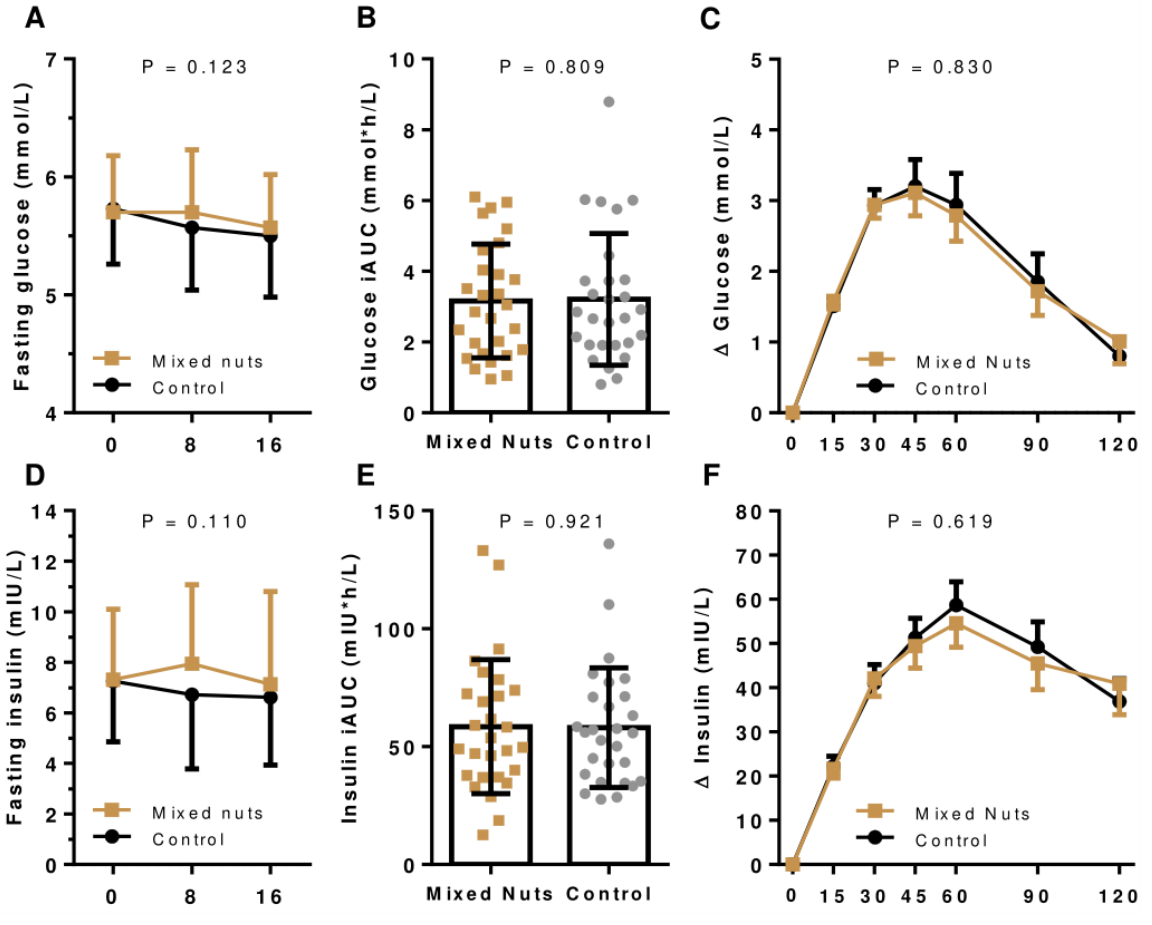


Figure 2 – Markers of peripheral insulin sensitivity following the mixed nuts intervention and control period (n = 28). (**A**) Mean (±SD) fasting plasma glucose concentrations assessed during all visits, and (**B**) mean (±SD) glucose incremental area under the curve (iAUC) and (**C**) mean (±SEM) changes in glucose concentrations during the 7-point oral glucose tolerance test (OGTT) at follow-up. (**D**) Mean (±SD) fasting serum insulin concentrations assessed during all visits, and (**E**) mean (±SD) insulin iAUC and (**F**) mean (±SEM) changes in insulin concentrations during the OGTT. *P*-values for treatment effects were analyzed with linear mixed model analysis with a random-intercept, period, sex, and treatment as fixed factors and participant as random factor.

Table 2 – Markers of lipid and lipoprotein metabolism, systemic inflammation and neurogenesis following the mixed nut intervention and control period in older adults[1].

	Mixed nut intervention			Control intervention			Treatment effect[2]
	Week 0	Week 8	Week 16	Week 0	Week 8	Week 16	
Lipid and lipoprotein metabolism							
TC (mmol/L)	6.1 ± 1.0	5.9 ± 0.9	5.9 ± 1.0	6.2 ± 1.1	6.1 ± 1.0	6.2 ± 1.1	-0.3 [-0.5, 0.0], **p = 0.024**
LDL-C (mmol/L)	4.4 ± 0.9	4.1 ± 0.7	4.1 ± 0.9	4.4 ± 0.9	4.4 ± 0.8	4.4 ± 0.9	-0.2 [-0.4, 0.0], **p = 0.019**
HDL-C (mmol/L)	1.5 ± 0.4	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.3	0.0 [0.0, 0.1], p = 0.700
TC:HDL-C ratio	4.2 ± 1.0	3.9 ± 0.8	4.0 ± 0.9	4.1 ± 0.9	4.2 ± 1.0	4.2 ± 0.9	-0.3 [-0.4, -0.1], **p = 0.003**
Low-grade systemic inflammation							
hsCRP (mg/L)	1.4 [0.8-3.9]	1.8 [0.9-4.6]	1.3 [0.6-2.7]	1.2 [0.6-2.7]	1.9 [0.5-2.4]	1.5 [0.8-3.1]	0.1 vs. -0.1, 0.0 vs. 0.2
Brain-derived neurotrophic factor							
Serum BDNF (ng/mL)	41 ± 10	-	40 ± 11	42 ± 11	-	41 ± 11	-1 [-6, 4], p = 0.759
Plasma BDNF (ng/mL)	10.4 ± 3.6	-	10.8 ± 3.4	9.4 ± 4.7	-	11.8 ± 4.0	-1.5 [-3.1, 0.1], p = 0.061

[1] Values are means ± SDs or medians [25–75th percentile]; n = 28,. TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TAG, triacylglycerol; hsCRP, high-sensitive C-reactive protein; BDNF, brain-derived neurotrophic factor.

[2] Linear mixed model analysis with random-intercept. Period, sex, time, treatment, and time*treatment interaction were used as fixed factors, participant as random factor, and baseline values as covariate. The interaction term was not statistically significant and therefore omitted from the final model. *P*-values for the effect of treatment (mean difference [95% CI]) were reported.

DISCUSSION

In this randomized, controlled, crossover trial involving older adults with overweight or obesity, the effects of a dietary intervention on regional brain insulin sensitivity were investigated for the first time. Longer-term mixed nut consumption significantly improved insulin responsiveness within occipital and frontal brain regions in participants who were requested and instructed to adhere to the Dutch food-based dietary guidelines. More specifically, the mixed nut intervention lowered CBF responses to intranasal insulin bilaterally in occipital regions (e.g. lateral occipital cortex, lingual gyrus, and cuneal cortex) that are involved in visuospatial processing and food cue perception [30, 31]. These regions are part of the default mode network (DMN), which is a network of closely interacting brain regions in the modulation of metabolic processes essential

for reward processing of food-related cues and eating behavior [5, 31]. Akintola and colleagues have also reported reduced occipital CBF responses following nasal insulin in younger adults as compared to older counterparts [32]. Hence, our results demonstrate that mixed nut consumption may improve age-related insulin responsiveness of occipital regions. The intervention also reduced insulin-induced activation of frontal brain regions, including both the precentral gyrus and middle frontal gyrus, which are involved in food-related inhibitory control [31]. Kullmann and colleagues also observed lowered CBF responses in the middle frontal gyrus after spray application in adults with normal-weight as compared to those with overweight or obesity [4]. This suggests regional brain insulin resistance associated with overweight and obesity, which can thus be beneficially affected by dietary interventions. Altogether, mixed nut consumption improved insulin sensitivity in specific brain regions showing a reduced insulin-induced activation within older adults with overweight or obesity [7].

Underlying mechanisms of nut consumption on brain insulin signaling are not known. A recent randomized controlled trial (RCT) within our research group in males with abdominal obesity has revealed that acute inorganic nitrate intake, which is well-known to increase nitric oxide (NO) bioavailability, resulted in higher CBF responses to nasal insulin in regions involved in the DMN [29]. Nut consumption has also been shown to increase NO bioavailability [33], suggesting a potential pathway to enhance neurovascular coupling [34]. We have already reported that mixed nut consumption improved vascular function of frontal and parietal regions, which may relate to the beneficial effects observed on cognitive performance [12]. We now showed that insulin responsiveness improved in other brain regions important for the modulation of metabolic processes. Since brain insulin signaling does not result in direct vasoactive effects, the observed changes in brain insulin responsiveness should reflect other underlying mechanisms, such as altered neuronal activity via the concept of functional hyperemia [35]. Alternatively, brain insulin signaling plays an important role in neurogenesis and synaptic plasticity, which can for example be reflected by changes in BDNF concentrations [1]. The mixed nut intervention did however not affect serum or plasma BDNF concentrations, which is in line with previous trials showing no effects of dietary interventions on BDNF [36, 37]. However, we cannot exclude the possibility that BDNF concentrations changed in the brain. Overall, this study provided evidence that nut consumption improved differential underlying aspects of brain function in the modulation of metabolic and cognitive processes in older adults with overweight or obesity.

A previous meta-analysis of 40 RCTs showed no effects of nut consumption on OGTT-derived indicators of peripheral insulin sensitivity, which is in line with our findings. They did find reductions in fasting insulin and $HOMA_{IR}$, but effects may only be evident in adults with (pre-)diabetes compared to normoglycemic controls [14]. Accordingly, a more recent meta-analysis showed no effects on fasting glycemic markers in metabolically healthy adults with overweight or obesity, independent of the type and dose of nut(s) consumed [38], which may explain the

absence of effects in the current study population. The relationship between peripheral and brain insulin resistance is complex and the exact contributions to metabolic and cognitive processes in the brain are unclear [1]. Various studies have demonstrated that individuals with peripheral insulin resistance, including those with obesity or T2D, may also exhibit impairments in brain insulin signaling and function [39, 40]. Conversely, regional brain insulin resistance can also manifest independently from peripheral insulin resistance, which has already been observed in patients with mild-cognitive impairment and dementia [41]. Therefore, dietary interventions have the potential to improve brain insulin sensitivity without peripheral effects, further highlighting the relevance of investigating insulin signaling in the brain.

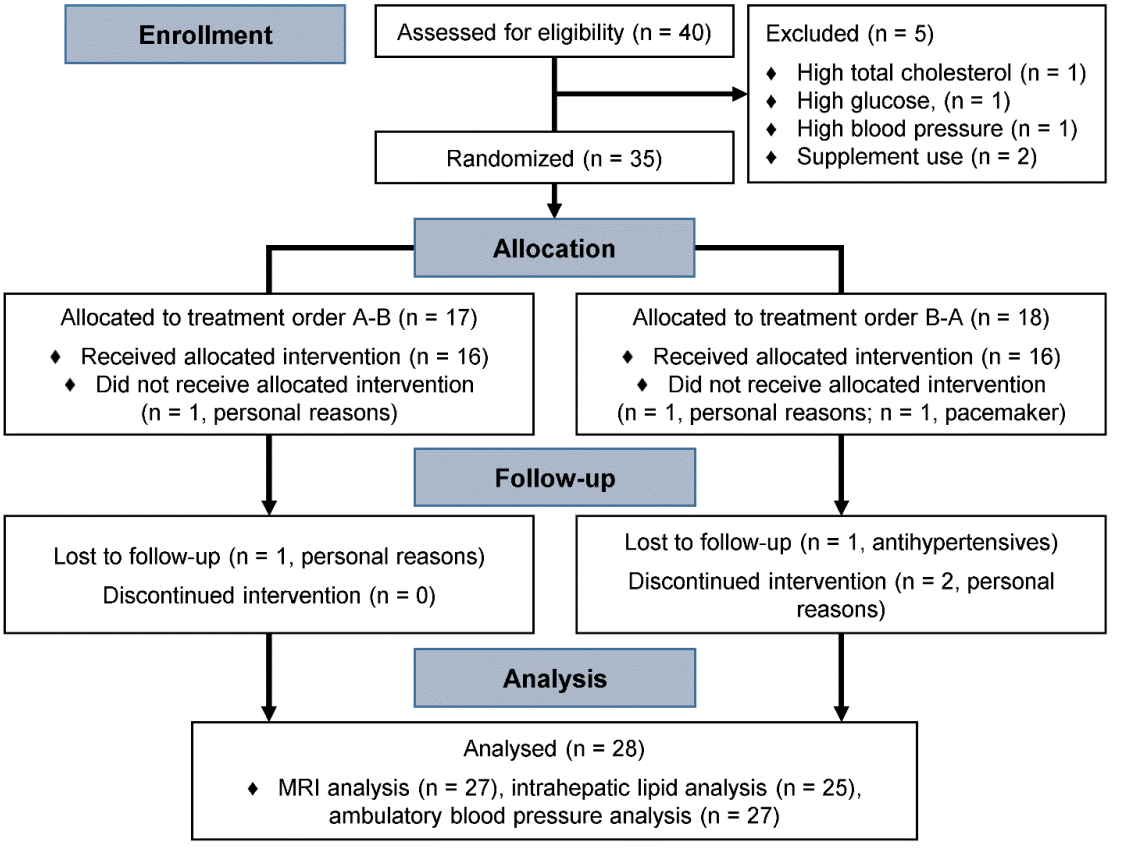
The mixed nut intervention led to a clinically relevant reduction of about 15% in intrahepatic lipid content, thereby supporting evidence for the beneficial effects of dietary interventions on liver fat independent of weight loss [42]. A previous study conducted by Cueto-Galàn *et al.* as part of the PREDIMED trial demonstrated that the inclusion of nuts into the Mediterranean diet improved the fatty liver index, an indirect marker of hepatic lipid content, in older adults at high cardiovascular disease risk after a 6-year follow-up [43]. However, other studies in healthy adults or populations with prediabetes failed to show any effects on intrahepatic lipid content [44, 45]. Our study provided for the first time some evidence for a similar relationship between intrahepatic lipid content and brain insulin sensitivity. Another recent study found that the reduction in intrahepatic lipid content following treatment with empagliflozin, an SGLT2-inhibitor, was mediated through improved hypothalamic insulin sensitivity [46]. This provides some evidence for the existence of the brain-liver axis, in which brain insulin-mediated signals may improve hepatic metabolism independently of peripheral insulin signaling [2], but further research is clearly warranted to establish this concept. The mixed nut intervention did however not affect serum TAG concentrations. In contrast, we observed reductions in TC and LDL cholesterol concentrations of 0.27 mmol/L and 0.24 mmol/L, respectively, which is in line with a meta-analysis showing pronounced effects of nut consumption on these markers with intakes over 60 g/d [13]. Possible mechanisms responsible for the cholesterol-lowering effects of nuts and its bioactive compounds include inhibition of intestinal cholesterol absorption and increased bile production [47]. We did not observe any differences in hsCRP, which is in line with a meta-analysis showing no overall effects of nut consumption on markers of low-grade systemic inflammation [48]. However, mixed nut consumption reduced different markers of office brachial and central BP, consistent with findings from previous trials [49]. Furthermore, we observed a decrease in ambulatory BP, particularly during daytime, indicating improvements in BP profiles also under free-living daily conditions. The potential antihypertensive properties of nuts may be attributed to its non-fatty acid compounds and bioactive substances, such as L-arginine that enhances NO production, which is a potent vasodilator [33].

Study strengths and limitations have been described before [12]. The study was statistically powered to detect changes in brain vascular function. Based on data from a previous study within our department [29], the current study was also adequately powered to detect differential changes in brain insulin action between treatments. Specifically, we have here observed longer-term improvements in regional brain insulin responsiveness following a dietary intervention, which have been hypothesized to translate into an improved food intake regulation that may account for the observed energy compensation as we have already reported [12]. Unfortunately, no functional outcomes specifically related to appetite control or eating behavior were measured, which clearly needs further investigation. It must however be noted that effects of nut consumption can never be disentangled from those due to the food products replaced. Moreover, participants were requested and instructed to adhere to the Dutch dietary guidelines throughout both study periods, but adherence to these guidelines could unfortunately not objectively be quantified. Furthermore, although the possibility of systemic spillover of insulin affecting the outcomes cannot be entirely ruled out, our recent systematic review suggested that the impact of spillover on brain function is minimal [7]. Moreover, a previous trial within our research group utilizing the same spray protocol and dose found no evidence of systemic spillover [29].

CONCLUSION

In conclusion, the longer-term daily intake of mixed nuts for 16 weeks improved brain insulin action in occipital and frontal regions. Regional brain insulin resistance observed in older adults with overweight or obesity can therefore be beneficially affected by intervention strategies, which may be important for the prevention of age-related metabolic diseases. Further intervention studies are needed to elucidate mechanisms underlying the effects observed on brain insulin responsiveness, and investigate whether our findings can be extrapolated to other population groups.

SUPPLEMENTAL MATERIAL



Supplemental Figure 1 – CONSORT flow diagram. In total, 35 subjects were eligible to participate who were randomized for treatment order (A = Mixed nuts intervention, B = Control intervention). During the intervention seven participants dropped out, resulting in a total of 28 subjects for the analysis. MRI data was unavailable for one participant due to missing data. Intrahepatic lipid quantification was not performed for two participants and three participants were not analyzed due to insufficient quality of the images obtained. One participant was unwilling to perform the ambulatory blood pressure measurement. This is a figure adapted from our previous manuscript [12].

Supplemental Table 1. Baseline participant characteristics[1].

	Participants (*n* = 28)
Men / women (%)	50 / 50
Age (years)	65 ± 3
Body weight (kg)	83 ± 10
BMI (kg/m^2)	27.9 ± 2.3
Systolic BP (mmHg)	129 ± 13
Diastolic BP (mmHg)	84 ± 7
Glucose (mmol/L)	5.6 ± 0.5
Triacylglycerol (mmol/L)	1.2 ± 0.6
Total cholesterol (mmol/L)	5.8 ± 1.0

[1] Values are means ± SDs. BMI, Body Mass Index; BP, blood pressure.

Supplemental Table 2. Daily energy and nutrient intake following the mixed nut intervention and control period in older adults[1].

	Mixed nut intervention	**Control intervention**	**Treatment effect[2]**
Total energy (Kcal)	2277 ± 507	2301 ± 495	-25 [-297, 247], p = 0.853
Protein (En%)	16.5 ± 2.3	16.1 ± 2.1	0.5 [-0.7, 1.6], p = 0.394
Carbohydrates (En%)	37.2 ± 3.8	42.0 ± 5.2	-4.9 [-7.1, -2.5], p < 0.001
Total fat (En%)	42.1 ± 3.8	36.5 ± 5.6	5.6 [3.2, 8.1], p < 0.001
Total SFA (En%)	10.9 ± 3.0	12.8 ± 2.3	-1.8 [-3.2, -0.5], p = 0.010
Total cis-MUFA (En%)	16.5 ± 2.1	13.0 ± 2.8	3.4 [2.2, 4.7], p < 0.001
Total cis-PUFA (En%)	10.8 ± 1.8	7.3 ± 2.4	3.6 [2.5, 4.7], p < 0.001
Linoleic acid (En%)	9.2 ± 1.6	5.9 ± 1.9	3.3 [2.4, 4.2], p < 0.001
α-Linolenic acid (En%)	0.9 ± 0.3	0.7 ± 0.3	0.2 [0.1, 0.3], p < 0.001
Cholesterol (mg/MJ)	27.0 ± 7.8	29.8 ± 7.6	-2.7 [-6.6, 1.1], p = 0.160
Fibers (g)	27.4 ± 5.2	26.0 ± 5.7	1.4 [-1.4, 4.2], p = 0.334

[1] Values are means ± SDs; n = 28. The investigational product was included, and data after 8 weeks and follow up were averaged. En%, energy percentage. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[2] Linear mixed model analysis with random-intercept. Period, gender, and treatment were used as fixed factors, and participant as random factor. P-values for the effect of treatment (mean difference [95% CI] between the mixed nut and control intervention) were reported.

Supplemental Table 3 – (Regional) cerebral blood flow (CBF, mL/100 g/min) pre- and post-intranasal insulin administration following the mixed nut intervention and control period[1].

	Mixed nuts		Control		Treatment effect[2]	
	Pre	Post	Pre	Post	Treatment	Treatment *Insulin
Global CBF	39.6 ± 6.0	39.3 ± 6.6	38.6 ± 6.8	38.6 ± 6.9	0.164	0.801
Grey matter CBF	47.2 ± 7.1	46.7 ± 7.5	45.8 ± 7.7	45.8 ± 8.0	0.094	0.800
Cortical CBF	51.9 ± 7.9	51.3 ± 8.3	50.4 ± 8.6	50.4 ± 8.8	0.129	0.744
Subcortical CBF	31.9 ± 6.6	31.8 ± 7.2	31.1 ± 7.2	31.4 ± 8.1	0.206	0.683

[1]Values are means ± SDs; n = 27. CBF, cerebral blood flow.

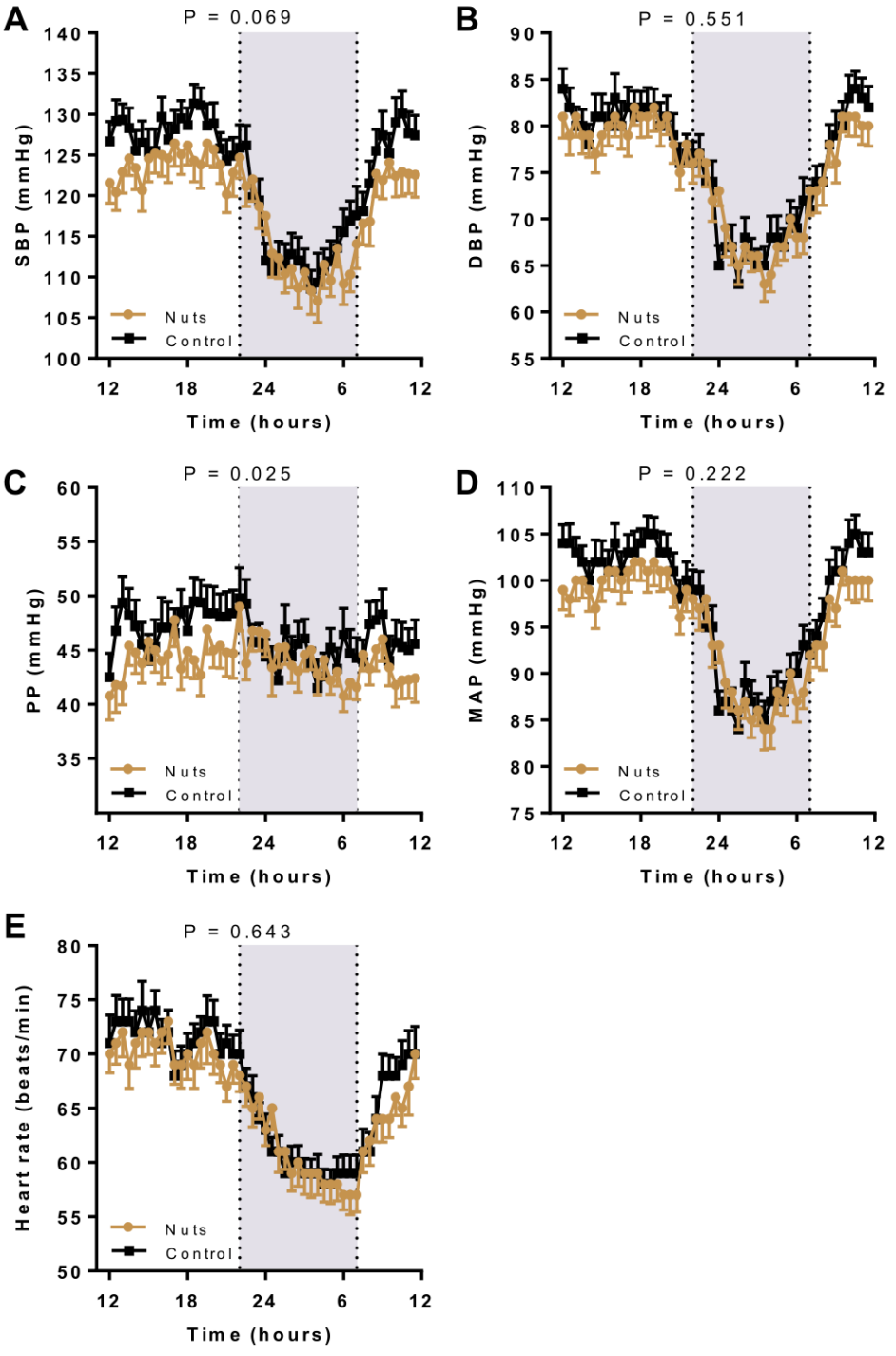
[2]Treatment effect (95% CI) for regional approach (random-intercept model with treatment, insulin, treatment*insulin, period and sex as fixed factors).

Supplemental Table 4 – Peripheral insulin sensitivity markers derived from the oral glucose tolerance test and intrahepatic lipid content following the mixed nut intervention and control period[1].

	Mixed nuts	Control	Treatment effect[2]
$HOMA_{IR}$ (AU)	1.8 ± 1.0	1.6 ± 0.7	0.2 [-0.1, 0.4], p = 0.222
HOMA-β (AU)	70.0 ± 34.4	68.4 ± 29.1	1.4 [-7.8, 10.6], p = 0.762
Matsuda Index (AU)	17.8 ± 8.0	17.6 ± 7.6	0.2 [-2.5, 2.9], p = 0.897
Disposition Index (AU)	385 ± 165	376 ± 160	8 [-51, 67], p = 0.780
HIRI (AU)	313 ± 161	296 ± 142	18 [-30, 66], p = 0.452
MISI (AU)	0.20 ± 0.12	0.18 ± 0.11	0.02 [-0.03, 0.06], p = 0.478

[1] Values are means ± SDs; n = 28. iAUC, incremental area under the curve; $HOMA_{IR}$, homeostatic model assessment for insulin resistance; HOMA-β, homeostatic model assessment of β-cell function; HIRI, hepatic insulin resistance index; MISI, muscle insulin sensitivity index.

[2] Linear mixed model analysis with random-intercept. Period, sex, and treatment were used as fixed factors and participant as random factor. *P*-values for the effect of treatment (mean difference [95% CI] between the mixed nut and control intervention) were reported.



Supplemental Figure 2 – Mean (± SEM) ambulatory blood pressure following the mixed nut and control period (n = 27). (A) Systolic blood pressure (SBP), (B) Diastolic blood pressure (DBP), (C) Pulse pressure (PP), (D) Mean arterial pressure (MAP), and (E) Heart rate (HR).

Supplemental Table 5 – Daytime and nighttime ambulatory blood pressure, blood pressure variability and dipping following the mixed nut intervention and control period[1].

	Mixed nuts	Control	Treatment effect[2]
Daytime blood pressure			
SBP (mmHg)	124 ± 10	127 ± 19	-4 [-7, 0], p = 0.051
DBP (mmHg)	79 ± 8	80 ± 9	-1 [-4, 2], p = 0.566
PP (mmHg)	44 ± 7	47 ± 7	-2 [-5, -1], p = 0.012
MAP (mmHg)	100 ± 8	102 ± 8	-2 [-5, 1], p = 0.172
HR (beats/min)	69 ± 7	70 ± 8	-1 [-3, 1], p =0.447
Nighttime blood pressure			
SBP (mmHg)	111 ± 10	112 ± 10	-1 [-5, 3], p = 0.562
DBP (mmHg)	68 ± 7	68 ± 7	0 [-2, 3], p = 0.829
PP (mmHg)	43 ± 6	44 ± 7	-1 [-3, 1], p = 0.433
MAP (mmHg)	88 ± 8	88 ± 8	0 [-3, 3], p = 0.849
HR (beats/min)	59 ± 8	59 ± 7	0 [-3, 3], p = 0.825
Blood pressure variability and dipping			
SBP variability (mmHg)	15 ± 3	15 ± 3	0 [-2, 1], p = 0.526
DBP variability (mmHg)	11 ± 2	11 ± 2	-1 [-2, 1], p = 0.397
PP variability (mmHg)	12 ± 4	13 ± 3	-1 [-2, 1], p = 0.292
MAP variability (mmHg)	11 ± 2	11 ± 2	0 [-1, 1], p = 0.598
HR variability (mmHg)	10 ± 4	11 ± 3	-1 [-3, 1], p = 0.585
Dipping SBP (%)	13 ± 6	10 ± 6	3 [0, 5], p = 0.068
Dipping DBP (%)	15 ± 7	14 ± 7	2 [-2, 5], p = 0.328

[1] Values are means ± SDs; n = 27. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate.

[2] Linear mixed model analysis with random-intercept. Period, sex, and treatment were used as fixed factors and participant as random factor. *P*-values for the effect of treatment (mean difference [95% CI] between the mixed nut and control intervention) were reported.

ACKNOWLEDGEMENTS

The authors thank all volunteers for participating in this study, E. Vanbrabant, L. van Brakel, and N.C.C. Thijssen for practical support throughout this study and M. Beckers for the biochemical analyses.

FUNDING STATEMENT

This study was supported by a grant obtained from the International Nuts and Dried Fruit Council (INC). The INC had no role in the study design, data collection or analysis, decision to publish, or preparation of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

RPM, JP and PJJ designed research; KMRN conducted research; KMRN, RPM, and PJJ analyzed data and performed and discussed statistical analysis; KMRN, RPM, and PJJ wrote the manuscript; DI and HP reviewed the manuscript. RPM, JP, and PJJ had primary responsibility for final content; All authors read and approved the final manuscript.

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CHAPTER 4

Longer-term mixed nut consumption improves brain vascular function and memory: a randomized, controlled crossover trial in older adults

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Clinical Nutrition, June 2023

ABSTRACT

Introduction

Nut consumption may reduce age-related cognitive decline, but underlying mechanisms are unclear. Therefore, we investigated in older adults the longer-term effects of mixed nut consumption on brain vascular function, which may underlie improvements in cognitive performance.

Methods

Twenty-eight healthy individuals (age 65 ± 3 years; BMI: 27.9 ± 2.3 kg/m^2) were included in a randomized, single-blinded, cross-over trial with a 16-week intervention (60 g/d mixed nuts: walnuts, pistachio, cashew, and hazelnuts) and control period (no nuts), separated by 8 weeks of washout. Participants followed the Dutch food-based dietary guidelines. At the end of each period, cerebral blood flow (CBF), a marker of brain vascular function, was quantified using arterial spin labeling magnetic resonance imaging. Effects on endothelial function, arterial stiffness, and the retinal microvasculature were also assessed. Cognitive performance was measured using the Cambridge Neuropsychological Test Automated Battery.

Results

Body weight remained stable during the study. As compared to the control period, the mixed nut intervention resulted in a higher regional CBF in the right frontal and parietal lobes (treatment effect: 5.0 ± 6.5 mL/100g/min; P<0.001), left frontal lobe (5.4 ± 7.1 mL/100g/min; P<0.001), and bilateral prefrontal cortex (5.6 ± 6.6 mL/100g/min; P<0.001). Carotid artery reactivity (0.7 PP; 95%CI: 0.2 to 1.2; P=0.007), brachial flow-mediated vasodilation (1.6 PP; 95%CI: 1.0 to 2.2; P<0.001) and retinal arteriolar calibers were higher (2 µm; 95%CI: 0 to 3; P=0.037), and carotid-to-femoral pulse wave velocity lower (-0.6 m/s; 95%CI: -1.1 to -0.1; P=0.032). Further, visuospatial memory (-4 errors [16%]; 95%CI: -8 to 0; P=0.045) and verbal memory (+1 correct [16%]; 0 to 2; P=0.035) improved, but executive function and psychomotor speed did not change.

Conclusions

Longer-term mixed nut consumption as part of a healthy diet beneficially affected brain vascular function, which may relate to the observed beneficial effects on memory in older adults. Moreover, different characteristics of the peripheral vascular tree also improved.

Trial registration number: NCT04210869 (ClinicalTrials.gov).

INTRODUCTION

Effective nutritional interventions are highly needed to prevent or reduce the global burden of age-related conditions, such as cardiovascular disease (CVD) and cognitive decline [1]. Besides the well-known beneficial effects on CVD risk, studies have also suggested that nut consumption protects against cognitive impairments [2], but underlying mechanisms remain to be elucidated. Nuts are nutrient-dense foods that are rich in bioactive components, including unsaturated fatty acids, polyphenols, fibers, phytosterols, tocopherols and proteins, which may all affect cognitive performance [3]. In fact, studies that incorporated mixed nuts into the Mediterranean diet have shown beneficial effects on cognitive performance in older adults [4, 5].

Randomized controlled trials (RCTs) have already shown nut-induced beneficial effects on impaired vascular function, which is a common denominator for CVD and cognitive decline [6]. Most of these trials have however focused on vascular function of peripheral arteries [7, 8] and evidence for effects on central arteries in closer proximity to the brain is limited. Therefore, an integrated approach combining different non-invasive vascular function measurements will add to a better understanding of the effects of nut consumption on characteristics of the vascular tree. Also, it is highly relevant to study if the beneficial effects on peripheral arteries can be extended to the brain vasculature, which is more directly associated with cognitive performance [9]. Recently, the Walnuts And Healthy Aging (WAHA) trial showed that two years of daily ±40 g walnut consumption did not improve cognitive performance in healthy adults. Post-hoc analyses however showed that cognitive benefits were observed following the intervention in a subgroup of participants at higher risk of cognitive decline [10], but effects on whole-brain cerebral blood flow (CBF) - a well-known physiological marker for brain vascular function [11] - were not observed. However, regional CBF can either be increased or decreased, which could explain the absence of an effect at the whole-brain level. The non-invasive magnetic resonance imaging (MRI) perfusion method pseudo-continuous arterial spin labeling (pCASL) can be used to quantify changes in regional CBF, which may underlie effects on changes in cognitive performance [12]. Therefore, the primary aim of this randomized, controlled, single-blinded crossover trial was to study for the first time the effects of longer-term nut consumption on regional CBF in older adults. As part of a recommended diet, a mixture of nuts (60 g/day: 15 g of walnuts, pistachio, cashew, and hazelnuts) was given to provide a rich matrix of bioactive compounds. Further, endothelial function, arterial stiffness, and retinal microvascular calibers were non-invasively assessed. Finally, cognitive performance was assessed using the Cambridge Neuropsychological Test Automated Battery (CANTAB).

METHODS

Study population

Apparently healthy men and postmenopausal women were recruited via online advertisements and posters in university and hospital buildings. Also, adults who participated in our previous intervention studies and agreed to be contacted for future studies were approached. After providing and discussing study information, volunteers were invited for a screening visit. During screening, a medical questionnaire and MRI safety screening list were completed, anthropometrics and office blood pressure were measured, and a fasting blood sample was drawn. Volunteers were eligible to participate if they met the following inclusion criteria: aged between 60 and 70 years; BMI between 25 and 35 kg/m^2; fasting plasma glucose <7.0 mmol/L; fasting serum total cholesterol <8.0 mmol/L; fasting serum triacylglycerol <4.5 mmol/L; systolic blood pressure <160 mmHg and diastolic blood pressure <100 mmHg; stable body weight (weight gain or loss <3 kg within 3 months); no blood donation 8 weeks prior to the screening visit and during the study. The exclusion criteria were: nut allergy or intolerance; left-handedness; current smoker or smoking cessation <12 months; medical conditions that interfered with the study outcomes (e.g., diabetes or active cardiovascular disease); familial hypercholesterolemia; drug or alcohol abuse; use of medication or dietary supplements known to interfere with the study outcomes; MRI contra-indications; participation in another biomedical intervention study within one month prior to screening. Written informed consent was obtained from all volunteers. The study was approved by the medical ethics committee of the University Hospital Maastricht and Maastricht University, registered on ClinicalTrials.gov on December 26th, 2019 as NCT04210869, and conducted from January 2020 until December 2021 according the guidelines of the Declaration of Helsinki.

Study design

The study had a randomized controlled, single-blinded, cross-over design with a 16-week intervention and control period, separated by a washout period of 8 weeks. Study participants were allocated to start either in the intervention or control period (no nuts) based on randomization stratified by sex in blocks of two or four. During the intervention period, participants consumed daily sachets providing 60 g unsalted and unroasted mixed nuts (BasBoerNoten, Ridderkerk, the Netherlands). This amount was chosen to provide ~15% of energy, which is similar to that used in the WAHA trial [10] and showed the most pronounced cardiovascular benefits in a meta-analysis [13]. Each sachet contained 15 g of walnut, cashew, hazelnut and pistachio (**Supplemental Table 1**). The study products were provided by a research assistant at the start of the period and after 8 weeks, and had to be stored at room temperature. Participants did not receive instructions to consume the study product at specific timepoints. Empty and

unused sachets had to be returned during the visits at 8 and 16 weeks to assess compliance. Throughout the study, participants were requested to adhere to the Dutch food-based dietary guidelines [14]. Study volunteers were not allowed to consume any products from a predefined list of food products with relatively high amounts of *n*-3 PUFA (e.g., other nuts, seeds, or fish oil capsules). Finally, participants were requested to record in diaries any protocol deviations or changes in their health status, medication use, and alcohol intake.

Measurements were performed at the start of the control and intervention periods (week 0), halfway (week 8), and during two follow-up days at the end of each period (week 16) with an interval of at least 3 days. All measurements were performed in a quiet and temperature-controlled room (20 °C). Before each visit, participants were requested to fast overnight for 12 h, to abstain from alcohol and heavy exercise 48 h preceding the measurement days and came to the Metabolic Research Unit Maastricht by car or public transport to standardize measurements as much as possible. Height was measured once during the screening visit using a stadiometer. During all visits, anthropometrics were measured as changes in body composition could interfere with the effects observed [15]. Body weight was measured and BMI was calculated. Body fat distribution was assessed by measuring the waist and hip circumference to calculate the waist-to-hip ratio (WHR). Furthermore, skinfold thickness of the biceps, triceps, subscapular and supra-iliac areas were measured to calculate body fat percentage based on the Durnin and Womersley formula [16]. During each visit, a validated FFQ was filled out to assess energy and nutrient intakes over the past month based on the Dutch Food Composition Database [17]. At the end of the period, measurements of brain and peripheral vascular function, and cognitive performance were performed and a fasting blood sample was collected in Na_2EDTA-containing tubes for the quantification of the fatty acid composition of plasma phospholipids as physiological biomarker for compliance. Tubes were directly placed on ice after withdrawal and immediately centrifuged (10 min at 1300g at 4 °C). After centrifugation, plasma samples were distributed in aliquots, snap frozen, and stored at -80°C until analysis at the end of the study. Gas chromatography–triple quadrupole mass selective detection (GC-TQMS, Agilent 7000 TQMS) was used for the quantification of the fatty acid composition of plasma phospholipids, as described previously [18].

Brain vascular function

MRI measurements were performed in supine-position using a 3T MAGNETOM Prisma Fit MRI-system and a 64-channel head-neck coil (Siemens Medical Solution, Erlangen, Germany) following 15 min of rest at the Scannexus research facilities in Maastricht. CBF was measured using pseudo-continuous ASL (pCASL), of which the acquisition and processing have been described in detail before [19]. In short, the scan was performed with background-suppressed segmented 3D gradient and spin echo readouts (TR 4300 ms, TE 13.6 ms, GRAPPA 2, labeling duration 1750 ms, post-labeling delay 2000 ms, segmentation factor 6, ten label-control repetition

with 19 slices and 3.0 mm isotropic voxel resolution). Preceding each pCASL, a high-resolution anatomical 3D magnetization-prepared rapid acquisition with gradient echo (MPRAGE) scan (TR 2400 ms, TE 2.19 ms, TI 1040 ms, 1.0 mm isotropic resolution, 8 degrees flip angle and 160 sagittal slices) was performed. pCASL-images were analyzed using FSL (Version 6.0) and the BASIL toolbox (Version 4.0.15) [20, 21]. Individual pCASL-images were distortion corrected with TopUp using M_0 images with opposite phase-encoding direction and a TR of 20 s. CBF quantification was performed based on the ASL White Paper [22] and assuming a labeling efficacy of 0.64 (four background suppression pulses; 0.934), a T1 of gray matter of 1330 ms, and the T1 of blood was calculated using the hemoglobin blood concentrations of participants measured during that visit. CBF was averaged in predefined regions: whole-brain, gray-matter, cortical and subcortical after Boundary-Based co-registration to the MPRAGE image, which was segmented using Volbrain [20].

Endothelial function

Ultrasound echography in B-mode using a 13-MHz transducer (MyLab Gamma, Esaote, Maastricht, the Netherlands) with continuous recording was used to visualize the left common carotid artery proximal to the bulbous. The carotid artery reactivity (CAR) response to a cold pressure test was determined, which consisted of a 1-min baseline period and 3-min immersion of the hand in a bucket of ice water (~4 °C). Images were analyzed offline using a custom-written Matlab program with automated edge-detection and wall tracking (MyFMD; Dr. AP Hoeks, Dept of Biomedical Engineering, MUMC+, Maastricht, the Netherlands). The baseline carotid artery diameter was averaged over the first min and diameters were averaged over 20-s intervals during immersion. The maximal percentage change in post-immersion diameter relative to baseline was calculated [23]. Ultrasound echography was also used to assess brachial artery flow-mediated vasodilation (FMD). Following a 3-min baseline period, distal hypoxia was induced by inflating a pneumatic cuff around the forearm to 200 mmHg for 5-min, followed by a 5-min post-occlusive reactive hyperemia response. The FMD was analyzed using the same software as for the CAR. The FMD response was quantified as the maximal percentage diameter change post-occlusion relative to the baseline diameter. Ratio-scaled FMD was reported based on the recommendations of an expert consensus [24], but allometric scaling was also performed to correct for changes in the baseline diameter [25].

Arterial stiffness

Carotid-to-femoral pulse wave velocity ($PWV_{c\text{-}f}$) was assessed in triplicate with a tonometer (SphygmoCor v9, AtCor Medical, West Ryde, Australia) according to the current guidelines [26] by using the direct distance between the left carotid and femoral artery. Radial artery pulse wave analysis was performed in triplicate to assess the central augmentation index adjusted for heart

rate (cAIxHR75) [26]. Furthermore, five-to-six heartbeats of the baseline period of the CAR measurement were analyzed to determine the systolic and diastolic diameters of the carotid artery using a custom-written MATLAB program (VidArt V13.5, Dr. AP Hoeks, Department of Biomedical Engineering, Maastricht University Medical Centre, Maastricht, the Netherlands). Radial tonometry-derived central blood pressure was used for the assessment of the carotid β_0-stiffness index, carotid compliance, and carotid distensibility, as described previously [27].

Retinal microvasculature

Retinal images were taken using a retinal camera (Topcon TRC-NW300; Topcon Co., Tokyo, Japan) that focused on the right optic disc to assess microvascular structure [28]. Images were analyzed using the semi-automated interactive vessel analysis software (IVAN, University of Wisconsin, Wisconsin, USA). Diameters of at least three arteriolar and venular segments were analyzed digitally to assess the arteriolar (CRAE) and venular caliber (CRVE), and the arteriolar-to-venular ratio (AVR) using the Parr-Hubbard formula [29]. The analyzed vascular segments had to be exactly the same for a particular participant at both measurements.

Cognitive performance

Cognitive performance was assessed at the end of each period using CANTAB [30]. These validated, computerized assessments measured performance in cognitive domains of memory, psychomotor speed and executive function, of which a comprehensive overview of specific cognitive tests and reported variables is provided in **Supplemental Table 2**. In brief, memory was assessed using the Delayed Matching to Sample (DMS), Paired Associates Learning (PAL), and Verbal Recognition Memory (VRM), psychomotor speed was assessed by the Reaction Time (RTI) task, and executive function was assessed by the Multitasking Test (MTT). Furthermore, we assessed mood using the single-item Affect Grid [31], quality of life using a 32-item questionnaire across seven domains [32], non-specific stress with the 10-item Perceived Stress Scale [33], while the Pittsburgh Sleep Quality Index was used to measure sleep quality [34].

Statistical analyses

Data are presented as means ± SDs unless otherwise indicated. It was determined before the start of the study, that 27 participants were needed to detect a 7.5% change in CBF, which was the primary outcome, with a within-subject variability of 12%, 90% power and a two-sided alpha of 0.05. For the evaluation of the effects of mixed nuts on regional CBF, voxel-wise comparison was performed after (non-)linear co-registration to the Montreal Neurological Institute (MNI; 2 mm) template to account for small differences in sulci or gyri across participants. FLAME stage 1 and 2 were run and cluster-wise interference was performed on the whole-brain without prior selection of predefined regions of interest. This approach has the advantage to limit the analyses

not to specific brain regions and to avoid that potentially relevant changes in other areas in the brain are missed [35]. The cerebellum was excluded due to issues with co-registration to the common space. We used a Z-threshold of 2.1, a voxel 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.

For all outcomes, the residuals were normally distributed based on the Shapiro-Wilk test. Differences between the intervention and control period over time were examined using linear mixed model analyses including treatment, time, time*treatment, period, sex as fixed factors, participant as random factor, and baseline values as covariate. The time*treatment interaction provided information if the effect of treatment was comparable at all timepoints (week 8 and week 16). However, the interaction was omitted from all statistical models as it never reached statistical significance. Residual covariance structures were selected based on maximum likelihood estimation using Akaike's Information Criteria (AIC). For parameters only assessed at the end of each period, random-intercept model analyses were performed including treatment, period, and sex as fixed factors. Carry-over effects were examined by including treatment order as fixed factor, but no significant effects were found and the factor was therefore omitted from all models. All analyses were completed using SPSS (IBM Corp., IBM SPSS Statistics, V26, USA). P-values ≤ 0.05 were considered statistically significant.

RESULTS

Study participants

The CONSORT flow diagram of study participants is shown in **Supplemental Figure 1**. Thirty-five men and women were eligible to participate. Five participants dropped out due to personal reasons, and two others received either a pacemaker or blood pressure medication. In total, 14 males and 14 females completed the study. For one participant, one MRI measurement could not be performed due to personal reasons. Retinal microvascular calibers could not be analyzed for three participants due to insufficient quality of the images and one CAR measurement could also not be analyzed due to technical issues. Baseline participant characteristics are reported in **Supplemental Table 3**. Study participants had a mean age of 64.6 ± 3.2 years and BMI of 27.9 ± 2.3 kg/m^2. No serious adverse events or protocol deviations were reported and mixed nut intake was well-tolerated. Compliance was excellent, with a median of 98% (IQR: 93-100%) of the sachets were consumed during the intervention period. No differences in body weight, BMI waist circumference, WHR and body fat percentage were observed between treatments (**Table 1**).

Table 1 – Anthropometrics during the mixed nut and control intervention[1].

	Mixed nut intervention			Control intervention			Treatment effect[2]
	Week 0	Week 8	Week 16	Week 0	Week 8	Week 16	
Weight (kg)	83 ± 10	82 ± 9	83 ± 10	83 ± 11	82 ± 10	82 ± 10	0.3 [-0.4, 1.0], p = 0.405
BMI (kg/m^2)	28.0 ± 2.3	28.0 ± 2.3	28.0 ± 2.5	28.0 ± 2.6	27.9 ± 2.6	27.8 ± 2.5	0.2 [-0.1, 0.4] p = 0.251
WC (cm)	97 ± 8	95 ± 7	97 ± 9	96 ± 8	97 ± 8	96 ± 8	-0.1 [-0.9, 0.7], p = 0.845
WHR	0.91 ± 0.06	0.90 ± 0.06	0.91 ± 0.06	0.91 ± 0.06	0.91 ± 0.06	0.91 ± 0.06	0.00 [-0.01, 0.01], p = 0.817
Body fat (%)	33.4 ± 5.8	33.1 ± 6.0	32.4 ± 5.8	33.5 ± 5.3	32.9 ± 5.3	31.8 ± 5.5	0.5 [-0.2, 1.1], p = 0.168

[1] Values are means ± SDs; n = 28. WC, waist circumference; WHR, waist-to-hip ratio.

[2] For each variable, the first linear mixed model included period, sex, time, treatment, and time*treatment interaction as fixed factors, participant as random factor, and baseline values as covariate. The interaction term however never reached statistical significance and therefore omitted from the final model. *P*-values for the effect of treatment (mean difference [95% CI] between the mixed nut and control intervention) were reported.

The FFQs, which included the investigational product, showed that total energy and protein intakes were not different between interventions (**Supplemental Table 4**). However, mixed nut consumption decreased carbohydrate (-4.3 En%; 95%CI: -5.5 to -3.1; P<0.001) and cholesterol intake (-2.6 mg/MJ; 95%CI: -4.2 to -0.9; P=0.004), and increased fiber intake (1.6 g; 95%CI: 0.3 to 3.0; P=0.019) compared with the control. In contrast, total fat intake was 5.4 En% higher (95%CI: 4.1 to 6.8; P<0.001), with lower intakes of SFA, but higher intakes of cis-MUFA, cis-PUFA, linoleic acid (LA) and alpha-linolenic acid (ALA) (all, P<0.001). Compliance was further confirmed by plasma phospholipid analyses. SFA were 0.9%-point (PP) lower (95%CI: -1.2 to -0.5; P=0.007) following the mixed nut intervention, mainly due to a decrease in palmitic acid (0.8 PP; 95%CI: -1.4 to -0.3; P=0.002) (**Table 2**). Total MUFA did not change, but total PUFA was 1.3 PP higher (95%CI: 0.7 to 1.8; P<0.001). Total n-3 PUFA levels were not different between interventions, but higher proportions of ALA (0.02 PP; 95%CI: 0.00 to 0.03; P=0.041) and lower proportions of DHA (-0.28 PP; 95%CI: -0.47 to -0.08; P=0.007) were found after mixed nut intake. Total n-6 PUFAs were 1.4 PP higher after the intervention (95%CI: 0.8 to 2.1; P<0.001); LA significantly increased with 2.2 PP (95%CI: 1.3 to 2.8; P<0.001), while arachidonic acid was 0.3 PP lower (95%CI: -0.7 to 0.0; P=0.037).

Table 2 – Plasma phospholipid fatty acid profiles after the mixed nut and control intervention[1].

	Mixed nut intervention	Control intervention	Treatment effect[2]
Total SFA (%)	51.0 ± 1.0	51.9 ± 0.9	-0.9 [-1.2, -0.5], **p < 0.001**
Palmitic acid (C16:0, %)	36.9 ± 1.6	37.8 ± 1.6	-0.8 [-1.4, -0.3], **p = 0.005**
Stearic acid (C18:0, %)	13.3 ± 1.0	13.3 ± 1.2	0.0 [-0.3, 0.4], p = 0.952
Total MUFA (%)	11.2 ± 1.0	11.5 ± 0.8	-0.4 [-0.8, 0.1], p = 0.105
Oleic acid (C18:1, %)	9.0 ± 1.0	9.3 ± 0.7	-0.3 [-0.7, 0.2], p = 0.289
Total PUFA (%)	37.8 ± 1.1	36.5 ± 1.3	1.3 [0.7, 1.8], **p < 0.001**
Total *n*-3 PUFA (%)	2.8 ± 1.0	3.0 ± 1.1	-0.2 [-0.6, 0.1], p = 0.132
ALA (C18:3, %)	0.2 ± 0.0	0.2 ± 0.0	0.02 [0.00, 0.03], **p = 0.039**
EPA (C20:5, %)	0.7 ± 0.6	0.7 ± 0.6	-0.05 [-0.23, 0.14], p = 0.615
DHA (C22:6, %)	1.6 ± 0.6	1.9 ± 0.7	-0.28 [-0.47, -0.08], **p = 0.003**
Total *n*-6 PUFA (%)	35.0 ± 1.5	33.4 ± 2.0	1.4 [0.8, 2.1], **p < 0.001**
LA (C18:2, %)	25.2 ± 2.2	22.9 ± 2.4	2.2 [1.3, 2.8], **p < 0.001**
AA (C20:4, %)	7.6 ± 1.2	8.0 ± 1.2	-0.3 [-0.7, 0.0], **p = 0.049**

[1] Values are means ± SDs; n = 28. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ALA, alpha linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid.

[2] Linear mixed model analysis with random-intercept. Period, sex, and treatment, were used as fixed factors, and participant as random factor. P-values for the effect of treatment (mean difference [95% CI] between the mixed nut and control intervention) were reported.

Brain vascular function

No significant differences between treatments were observed for whole-brain CBF, gray-matter CBF, cortical CBF, and subcortical CBF (**Table 3** & **Supplemental Figure 2**). Cluster-wise analysis showed significantly higher regional CBF in three clusters following the mixed nut intervention (**Figure 1** & **Table 3**). Cluster 1 was the largest cluster with a volume of 6920 mm^3 and CBF increased by 5.0 ± 6.5 mL/100 g/min (17%; P<0.001). Based on the Harvard-Oxford atlas, the average probability of location was in the right lateral occipital cortex (12%), precentral gyrus (10%), superior frontal gyrus (10%), superior parietal lobe (8%), postcentral gyrus (5%) and middle frontal gyrus (3%). Regional CBF in cluster 2 increased by 5.4 ± 7.1 mL/100 g/min (16%; P<0.001). This cluster had a volume of 4352 mm^3 and the average probability of location was in the left precentral gyrus (27%), superior frontal gyrus (12%), middle frontal gyrus (8%), and postcentral gyrus (4%). Cluster 3 had a volume of 2952 mm^3 and regional CBF increased by 5.6 ± 6.6 mL/100 g/min (13%; P<0.001). The average probability of location was found bilaterally in the frontal medial cortex (43%), paracingulate gyrus (16%), frontal pole (8%) and cingulate gyrus (2%).

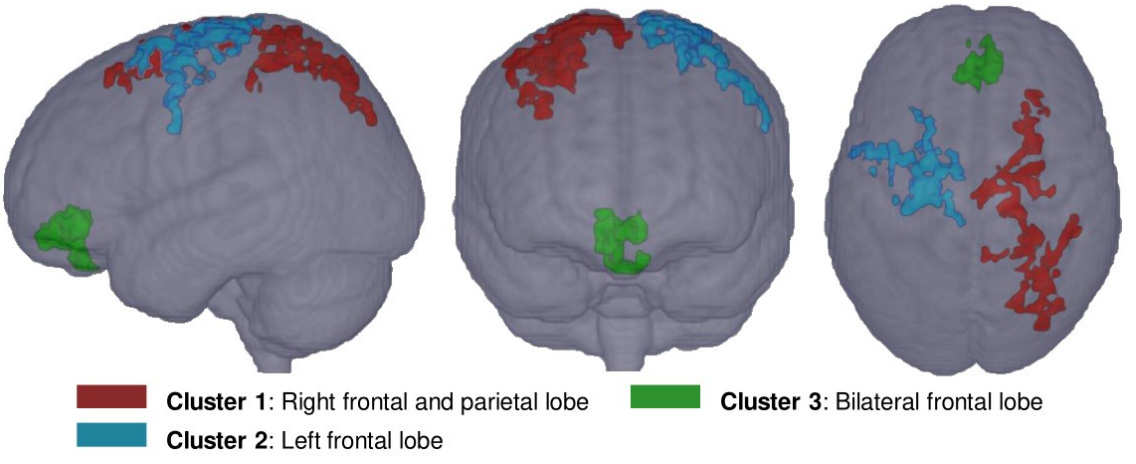


Figure 1 – Results of voxel-wise comparisons of cerebral blood flow (CBF) data in the 3-dimensional Montreal Neurological Institute template in older adults. CBF increased in three clusters after mixed nut intake as compared with the control period (family-wise error corrected, n = 27). Cluster 1: right frontal and parietal lobe, Δ5.0 ± 6.5 mL/100 g tissue/min (17%), volume 6,920 mm3, P < 0.001; Cluster 2: left frontal lobe, Δ5.4 ± 7.1 mL/100 g tissue/min (16%), volume 4,352 mm3, P < 0.001; Cluster 3: bilateral frontal lobe, Δ5.6 ± 6.6 mL/100 g tissue/min (13%), volume 2,952 mm3, P < 0.001.

Table 3 – Cerebral blood flow (CBF, ml/100g/min) after the mixed nut and control intervention[1].

	Mixed nut intervention	Control intervention	Treatment effect[2]
Whole-brain CBF	39.6 ± 6.0	38.6 ± 6.8	1.0 [-1.1, 3.2], p = 0.324
Gray matter CBF	47.2 ± 7.1	45.8 ± 7.7	1.5 [-1.2, 4.1], p = 0.256
Cortical CBF	51.9 ± 7.9	50.4 ± 8.6	1.6 [-1.4, 4.6], p = 0.285
Subcortical CBF	31.9 ± 6.6	31.4 ± 7.2	0.9 [-0.7, 2.5], p = 0.267
Cluster 1 CBF	38.8 ± 8.4	33.8 ± 7.3	5.0 ± 6.5, **p < 0.001**
Cluster 2 CBF	44.6 ± 9.0	39.3 ± 8.0	5.4 ± 7.1, **p < 0.001**
Cluster 3 CBF	53.6 ± 8.8	48.0 ± 8.3	5.6 ± 6.6, **p < 0.001**

[1] Values are means ± SDs; n = 27.

[2] Linear mixed model analysis with random-intercept for regional approach. Period, sex, and treatment, were used as fixed factors, and participant as random factor. *P*-values for the effect of treatment (mean difference [95% CI] between the mixed nut and control intervention) were reported. For cluster-wise comparison, repeated measured effects analysis using a general linear model with a single group paired difference (FLAME stage 1 and 2) were applied and family-wise corrected (FWE). *P*-values for the effect of treatment (mean difference [± SDs] between the mixed nut and control period) was reported.

Endothelial function, arterial stiffness and retinal microvasculature

Effects on endothelial function, arterial stiffness and the retinal microvasculature are shown in **Table 4**. After 16 weeks of mixed nut consumption, CAR was 0.7 PP higher (95%CI: 0.2 to 1.2; P=0.007) compared with control, but baseline carotid artery diameter did not change (**Figure 2A&B**). The mixed nut intervention also significantly lowered $PWV_{c\text{-}f}$ by 0.6 m/s (95%CI: -1.1 to -0.1; P=0.032). However, cAIxHR75, radial strain, carotid β_0-stiffness index, arterial distensibility, and arterial compliance did not change. Furthermore, brachial artery FMD was 1.6 PP higher (95%CI: 0.8 to 2.2; P<0.001; **Figure 2C&D**) after the intervention and brachial baseline diameter did not change. Allometric scaling did not affect the FMD outcomes. Finally, CRAE (2 µm; 95%CI: 0 to 3; P=0.037) and AVR (0.01; 95%CI: 0.00 to 0.01; P=0.023) were significantly higher after the intervention, but no differences were observed for CRVE.

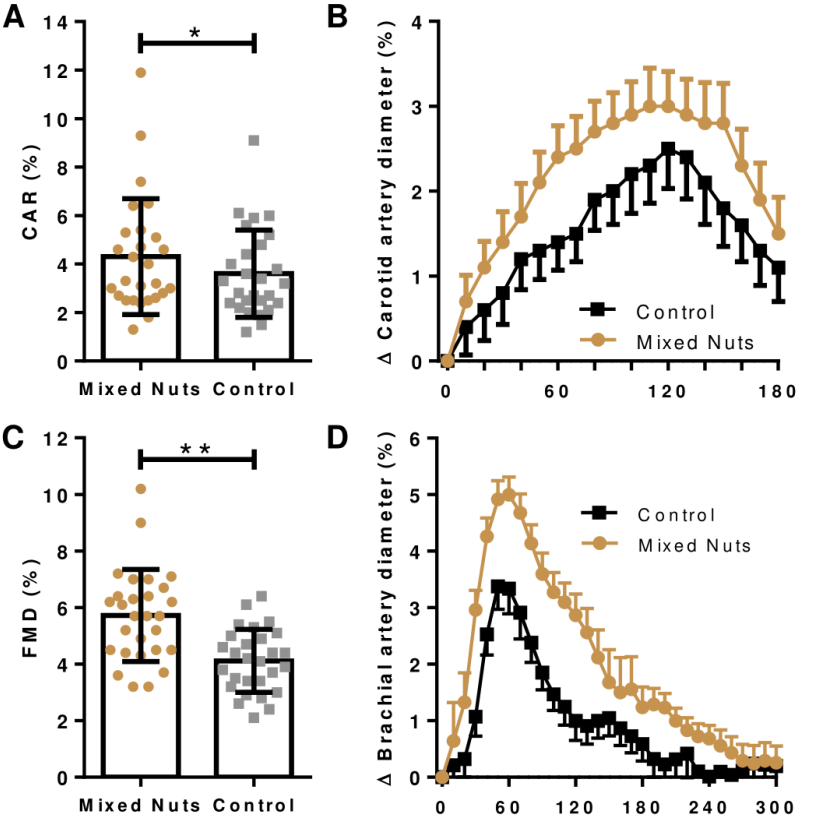


Figure 2 – Markers of endothelial function following the mixed nut and control period in older adults. (**A**) Mean (± SD) and individual carotid artery reactivity (CAR; n = 27) and (**B**) mean (± SEM) carotid artery diameter changes averaged for each 20 seconds during the cold pressor test. (**C**) Mean (± SD) and individual brachial artery flow-mediated dilation (FMD; n = 28) and (**D**) mean (± SEM) brachial artery diameter changes averaged for each 10 seconds post-occlusion.

Table 4 – Markers of endothelial function, arterial stiffness and the retinal microvasculature following the mixed nut and control intervention[1].

	Mixed nut intervention	Control intervention	Treatment effect[2]
Endothelial function			
CAR (%)[3]	4.3 ± 2.4	3.6 ± 1.8	0.7 [0.2, 1.2], **p = 0.007**
Baseline carotid artery diameter (mm)[3]	9.1 ± 1.5	9.2 ± 1.4	-0.1 [-0.2, 0.1], p = 0.455
Brachial artery FMD (%)	5.7 ± 1.6	4.1 ± 1.1	1.6 [1.0, 2.2], **p < 0.001**
Baseline brachial artery diameter (mm)	5.2 ± 1.0	5.2 ± 1.0	0.0 [-0.1, 0.2], p = 0.897
Arterial stiffness			
$PWV_{c\text{-}f}$ (m/s)	10.0 ± 1.8	10.6 ± 1.8	-0.6 [-1.1, -0.1], **p = 0.032**
cAIxHR75 (%)	24.5 ± 8.1	25.4 ± 7.1	-0.9 [-2.6, 0.9], p = 0.339
Radial strain (%)[3]	4.7 ± 1.4	5.0 ± 1.5	-0.3 [-0.9, 0.3], p = 0.343
Carotid β_0-stiffness index[3]	9.2 ± 3.0	9.5 ± 2.9	-0.2 [-1.6, 1.1], p = 0.720
Carotid arterial distensibility (1/mPa)[3]	19.4 ± 6.8	17.9 ± 5.6	1.4 [-0.8, 3.7], p = 0.203
Carotid arterial compliance (mm^2/kPa)[3]	0.94 ± 0.32	0.89 ± 0.30	0.05 [-0.06, 0.16], p = 0.353
Retinal microvascular calibers[4]			
CRAE (µm)	133 ± 20	131 ± 19	2 [0, 3], **p = 0.037**
CRVE (µm)	228 ± 14	228 ± 14	0 [-1, 1], p =0.338
Retinal AVR	0.58 ± 0.06	0.57 ± 0.06	0.01 [0.00, 0.01], **p = 0.023**

[1] Values are means ± SDs; n = 28. CAR, carotid artery reactivity; cAIxHR75, central augmentation index corrected for heart rate; $PWV_{c\text{-}f}$, Carotid-to-femoral pulse wave velocity; FMD, brachial artery flow-mediated vasodilation; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; AVR, arteriolar-to-venular ratio.

[2] Linear mixed model analysis with random-intercept. Period, sex, and treatment, were used as fixed factors, and participant as random factor. *P*-values for the effect of treatment (mean difference [95% CI] between the mixed nut and control intervention) were reported.

[3] Data missing for one participant. [4] Data missing for three participants.

Cognitive performance

Effects on cognitive performance after the mixed nut intervention and control period are summarized in **Table 5**. Performance on the PAL task, assessing visuospatial memory, was higher following the mixed nut intervention, as the total errors were 16% lower (-4; 95%CI: -8 to 0; P=0.045) compared with the control period. Furthermore, the total number of correct words during the free recall phase of the VRM, assessing verbal memory, was 16% higher (1.1; 95%CI: 0.1 to 2.2; P=0.035), but no differences were observed for immediate or delayed word recognition. No differences between treatments were observed for psychomotor speed or executive function. Finally, no differences were observed for mood, quality of life, stress, or sleep quality (**Supplemental Table 5**).

Table 5 – Cognitive performance following the mixed nut and control intervention[1].

	Mixed nut intervention	Control intervention	Treatment effect[2]
Memory			
DMS (total correct)	12.1 ± 1.9	12.0 ± 1.6	0.1 [-0.8, 1.0], p = 0.800
PAL (1st attempt memory score)	11.4 ± 3.5	10.5 ± 3.3	0.9 [-0.2, 2.4], p = 0.095
PAL (total error)	18.6 ± 12.5	22.1 ± 13.2	-3.5 [-8.1, -0.1], **p = 0.045**
VRM Free-recall (total correct)	8.0 ± 2.4	6.9 ± 2.3	1.1 [0.1, 2.2], **p = 0.035**
VRM Immediate (total correct)	32.1 ± 2.3	32.6 ± 2.7	-0.4 [-1.4, 0.5], p = 0.356
VRM Delayed (total correct)	31.3 ± 2.7	31.9 ± 3.0	-0.6 [-1.6, 0.4], p = 0.206
Psychomotor speed			
RTI movement time (ms)	301 ± 53	295 ± 60	6 [-17, 15], p = 0.338
RTI reaction time (ms)	378 ± 36	381 ± 32	-3 [-16, 10], p = 0.645
Executive function			
MTT incongruency cost (ms)	114 ± 75	97 ± 51	17 [-8, 39], p = 0.187
MTT multitasking cost (ms)	294 ± 162	295 ± 157	-1 [-62, 54], p = 0.782
MTT reaction latency (ms)	766 ± 110	760 ± 102	7 [-29, 38], p = 0.887
MTT (total error)	6 ± 7	5 ± 5	1 [-3, 4], p = 0.772

[1] Values are means ± SDs; n = 28. DMS, delayed matching samples; MOT, motor screening task; MTT, multitasking task; PAL, paired association learning; RTI, reaction time; VRM, verbal recognition memory.

[2] Linear mixed model analysis with random-intercept. Period, sex, and treatment, were used as fixed factors, and participant as random factor. *P*-values for the effect of treatment (mean difference [95% CI] between the mixed nut and control intervention) were reported.

DISCUSSION

In this randomized, controlled cross-over trial involving older adults, we examined for the first time the longer-term effects of mixed nut consumption on regional CBF using a non-invasive MRI technique. We found that mixed nut consumption as part of a recommended diet increased CBF within three clusters located in the right frontal and parietal lobe, left frontal lobe, and bilateral prefrontal cortex. Cognitive performance also improved within the memory domain, but no differences were observed for executive function or psychomotor speed. Moreover, mixed nut consumption also improved different characteristics of the peripheral vascular tree.

In line with our study, nut consumption did not significantly affect whole-brain CBF in the WAHA trial (± 40 g walnuts/day) [10], which could relate to the fact that regional CBF may either increase or decrease. For the first time, we therefore examined effects of nut consumption on regional CBF and found increased CBF within bilateral frontal and parietal brain regions. Recently, it has been shown that consumption of the Mediterranean diet for 4 weeks also increased regional CBF in multiple brain regions, including the left frontal lobe as in our study, as compared to a Western diet [36]. Nuts are part of the Mediterranean diet and may therefore

be at least partly responsible for the effects observed in that study [36]. Effects could be related to specific bioactive components of nuts, such as polyphenols, which have already been shown to increase NO bioavailability [22, 37] thereby possibly improving CBF in brain regions that are important for cognitive processes. In fact, acute studies have already shown elevated CBF after consumption of flavonoid-rich products in regions of the frontal and parietal regions [37], while 12 weeks of blueberry supplementation increased CBF within parietal and occipital regions [38]. Furthermore, the amounts or types of proteins or unsaturated fatty acids in food products may also contribute to effects observed on CBF. We have previously found increased CBF in frontal and parietal regions in older adults following a 16-week soy nut intervention, which was relatively high in protein [19]. Moreover, a 12-week high-oleic peanut consumption, high in protein and *cis*-MUFAs, also improved cerebral perfusion that was indirectly measured using transcranial doppler echography [39]. The mixed nut intervention also increased plasma LA, but effects of changing LA intake on regional CBF have not been studied so far. However, the majority of LA entering the brain is converted into oxidized metabolites that may increase CBF [40]. We also found increased plasma phospholipid levels of ALA, a fatty acid that can be converted in limited amounts into the long-chain *n*–3 PUFAs EPA and DHA [41], which however was not reflected in the plasma phospholipid fatty acid profile. RCTs have already shown that long-chain *n*-3 PUFA supplementation increased resting CBF within the parietal cortex and cingulate gyrus, that typically show lower CBF with cognitive decline [42], and prefrontal CBF during a cognitive task [43, 44]. Potential mechanisms include effects on cholinergic activity, neurovascular coupling and NO bioavailability [45]. Changes in regional CBF may be further attributed to additive or synergistic effects of other known bioactive components in nuts, including fibers, phytosterols, and tocopherols, but these effects have not been studied in human intervention studies so far.

The mixed nut intervention also improved performance on verbal and visuospatial memory tasks, but psychomotor speed and executive function were not affected. This is in line with two clinical trials from the PREDIMED cohort that also showed improved memory in older adults at high CVD risk after a Mediterranean diet supplemented with mixed nuts (15 g walnuts, 7.5 g hazelnuts, and 7.5 g almonds) for 4 to 6 years [4, 5]. Interestingly, we found bilateral increases in CBF of the precentral gyrus that has repeatedly been implicated in the retention of verbal short-term memory [46]. Also, CBF increased in the superior/middle frontal gyrus that has been associated with visuospatial memory [47]. CBF also increased within prefrontal regions (e.g., frontal medial cortex and paracingulate gyrus). These regions have been associated with reward-based decision making and executive function [48], which was however not reflected by the outcomes of the cognitive tasks. Overall, the addition of mixed nuts to a healthy dietary pattern increased CBF in brain regions of older adults that may be related the observed improved memory performance.

In the current study, we have found that longer-term mixed nut consumption improved different characteristics of the peripheral vascular tree that have already been associated to cognitive performance and CBF [9]. Several studies have already reported effects of nut consumption on vascular function of peripheral arteries [7, 8]. In line with these studies, we found that mixed nuts significantly improved peripheral endothelial function, which has been explained by enhanced NO bioavailability [8]. We also provided evidence for a higher CAR response to a cold pressor. This indicates an improved endothelium-mediated vasodilation of a central artery in closer proximity to the brain and effects may thus be more directly related to CBF changes [9]. Furthermore, in line with other nut interventions [7], we also found a reduction in $PWV_{c\text{-}f}$, which is a measure for central arterial stiffness, and has already been inversely related to CBF and cognitive outcomes in many studies [49]. Finally, arteriolar calibers in the retina, which shares similarities with the brain, were higher following the mixed nut intervention. Characteristics of the retinal microvasculature have also already been associated to changes in CBF and cognitive performance before [50]. Taken together, effects on endothelial function, arterial stiffness, and the retinal microvasculature provide strong evidence for beneficial effects of mixed nuts on vascular function.

A key strength of the current study was the controlled design in which older men and women had to adhere to food-based dietary guidelines [14], meaning that mixed nut effects were as part of a recommended diet. Moreover, we deliberately chose a mixture of nuts that may have provided a rich mix of bioactive compounds. It is estimated that the Dutch population consumes on average lower amounts of nuts per day (5 g [1–2 En%]), which is lower than the recommended daily intake of at least 15 g (~4 En%) [14]. However, previous studies showed more pronounced cardiovascular benefits when nuts accounted for about 15% of daily energy intake [13], which is in line with the amount provided in the current study. The intervention did not result in changes in body composition and total energy intake as compared to the control period, which indicates that participants compensated for the extra energy from the intake of the nuts, possibly due to their high satiety value [15]. However, effects of the nuts cannot be disentangled from those due to the replacement of food products by the intake of the nuts. Moreover, the mixed nut intervention was well-tolerated without any side effects and compliance was excellent. The fatty acid composition of plasma phospholipids was also analyzed as a physiological biomarker following nut consumption, which confirmed dietary compliance. Specifically, we found a higher plasma content of PUFA, especially LA and ALA, and lower content of plasma SFA following mixed nut consumption. Finally, we used the MRI perfusion method pCASL, which is considered the non-invasive gold standard [12], to quantify changes in CBF, and CANTAB was used as a standardized and sensitive method to detect changes in cognitive performance following dietary interventions [30]. An immanent limitation was that participants could not be blinded. Except for the research assistant, however, researchers were blinded. Moreover, we focused on a well-

defined population at increased risk for cognitive decline, but not yet diagnosed or treated for chronic diseases. Therefore, it is not known whether our findings can be extrapolated to other population groups.

CONCLUSION

In conclusion, longer-term mixed nut consumption as part of a healthy diet beneficially affected brain vascular function, as regional CBF was higher, which may relate to the observed beneficial effects on memory in older adults. Moreover, different characteristics of the peripheral vascular tree also improved.

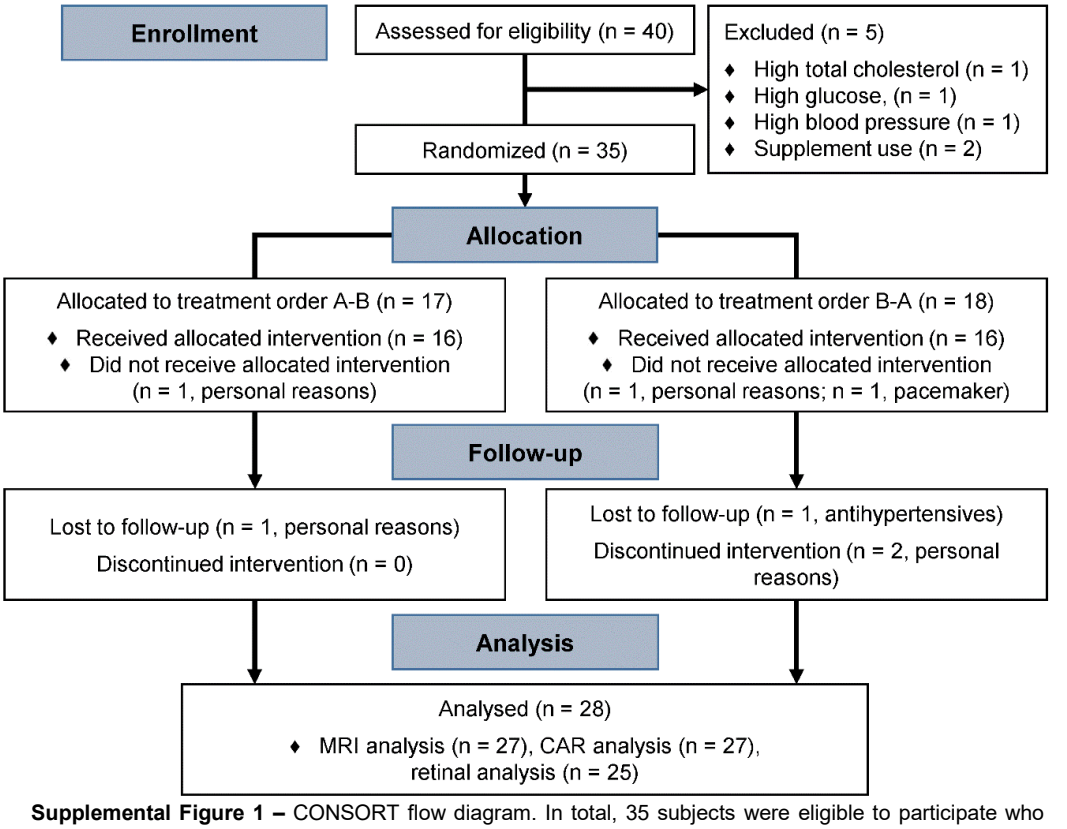
SUPPLEMENTAL MATERIAL

Supplemental Table 1 – Nutritional composition of mixed nuts (BasBoerNoten; Ridderkerk, the Netherlands).

	Per portion (60 g)	Walnut (15 g)	Cashew (15 g)	Hazelnut (15 g)	Pistachio (15 g)
Total energy (kcal)	359	98	83	94	84
Protein (g)	10.3	2.3	2.7	2.2	3.0
Carbohydrates (g)	13.2	2.1	4.5	2.5	4.1
Total fat (g)	32.3	9.8	6.6	9.1	6.8
Total saturated fatty acids (g)	3.6	0.9	1.2	0.7	0.9
Total monounsaturated fatty acids (g)	15.3	1.3	3.6	6.9	3.5
Total polyunsaturated fatty acids (g)	11.6	7.1	1.2	1.2	2.2
Total linoleic acid (g)	10.0	5.7	1.2	2.0	1.2
Total alpha-linolenic acid (g)	1.4	1.4	0.0	0.0	0.0
Fibers (g)	4.5	1.0	0.5	1.5	1.6

Supplemental Table 2 – Summary of cognitive tests and reported variables.

Cognitive domain	Cognitive test	Outcomes
Memory		
Visual matching ability	Delayed Matching to Sample (DMS)	Total correct (TC) responses for all delays
Visual memory	Paired Associates Learning (PAL)	First attempt memory score (FAMS) Total errors (TE)
Verbal memory	Verbal Recognition Memory (VRM)	Total correct (TC) during free-recall phase Total correct (TC) during immediate word recognition Total correct TC) during delayed word recognition
Psychomotor speed		
Motor and mental response speed	Reaction Time (RTI)	Movement time (MT) from button release to target selection Reaction time (RT) from target appearance to button release
Executive function		
Flexibility and response inhibition	Multitasking task (MTT)	Incongruency cost (IC); median latency of congruent minus incongruent trials Multitasking cost (MTC); median latency of two-rule condition (direction and side) minus single-rule blocks Reaction latency (RL) of all correct trials Total errors (TE)
Questionnaires		
Mood	Single-item Affect Grid	Pleasantness (range 1-19; higher is better) Arousal (range 1-19; higher is better)
Quality of Life	32-item Quality of Life (QoL) questionnaire	QoL-score (higher is better) across social, spiritual, emotional, cognitive, physical, daily life, and integrated domains
Perceived stress	10-tem Perceived Stress Scale (PSS)	PSS-score (lower is better) calculated by reversing four positive items that were summed along with six negative items
Sleep quality	Pittsburgh Sleep Quality Index (PSQI)	PSQI-score (range 0-21; lower is better) across seven subscales



Supplemental Figure 1 – CONSORT flow diagram. In total, 35 subjects were eligible to participate who were randomized for treatment order (A = Mixed nuts intervention, B = Control intervention). During the intervention seven participants dropped out, resulting in a total of 28 subjects for the analysis. MRI data was unavailable for one participant due to missing data. CAR analyses could not have been performed for one subject due to technical issues. Retinal calibers were not analyzed for three participants due to insufficient quality of the images obtained.

Supplemental Table 3 – Baseline participant characteristics[1].

	Participants (*n* = 28)
Men / women (%)	50 / 50
Age (years)	65 ± 3
Body weight (kg)	83 ± 10
Body Mass Index (kg/m^2)	27.9 ± 2.3
Systolic blood pressure (mmHg)	129 ± 13
Diastolic blood pressure (mmHg)	84 ± 7
Glucose (mmol/L)	5.6 ± 0.5
Triacylglycerol (mmol/L)	1.2 ± 0.6
Total cholesterol (mmol/L)	5.8 ± 1.0

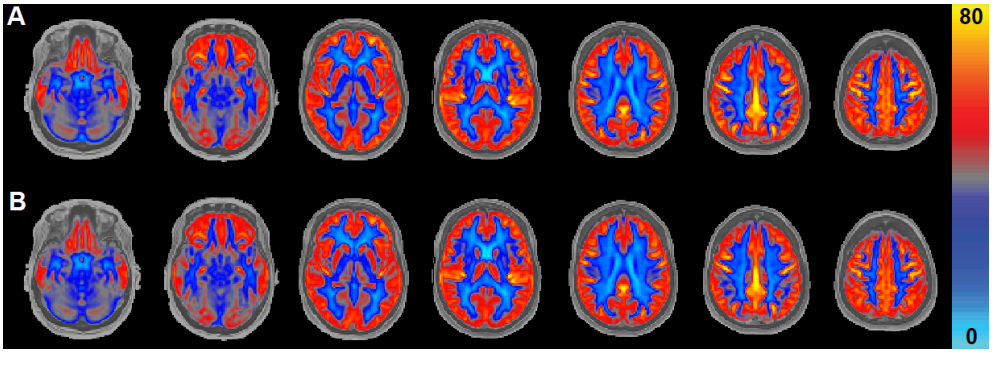
[1] Values are means ± SDs.

Supplemental Table 4 – Daily energy and nutrient intake after the mixed nut and control intervention[1].

	Mixed nut intervention	Control intervention	Treatment effect[2]	Contribution of mixed nuts to total energy intake (En%)
Total energy (Kcal)	2291 ± 500	2286 ± 495	15 [-56, 87], p = 0.659	16.4 ± 3.5
Protein (En%)	16.2 ± 2.1	15.9 ± 2.2	0.3 [-0.1, 0.7], p = 0.197	1.9 ± 0.4
Carbohydrates (En%)	36.9 ± 3.4	41.2 ± 4.7	-4.3 [-5.5, -3.1], **p < 0.001**	2.4 ± 0.5
Total fat (En%)	42.5 ± 4.1	37.1 ± 5.4	5.4 [4.1, 6.8], **p < 0.001**	13.3 ± 2.8
Total SFA (En%)	11.2 ± 3.0	12.3 ± 3.2	-1.1 [-1.8, -0.5], **p < 0.001**	1.5 ± 0.3
Total cis-MUFA (En%)	15.7 ± 3.5	12.8 ± 3.5	2.9 [2.1, 3.8], **p < 0.001**	6.3 ± 1.3
Total PUFA (En%)	10.2 ± 2.5	7.1 ± 2.5	3.2 [2.5, 3.8], **p < 0.001**	4.8 ± 1.0
Total ω-6 PUFA (En%)	8.7 ± 2.1	5.9 ± 2.0	2.8 [2.3, 3.4], **p < 0.001**	3.1 ± 0.7
Linoleic acid (En%)	7.8 ± 2.0	5.8 ± 2.0	2.0 [1.6, 2.5], **p < 0.001**	1.9 ± 0.4
Total ω-3 PUFA (En%)	1.1 ± 0.3	0.9 ± 0.3	0.2 [0.1, 0.4], **p < 0.001**	0.3 ± 0.1
α-Linolenic acid (En%)	0.9 ± 0.3	0.7 ± 0.3	0.2 [0.1, 0.3], **p < 0.001**	0.1 ± 0.0
Cholesterol (mg/MJ)	27.3 ± 7.2	29.9 ± 7.1	-2.6 [-4.2, -0.9], **p = 0.004**	0.0 ± 0.0
Fibers (g)	26.4 ± 6.8	24.8 ± 7.1	1.7 [0.3, 3.0], **p = 0.019**	0.4 ± 0.1

[1] Values are means ± SDs; n = 28. The investigational product was included. En%, energy percentage. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[2] Linear mixed model analysis with random-intercept. Period, gender, and treatment were used as fixed factors, and participant as random factor. *P*-values for the effect of treatment (mean difference [95% CI] between the mixed nut and control intervention) were reported.



Supplemental Figure 2 – Mean cerebral blood flow (CBF) maps following the (**A**) mixed nuts intervention and (**B**) control period after nonlinear co-registration to the Montreal Neurological Institute template in older adults (n = 27). CBF is displayed in mL/100 g tissue/min (scale shown by color bar). No differences were observed between treatments in global CBF (P = 0.324), gray matter CBF (P = 0.256), cortical CBF (P = 0.285), and subcortical CBF (P = 0.267).

Supplemental Table 5 – Mood, quality of life, stress, and sleep quality following the mixed nut intervention and control period in older adults[1].

	Mixed nut intervention	Control intervention	Treatment effect[2]
Pleasantness	13 ± 4	14 ± 3	-1 [-2, 1], p = 0.263
Arousal	12 ± 4	12 ± 4	0 [-2, 3], p =0.816
Quality of Life score	17 ± 3	17 ± 3	0 [-1, 1], p =0.942
Perceived Stress Scale score	21 ± 7	23 ± 4	-2 [-4, 1], p = 0.125
Pittsburgh Sleep Quality Index	5 ± 2	5 ± 3	0 [-1, 1], p = 0.800

[1] Values are means ± SDs; n = 28.

[2] Linear mixed model analysis with random-intercept. Period, gender, and treatment, were used as fixed factors, and participant as random factor. *P*-values for the effect of treatment (mean difference [95% CI] between the mixed nut and control intervention) were reported.

ACKNOWLEDGEMENTS

The authors thank all volunteers for participating in this study, E. Vanbrabant, L. van Brakel, and N.C.C. Thijssen for practical support throughout this study, M.C.J.M. Konings for the biochemical analyses, and J. Op't Roodt for analyzing arterial stiffness parameters.

FUNDING STATEMENT

This study was supported by a grant obtained from the International Nuts and Dried Fruit Council (INC). The INC had no role in the study design, data collection or analysis, decision to publish, or preparation of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

RPM, JP and PJJ designed research; KMRN conducted research; KMRN, RPM, and PJJ analyzed data and performed and discussed statistical analysis; KMRN, RPM, and PJJ wrote the manuscript; RPM, JP, and PJJ had primary responsibility for final content; All authors read and approved the final manuscript.

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CHAPTER 5

Longer-term effects of the egg-protein hydrolysate NWT-03 on arterial stiffness and cardiometabolic risk markers in adults with metabolic syndrome: A randomized, double-blind, placebo-controlled, crossover trial.

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European Journal of Clinical Nutrition, June 2023

ABSTRACT

Introduction

Short-term intake of egg-derived protein hydrolysates, such as NWT-03, suggest improvements in arterial stiffness and metabolic profiles, but longer-term trials are lacking. This study therefore examined the longer-term effects of NWT-03 on arterial stiffness and cardiometabolic markers in men and women with metabolic syndrome.

Methods

Seventy-six adults with metabolic syndrome (age 61 ± 10 years; BMI 31.7 ± 4.0 kg/m^2) participated in a randomized, controlled, double-blind, cross-over trial with a 27-day intervention (5 g/day NWT-03) or placebo period, separated by two-to-eight weeks of washout. At the start and end of both periods, measurements were performed in the fasting state and 2-hours following acute NWT-03 intake. Arterial stiffness was assessed by carotid-to-radial (PWV_{c-r}), carotid-to-femoral pulse wave velocity (PWV_{c-f}), and central augmentation index (CAIxHR75). Moreover, cardiometabolic markers were assessed.

Results

Compared with control, longer-term NWT-03 supplementation did not affect fasting PWV_{c-r} (0.1 m/s; -0.2 to 0.3; P=0.715) or PWV_{c-f} (-0.2 m/s; -0.5 to 0.1; P=0.216). Fasting pulse pressure (PP) was however reduced by 2 mmHg (95%CI: -4 to 0; P=0.043), but other fasting cardiometabolic markers were not affected. No effects were observed following acute NWT-03 intake at baseline. However, acute intake of NWT-03 after the intervention significantly lowered CAIxHR75 (-1.3 %-point; -2.6 to -0.1; P=0.037) and diastolic BP (-2 mmHg; -3 to 0; P=0.036), but other cardiometabolic markers did not change.

Conclusions

Longer-term NWT-03 supplementation did not affect arterial stiffness, but modestly improved fasting PP in adults with metabolic syndrome. Acute intake of NWT-03 after the intervention also improved CAIxHR75 and diastolic BP.

Trial registration number: NCT02561663 (ClinicalTrials.gov).

INTRODUCTION

Metabolic syndrome is a clustering of (metabolic) risk markers that substantially promote arterial stiffening, and increases the risk to develop cardiovascular disease (CVD) and type 2 diabetes (T2D) [1]. Therefore, the need for effective nutritional approaches for reducing arterial stiffness and the improvement of metabolic profiles is key. Foods containing functional ingredients, such as protein hydrolysates, may be of potential interest in metabolic syndrome risk reduction [2, 3]. NWT-03 is a dietary egg-protein hydrolysate, derived from the digestion of lysozyme with Alcalase. It has been identified as a potential inhibitor of angiotensin-converting enzyme (ACE) [4]. Recently, we have demonstrated a blood pressure (BP)-lowering effect in mild-hypertensive adults following the daily intake of 2 g NWT-03 for one week, but no effects were observed at lower (1 g) or higher doses (5 g) [5]. In adults with impaired glucose tolerance (IGT) or T2D, the administration of 5 g NWT-03 for two days did however not affect carotid-to-femoral pulse wave velocity ($PWV_{c\text{-}f}$), which is the gold standard to assess regional arterial stiffness [6, 7]. However, carotid-to-radial pulse wave velocity ($PWV_{c\text{-}r}$) was reduced, which suggests changes in arterial stiffness of peripheral muscular arteries that are more sensitive to vasoactive agents that central elastic arteries due to their differential composition [6, 8]. Interestingly, $PWV_{c\text{-}r}$ improvement was not accompanied by changed in BP, suggesting that the decrease in $PWV_{c\text{-}r}$ may be related to mechanisms other than ACE-inhibition [6]. In this context, *in vitro* studies have shown that NWT-03 also inhibits the enzyme dipeptidyl peptidase 4 (DPP-IV), which plays a major role in glucose and lipid metabolism [4, 9-11]. Accordingly, marked improvements in cardiometabolic risk markers (i.e., glucose, lipid and lipoprotein metabolism) were observed after intake of 5 g NWT-03 for two days in adults with IGT or T2D, which may contribute to the observed reduction in $PWV_{c\text{-}r}$ [6]. While our previous studies primarily focused on acute or short-term NWT-03 intake in overweight or obese adults with or without (pre)diabetes (5, 6), it is also relevant to examine the immediate physiological responses in adults with metabolic syndrome that exhibit a more heterogenous risk profile. Moreover, it is important to investigate also longer-term effects for a comprehensive understanding of the sustained impact on arterial stiffness and cardiometabolic markers to better evaluate the clinical relevance of NWT-03 supplementation in reducing CVD and T2D risk [12]. Therefore, the primary objective of this randomized, placebo-controlled, double-blind, cross-over trial was to evaluate the acute (2 hours) and longer-term (27 days) effects of daily 5 g NWT-03 supplementation on arterial stiffness in adults with metabolic syndrome. Furthermore, effects on cardiometabolic risk markers were explored.

METHODS

Study population

Potential participants were recruited via posters in university and hospital buildings or advertisements in local newspapers. Additionally, participants of previous studies were approached if they had given written consent to contact them again. Potential volunteers were contacted by telephone and invited for a screening visit. Adults aged between 18 and 75 years were included when they met the International Diabetes Federation's (IDF) harmonized criteria for the presence of the metabolic syndrome [13], defined as having at least three of five risk components: abdominal obesity (waist circumference >94 cm for men or >80 cm for women) or a Body Mass Index (BMI) >30 kg/m^2; raised fasting triacylglycerol (TAG) concentrations ≥1.7 mmol/L; reduced fasting HDL-cholesterol (HDL-C) concentrations (<1.03 mmol/L for men or <1.29 mmol/L for women); raised fasting plasma glucose concentrations (> 5.6 mmol/L); raised BP (systolic BP [SBP] ≥130 mmHg or diastolic BP [DBP] ≥85 mmHg). The exclusion criteria were: hypersensitivity to study product; instable body weight (weight gain or loss ≤5 % within 3 months); current smoker or smoking cessation <12 months; medical conditions that could interfere with the main study outcomes (e.g. chronic kidney disease, endocrinological or immunological disorders); use of medication or dietary supplements known to interfere with the main study outcomes; pregnancy or lactation; drug or alcohol abuse (men >21 units/week and women >14 units/week); blood donation within 8 weeks prior to screening or during the study; participation in another biomedical study within 60 days before or during the study. Written informed consent was obtained from all volunteers. The study was conducted according the guidelines of the Declaration of Helsinki and approved by the Medical Ethics Committee of Maastricht University Medical Center (METC153021). The study was registered on September 28, 2015 at ClinicalTrials.gov as NCT02561663.

Study design

The study had a randomized, placebo-controlled, double-blind, cross-over design with an intervention and control period of both 27 days, separated by a washout period of two to eight weeks (**Figure 1**). Study participants were allocated by a research assistant to start either in the intervention or control period based on a computer-generated randomization scheme. During the intervention period, subjects consumed daily 5 g NWT-03, which was produced and provided by Newtricious R&D BV as described previously [5]. Both NWT-03 and the placebo (maltodextrin) were packaged in dry-powder sachets. Sachets had to be stored at room temperature (15-25°C). Before consumption, the study product had to be dissolved in 200 mL water and was consumed 30 minutes before breakfast in the fasting state.

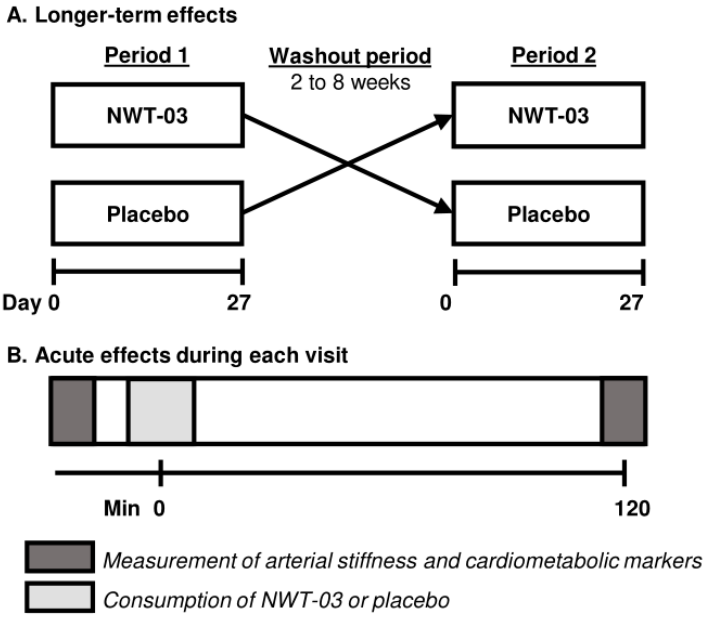

Figure 1 – Overview of the study design. (**A**) Longer-term effects were determined by comparing the difference in fasting values after the 27-day NWT-03 of placebo intervention. (**B**) During each visit, acute effects were determined 2 h after the intake of NWT-03 or placebo.

Both periods included visits at baseline and after 27 days. All measurements were performed in quiet and temperature-controlled (20 °C) rooms. Measurements were performed by several researchers, but measurements for a specific participant were always performed by the same observer. On the day preceding each visit, participants were provided a standardized meal (commercially available lasagna) to minimize the variation due to the influence of the last evening meal on metabolic and vascular outcomes. Participants were requested to fast overnight for at least 10 hours and to abstain from alcohol before the study visits. Weight and height were measured at each visit using a wall-mounted stadiometer. Subsequently, participants had to rest in the supine position for at least 10 min before vascular measurements were performed. Moreover, a fasting venous blood sample was collected. Subjects were then requested to take their first daily dose of the investigational product under supervision of the study investigator. Acute effects were determined by repeating the same set of measurements two hours after intake. During the entire study period, all participants were requested to record daily in study diaries any signs or symptoms of illnesses, use of medication, alcohol consumption, any protocol deviations and any other complaints. Volunteers was asked to fill in a validated food-frequency questionnaire (FFQ) to assess energy and nutrient intakes over the past four weeks. Energy and nutrient intakes were calculated using the Dutch Food Composition Database (NEVO) [14].

Arterial stiffness

Regional arterial stiffness was assessed in triplicate using a tonometer (SphygmoCor v9, AtCor Medical, West Ryde, Australia) by detecting the delayed pulse wave arrival compared to the R-wave of the electrocardiogram at the carotid and radial artery for $PWV_{c\text{-}r}$, and at the carotid and femoral artery for $PWV_{c\text{-}f}$. These parameters were calculated automatically by the program provided by the manufacturer by dividing the timeframe of delay by the direct carotid-to-radial and carotid-to-femoral distance [15, 16]. Furthermore, radial artery pulse wave analysis was performed in triplicate using the same tonometer applied to the radial artery. The central arterial waveform was derived from the peripheral waveform with a validated transfer function. The central augmentation index adjusted for heart rate (CAIxHR75) was defined as the difference between the first and second peak of the waveform, expressed as a percentage of the pulse pressure (PP) and further corrected for heart rate [16].

Cardiometabolic markers

Using a validated semi-automatic device (Omron M7 IntelliSenseTM, Omron, Hoofddorp, The Netherlands) office brachial SBP and DBP were measured in a supine position four times, separated by at least one minute between measurements. The first measurement was discarded and the last three measurements were averaged. PP was calculated by subtracting SBP from DBP, and mean arterial pressure (MAP) was calculated by the following formula: MAP = 1/3 * SBP + 2/3 * DBP.

Venous blood samples were drawn into serum separator tubes and sodium fluoride (NaF)-containing tubes (Becton, Dickinson and Company, Franklin Lanes, New York, USA). Serum tubes were allowed to clot at room temperature for 30 to 60 min after withdrawal and centrifuged (15 min at 1300g at 21 °C). Plasma tubes were directly placed on ice after withdrawal and immediately centrifuged (15 min at 1300g at 4 °C). After centrifugation, plasma and serum samples were distributed in aliquots, snap frozen in liquid nitrogen, and stored at -80°C until further analysis. Serum total cholesterol (TC) concentrations (CHOD-PAP method; Roche Diagnostics System, Mannheim, Germany), HDL-C concentrations (precipitation method, Roche Diagnostics System, Mannheim, Germany), TAG concentrations corrected for free glycerol (GPO-Trinder, Sigma Diagnostics, St Louis, USA), and high-sensitivity C-reactivity protein (hsCRP; immunoturbidimetric assay, Horiba ABX, Montpeillier) were measured in all samples. Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated using the Friedewald formula [17]. Serum insulin concentrations (Millipore Corporation, Billerica, USA) and plasma glucose concentrations (Horiba, ABX, Montpellier, France) were measured in all samples. $HOMA_{IR}$, a marker for peripheral insulin sensitivity was calculated [18]. All technicians were blinded to the treatments of the subjects.

Statistical analyses

Data were presented as means ± standard deviations (SDs) unless otherwise indicated. Based on previous research, it was determined that 72 participants were needed to detect a true difference of at least 0.84 m/s (expected SD = 1.76 m/s) in $PWV_{c\text{-}r}$, which was the primary study outcome, with 80% power and a two-sided alpha of 0.05 [6]. Linear mixed models were used to examine differences between the intervention and control periods over time. Treatment, period, and gender were included as fixed factors and a subject-specific random intercept was included. Long-term effects were determined as the difference between fasting values at the end of the treatments using day 0 values as covariate. Acute effects were evaluated at baseline and after the intervention by comparing differences between treatments before and two hours after intake of the study product using the corresponding fasting values as covariate. Carry-over effects were examined by including the order of the treatment as fixed factor in all models, but no significant effects were found and treatment order was therefore omitted from all models. SPSS was used to perform all statistical analyses (IBM Corp., IBM SPSS Statistics, V26, Armonk, NY, USA). A P-value < 0.05 was considered to be statistically significant.

RESULTS

A Consolidated Standards of Reporting Trials flow (CONSORT) diagram of the study is shown in **Figure 2**. After screening, a total of 79 subjects fulfilling the criteria of the metabolic syndrome were eligible for participation in this trial. Two participants dropped out during the first period due to personal reasons or digestive problems, and one subject was excluded from analyses due to unjustified inclusion not meeting the IDF harmonized criteria for the metabolic syndrome. Therefore, 76 participants (46 men and 30 women) completed the study. Baseline subject characteristics are presented in **Table 1**. The mean age of participants was 61 ± 10 years and their BMI was 31.7 ± 4.0 kg/m^2. NWT-03 supplementation did not significantly change weight (P=0.806) or BMI (P=0.862) compared to placebo. No (serious) adverse events were reported regarding the intake of NWT-03 or placebo. Total energy and nutrient intakes were comparable during the experimental and placebo periods (**Supplemental Table 1**).

Arterial stiffness

No significant differences were observed in fasting $PWV_{c\text{-}r}$, $PWV_{c\text{-}f}$ or CAIxHR75 after longer-term supplementation with NWT-03 (**Table 2**). However, CAIxHR75 was 1.3 %-point lower (95%CI: -2.6 to -0.1; P=0.037) after acute intake of NWT-03 compared to placebo at day 27, whereas no acute effects were observed for $PWV_{c\text{-}r}$ or $PWV_{c\text{-}f}$ (**Table 3**). There were no acute effects of NWT03 intake on markers of arterial stiffness at baseline (**Supplemental Table 2**).

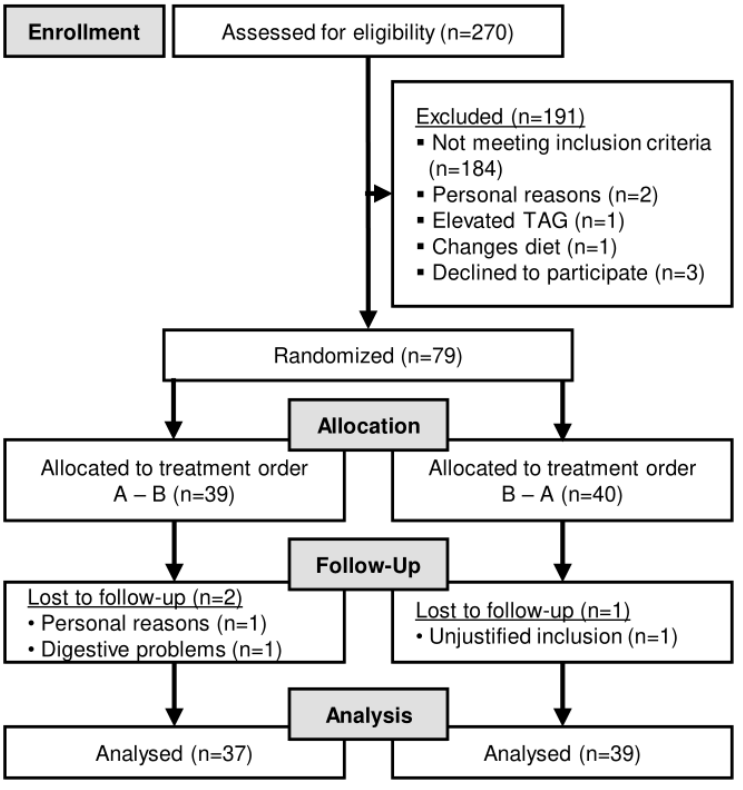

Figure 2 – CONSORT flow diagram. In total, 79 subjects were eligible to participate who were randomized for treatment order (A = Placebo, B = NWT-03). During the intervention three participants dropped out, resulting in a total of 76 subjects for the analysis.

Cardiometabolic markers

The longer-term NWT-03 intervention significantly lowered fasting PP by 2 mmHg (95%CI: -4 to 0; P=0.043), but other fasting cardiometabolic markers did not change (**Table 2**). After 27 days, DBP was 2 mmHg (95%CI: -3 to 0; P=0.036) lower following acute intake of NWT-03 compared to placebo (**Table 3**). Furthermore, the acute intake of NWT-03 after the 27-day intervention tended to lower MAP (-1 mmHg; -3 to 0; P=0.095), TC (-0.07 mmol/L; -0.15 to 0.01; P=0.097), and LDL-C (-0.06 mmol/L; 95%CI: -0.14 to 0.01; P=0.089). No acute effects were observed for glucose, insulin, $HOMA_{IR}$, HDL-C, and TAG. Also, no acute effects on cardiometabolic markers were observed at baseline (**Supplemental Table 2**).

Table 2 – Longer-term effects of NWT-03 or placebo intake on fasting arterial stiffness and cardiometabolic markers in adults with metabolic syndrome[1].

	NWT-03		Placebo		Treatment effect
	Day 0	Day 27	Day 0	Day 27	[95%CI][2]
Arterial stiffness					
$PWV_{c\text{-}r}$ (m/s)	7.5 ± 1.3	7.5 ± 1.1	7.5 ± 1.2	7.5 ± 1.1	0.1 [-0.2, 0.3], p=0.715
$PWV_{c\text{-}f}$ (m/s)	8.7 ± 1.7	8.6 ± 1.6	8.6 ± 1.5	8.7 ± 1.6	-0.2 [-0.5, 0.1], p=0.216
CAIxHR75 (%)	21.7 ± 8.4	21.7 ± 8.1	22.7 ± 8.3	21.5 ± 8.1	0.8 [-0.5, 2.1], p=0.186
Cardiometabolic markers					
SBP (mmHg)	131 ± 13	129 ± 13	130 ± 13	130 ± 14	-2 [-4, 1], p=0.180
DBP (mmHg)	86 ± 6	85 ± 8	85 ± 8	85 ± 9	0 [-1, 2], p=0.778
PP (mmHg)	45 ± 10	44 ± 10	45 ± 10	45 ± 10	-2 [-4, 0], **p=0.043**
MAP (mmHg)	101 ± 9	100 ± 9	100 ± 9	100 ± 9	0 [-2, 1], p=0.616
Glucose (mmol/L)	6.0 ± 0.7	6.0 ± 0.7	6.0 ± 0.7	6.0 ± 0.7	-0.1 [-0.1, 0.0], p=0.203
Insulin (mmol/L)	15.7 ± 6.3	16.4 ± 8.8	15.0 ± 6.2	16.2 ± 8.3	-0.4 [-2.1, 1.3], p=0.636
$HOMA_{IR}$	4.3 ± 2.0	4.5 ± 2.7	4.0 ± 1.9	4.4 ± 2.7	-0.2 [-0.7, 0.4], p=0.519
TC (mmol/L)	5.59 ± 0.98	5.55 ± 1.03	5.60 ± 1.00	5.59 ± 1.01	-0.03 [-0.18, 0.12], p=0.682
HDL-C (mmol/L)	1.10 ± 0.24	1.11 ± 0.27	1.08 ± 0.31	1.11 ± 0.25	-0.01 [-0.04, 0.03], p=0.607
LDL-C (mmol/L)	4.09 ± 0.91	4.07 ± 0.94	4.12 ± 0.95	4.11 ± 0.91	-0.01 [-0.14, 0.12], p=0.887
TAG (mmol/L)	2.03 ± 1.01	1.84 ± 0.82	1.94 ± 0.92	1.82 ± 0.80	-0.02 [-0.04, 0.11], p=0.817
hsCRP (mg/L)	2.2 [1.2, 4.0]	2.2 [1.0, 4.1]	2.0 [1.0, 3.8]	2.4 [1.2, 4.1]	-0.5 [-1.1, 0.1], p=0.103

[1] Values are means ± SDs or median [IQR]; n = 76. $PWV_{c\text{-}r}$, carotid-to-radial pulse wave velocity; $PWV_{c\text{-}f}$, carotid-to-femoral pulse wave velocity; cAIxHR75, central augmentation index corrected for heart rate. SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; $HOMA_{IR}$, homeostatic model assessment of insulin resistance; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TAG, triacylglycerol; hsCRP, high-sensitive C-reactive protein.

[2] Linear mixed model analysis with treatment, period and gender as fixed factors, participant as random factor and day 0 values as covariate. *P*-values for the effect of treatment (mean difference [95% CI] between the NWT-03 and placebo intervention) were reported. P<0.05 was considered statistically significant.

Table 3 – Acute effects of NWT-03 or placebo intake on arterial stiffness and cardiometabolic markers following the longer-term intervention in adults with metabolic syndrome[1].

Day 27	NWT-03		Placebo		Treatment effect
	0h	2h	0h	2h	[95%CI][2]
Arterial stiffness					
PWV_{c-r} (m/s)	7.5 ± 1.1	7.4 ± 1.1	7.5 ± 1.1	7.5 ± 1.1	0.0 [-0.3, 0.2], p=0.778
PWV_{c-f} (m/s)	8.6 ± 1.6	8.8 ± 1.6	8.7 ± 1.6	8.9 ± 1.7	-0.2 [-0.3, 0.3], p=0.785
CAIxHR75 (%)	21.7 ± 8.1	21.9 ± 8.1	21.5 ± 8.1	23.0 ± 8.5	-1.3 [-2.6, -0.1], **p=0.037**
Cardiometabolic markers					
SBP (mmHg)	129 ± 13	133 ± 13	130 ± 14	135 ± 14	-1 [-3, 2], p=0.532
DBP (mmHg)	85 ± 8	86 ± 9	85 ± 9	87 ± 8	-2 [-3, 0], **p=0.036**
PP (mmHg)	44 ± 10	47 ± 11	45 ± 10	48 ± 10	1 [-1, 3], p=0.507
MAP (mmHg)	100 ± 9	102 ± 9	100 ± 9	103 ± 9	-1 [-3, 0], p=0.095
Glucose (mmol/L)	6.0 ± 0.7	5.7 ± 0.5	6.0 ± 0.7	5.6 ± 0.6	0.0 [0.0, 0.1], p=0.268
Insulin (mmol/L)	16.4 ± 8.8	12.9 ± 6.0	16.2 ± 8.3	13.1 ± 6.5	-0.3 [-1.4, 0.7], p=0.507
$HOMA_{IR}$	4.5 ± 2.7	3.3 ± 1.7	4.4 ± 2.7	3.3 ± 1.9	-0.1 [-0.4, 0.3], p=0.765
TC (mmol/L)	5.55 ± 1.03	5.46 ± 1.01	5.59 ± 1.01	5.57 ± 1.01	-0.07 [-0.15, 0.01], p=0.097
HDL-C (mmol/L)	1.11 ± 0.27	1.09 ± 0.24	1.11 ± 0.25	1.10 ± 0.26	-0.01 [-0.03, 0.02], p=0.530
LDL-C (mmol/L)	4.07 ± 0.94	3.99 ± 0.93	4.11 ± 0.91	4.10 ± 0.93	-0.06 [-0.14, 0.01], p=0.089
TAG (mmol/L)	1.84 ± 0.82	1.86 ± 0.83	1.82 ± 0.80	1.82 ± 0.83	0.02 [-0.06, 0.09], p=0.685
hsCRP (mg/L)	2.2 [1.0, 4.1]	2.1 [1.0, 4.0]	2.4 [1.2, 4.1]	2.2 [1.1, 3.8]	0.1 [-0.13, 0.27], p=0.517

[1] Values are means ± SDs or median [IQR]; n = 76. PWV_{c-r}, carotid-to-radial pulse wave velocity; PWV_{c-f}, carotid-to-femoral pulse wave velocity; cAIxHR75, central augmentation index corrected for heart rate. SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; $HOMA_{IR}$, homeostatic model assessment of insulin resistance; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TAG, triacylglycerol; hsCRP, high-sensitive C-reactive protein.

[2] Linear mixed model analysis with treatment, period and gender as fixed factors, participant as random factor and 0h values as covariate. *P*-values for the effect of treatment (mean difference [95% CI] between the NWT-03 and placebo intervention) were reported. P<0.05 was considered statistically significant.

DISCUSSION

The intake of egg-protein-derived hydrolysates, such as NWT-03, has already been shown to exert several cardiometabolic benefits in animal studies [3, 4]. However, the number of well-designed human intervention trials investigating these functional ingredients is limited and mostly restricted to short-term interventions. We evaluated here for first time the longer-term effects of NWT-03 on regional arterial stiffness and cardiometabolic markers in adults with unfavorable metabolic profiles. Our findings indicate that supplementation of 5 g NWT-03 for 27 days did not affect fasting regional arterial stiffness. However, longer-term beneficial effects on fasting PP were observed, whereas the acute intake of NWT-03 after the 27 days intervention significantly improved CAIxHR75 and DBP.

The current study showed that the 27-day consumption of NWT-03 did not affect markers of arterial stiffness in this population consisting of adults with metabolic syndrome. In line with the current findings, no effects were observed on $PWV_{c\text{-}f}$ in our previous study following two days of 5 g NWT-03 intake in subjects with IGT or T2D [6]. Lucey *et al.* also failed to show any effects on $PWV_{c\text{-}f}$ in adults with mild hypertension after the 6-week supplementation of an egg ovalbumin-derived protein hydrolysate [19]. Previous studies using milk-derived protein hydrolysates showed improvements in $PWV_{c\text{-}f}$ with supplementation periods longer than 6 weeks, suggesting that longer trials may be necessary to observe beneficial effects [20]. In contrast, in a previous trial we reported an improved $PWV_{c\text{-}r}$ after two days of 5 g NWT-03 intake in adults with IGT or T2D [6], but no effects were observed on $PWV_{c\text{-}r}$ in the current study. Effects on $PWV_{c\text{-}r}$ suggest changes in stiffness of peripheral muscular arteries that are more sensitive to vasoactive agents (i.e., nitric oxide (NO)) than central elastic arteries due to their differential composition [8]. Thus, increased NO-availability leads to a greater vasodilation, thereby improving $PWV_{c\text{-}r}$ [21]. Unfortunately, no human trials to date have focused on NO-dependent vasodilation as a potential explanation for the observed discrepancies between the outcomes of the different studies evaluating effects of NWT-03. Animal models have already reported beneficial effects of NWT-03 on endothelium-dependent vasodilation and vascular resistance [4, 22]. Moreover, trials using protein hydrolysates from other food sources already have demonstrated beneficial effects on the NO-dependent endothelial function after 2 weeks of supplementation [20]. Altogether, based on these findings, effects of egg-derived protein hydrolysates on arterial stiffness are not convincing and further longer-term trials are warranted to study whether NWT-03 intake affects markers of endothelial function in humans.

In our current study, we observed a reduction in fasting brachial PP following NWT-03 supplementation. Although the magnitude of the PP reduction may appear to be small, it is important to recognize that lower PP has been independently associated with a reduced risk of CVD and all-cause mortality [23]. In the context of our findings, it can however be questioned whether the observed decrease in brachial PP translates to a clinically relevant CVD risk

reduction. The underlying physiological explanation driving the PP change remains to be elucidated. With advancing age, PP amplification occurs as a result of arterial stiffening, which was not affected by NWT-03 [21, 23]. An alternative explanation for the observed changes in PP could however be potential effects on other markers of the vasculature, such as endothelial function, which was not assessed in the current study. Regarding the potential link between the PP reduction and cardiovascular health, it is important to note that the effects of egg-derived protein hydrolysates on BP regulation have yielded inconsistent findings. While animal studies have shown that egg-derived protein hydrolysates exert modest ACE-inhibitory effects thereby reducing BP [24], these effects are more controversial in humans. In two studies, no effects were observed [6, 19], while in one trial a daily supplement of 2 g NWT-03 for 7 days decreased 36-h ambulatory SBP and DBP, but only in mild-hypertensive adults [5]. The discrepancies in the effects of NWT-03 on BP may be attributed to several factors, including the dosage and characteristics of the study population. Indeed, positive effects of protein hydrolysates depend on the type of hydrolysate and characteristics of the study population, such as baseline BP [25]. Whereas trials with egg-derived protein hydrolysates show inconsistent effects on BP [6, 19], a recent meta-analysis has demonstrated beneficial effects of protein hydrolysates from other food sources, especially milk-derived lactotripeptides [26]. Interestingly, studies that observed improvements in $PWV_{c\text{-}f}$ following the longer-term intake of casein-derived lactotripeptides simultaneously observed changes in BP [27, 28]. In our study, we also showed that after the longer-term intervention but not at baseline, acute intake of NWT-03 reduced CAIxHR75 by 1.3 %-point, which reflects a decreased wave reflection that may indicate a lower amplitude of pressure waves in peripheral arteries that is associated with lower CVD risk [29, 30]. However, the use of CAIxHR75 as a surrogate for arterial stiffness has been challenged, as the parameter is dependent on several confounding factors, including changes in reflection sites and DBP [29, 31]. Accordingly, we found that acute NWT-03 intake after the longer-term intervention also lowered DBP, which could to some extent explain the reduction in CAIxHR75 [31].

Our results did not show any effects on glucose homeostasis or insulin sensitivity. However, in a previous trial we found that 5 g NWT-03 for 2 days improved fasting glucose and insulin concentrations in adults with IGT or T2D [6]. In agreement with the present study, a 6-week supplementation of an egg-ovalbumin-derived protein hydrolysate did also not show any effects in a population with normal blood glucose profiles [19]. This may imply that NWT-03 exerts more pronounced glucoregulatory effects in those with IGT or T2D, in which the current study population may have been too heterogenous to detect changes. *In vitro* studies suggest that egg-derived proteins may beneficially affect glucose homeostasis by inhibition of α-glucosidase to reduce intestinal carbohydrate absorption, and by DPP-IV-inhibition, leading to increased incretin levels that inhibit glucagon synthesis and stimulate insulin production to lower plasma glucose [4, 9, 10]. However, animal studies show little to no change in blood glucose or insulin

concentrations following intakes of egg-protein hydrolysates [32]. No longer-term effects of NWT-03 intake were observed on fasting lipid or lipoprotein concentrations in the current study, which is in line with Lucey *et al.* that did not show any effects [19]. At the end of the current intervention, acute NWT-03 intake did however tend to lower serum TC and LDL-C concentrations compared to placebo. Comparable results have already been reported in other studies with protein hydrolysates showing acute improvements in lipid and lipoprotein metabolism [6, 33]. Mechanisms of hypolipidemic activity of food protein hydrolysates have already been reviewed before [34], but it must be noted that evidence is mainly based on *in vitro* and animal studies. Protein hydrolysates are thought to alter the enterohepatic bile acid circulation and as such elevate cholesterol catabolism, thereby inducing a compensatory increase in hepatic cholesterol uptake and consequently lower serum TC and LDL-C concentrations. Other postulated mechanisms for the hypolipidemic activity include disruption of micellar solubility and the regulation of lipogenic proteins and genes [34].

A key strength of the current trial was the double-blinded, controlled design with low dropout rates, excellent compliance and no changes in the background diet of participants. In addition, the study used a comprehensive assessment of arterial stiffness at both carotid-femoral and carotid-radial sites using the recommended guidelines. Moreover, the study focused on a well-defined population with metabolic syndrome who had not yet been diagnosed or treated for chronic or metabolic diseases. This also implies that it remains unknown whether our findings can be extrapolated to other population groups. Finally, the study intervention duration was relatively short. Nevertheless, a strength of the study was the inclusion of analyzing both acute and chronic effects of the intervention side by side.

CONCLUSION

In conclusion, the present findings indicate that NWT-03 supplementation for 27 days does not affect arterial stiffness in adults with metabolic syndrome. However, an improvement in fasting PP was observed, but it remains to be elucidated whether this moderate change is clinically relevant. Future well-designed trials involving diverse study populations are warranted to validate the potential longer-term cardiometabolic benefits of egg-protein hydrolysates.

SUPPLEMENTAL MATERIAL

Supplemental Table 1 – Daily energy and nutrient intakes during the 27-day NWT-03 or placebo treatment in a randomized controlled cross-over trial with 76 individuals with metabolic syndrome.

	NWT-03	Placebo	P-value
Total energy (MJ)	10.1 ± 2.5	10.2 ± 2.8	P > 0.05
Carbohydrates (En%)	40 ± 5	40 ± 6	P > 0.05
Protein (En%)	16 ± 3	16 ± 2	P > 0.05
Total fat (En%)	39 ± 5	39 ± 5	P > 0.05
Saturated FA (En%)	13 ± 3	13 ± 3	P > 0.05
Monounsaturated FA (En%)	14 ± 3	14 ± 3	P > 0.05
Polyunsaturated FA (En%)	8 ± 2	8 ± 2	P > 0.05
Fiber (g/day)	28 ± 9	29 ± 8	P > 0.05
Cholesterol (mg/day)	275 ± 96	260 ± 82	P > 0.05

Values are means ± SDs; n = 76. En%: energy percentage; FA, fatty acid.

Supplemental Table 2. Acute effects of NWT-03 or placebo intake on arterial stiffness and cardiometabolic markers at baseline in adults with metabolic syndrome[1].

Day 0	NWT-03		Placebo		Treatment effect
	0h	2h	0h	2h	[95%CI][2]
Arterial stiffness					
$PWV_{c\text{-}r}$ (m/s)	7.5 ± 1.3	7.5 ± 1.1	7.5 ± 1.2	7.6 ± 1.2	-0.1 [-0.3, 0.2], p=0.440
$PWV_{c\text{-}f}$ (m/s)	8.7 ± 1.7	8.8 ± 1.7	8.6 ± 1.5	8.7 ± 1.5	0.0 [-0.2, 0.3], p=0.764
CAIxHR75 (%)	21.7 ± 8.4	22.1 ± 8.0	22.7 ± 8.3	22.6 ± 7.5	-0.2 [-2.6, -0.1], p=0.701
Cardiometabolic markers					
SBP (mmHg)	131 ± 13	136 ± 15	130 ± 13	136 ± 15	-1 [-3, 1], p=0.278
DBP (mmHg)	86 ± 6	88 ± 8	85 ± 8	87 ± 9	0 [-2, 2], p=0.876
PP (mmHg)	45 ± 10	48 ± 12	45 ± 10	49 ± 12	-1 [-3, 1], p=0.231
MAP (mmHg)	101 ± 9	104 ± 10	100 ± 9	103 ±10	0 [-2, 1], p=0.668
Glucose (mmol/L)	6.0 ± 0.7	5.7 ± 0.5	6.0 ± 0.7	5.6 ± 0.6	0.0 [0.0, 0.1], p=0.208
Insulin (mmol/L)	15.7 ± 6.3	12.4 ± 5.3	15.0 ± 6.2	12.4 ± 5.3	-0.4 [-1.2, 2.1], p=0.278
$HOMA_{IR}$	4.3 ± 2.0	3.2 ± 1.5	4.0 ± 1.9	3.1 ± 1.5	0.1 [-0.1, 0.3], p=0.281
TC (mmol/L)	5.59 ± 0.98	5.55 ± 0.99	5.60 ± 1.00	5.56 ± 1.00	0.00 [-0.07, 0.08], p=0.948
HDL-C (mmol/L)	1.10 ± 0.24	1.10 ± 0.25	1.08 ± 0.31	1.10 ± 0.27	-0.01 [-0.04, 0.03], p=0.742
LDL-C (mmol/L)	4.09 ± 0.91	4.06 ± 0.92	4.12 ± 0.95	4.08 ± 0.91	-0.01 [-0.08, 0.06], p=0.767
TG (mmol/L)	2.03 ± 1.01	1.99 ± 0.88	1.94 ± 0.92	1.89 ± 0.84	0.04 [-0.04, 0.11], p=0.334
hsCRP (mg/L)	2.2 [1.2, 4.0]	2.2 [1.1, 3.6]	2.0 [1.0, 3.8]	2.0 [0.9, 3.8]	0.1 [0.0, 0.12], p=0.133

[1] Values are means ± SDs or median [IQR]; n = 76. $PWV_{c\text{-}r}$, carotid-to-radial pulse wave velocity; $PWV_{c\text{-}f}$, carotid-to-femoral pulse wave velocity; cAIxHR75, central augmentation index corrected for heart rate. SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; $HOMA_{IR}$, homeostatic model assessment of insulin resistance; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; hsCRP, high-sensitive C-reactive protein.

[2] Linear mixed model analysis with treatment, period and gender as fixed factors, participant as random factor and 0h values as covariate. *P*-values for the effect of treatment (mean difference [95% CI] between the NWT-03 and placebo intervention) were reported. P<0.05 was considered statistically significant.

ACKNOWLEDGEMENTS

We thank all volunteers for participating in the study, and Maud Beckers for technical support. We thank Sanne van der Made for critically reading the study protocol and Resy Smeets, Dorien Pieters, and Bibi Waterval for their practical work in the study.

FUNDING STATEMENT

This study was sponsored by the SME Newtricious, Oirlo, The Netherlands. Newtricious provided the hydrolysate but had no further role in the design, analysis or writing of this article.

CONFLICT OF INTEREST

The authors declare no competing financial interests in relation to the work described.

ETHICAL APPROVAL

The study was conducted according the guidelines of the Declaration of Helsinki and approved by the Medical Ethics Committee of Maastricht University Medical Center (METC153021).

AUTHOR CONTRIBUTIONS

Conceptualization was performed by JP. All authors contributed to the data analysis, curation and visualization, and contributed to the original draft of the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CHAPTER 6

General discussion

Consuming a healthy diet is important for reducing the risk of age-related disorders, such as cardiovascular disease (CVD), type 2 diabetes (T2D), and dementia [1, 2], which are all strongly associated with cognitive decline. Changes in the more traditional risk factors, such as serum lipids or blood pressure (BP) only partly explain the underlying mechanisms through which dietary interventions can reduce the risk of these disorders [3]. Therefore, in this dissertation a comprehensive approach was used by evaluating dietary effects on multiple vascular function markers across the vascular tree [4], and on whole-body insulin sensitivity [5, 6]. Effects were studied beyond those on peripheral markers and we extended our focus on vascular function and insulin sensitivity in the brain as potential targets for preventing age-related cognitive decline [7, 8]. An overview of the main findings is presented in **Table 1**.

COGNITIVE PERFORMANCE

Cognitive performance includes a variety of domains such as attention and psychomotor speed, memory, and executive function, which are all essential for decision-making, problem-solving, and daily functioning [9]. Cognitive decline progresses in different stages, with some decline observed in middle age for psychomotor speed, and in adults older than 60 years for memory and executive function [10]. The assessment of cognitive performance in specific domains presents challenges that requires standardized methods. These methods should be accurate, valid, reliable, and should have high sensitivity and specificity for the outcomes [11]. In our systematic review (**Chapter 2**), many different questionnaires have been used to assess cognitive performance. However, in the study described in **Chapter 4** the Cambridge Neuropsychological Test Automated Battery (CANTAB), a standardized, validated, and sensitive touchscreen-based approach was used to detect dietary changes in cognitive performance across the different domains [11]. CANTAB has been developed to investigate populations with different cognitive abilities, avoiding floor and ceiling effects [12]. For our study, five cognitive tasks were selected that assessed cognitive performance in domains of psychomotor speed (five-choice reaction time test), executive function (multitasking test), visuospatial memory (delayed matching to sample and paired associates learning), and verbal memory (verbal recognition memory test). These tasks are sensitive to various dietary interventions [11, 13], making them suitable to address potential underlying mechanisms, such as vascular function [13] or insulin sensitivity in the brain (**Chapter 2**), which will be discussed in the following paragraphs. Test duration was limited to a maximum of 70 minutes to avoid the potential effects of fatigue [14]. Besides the type and duration of the intervention, it is important to consider the general well-being of the participants, which can affect the outcomes of dietary interventions as well [11]. However, no effects on quality of life, perceived stress, sleep quality or mood were observed in **Chapter 4**.

Table 1 – Overview of the main findings and conclusions of the studies presented in this thesis.

Ch	Study design	Main findings
2	**Systematic review** Randomized, controlled trials involving adults were included that investigated the effects of intranasal insulin on (regional) CBF.	» Region-dependent increases or decreases in cerebral blood flow following intranasal insulin in healthy adults. » Brain insulin responsiveness is affected by normal aging, obesity, and type 2 diabetes. » The relationship between brain insulin sensitivity and cognitive performance warrants further study, while systemic spillover of intranasal insulin is minimal.
3	**Mixed nut study** Randomized, cross-over trial in 28 men and women, age (mean ± SD): 65 ± 3 yr, BMI: 27.9 ± 2.3 kg/m^2. The effects of 60 g/day mixed nuts or no nuts as part of a recommended diet (Dutch Wheel of Five) for 16 weeks (washout: 8 weeks) were investigated.	» Mixed nut consumption improved brain insulin sensitivity in occipital and frontal regions, but did not affect markers of peripheral insulin sensitivity. » The mixed nut intervention reduced intrahepatic lipid content, total and LDL-cholesterol, and office and 24-h ambulatory blood pressure.
4		» Mixed nut consumption improved brain vascular function in frontal and parietal regions that may underlie the observed beneficial effects on memory performance. Domains of executive function and psychomotor speed were unaffected. » Strong effects of mixed nuts on vascular function along the arterial tree were found with improved endothelial function of the brachial and carotid artery, reduced arterial stiffness, and improved retinal microvascular function.
5	**NWT-03 study** Randomized, cross-over trial in 76 metabolic syndrome patients, age: 61 ± 10 yr, BMI: 31.7 ± 4.0 kg/m^2. The effects of 5 g/day NWT-03 or placebo for 4 weeks (washout: 2–8 weeks) were studied.	» Long-term NWT-03 supplementation did not affect markers of arterial stiffness, but modestly improved pulse pressure. No long-term effects were found on cardiometabolic parameters. » Acute NWT-03 intake following the intervention improved diastolic blood pressure and an indirect marker of arterial stiffness.

BMI, body mass index; Ch, Chapter; LDL, low-density lipoprotein; RCT, randomized controlled trial.

VASCULAR FUNCTION MARKERS ALONG THE ARTERIAL TREE

The early detection of CVD risk markers is important to attenuate disease progression [15]. While changes in more traditional cardiometabolic risk markers play an important role in assessing reductions in CVD risk, they may not fully elucidate the underlying mechanisms following dietary interventions [3]. Therefore, non-invasive vascular markers have emerged as valuable additions [4]. While many previous studies have examined vascular function in specific regions, a comprehensive approach that includes multiple markers is necessary to fully understand the impact of diet across the arterial tree [4]. In this dissertation, the focus was on dietary effects on different segments, such as central (e.g., carotid artery), peripheral (e.g., brachial artery), and retinal microvascular arteries, and the brain vasculature. As many factors such as dietary intake, physical activity, and alcohol consumption can affect vascular parameters [16, 17], measurements were standardized as much as possible (**Chapters 4 & 5**). Participants were instructed to fast overnight for 12 h, and to abstain from consuming alcoholic beverages or performing strenuous physical exercise. Finally, care was taken that measurements were consistently performed by the same researcher to reduce inter-operator variability and at the same time of the day for each participant.

Brain vascular function

Brain vascular function plays an important role in maintaining optimal cerebral blood flow (CBF) to ensure not only delivery of adequate amounts of oxygen and essential nutrients to the brain, but also to remove carbon dioxide and other metabolic products. Resting CBF values typically range from 40 to 60 mL/100g of brain tissue/min, but CBF in multiple cortical brain regions tends to decline with age with an estimated decrease of about 0.5-1.0% per year after the age of 60 [18]. Accordingly, in our study involving adults aged 60 to 70 years, the average whole-brain resting CBF was 39 (SD: 6) mL/100g/min (**Chapter 4**). Despite this small age range, a significant negative correlation was found between whole-brain CBF and age ($r = -0.420$, $P = 0.029$; **Figure 1A**). Reductions in whole-brain CBF have been consistently observed in dementia and are strongly associated with cognitive performance [19]. However, in our study, no significant correlations were found between whole-brain CBF and cognitive performance. Interestingly, significant negative associations were observed between whole-brain CBF and total white matter hyperintensity (WMH) counts ($r = -0.490$, $P = 0.009$; **Figure 1B**). WMHs are commonly observed in older adults and are indicative of chronic ischemia related to cerebral small vessel disease. Importantly, WMHs have been associated with progressive cognitive decline and a twofold increase in the risk of dementia [20]. Due to the relatively short supplementation period of four months, it is however unlikely that mixed nut consumption - or any other lifestyle intervention – could have affected WMH structures.

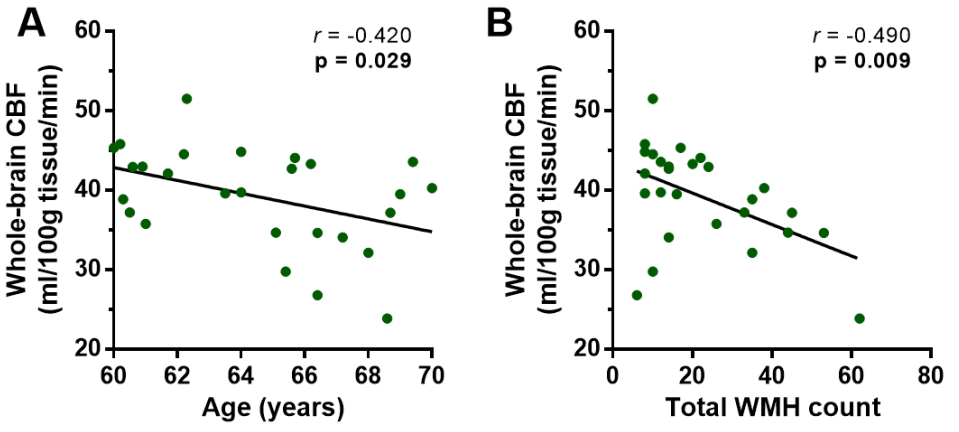

Figure 1 – The associations between whole-brain cerebral blood flow (CBF) and (**A**) age and (**B**) total WMH count in older adults with overweight or obesity (n = 27). The data are derived from **Chapter 3**.

There are several techniques available to quantify CBF, including positron emission tomography (PET) imaging, which is the most accurate to assess CBF. However, this requires intravenous injection of a radioactive contrast agent that diffuses through the blood-brain barrier (BBB) [21]. The focus of this dissertation was therefore on the non-invasive arterial spin labeling (ASL)-MRI technique (**Chapter 2–4**), which utilizes magnetically-labelled arterial blood as an endogenous tracer to quantify CBF within approximately 10 minutes. This method employs radiofrequency pulses to label blood protons within the carotid and vertebral arteries, which are labeled perpendicular based on an angiogram. Ten labeled images were acquired with a resolution of 3 mm isotropic when the labeled blood has reached the brain, while ten separate control images were obtained without prior labeling. The difference between control and labeled images estimates the amount of labelled blood delivered to the tissue by perfusion. After calibration, this technique generates a three-dimensional CBF map. Studies have demonstrated the accuracy of ASL-MRI in measuring CBF in both healthy and diseased populations, making it an useful tool to study brain vascular function [22]. Overall, ASL-MRI exhibits good reproducibility and can be used to explore the effects of dietary interventions aimed at improving CBF [7]. The coefficient of variation of scan-rescan reproducibility should however be taken into account, for which we observed a between-session coefficient of 13% in our study (**Chapters 3 and 4**).

The main challenge of ASL techniques is the long acquisition time due to the intrinsically low signal-to-noise ratio (SNR). The pseudo-continuous ASL (pCASL) approach was used, which is recommended by the consensus paper of Alsop et al. [7]. For pCASL, labeling occurs over a longer period with over 1000 radiofrequency pulses applied at a rate of one per second. In contrast, pulsed (p)ASL uses a single short pulse or a limited number of pulses to invert a thick slab of arterial water spins. However, the SNR is higher for pCASL due to the temporal duration of the labeled bolus and lower magnetization effects. Importantly, multishot three-dimensional

gradient and spin echo (GRASE) sequences were used that increase temporal SNR as compared to two-dimensional readouts [23]. Further improvement of the SNR ratio can be obtained by modular features, including background suppression that reduces noise from fluctuations in the static tissue signal [7]. In our study, four background suppression pulses were used and individual pCASL-images were distortion corrected using images with opposite phase-encoding direction. Due to the large field of view, the entire brain was imaged excluding the cerebellum, while other studies often focus on only specific brain regions. It is important to consider the arterial transit time, as post labeling delay is ideally just longer than the longest vale of arterial transit time in the participants. A post labeling delay of 2000 ms was used, which is recommended for older participants to account for expected longer arterial transit time. Furthermore, studies should consider the relaxation time (T_1) of labeled blood, which largely depends on blood water content. The T_1 of blood was estimated by measuring the plasma hemoglobin concentrations, which may affect CBF up to 35% [24].

There are several approaches to analyze CBF data obtained from pCASL-MRI. For region-of-interest (ROI) analysis, CBF values are extracted from predetermined brain regions based on anatomical criteria, which is used in hypothesis-driven research. Within our studies, CBF values were first calculated in the native space of predefined regions using the Harvard-Oxford atlas and Volbrain subcortical segmentations. However, no differences in regional CBF were found using this approach (**Chapter 4**). In contrast, voxel-wise comparison analyzes CBF values for individual voxel across the entire brain, which captures spatial heterogeneity and is not constrained by the size or boundaries of predefined brain regions, making it more sensitive to subtle dietary effects [25]. Voxel-wise analysis were performed after non-linear registration to the Montreal Neurological Institute (MNI)-atlas with a 2 mm voxel-size. Statistical comparisons were performed at each voxel to identify clusters of regions with significant differences between interventions and within subjects. In our study, default settings of a connectivity of 26 voxels and a Z-threshold of 2.1 were used. Choosing a Z-threshold is subjective and can influence the results. Setting the threshold too high may not capture subtle dietary changes in voxel intensities, while a low threshold may increase the risk of false positives. A family-wise error correction was however used to deal with multiple-comparisons. Finally, the power calculation was based on changes in whole-brain CBF. Our voxel-wise analyses revealed that effect sizes and variability of individual clusters were comparable. Therefore, future studies targeting specific brain regions may also consider powering based on regional CBF, while ensuring that statistical assumptions are met.

Peripheral vascular function

The presence of peripheral vascular risk markers may also contribute to the age-related reduction in CBF and related cognitive decline [26]. First, two independent markers of peripheral endothelial function were assessed. Flow-mediated vasodilation (FMD) measures NO-dependent vasodilation of the brachial artery in response to increased blood flow after temporary occlusion of the artery [16]. Longer-term mixed nut consumption improved FMD by 1.6 %-point (**Chapter 4**), translating to a 13–21% reduced risk of cardiovascular events [27]. Moreover, evidence was provided for a higher carotid artery reactivity (CAR) response to a cold pressor following mixed nut consumption (**Chapter 4**). This indicates an improved endothelium-mediated vasodilation of a central artery in closer proximity to the brain and effects may thus be more directly related to CBF or cognitive changes [28]. However, no relationship was observed between whole-brain CBF and CAR (r = -0.011, P = 0.956; **Figure 2A**). Interestingly, a significant negative association was observed between whole-brain CBF and intima-media thickness of the carotid artery (r = -0.422, P = 0.040; **Figure 2B**). While it is important to consider the structural properties of arteries when interpreting vascular function in the brain and periphery, it is unlikely that dietary interventions would affect the intima-media thickness over relatively short periods of time. Moreover, CAR-responses can be dichotomized into carotid constriction or dilation, with CAR-constrictors showing a 4-fold higher risk for cardiovascular events and clinical progression [29]. It should however be noted that none of the participants included in **Chapter 4** were identified as constrictors.

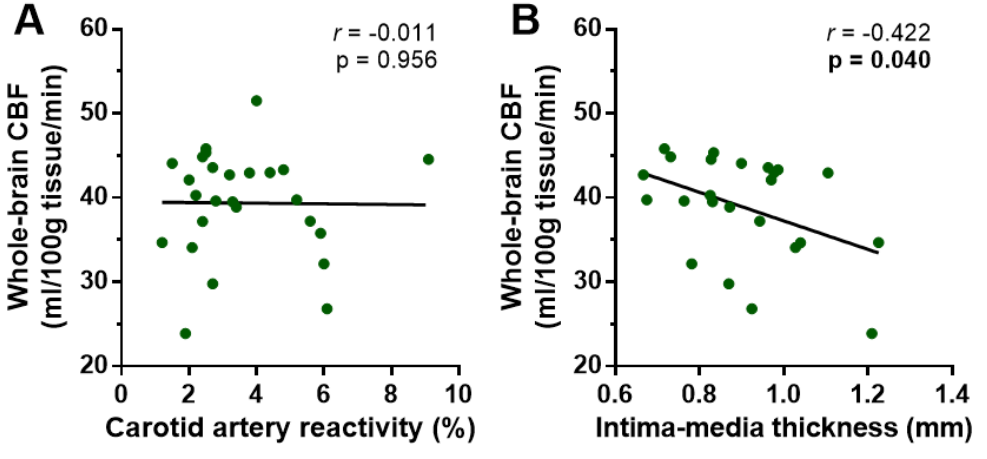

Figure 2 – The associations between whole-brain cerebral blood flow (CBF) and (**A**) carotid artery reactivity and (**B**) intima-media thickness in older adults with overweight or obesity (n = 27). The data are derived from **Chapter 3**.

Arterial stiffness, which refers to the elasticity of the arterial walls, is an important factor in maintaining BP and has been inversely related to CBF and cognitive outcomes in many studies [30]. The gold-standard approach – carotid-femoral pulse wave velocity (PWV_{c-f}) – was used to non-invasively assess regional arterial stiffness [31]. In **Chapters 4 and 5**, these parameters were measured according to the expert recommendations, and strict quality control was used with at least three repeated measures that should not differ more than 1.0 m/s from the average value [17]. Mixed nut consumption improved PWV_{c-f} by 0.6 m/s (**Chapter 4**), translating to a clinically-relevant reduction of 8% of future cardiovascular events [32], while NWT-03 supplementation did not affect markers of arterial stiffness (**Chapter 5**).

The retinal microvasculature shares anatomical and physiological similarities with the microvasculature in the brain. Consequently, abnormalities in retinal vascular caliber, characterized by narrower arterioles and wider venules, may indicate the presence of systemic microvascular disease, and have been associated to CBF [33] and cognitive performance [34]. Such associations were not found in our studies, possibly due to the limited sample sizes. Our fundus images were digitally analyzed with semi-automated software to identify at least two segments of retinal arterioles and venules [35]. It is important to match exactly the same segments for repeated visits in each participant to increase the internal validity [36]. In **Chapter 4**, the Parr-Hubbard formulas were used to estimate the central retinal arteriolar (CRAE) and venular equivalents (CRVE), and the arteriolar-to-venular ratio (AVR), and improvements were found in the CRAE and AVR. These parameters describe the projected calibers of the central retinal vessels, which enter and leave the eye through the center of the optic nerve and receive blood supply from the internal carotid artery that also supplies the brain [37].

Office brachial BP was assessed in the fasted state according to expert guidelines [38]. Given the strong association between office BP and peripheral vascular markers, it is important to consider intervention effects on BP when interpreting effects on vascular function. In **Chapter 4**, it was reported that longer-term mixed nut consumption reduced both PWV_{c-f} and CRAE, which are closely related to mean arterial pressure (MAP) [17]. Importantly, even after statistically adjusting both parameters for the change in MAP, the observed effect on MAP remained significant. Additionally, in **Chapter 5**, acute NWT-03 intake was found to decrease the central augmentation index (cAIxHR75), reflecting peripheral wave reflections, which may be attributed to the simultaneous drop in diastolic BP [39, 40]. Furthermore, mixed nut consumption lowered daytime BP (**Chapter 4**), indicting improvements in BP under real-life conditions, which are less commonly observed compared to office BP effects.

INSULIN RESISTANCE IN PERIPHERAL TISSUES AND IN THE BRAIN

Impaired glucose metabolism is an important contributor to the pathophysiology of age-related disorders and cognitive decline. The quantification of insulin resistance is therefore an important aspect of characterizing the metabolic risk profile of individuals at risk of developing these disorders. Within this dissertation, a comprehensive approach was used to study dietary effects on peripheral insulin sensitivity, including tissue-specific indices for the liver and muscle [5, 6], but also on the impact of diet on brain insulin sensitivity for the first time

Brain insulin sensitivity

In contrast to earlier concepts that considered the brain as an insulin-insensitive organ, it is now widely recognized that insulin action plays an important role in the central nervous system (CNS) [8, 41]. Insulin receptors are expressed in various brain regions, with high concentrations in the hypothalamus, hippocampus, and cerebral cortex. Circulating insulin crosses the BBB through active receptor-mediated transport. Insulin does not directly affect brain glucose transport or metabolism [42]. Instead, it serves as a peripheral feedback signal, regulating appetite and contributing to body weight control and eating behavior. Insulin is part of a neuropeptidergic signaling network within the hypothalamus that regulates appetite [43, 44]. Furthermore, insulin has an important role in regions beyond the hypothalamus for the regulation cognitive processes [45], which was a scope of the current dissertation.

Measuring and differentiating brain insulin action from peripheral insulin effects in humans is challenging. Various techniques are available for assessing brain insulin action, including methods that stimulate endogenous insulin release (e.g. oral glucose tolerance test [OGTT] or intravenous glucose infusion) or exogenous insulin infusion (e.g. hyperinsulinemic-euglycemic clamp). However, these techniques elicit insulin-stimulated effects in most tissues throughout the body [41, 46]. In contrast, intranasal insulin administration has been used to isolate brain insulin responses from peripheral effects. Nasal insulin effectively reaches distinct brain areas within 30-60 min through paracellular transport or bulk flow along olfactory nerves and trigeminal perivascular channels [47, 48]. Whereas circulating insulin enters the cerebrospinal fluid (CSF) via a saturable transport mechanism, nasal insulin directly bypasses the BBB [49]. Combining intranasal insulin with neuroimaging methods enables the quantification of brain insulin action. The pCASL-MRI technique, as discussed earlier, also offers a suitable approach to study the effects diet on brain insulin responsiveness by quantifying the absolute change in CBF before and after intranasal insulin. Depending on the study objectives, functional MRI can also be used combined with the spray to capture hemodynamic changes related to neuronal activity using blood-oxygen-level-dependent (BOLD) imaging [8, 41].

Brain insulin resistance refers to impaired insulin signaling in the CNS. Unlike the periphery, there is currently no universal definition or established cut-off value for brain insulin resistance.

In **Chapter 2**, a systematic review was performed investigating the effects of intranasal insulin on CBF using ASL-MRI. In healthy adults, brain insulin sensitivity can be defined as region-specific increases (e.g. insular cortex and dorsal striatum) or decreases (e.g. hypothalamus and middle frontal gyrus) in CBF following intranasal insulin application. Interestingly, obesity, T2D, and aging have been identified as important determinants of CBF responses to the spray, suggesting impaired insulin-induced (de-)activation patterns and regional brain insulin resistance [50, 51]. Further research is clearly warranted to establish brain insulin responsiveness in other populations, such as dementia, and to relate differences in brain insulin responsiveness to functional outcomes. Intranasal insulin further has the potential to beneficially affect memory and executive function (**Chapter 2**). This is in line with preclinical trials showing that brain insulin signaling exerts neuromodulatory actions that have implications for cognitive performance. For example, insulin can regulate the expression of neurotransmitters and increase cortical cerebral glucose metabolism in brain regions important for memory [52]. However, a causal relationship between regional brain insulin sensitivity and cognitive performance still needs to be established in humans in the maintenance of age-related cognitive health.

This dissertation provided first evidence suggesting that long-term dietary interventions have the potential to improve brain insulin sensitivity (**Chapter 3**). Tschritter and colleagues were the first to demonstrate that individuals with high brain insulin sensitivity experienced greater reductions in body fat, particularly visceral fat, following a 24-month lifestyle intervention compared to those with lower brain insulin sensitivity [53-55]. These findings were attributed to altered hypothalamic insulin action, which was positively correlated with visceral adipose tissue [55]. Furthermore, recent trials have shown that aerobic exercise training and pharmacological interventions can improve hypothalamic insulin sensitivity [55, 56]. Interestingly, our study revealed that mixed nut consumption improved insulin sensitivity in frontal and occipital brain regions (**Chapter 3**), which also displayed impaired insulin-induced activation patterns associated with aging and obesity (**Chapter 2**). While it cannot be ruled out that insulin action within these regions may affect cognitive performance, these regions are part of a brain network associated with feeding and reward behaviors [57, 58]. Therefore, incorporating fMRI BOLD-techniques in future dietary intervention studies to evaluate insulin-related appetite control and satiety is of great interest.

Peripheral insulin sensitivity

Various methods and indices are available to assess peripheral insulin sensitivity, each with their own advantages and limitations [59]. The two-step hyperinsulinemic euglycemic clamp technique is considered the gold standard for determining whole-body insulin resistance, but is rather invasive, costly, and time-consuming [60]. In this dissertation, we quantified peripheral insulin sensitivity using parameters derived from fasting glucose homeostasis markers (**Chapter 3 & 5**) and the OGTT (**Chapter 3**), which are more practical and are commonly used in clinical practice. The HOMA-IR [61] and Matsuda index [62] were used to assess whole-body insulin sensitivity. Moreover, the hepatic insulin resistance index (HIRI) and muscle insulin sensitivity index (MISI) [63] quantified tissue-specific insulin resistance, which have been validated against the hyperinsulinemic euglycemic clamp technique. OGTT-derived parameters do however show discrepancies. Oral glucose delivery can stimulate insulin secretion through gastrointestinal factors, including the rate of intestinal glucose absorption and the incretin response (e.g. GLP-1 and PYY) [64]. Consequently, oral glucose administration during an OGTT leads to higher plasma insulin concentrations compared to intravenous glucose infusion during a clamp using the same glucose load [64]. Nonetheless, OGTT-related measures may provide a closer reflection of human physiology in daily life. Our results from both studies however indicate that markers of peripheral insulin sensitivity did not change (**Chapter 3 & 5**). Overall, the long-term effects of nut consumption and egg-protein hydrolysates on markers of glycemic control and peripheral insulin sensitivity were not convincing.

In **Chapter 3**, mixed nut consumption improved insulin sensitivity in the brain without concurrent effects in the periphery. Next to effects on whole-body insulin sensitivity, it is therefore highly relevant for future dietary interventions to consider brain insulin responses and its implications for metabolic health. No association between peripheral and brain insulin sensitivity was found in **Chapter 3**, but it should be noted that this study was not statistically powered to detect such correlations. The complex relationship between peripheral and brain insulin resistance may depend on distinctive characteristics of different brain regions, such as variations in insulin receptor density and BBB permeability across brain areas. Regions with higher receptor density or BBB permeability, such as the hypothalamus, may be more susceptible to changes in insulin signaling. Individuals with peripheral insulin resistance, including those with obesity or T2D, may also exhibit impaired brain insulin signaling and function [65, 66]. Peripheral insulin resistance can impact brain insulin sensitivity through several mechanisms, including decreased saturated insulin transport capacity across the BBB, reduced sensitivity of insulin receptors or chronic inflammation [67]. Studies using ASL-MRI combined with a hyperinsulinemic euglycemic clamp to measure insulin sensitivity in both the brain and periphery have demonstrated strong associations between peripheral insulin sensitivity and brain insulin responses in the hypothalamus [46, 51, 68] and striatum [68]. This suggests region-specific associations between

systemic insulin resistance and the brain, indicating that the impact on cognitive processes and metabolic regulation may vary depending on the brain regions involved. In contrast, brain insulin resistance can manifest independently of the periphery, as lower ratios of CSF insulin to plasma concentrations were observed in older adults and patients with neurodegenerative diseases like dementia [69]. Furthermore, a modest relationship was observed between changes in regional brain insulin responses and those in intrahepatic lipid content following mixed nut consumption (**Chapter 3**). Consistent with these findings, a previous study in healthy adults found that nasal insulin reduced hepatic lipid content independently of systemic hyperinsulinemia [70]. However, the potential existence and importance of an insulin-related brain-liver axis need to be further established in future studies.

DIETARY INTERVENTION STRATEGIES

The results presented in this thesis show that healthy dietary interventions can beneficially affect markers of vascular function and insulin sensitivity in the periphery and the brain.

Consumption of mixed nuts

Nuts are nutrient-dense foods that are rich in bioactive components, including unsaturated fatty acids, polyphenols, fibers, phytosterols, tocopherols, and proteins. Nut consumption have been extensively studied for their potential effects on traditional CVD risk factors [71-73]. It remains however unclear whether these effects can be extended to the brain, which may be more closely related to cognitive performance [74]. Our study therefore investigated the effects of a sixteen-week mixed nut intervention (60 g/day: 15 g of walnuts, cashew, pistachio, and hazelnut) on insulin sensitivity and vascular function in the brain and periphery of older adults with overweight or obesity (**Chapter 3 & 4**). A summary of the main results is depicted in **Figure 3**. In short, besides effects on traditional CVD risk factors and peripheral vascular function, beneficial effects of mixed nut consumption were found on brain health. Specifically, the intervention led to increased resting CBF in frontal and parietal brain regions, which may underlie the observed effects on memory. On the other hand, mixed nut consumption improved brain insulin responsiveness in occipital and frontal regions, which is hypothesized to be related to appetite control and satiety, as described earlier.

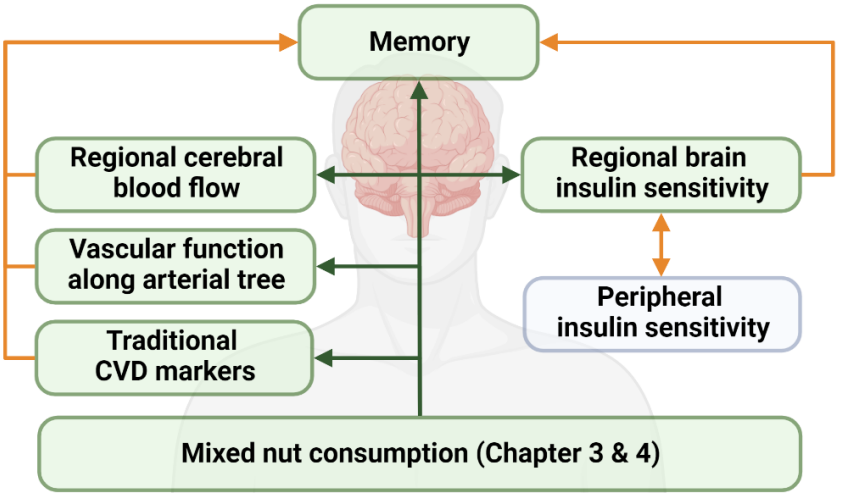

Figure 3 – An overview of the key findings of the mixed nut study (**Chapters 3 & 4**). Mixed nut consumption improved traditional cardiovascular disease (CVD) risk markers, such as serum total cholesterol concentrations and blood pressure, as well as different vascular function markers across the arterial tree. Mixed nuts also increased regional cerebral blood flow in brain regions that may explain the observed beneficial effects on memory performance. Furthermore, it was shown for the first time that diet can improve brain insulin responsiveness in regions that had previously displayed impaired insulin-induced activation patterns associated with aging and obesity (**Chapter 2**). The green arrows indicate the identified improvements, and orange arrows are hypothesized relationships that require further study.

An important consideration is that participants were requested to adhere to the Dutch food-based dietary guidelines [75], meaning that effects of mixed nuts were evaluated as part of a recommended diet. Furthermore, a specific mixture of nuts was deliberately chosen, aiming to provide a rich combination of bioactive compounds. Notably, the average daily nut intake in the Dutch population is relatively low, around 5 g (1-2 En%), which falls below the recommended daily intake of at least 15 g unsalted and unroasted nuts (~4 En%) [75]. However, previous studies have demonstrated more pronounced cardiovascular benefits when nuts accounted for approximately 15% of daily energy intake [76], aligning with the dose provided in our study. No further guidelines were provided on how to include the nuts into the diet to mimic real-life conditions as much as possible. Based on our results, it should be emphasized that participants compensated for the extra energy provided by the mixed nuts, because energy intake and markers of body composition between the intervention and control periods did not differ. However, it is important to acknowledge that effects of the nuts as part of a recommended diet cannot be disentangled from those due to the replacement of food products by the intake of the nuts.

In a previous trial conducted at our facilities, it was observed that long-term almond consumption (50 g/day) showed adverse effects on peripheral insulin sensitivity and glucose metabolism in adults with prediabetes, which were independent of BMI changes. Surprisingly, total energy intake was increased during the intervention period, suggesting that the almond consumption was not fully compensated by decreasing the intake of other food items [77]. To explore potential influences of glycemic status in our findings, subgroup analyses were conducted based on the American Diabetes Association' s criteria of prediabetes for fasting glucose values (≥5.6 mmol/L) [78] (**Table 2, Chapter 3 & 4**). Among the 28 participants, 13 were classified as prediabetic. The improvements in total and LDL-cholesterol levels, as well as intrahepatic lipid content, appeared to be more pronounced in participants with normal glucose homeostasis. Interestingly, non-significant increases were observed for weight, BMI and total energy intake in this subgroup, while markers of peripheral insulin sensitivity were not different. This may suggest that the underlying metabolic disturbances in prediabetes may reduce the efficacy of nut consumption to improve lipid metabolism. These findings should however be interpreted with caution due to insufficient statistical power and warrant further confirmation with larger sample sizes. The timing of nut consumption may also affect the study outcomes. Previous research has indicated that consuming nuts primarily in the evening as compared to the morning may diminish their potential benefits on acute and second meal glycemic responses as well as satiety [79]. In our study population, approximately 70% of participants consumed nuts in the morning or throughout the day, while 30% consumed them in the afternoon or evening. However, the sample sizes within these subgroups were too small to conduct meaningful analyses. Future studies with larger sample sizes should consider investigating the optimal timing of nut consumption to maximize the health benefits.

Table 2 – Subgroup analyses based on glucose status

	NFG (<5.6 mmol/L, n = 15)	**IFG (≥5.6 mmol/L, n = 13)**
Weight, kg	-0.2 (-1.1, 0.7); P = 0.708	0.9 (-0.4, 2.2); P = 0.168
BMI, kg/m^2	0.0 (-0.3, 0.3); P = 0.945	0.4 (-0.1, 0.9); P = 0.118
Total energy intake, kcal/d	-68 (-186, 51); P = 0.293	76 (-14, 166); *P = 0.092*
Fasting glucose, mmol/L	0.1 (-0.1, 0.3); P = 0.230	0.1 (-0.1, 0.4); P = 0.345
$HOMA_{IR}$	-0.1 (-0.3, 0.2); P = 0.575	0.4 (-0.1, 0.8); P = 0.113
Matsuda Index	0.3 (-4.0, 4.5); P = 0.887	0.4 (-0.1, 0.8); P = 0.659
Total cholesterol, mmol/L	-0.47 (-0.84, -0.11); **P = 0.012**	-0.01 (-0.31, 0.28); P = 0.922
LDL-cholesterol, mmol/L	-0.42 (-0.73, -0.12); **P = 0.009**	-0.02 (-0.28, 0.25); P = 0.894
Triacylglycerol, mmol/L	-0.20 (-0.47, 0.06); P = 0.126	-0.02 (-0.28; 0.23); P = 0.863
Intrahepatic lipid content, %	-0.8 (-1.5, -0.1); **P = 0.022**	-0.4 (-2.0, 0.9); P = 0.423

BMI, Body Mass Index; $HOMA_{IR}$, Homeostatic Model Assessment of Insulin Resistance. LDL, low-density lipoprotein; NFG, normal fasting glucose; IFG, impaired fasting glucose.

Supplementation with the egg-protein hydrolysate NWT-03

NWT-03 is a dietary egg-protein hydrolysate obtained through lysozyme digestion with alcalase [80, 81]. Acute and short-term intake of NWT-03 has shown BP-lowering effects in individuals with mild hypertension [82], and improved cardiometabolic markers in adults with impaired glucose tolerance or T2D [83]. However, the clinical relevance of egg-derived protein hydrolysates in long-term trials remains to be determined [84]. In our study, the longer-term effects of egg-derived protein hydrolysates on arterial stiffness were inconclusive, although a modest reduction in fasting pulse pressure was observed (**Chapter 5**). However, the translation of this decrease in brachial pulse pressure into a clinically significant reduction in CVD risk remains uncertain [85]. Inconsistencies in the effects of egg-derived protein hydrolysates on BP regulation may however be attributed to factors such as the amount supplemented and baseline characteristics of the study population, including baseline BP [86].

In a recently published article based on this study, it was demonstrated that the longer-term intake of 5 g/day NWT-03 improved cognitive performance within the executive function domain, as reflected by better performance on an anti-cue reaction time task [87]. The article suggested that these cognitive effects were possibly related to DPP-IV inhibition and improved insulin sensitivity. However, based on the current dissertation, it is unlikely that 5 g/day NWT-03 affects markers of peripheral insulin sensitivity. Instead, small protein fragments in NWT-03 may potentially cross the BBB [88] and directly influence regional brain processes involved in cognitive performance. This may suggest that future studies on protein hydrolysates should consider investigating brain insulin responsiveness. Additionally, markers of arterial stiffness were unaffected, suggesting that it is unlikely that NWT-03 exerts its cognitive effects via the peripheral vasculature as well.

The selection of an appropriate food matrix is crucial for determining the efficacy and bioavailability of egg-protein hydrolysates [89]. The bioactivity of these hydrolysates depends on their resistance to degradation by gastrointestinal peptidases and their absorption into the bloodstream [90, 91]. Powdered sachets of egg-protein hydrolysates have commonly been used due to their feasibility and simple blinding, as also used in **Chapter 5**. However, it is important to note that the choice of food vehicle, such as water, milk, or fruit juices, can potentially affects the physiological effects of the bioactive components, as other constituents within the food matrices can exert synergistic or antagonistic effects [92]. For instance, studies incorporating dairy peptides with fruit juices have demonstrated limited effects on cardiovascular outcomes [93], which questions the suitability of fruit juice as a food vehicle for bioactive peptides. Finally, the amount and quality of protein and the presence of fibre are main factors that increase the systemic bioavailability of food-derived peptides [94].

CONCLUSIONS AND FUTURE DIRECTIONS

The overall aim of the current dissertation was to investigate the effects of dietary intervention strategies on vascular function and insulin sensitivity of the brain and periphery in adults. In the first chapter, randomized, controlled trials involving adults were systematically reviewed that investigated the effects of intranasal insulin on (regional) CBF (**Chapter 2**). The next two chapters presented the results of a randomized, controlled crossover trial in older adults with overweight or obesity examining the effects of mixed nut consumption on vascular function and insulin sensitivity in the brain and periphery, and cognitive performance (**Chapters 3 and 4**). Furthermore, the impact of longer-term NWT-03 supplementation, an egg-protein hydrolysate, was investigated on arterial stiffness and cardiometabolic markers in adults with metabolic syndrome (**Chapter 5**). Based on these findings, the following conclusions and recommendations for future research can be drawn:

» In **Chapter 2**, it has been shown that brain insulin sensitivity can be defined as either a region-specific increase or decrease in CBF following nasal insulin in healthy adults. It was further concluded that regional brain insulin responsiveness is age-dependent and affected by obesity and T2D. Further research is warranted to further define the concept of brain insulin sensitivity, particularly in patient populations such as dementia. These studies should consider various determinants such as the insulin dose. Moreover, factors such as oxidative stress, neuroinflammation, and BBB permeability should be considered as potential contributors to changes in brain insulin sensitivity.

» For the first time, evidence was provided that long-term dietary interventions can beneficially affect regional brain insulin sensitivity in older adults with overweight or obesity (**Chapter 3**). Specifically, mixed nut consumption improved insulin sensitivity in specific brain regions that had previously shown impaired insulin-induced activation patterns associated with aging and obesity (**Chapter 2**). However, further research is needed to explore the mechanisms through which therapeutic and lifestyle interventions may improve brain insulin sensitivity, which may underlie changes in cognitive performance, appetite control and satiety, and weight maintenance. For this, combining non-invasive neuroimaging techniques, such as pCASL-MRI and BOLD-fMRI would provide additional insights to further evaluate the impact of diet on brain function.

» While mixed nut consumption improved brain insulin sensitivity, no concurrent effects on peripheral insulin sensitivity were observed (**Chapter 3**). Future studies should further explore the potential causal or bidirectional relationship between brain and peripheral insulin sensitivity. To elucidate this relationship, studies combining nasal insulin simultaneously with an OGTT or clamp should be conducted. These approaches allow for the assessment of tissue-specific insulin signaling in the periphery and provide novel insights into the impact of nasal insulin on peripheral insulin sensitivity.

» Mixed nut consumption also improved vascular function across multiple sites of the arterial tree, with the observed regional improvements in CBF may underlie beneficial effects on memory performance (**Chapter 4**). Large-scale, longer-term trials should investigate the potential causal relationship between lifestyle-induced changes in CBF and brain functional parameters. More research is warranted to understand how different dietary factors affect different cognitive domains. Further studies with larger sample sizes are however necessary to establish these causal relationships in healthy and diseased populations following dietary interventions.

» Longer-term NWT-03 supplementation did not affect markers of arterial stiffness, but modestly reduced pulse pressure in adults with metabolic syndrome (**Chapter 5**). Future studies investigating the long-term health effects of egg-protein hydrolysates should take into account multiple markers of vascular function. Moreover, future research should focus on the potential underlying mechanisms, such as brain insulin responsiveness to explain why NWT-03 may improve cognitive performance in the executive function domain.

In conclusion, this dissertation provides further evidence that dietary intervention strategies can reduce the risk of age-related metabolic disorders by effects on vascular function and insulin sensitivity in both the brain and periphery. These observed findings may contribute to beneficial effects on cognitive functioning. Additionally, mixed nut consumption also improved traditional risk factors such as blood pressure and cholesterol levels, as well as various markers of vascular function in periphery, thereby reducing the risk of CVD.

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Summary

A healthy diet is important in preventing the development of age-related disorders, such as cardiovascular disease (CVD), type 2 diabetes (T2D), and dementia, which are closely associated with cognitive decline. While previous dietary intervention trials have primarily focused on traditional CVD risk factors, underlying mechanisms involved in risk reduction of age-related disorders have not been fully explained. To gain a comprehensive understanding of the effects of diet, various markers of vascular function have emerged, including markers of endothelial function, arterial stiffness and retinal microvascular calibers. In addition, over the last decade the importance of evaluating brain health has been recognized in relation to dietary interventions. Arterial spin labeling magnetic resonance imaging (ASL-MRI) has emerged as a non-invasive tool to assess regional brain vascular function, which is closely related to cognitive performance. While many dietary interventions have focused on markers of peripheral insulin resistance using fasting values or an oral glucose tolerance test (OGTT), the effects of diet on brain insulin sensitivity remain an understudied area. Insulin signaling in the brain exerts region-specific effects on neural circuits involved in cognitive performance. Brain insulin responsiveness can be assessed with ASL-MRI combined with intranasal insulin application. The overall aim of this dissertation was to study the effects of dietary interventions on vascular function and insulin sensitivity in both the brain and periphery among adults.

Brain insulin resistance is an important hallmark of age-related conditions, including T2D and dementia. In **Chapter 2**, we therefore conducted a systematic review that summarized 58 randomized, placebo-controlled trials that investigated the acute effects of intranasal insulin on cerebral blood flow (CBF) using ASL-MRI in healthy and diseased populations to define brain insulin responsiveness. We also explored relationships between changes in brain insulin sensitivity and cognitive performance. Intranasal insulin did not affect whole-brain CBF in healthy adults, but increased regional CBF of the inferior frontal gyrus, dorsal striatum, and insular cortex, and reduced CBF around the middle frontal gyrus and hypothalamus. These regions have typically been related to cognitive functioning, and feeding and reward behaviors. Important determinants of the CBF response to the intranasal spray were obesity, T2D, and normal human aging, which indicates altered brain insulin sensitivity. Obese adults showed increased CBF following nasal insulin for the middle frontal gyrus but decreased CBF for hypothalamic and cortico-limbic regions. Furthermore, CBF responses were higher for the insular cortex in T2D patients and for occipital and thalamic regions in older adults. Intranasal insulin also improved memory and executive function, but a causal relation with regional CBF still needs to be established. Finally, nasal insulin at frequently used doses resulted in only a small amount of systemic spill-over, which is unlikely to have an impact on the observed findings. Future studies should investigate longer-term effects of nasal insulin and explore associations between effects on CBF and cognitive performance.

The next two chapters presented the findings of a randomized, single-blinded, controlled cross-over trial that investigated the effects of long-term mixed nut consumption. **Chapter 3** focused on the outcomes related to brain and peripheral insulin sensitivity, as well as cardiometabolic risk markers, while **Chapter 4** described the effects on brain and peripheral vascular function, and cognitive performance. The study involved twenty-eight older adults, aged 65 ± 3 years (mean ± SD), with overweight or obesity (BMI: 27.9 ± 2.3 kg/m^2). Participants were randomly assigned to either a sixteen-week mixed nut intervention (60 g/d mixed nuts: walnuts, pistachio, cashew, and hazelnuts) or a control period without nuts, separated by an 8-week washout. The main outcomes were measured at the end of both periods. Throughout the study, participants adhered to the Dutch food-based dietary guidelines. No serious adverse events or protocol deviations were reported in the diaries and mixed nut intake was well-tolerated. Compliance was excellent, with a median of 98% (IQR: 93-100%) of the sachets consumed. Body weight and composition did not change throughout the study. Food-frequency questionnaires revealed that total energy and protein intakes were not different between intervention periods. However, mixed nut consumption lowered carbohydrate (-4.3 En%; 95%CI: -5.5 to -3.1; P < 0.001) and cholesterol intake (-2.6 mg/MJ; 95%CI: -4.2 to -0.9; P = 0.004), and increased fiber intake (1.6 g; 95%CI: 0.3 to 3.0; P = 0.019) compared with the control. In contrast, total fat intake was 5.4 En% higher (95%CI: 4.1 to 6.8; P < 0.001), with lower intakes of saturated fatty acids, but higher intakes of *cis*-monounsaturated and *cis*-polyunsaturated fatty acids (all, P < 0.001). These dietary changes were further supported by the fatty-acid composition of plasma phospholipids.

In **Chapter 3**, we reported that mixed nut consumption improved regional brain insulin action in six brain clusters, as assessed by quantifying acute effects of nasal insulin on regional CBF, a marker for brain insulin sensitivity, using ASL-MRI. Five clusters were located in the left (-4.5 ± 4.7 mL/100g/min; P < 0.001; -4.6 ± 4.8 mL/100g/min; P < 0.001; and -4.3 ± 3.6 mL/100g/min; P = 0.007) and right occipital lobe (-4.3 ± 5.6 mL/100g/min; P = 0.028). Another cluster was part of the left frontal lobe (-4.9 ± 4.6 mL/100g/min; P < 0.001). Markers of peripheral insulin sensitivity during the oral glucose tolerance test were not affected. Intrahepatic lipid content (-0.7 %-point; (-1.3 to -0.1; P = 0.027), serum low-density lipoprotein cholesterol concentrations (-0.24 mmol/L; 95%CI: -0.44 to -0.04; P = 0.019), and systolic blood pressure (-5 mmHg; 95%CI: -8 to -1; P = 0.006) were reduced after the intervention as compared to the control period. In **Chapter 4**, we observed that mixed nut consumption resulted in a higher resting CBF in the right frontal and parietal lobes (5.0±6.5 mL/100g/min; P < 0.001), left frontal lobe (5.4 ± 7.1 mL/100g/min; P < 0.001), and bilateral prefrontal cortex (5.6 ± 6.6 mL/100g/min; P < 0.001). Effects on endothelial function, arterial stiffness, and the retinal microvasculature were also assessed. Carotid artery reactivity (0.7 %-point; 95%CI: 0.2 to 1.2; P = 0.007), brachial flow-mediated vasodilation (1.6 %-point; 95%CI: 1.0 to 2.2; P < 0.001) and retinal arteriolar calibers

were higher (2 μm; 95%CI: 0 to 3; P = 0.037), and carotid-to-femoral pulse wave velocity lower (-0.6 m/s; 95%CI: -1.1 to -0.1; P = 0.032). Finally, cognitive performance was measured using the Cambridge Neuropsychological Test Automated Battery, for which visuospatial memory (-4 errors [16%]; 95%CI: -8 to 0; P = 0.045) and verbal memory (+1 correct [16%]; 0 to 2; P = 0.035) improved, but executive function and psychomotor speed did not change. Based on these two chapters, we concluded that longer-term mixed nuts consumption as part of a healthy diet improved insulin sensitivity in specific brain regions involved in metabolic and cognitive processes in older adults with overweight and obesity. Regional brain vascular function also improved, which may relate to the observed beneficial effects on memory performance. Furthermore, different vascular function markers along the peripheral arterial tree also improved, and beneficial effects on intrahepatic lipid content, cholesterol concentrations, and blood pressure were observed.

In **Chapter 5**, the results from another randomized, double-blinded, placebo-controlled, cross-over trial investigating the longer-term effects of NWT-03 supplementation, an egg-protein hydrolysate, on arterial stiffness and cardiometabolic markers were reported. The study involved seventy-six adults with metabolic syndrome, aged 61 ± 10 years and a mean BMI of 31.7 ± 4.0 kg/m^2. Participants were randomly assigned to either a 27-day intervention (5 g/day NWT-03) or placebo period, separated by two-to-eight weeks of washout. At the start and end of both periods, measurements were performed in the fasting state and 2-hours following acute NWT-03 intake. Compared with the placebo, longer-term NWT-03 intake did not affect pulse-wave velocity, a marker of arterial stiffness. Fasting pulse pressure was however reduced by 2 mmHg (95%CI: -4 to 0; P = 0.043), but other fasting cardiometabolic risk markers were not affected. No effects were observed following acute NWT-03 intake at baseline. However, acute intake of NWT-03 after the intervention significantly lowered the central augmentation index (-1.3 %-point; -2.6 to -0.1; P = 0.037), suggesting a decreased pressure wave reflection, and diastolic blood pressure (-2 mmHg; -3 to 0; P = 0.036), but other cardiometabolic markers did not change. Longer-term NWT-03 intake did not affect arterial stiffness, but modestly improved fasting pulse pressure in adults with metabolic syndrome. Acute intake of NWT-03 after the intervention also improved CAIxHR75 and diastolic BP.

In conclusion, this dissertation provides further evidence that dietary intervention strategies can reduce the risk of age-related metabolic disorders by effects on vascular function and insulin sensitivity in both the brain and periphery. These observed findings may contribute to beneficial effects on cognitive functioning. Additionally, mixed nut consumption also improved traditional risk factors such as blood pressure and cholesterol levels, as well as various markers of vascular function in periphery, thereby reducing the risk of CVD.

Nederlandse samenvatting

Het is belangrijk om een gezond voedingspatroon te volgen om de ontwikkeling van leeftijdsgebonden aandoeningen, zoals hart- en vaatziekten, type 2 diabetes en dementie, te voorkomen. Deze aandoeningen gaan samen met het verslechteren van het cognitieve functioneren. Tijdens een studie kunnen verschillende cognitieve functies worden gemeten, zoals het geheugen of het uitvoerend vermogen, ook wel executieve functie genoemd. Eerdere voeding-gerelateerde onderzoeken hebben zich voornamelijk gericht op de meer traditionele risicofactoren voor leeftijdsgebonden aandoeningen, zoals de bloeddruk of cholesterol-concentraties. Echter, deze risicofactoren verklaren de onderliggende mechanismen die betrokken zijn bij deze aandoeningen niet volledig. Om een meer volledig beeld te krijgen van de effecten van voeding, zijn er verschillende markers voor het functioneren van de bloedvaten (de vaatfunctie) beschikbaar, zoals endotheelfunctie, de stijfheid van de bloedvaten, en de structuur van kleine bloedvaten in het netvlies. Bovendien is er steeds meer aandacht voor de effecten van voeding op de gezondheid van de bloedvaten in de hersenen. Dit kan worden gemeten met behulp van “arterial spin labeling” magnetische resonantie beeldvorming (ASL-MRI). Met deze meting kan de hersendoorbloeding gemeten worden, wat een beeld geeft over de vaatfunctie van bloedvaten in specifieke hersenengebieden. Deze vaatfunctie is sterk geassocieerd met het cognitief functioneren. Terwijl veel voedingsinterventies zich hebben gericht op het verbeteren van de insulinegevoeligheid van spieren en de lever, is het effect van voeding op de gevoeligheid van de hersenen voor het hormoon insuline onderbelicht. Insuline kan in de hersenen specifieke effecten hebben op gebieden die betrokken zijn bij cognitief functioneren. De insulinegevoeligheid van de hersenen kan ook worden gemeten met ASL-MRI in combinatie met een insuline neusspray. Het doel van dit proefschrift was om de effecten van voedingsinterventies op de vaatfunctie en insulinegevoeligheid zowel in de hersenen als in de rest van het lichaam bij volwassenen te onderzoeken.

Het minder gevoelig worden van de hersenen voor insuline (insuline resistentie) is een belangrijk kenmerk van leeftijdsgebonden aandoeningen, zoals type 2 diabetes en dementie. In **Hoofdstuk 2** hebben we daarom op systematische wijze gerandomiseerde, placebo-gecontroleerde onderzoeken samengevat, die effecten van een insuline neusspray op de hersendoorbloeding hebben onderzocht met behulp van ASL-MRI. Dit hebben we bekeken bij gezonde jonge en oudere volwassenen, volwassenen met overgewicht of obesitas, en patiënten met type 2 diabetes. We hebben ook gekeken naar de relatie tussen veranderingen in de insulinegevoeligheid in bepaalde hersengebieden en het cognitief functioneren. De resultaten van deze systematische review toonden aan dat de insuline neusspray geen invloed heeft op de algehele doorbloeding van de hersenen. Echter, insuline zorgde wel voor veranderingen in de doorbloeding van specifieke hersengebieden die betrokken zijn bij het cognitief functioneren, en het gevoel van honger en verzadiging. De insuline neusspray verbeterde ook het geheugen en de executieve functie bij gezonde volwassenen. Of de veranderingen in regionale doorbloeding

van de hersenen een direct effect hebben cognitief functioneren moet nog verder worden onderzocht. Daarnaast hebben we gezien dat de werking van insuline op de specifieke hersengebieden verstoord is bij volwassenen met obesitas en type 2 diabetes, evenals bij oudere volwassenen. Ten slotte leidde de spray slechts tot een kleine hoeveelheid insuline die in het bloed terecht komt, hetgeen waarschijnlijk geen invloed heeft op de waargenomen bevindingen. Toekomstige studies moeten zich richten op de langetermijneffecten van de insuline neusspray en het verkennen van mogelijke verbanden tussen effecten op de hersendoorbloeding en cognitieve prestaties.

De volgende twee hoofdstukken gaven de resultaten weer van een gerandomiseerd, enkelblind, gecontroleerd cross-over onderzoek naar de effecten van langdurige consumptie van gemengde noten. **Hoofdstuk 3** richtte zich op de resultaten met betrekking tot de insulinegevoeligheid van de hersenen en meer traditionele risicomarkers voor leeftijdsgeboden aandoeningen, terwijl **Hoofdstuk 4** de effecten beschreef op de vaatfunctie van de hersenen en de rest van het lichaam, en het cognitief functioneren. Aan het onderzoek deden achtentwintig oudere volwassenen mee van 65 ± 3 jaar (gemiddelde ± SD) oud, met overgewicht of obesitas (BMI: 27,9 ± 2,3 kg/m^2). De deelnemers werden willekeurig toegewezen aan zowel een zestien weken durende interventie met gemengde noten (60 gram/dag gemengde noten: walnoten, pistachenoten, cashewnoten en hazelnoten) en een controleperiode zonder noten, met een tussenperiode van 8 weken. Gedurende het hele onderzoek volgden de deelnemers de Nederlandse richtlijnen goede voeding. Er werden geen nadelige effecten of protocolafwijkingen gemeld in de dagboekjes van de deelnemers en het eten van gemengde noten gaf geen problemen. De naleving was uitstekend en het lichaamsgewicht van de deelnemers veranderde niet. Uit voedingsvragenlijsten bleek dat de totale energie- en eiwitinname niet verschillend was tussen de interventie- en controleperiode. Gemengde notenconsumptie leidden echter tot een vermindering van de koolhydraat- en cholesterolinname, en een toename van de vezelinname. Daarentegen was de totale vetinname hoger, met een lagere inname van verzadigde vetzuren, maar een hogere inname van onverzadigde vetzuren.

In **Hoofdstuk 3** werd gevonden dat het eten van gemengde noten de insulinegevoeligheid in specifieke hersengebieden verbeterde. Dit werd bestudeerd door de acute effecten van een insuline neusspray te meten op de regionale hersendoorbloeding met behulp van ASL-MRI. Het eten van gemengde noten verbeterde de gevoeligheid van de hersenen voor het hormoon insuline in zes hersengebieden. Deze gebieden zijn betrokken bij de regulatie van verschillende metabole en cognitieve processen in de hersenen, en hebben een invloed op de voedselinname. De insulinegevoeligheid in de rest van het lichaam werd echter niet beïnvloed. Het vetgehalte in de lever, het cholesterolgehalte in het bloed en de bloeddruk namen af na de interventie in vergelijking met de controleperiode. In **Hoofdstuk 4** werd waargenomen dat het eten van gemengde noten de hersendoorbloeding, een maat voor de vaatfunctie in de hersenen, in rust

verhoogde in drie hersenregio's die betrokken zijn bij het cognitief functioneren. Ook werden de effecten op endotheelfunctie, vaatstijfheid en de functie van kleine bloedvaten in het netvlies onderzocht. De endotheelfunctie van de halsslagader en slagader in de arm verbeterde na het consumeren van gemende noten. Daarnaast zorgden de noten voor een verlaging van de vaatstijfheid en een grotere diameter van de kleine bloedvaten in het oog. Tot slot werd het cognitieve functioneren gemeten, waarbij twee verschillende maten van het geheugen verbeterden. De executieve functie en psychomotorische snelheid veranderden echter niet. Op basis van deze hoofdstukken werd geconcludeerd dat langdurige consumptie van gemengde noten als onderdeel van een gezond voedingspatroon de insulinegevoeligheid verbeterde in hersengebieden die betrokken zijn bij metabole en cognitieve processen bij oudere volwassenen met overgewicht en obesitas. De regionale vaatfunctie van de hersenen zonder het gebruik van insuline verbeterde ook, hetgeen verband kan houden met de waargenomen gunstige effecten op het geheugen. Bovendien verbeterden verschillende markers van vaatfunctie door het gehele lichaam en werden gunstige effecten waargenomen op de hoeveelheid levervet, het cholesterolgehalte in het bloed en de bloeddruk.

In **Hoofdstuk 5** staan de resultaten beschreven van een gerandomiseerd, dubbelblind, placebo-gecontroleerd cross-over onderzoek naar de langdurige effecten van Newtricious (NWT)-03-suppletie, wat een mengsel van kleinere eiwitten (eiwithydrolysaat) bevat, op de vaatstijfheid en meer traditionele risicomarkers. Aan het onderzoek deden zesenzeventig volwassenen met het metabool syndroom mee, met een gemiddelde leeftijd van 61 ± 10 jaar en een gemiddelde BMI van 31,7 ± 4,0 kg/m^2. De deelnemers werden willekeurig toegewezen aan een interventieperiode van 27 dagen (5 gram/dag NWT-03) of een placeboperiode, met een tussenperiode van twee tot acht weken. Aan het begin en het einde van beide perioden werden metingen uitgevoerd in nuchtere toestand en 2 uur na inname van NWT-03 om de acute effecten van NWT-03 in kaart te brengen. In vergelijking met de placebo had langdurige inname van NWT-03 geen invloed op de vaatstijfheid. De nuchtere polsdruk was echter lager, maar andere nuchtere risicomarkers werden niet beïnvloed. Er werden geen acute effecten waargenomen na inname van NWT-03 bij aanvang van het onderzoek. Echter, na de interventie resulteerde de inname van NWT-03 in een acute significante verlaging van de centrale augmentatie-index, wat een maat is voor een verlaging van de polsdruk als gevolg van minder weerstand van de bloedvaten, en de bloeddruk, maar andere markers veranderden niet. Geconcludeerd kan worden dat langdurige inname van NWT-03 geen invloed heeft op vaatstijfheid, maar de nuchtere polsdruk bij volwassenen met het metabool syndroom verbeterde. Acute inname van NWT-03 na de interventie verbeterde ook een maat voor de vaatstijfheid en de bloeddruk.

Samengevat levert dit proefschrift verder bewijs dat veranderingen in de samenstelling van voeding, zoals het consumeren van gemende noten, het risico op leeftijdsgebonden aandoeningen kan verminderen door een gunstige werking op de vaatfunctie en insulinegevoeligheid, zowel in de hersenen als in de rest van het lichaam. Deze waargenomen veranderingen dragen mogelijk bij aan gunstige effecten op het cognitief functioneren. Daarnaast heeft het eten van gemengde noten ook een positief effect op traditionele risicofactoren, zoals bloeddruk en cholesterolconcentraties, evenals op verschillende markers van vaatfunctie in het lichaam, hetgeen het risico op hart- en vaatziekten verlaagt.

Impact

In this dissertation, the results from two well-controlled human intervention studies and one systematic literature review have been described and discussed. The overall aim of the dissertation was to investigate the effects of dietary intervention strategies on vascular function and insulin sensitivity of the brain and periphery in adults. The potential impact of this research will be discussed from a scientific, socio-economic and environmental perspective, as well as the translation of the findings into practice.

Scientific relevance

Our research has made significant contributions to the scientific understanding of age-related metabolic and cognitive health, in particular through the further implementation of a non-invasive neuroimaging technique at the research facilities in Maastricht. This technique is sensitive for mapping nutritional effects. It allowed us to quantify (regional) cerebral blood flow (CBF) using arterial spin labeling magnetic resonance imaging (ASL-MRI) under resting conditions and following intranasal insulin administration to study respectively brain vascular function and brain insulin sensitivity [1]. Our randomized controlled trial with mixed nut supplementation indeed provided evidence that regional CBF was beneficially affected through this nutritional intervention, further highlighting the relevance of these measurements to investigate mechanisms through which a healthy diet can improve cognitive performance. The results further underscores the importance of studying brain health together with peripheral vascular and metabolic effects of dietary interventions, as age-related comorbidities, including cardiovascular disease (CVD), type 2 diabetes (T2D), and dementia, often share common risk factors. Especially, our research has shed light on specific brain regions that are activated in response to insulin, suggesting their important role in cognitive processes. Future research should however also focus on other functional outcomes, such as CBF in brain regions involved in the regulation of appetite and satiety, which were also affected. Moreover, our research has emphasized the importance of age and metabolic disorders, such as obesity and T2D, on brain insulin sensitivity. However, future investigations are warranted to further explore differences - and their importance - in brain insulin sensitivity across different patient populations. This will further unravel mechanisms through which therapeutic and lifestyle interventions may enhance brain insulin sensitivity, subsequently leading to improved cognitive performance and appetite regulation.

Socio-economic relevance

The population of adults aged 60 years and older is expected to double from 1.2 billion in 2022 to 2.1 billion by the year 2050 [2]. This demographic shift will have profound socio-economic and public health implications, primarily due to the expected rise in the prevalence and economic burden of age-related disorders, such as CVD, T2D, and dementia [3], which are all closely related to cognitive decline [4, 5]. Notably, the number of patients with dementia is expected to

triple worldwide from 57.4 million in 2019 to 152.8 million in 2050 [6]. The global economic burden associated with dementia is high, with an estimated global cost exceeding €250 billion in 2019, despite that only a fourth of cases is diagnosed and treated [7]. The costs can be split into 16% for direct medical costs, 34% for direct social sector costs, such as long-term care, and 50% for time and efforts of informal caregivers [8]. The direct global cost of dementia-related care is projected to reach €1.5–2.2 trillion by 2050 [7]. Additionally, it should be acknowledged that the total costs related to age-related cognitive decline extend beyond dementia and will be much higher. To address the global socio-economic burden of age-related disorders, it is important to promote a healthy diet - and lifestyle in general - for managing risk factors. Research has demonstrated that an unhealthy diet is responsible for up to 45% of cardiometabolic disease deaths [9]. Our trial provided insights in the beneficial effects of mixed nut consumption on cardiometabolic and brain health, and cognitive performance. These findings further support for the inclusion of nuts in the diet as a healthy aging strategy for the general population, which results in perceivable benefits that have implications for the prevention or delay of metabolic and cognitive disorders [10]. Moreover, lower nut consumption may contribute significantly to annual diet-related cardiometabolic costs [11], highlighting the relevance of nuts for improving not only health outcomes, but also for reducing the socio-economic burden of age-related disorders. Finally, even though we did not report convincing health benefits of long-term NWT-03 supplementation, an egg-protein hydrolysate, on (peripheral) arterial stiffness or cardiometabolic risk markers, a recent publication from our research group demonstrated that NWT-03 intake significantly improved cognitive performance within the executive function domain [12]. These findings suggest that NWT-03 intake may also contribute to the reduction of the burden of cognitive decline.

Environmental relevance

It is essential to consider aspects related to sustainability into dietary recommendations and the development of dietary guidelines [13]. Agricultural food production worldwide is responsible for about 30% of global greenhouse gas (GHG) emissions, contributes to nutrient pollution affecting ecosystems, and consumes a significant amount of freshwater resources [14]. When formulating dietary guidelines for nut consumption, it is important to account for their environmental footprint. The Netherlands Nutrition Centre recommends consuming nuts at a dose of 15-25 g/day [10], while the mixed nut study used a higher daily dose of 60 g. Although nuts generally have a low environmental impact in terms of GHG emissions and pollution compared to other food products, they do have a relatively high impact on scarcity-weighted water use [13]. For instance, the water requirements per kilogram of nuts are comparable to those of less environmentally sustainable food products like red meat. However, when considering the averaged relative environmental impact in relation to the relative risk of mortality, the environmental impact of nut consumption

may not be as significant as that of (un)processed red meat [13]. Furthermore, the environmental impact vary depending on the type of nut. Walnuts consistently demonstrate positive sustainability performance across various criteria, while cashews showed relatively poorer scores [15]. Finally, considering the potentially beneficial longer-term effects of NWT-03 on cognitive performance, it is important to discuss the environmental impact when increasing the production of NWT-03. This should take into account all environmental considerations at the production facilities, including operational and logistic aspects.

Target groups

The study on the effects of mixed nut consumption included older men and women aged 60 to 70 years old who were overweight or obese, while the NWT-03 study included adults with the metabolic syndrome. These populations are known to have an increased risk of metabolic diseases and to develop cognitive impairment [16]. Importantly, it should be emphasized that the participants in our studies did not have existing cognitive complaints. Furthermore, our systematic review revealed differences in regional brain insulin responsiveness associated with aging and obesity. Interestingly, we found that these regions were beneficially affected in our study population through the consumption of mixed nuts. However, future research is needed to generalize these results also to other study populations, such as individuals with or without (pre-)diabetes, subjective cognitive decline, and to explore its potential in reducing the progression to mild cognitive impairment (MCI) and ultimately dementia.

Translation into practice

Clinical studies involving humans play a crucial role in translating findings derived from cell or animal studies to real-life human settings. The described randomized, controlled intervention trials showed excellent study compliance and food products were well-tolerated, indicating that incorporating these products into the diet for extended periods is feasible and safe. To ensure long-term feasibility and success, the inclusion of nuts as part of healthy diet or egg-protein hydrolysates in other food products could be considered as an option for extended periods. Most research findings presented in this thesis have already been published in international open-access journals, such as Neuroendocrinology [17] and Clinical Nutrition [18], or are currently undergoing the peer-review process. As a result, the obtained knowledge is readily accessible to scientists, health professionals, patient organizations and the general public worldwide, thereby promoting further investigation into the effects of healthy foods and food products on brain and metabolic health. Furthermore, the clinical relevance of these results has been presented at national and international conferences, further disseminating the findings to relevant stakeholders. Thus, the research conducted in this dissertation is not only important from a health perspective, but also from socio-economic, environmental and public health perspectives. In this

context, these findings may also be relevant for policymakers in the development of healthy food-based dietary guidelines.

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Dankwoord

De afgelopen jaren heb ik met ontzettend veel passie en plezier dit promotieonderzoek tot een mooi eind gebracht. Naast de kennis en ervaring die ik heb opgedaan, waren er talloze momenten die dit tot een prachtige tijd hebben gemaakt. Hier hebben veel mensen aan bijgedragen die ik daarom graag zou willen bedanken.

Allereerst wil ik mijn promotieteam hartelijk bedanken voor het mogelijk maken van dit hele traject. **Peter**, mijn oprechte dank voor het vertrouwen dat je jarenlang in mij hebt gesteld en de kansen die je me hebt geboden om te groeien. Jouw sterke balans tussen een gepassioneerde wetenschapper en een vlotte spreker maakt jou een voorbeeld om naar op te kijken binnen de academische wereld. We hebben samen veel meegemaakt in de afgelopen jaren, met als hoogtepunt het ontdekken van de Japanse cultuur tijdens het voedingscongres. **Ronald**, in de loop der jaren heb ik enorm veel geleerd van jouw waardevolle en kritische inzichten, altijd met de beste bedoelingen. Of het nu ging om complexe statistiek, vakantieplannen of dat vervelende scherm dat weer niet aanging in de onderwijsruimte. Daarnaast wil ik je ook bedanken voor alle heerlijke automaatkoffies (*herinnering: 1760*) op de vroege ochtend, en dat je deur altijd wagenwijd openstaat voor wat voor reden dan ook. **Jogchum**, ook jou wil ik bedanken voor jouw waardevolle bijdrage aan dit proefschrift. Ik bewonder jouw spontaniteit en oprechtheid, waarmee je altijd de sfeer binnen de PHuN-groep tot een succes weet te maken. Ik ben verheugd dat dit nog niet het einde is van onze samenwerkingen binnen deze groep, en ik hoop dat we onze visies nog lange tijd kunnen en mogen voortzetten.

De leden van de beoordelingscommissie, **Prof. dr. C.G. Schalkwijk, Prof. dr. E.E. Blaak, Prof. dr. Ir. M. Visser, Dr. S. Kullmann** en **Dr. T.C. Adam**, wil ik graag bedanken voor het lezen en beoordelen van dit proefschrift.

Hoewel het uitvoeren van humaan onderzoek veel werk met zich meebrengt, wil ik graag alle onmisbare deelnemers aan dit onderzoek enorm bedanken voor hun inzet, inspirerende verhalen en vooral gezelligheid die het werk leuk maken. Daarnaast heb ik mijn onderzoek niet kunnen uitvoeren zonder de waardevolle hulp van anderen. I would like to express my gratitude to **Dimo** for his expertise regarding the MRI-scans. **Lieve**, **Nathalie**, **Sanne**, en **Maite**, bedankt dat jullie altijd klaarstonden om studieproducten aan te nemen of uit te geven en het helpen bij testdagen, zelfs wanneer de planning soms wat enthousiast of vooral vroeg in de ochtend was. **Maurice** en **Maud**, bedankt voor de tijd die jullie hebben gestoken in de labanalyses. Daarnaast wil ik mijn dank uitspreken aan de studenten die me hebben geholpen tijdens de onderzoeken, bedankt **Eva**, **Bernadett**, **Caro**, **Cédric**, **Romy** en **Raquel**.

Een speciaal dankwoord aan mijn paranimfen, de twee rotsen in de branding die altijd achter mij hebben gestaan en ik ook goede vrienden mag noemen. **Lieve**, bedankt dat je mij aan de start met open armen hebt ontvangen op het kantoor. Samen hebben we dit traject vrijwel ”zónder te klagen” met trots doorlopen, en hoe mooi is het dat uiteindelijk onze promoties vrijwel binnen dezelfde week plaatsvinden. Je maakt altijd tijd vrij om collega’s uit de brand te helpen, en jouw creativiteit ontgaat niemand, zoals práchtige verjaardagsposters of het schaamteloos maken van strikes met je pols in het gips. **Willem**, hoewel ik je eigenlijk al vrij lang ken, wil ik je enorm bedanken dat ik je de afgelopen jaren nog veel beter ben gaan leren kennen. Dank voor de trouwe koffiemomenten iedere ochtend en jouw oprechte gunfactor op en naast het werk. Ik geniet erg van jouw culinaire vaardigheden, alsook alle spontane avonden en het meesleuren naar de Gouv. Toppers, ik weet zeker dat onze gezamenlijke passies nog lang zullen voortduren!

Mijn voorgangers, **Jordi** en **Ellen**, bedankt voor jullie gezelligheid aan de start van mijn traject en daarvoor gedurende mijn wetenschappelijke stage, alsook jullie aanstekelijk enthousiasme waarmee jullie mij de MRI en vaatmetingen hebben bijgebracht. **Tanja**, hoewel je geen deel uitmaakte van het promotieteam, hebben onze onderzoekslijnen veel raakvlakken. Bedankt voor jouw enthousiaste en waardevolle onderzoeksblik en ideeën, die we gelukkig mogen voortzetten. **Maite**, bedankt voor alle fijne (lach)momenten op en naast het werk – weet dat ik altijd met plezier heb meegezocht naar je sleutels en pas. **Nathalie**, jij bent ook zeker een gemis op de werkvloer, ik zal nooit vergeten hoe we kleine Ize in beeld hebben mogen brengen met de echo. **Sanne**, samen de Master afgerond en doorgestroomd binnen de PHuN – bedankt voor jou altijd nuchtere blik, gezelligheid en het opruimen van het kantoor.

Alle (voormalige) collega’s binnen de PHuN-groep zijn vooral verantwoordelijk voor de goede werksfeer, bedankt **Dena**, **Ellen**, **Elske**, **Elze**, **Eva**, **Fatma**, **Herman**, **Jehad**, **Jogchum**, **Jordi**, **José**, **Lea**, **Lieve**, **Lucia**, **Maite**, **Marco**, **Maud**, **Maurice**, **Merel**, **Micah**, **Nathalie**, **Peter**, **Ronald**, **Sabine**, **Sanne**, **Sophia**, **Sultan**, **Tanja** en **Willem**! Bedankt voor alle gezellige koffiemomenten, de PHuN uitjes, VBW barbecues, kerstdiners en uitjes, alsook de soms wat uit de hand gelopen avonden waar ik hier niet verder op zal ingaan. Daarnaast ook dank aan secretaresses **Désirée** en **Yolanda**, computerspecialisten **Marc** en **Paul**, en de collega’s van VBW: **Anna**, **Charlotte**, **Dzhansel**, **Evi**, **Froukje**, **Guy**, **Ivo**, **Kay, Marit**, **Merel**, **Pip**, **Yvonne** en alle andere collega’s die hebben bijgedragen aan deze prettige werksfeer.

A special thanks to the Metabolic Neuroimaging research group, especially **Hubert** and **Stephanie**, at the University in Tübingen. I truly appreciate the opportunity you gave me to visit your lab over the last couple of months, sharing your expertise on the concept of brain insulin sensitivity. **Ralf**, thank you for your enthusiasm and time invested in teaching me the functional MRI analyses. Also, my gratitude extends to the other members of the group who contributed to making me feel at home so quickly. Thank you, **Anja**, **Jason**, **Lorenzo**, **Maike**, **Olga**, **Sixiu**, **Theresa**, and all the others – Herzlichen dank and I am looking forward to future collaborations!

Gedurende de jaren is het groepje studiegenoten dat na onze opleiding nog in en rondom Maastricht verbleef steeds kleiner geworden. **Anouk, Daphne, Dylan, Loes, Kristel, Nadieh** en **Veerle,** hartelijk dank voor de prachtige jaren en voor het feit dat we, ondanks dat we allemaal verschillende wegen zijn ingeslagen, nog steeds in contact zijn gebleven. In het bijzonder wil ik ook mijn huisgenoten in Maastricht over de jaren heen, **Anouk**, **Niek**, **Max**, **Tess** en **Zoë**, bedanken voor alle gezelligheid en avonden vol met etentjes of spellen.

Ook een grote dank aan de heren en aanhang van Kiët Simpel: **Bert, Lynn, Freek, Anne, Guus, Guus*, Melanie, Kai, Karel, Max, Nick, Christel, Roel, Roy, Siem, Stan** en **Stef**. Sinds de basisschool vormen we al een hechte vriendengroep. Jullie hebben altijd achter me gestaan en tonen oprecht interesse in mijn onderzoek, waarvan ik altijd probeer het begrijpelijk uit te leggen. Het is heel fijn om te weten dat ik na een lange week altijd bij jullie gezelligheid en afleiding kon vinden, vergezeld van heerlijk gekoelde versnaperingen en diepgaande gesprekken.

Pap, **mam**, **Marco**, **Rachel**, **Ruud**, **Angelique**, **Timo**, **Dana**, **Sven**, **opa Wim** en **oma Mia**, ook jullie wil ik ook enorm bedanken voor het onvoorwaardelijk achter mij staan en ondersteuning op hele verschillende vlakken. Mede door jullie support ben ik gekomen waar ik nu sta in het leven. Ik weet dat de deur altijd open zal staan bij jullie, dank jullie wel!

About the author

Kevin Marie Ruud Nijssen was born on April 29th, 1996, in Venlo, the Netherlands. He completed his secondary school education at Blariacumcollege in Venlo in 2014. From 2014 to 2017, he obtained a Bachelor's degree in Health Sciences at Maastricht University, specializing in Biology and Health, where he developed an interest in the impact of lifestyle and nutrition on human physiology. He completed an internship at the Department of Internal Medicine at Maastricht University and graduated in 2017. He continued with the Master Biomedical Sciences, specialization Nutrition, Physical Activity, and Metabolism, at Maastricht University, and graduated in 2019. During this time, he completed internships at the Department of Nutrition and Movement Sciences at Maastricht University and the Department of Physiology at RadboudUMC in Nijmegen. Both internships focused on the effects of lifestyle and diet on peripheral and brain vascular function in older adults.

Since September 2019, he has been working on his PhD project at the Department of Nutrition and Movement Sciences at Maastricht University, under the supervision of Prof. Dr. Ronald P. Mensink, Prof. Dr. Jogchum Plat, and Dr. Peter J. Joris. During his PhD, he conducted human intervention studies and a systematic literature review to investigate the role of a healthy diet on vascular function and insulin sensitivity in the brain and periphery. His primary focus was on developing and applying a non-invasive technique to assess brain insulin sensitivity using magnetic resonance imaging in combination with intranasal insulin. After PhD approval, he visited the Metabolic Neuroimaging Lab at the University of Tübingen from September to December to further integrate functional magnetic resonance imaging techniques. In December 2023, he was awarded the Kootstra Talent Fellowship grant that will allow him to further develop his research line at Maastricht University.

Since 2021, he has been a registered nutritional scientist at the Dutch Academy of Nutritional Sciences (NAV). Furthermore, he is part of the NAV-board, chair of the Young-NAV committee, and was part of the NUTRIM Division 1 committee, where he organizes symposia, panel discussions, and career events for (future) nutritional experts. He has also obtained his University Teaching Qualification and has been involved in differential educational activities. Some of the study results have already been published in international, peer-reviewed, open-access journals. Furthermore, he presented his research at multiple national and international conferences. For his work on the effects of mixed nuts on vascular function and insulin sensitivity in the brain, he was awarded the Early Career Award during the IUNS-ICN 2022 (Tokyo, Japan), and was nominated for the Foppe Ten Hoor Award during the NSD 2022 (Heeze, the Netherlands) and NAV Impact Prize 2023.

List of publications

Published manuscripts

- **Nijssen KMR**, Mensink RP, Plat J, Ivanov D, Preissl H. Joris PJ. Mixed nut consumption improves brain insulin sensitivity: a randomized, single-blinded, controlled, crossover trial in older adults with overweight or obesity. *Am J Clin Nutr*. 2023.

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Manuscripts in preparation

- **Nijssen KMR,** Mensink RP, Joris PJ. Effects of chlorogenic acid on flow-mediated vasodilation: a meta-analysis of randomized, placebo-controlled intervention studies.