Metabolic health and vascular function in adults

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Effects of a high-protein diet and soy nuts

Lea Tischmann

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CHAPTER 1

General introduction

Overweight and related co-morbidities

Globally, the prevalence of overweight and obesity is increasing rapidly, and in 2016, 39% of the total population was overweight and 13% obese [1]. According to the WHO, overweight is defined as a BMI >25 kg/m^2 and obesity as a BMI >30 kg/m^2 [1]. Overweight and obesity are often caused by an unhealthy lifestyle, with excessive energy intake, poor nutrient composition, and physical inactivity quickly creating a positive energy balance promoting weight gain [1]. Excess body weight has been, next to age, categorized as a major contributing factor for the development of non-communicable diseases such as cardiovascular disease (CVD) and type II diabetes mellitus (T2DM) [2] through a cascade of metabolic and inflammatory processes. Pro-inflammatory cytokines and other bioactive mediators are released by adipose tissue, negatively affecting blood lipid concentrations, adhesion molecules, coagulation, endothelial function, atherosclerosis, and insulin resistance [3].

Energy intake regulation

Chronic intake of excess energy causes a positive energy balance and weight gain in the longer term. Therefore, careful regulation of energy intake is of importance. However, dieting, weight loss, or long-term weight maintenance remain challenging as limited energy intake affects energy expenditure and often results in increased hunger sensation and desire to eat [4]. As fat mass and fat-free mass (FFM) decrease with weight loss, the total energy requirement is reduced, which often mitigates the negative energy balance caused by the limited energy intake. This indicates the importance of finding strategies to reduce energy intake next to simple caloric reduction. In this context, high-protein diets may help to counteract the loss of FFM while simultaneously affecting appetite sensation.

For appetite regulation, multiple pathways have been suggested to be acutely affected by high-protein diets, such as satiety-related anorexigenic gut peptides (e.g., GLP-1 and PYY), increased serum amino acid concentrations, and elevated diet-induced thermogenesis [4]. Other endogenous compounds suggested to be involved in energy intake regulation are the endocannabinoids [5].

Endocannabinoids

The endocannabinoid system (ECS) has been suggested to be involved in metabolic and cardiovascular health by influencing body composition, blood pressure (BP) regulation, and vasoactive properties [6-9]. The ECS is a complex system getting more and more interest lately. It consists of receptors (cannabinoid receptors 1 and 2), endogenous ligands, and enzymes ensuring synthesis and degradation of those ligands. The ligands are called endocannabinoids (e.g., anandamide (AEA) and 2-arachidonoylglycerol (2-AG)) and endocannabinoid-related compounds (e.g., oleoylethanolamide (OEA), palmitoylethanolamide (PEA), and pregnenolone (PREG)). Especially AEA and 2-AG have been suggested to exert blood pressure, vasoactive modifying properties, affect body composition, and metabolic markers such as blood lipoprotein concentrations and glucose metabolism [6,8,10,11]. Endocannabinoid concentrations, especially AEA and 2-AG, are increased in obesity and are not only positively associated with anthropometric measures such as BMI and body fat percentage but also with decreased insulin sensitivity and triacylglycerol concentrations (2-AG) [6,9]. Studies with cannabinoid receptor 1 antagonists showed anorexigenic effects, increased glucose uptake by muscles, and inhibition of lipogenic enzymes suggesting that endocannabinoids are involved in pathways regulating energy intake and metabolism. However, knowledge about exact mechanisms is still largely missing and under further investigation [5,7,9,12].

Type II diabetes mellitus

Impaired glucose metabolism and insulin resistance at an early stage can be categorized as pre-diabetes, a reversible condition where patients are at high risk of developing T2DM [13]. Overweight and obesity have been estimated to be responsible for around 90% of the T2DM cases. Excess weight causes increased circulating free fatty acids (FFA) concentrations via multiple routes (e.g., meal derived and enhanced lipolysis), which in turn stimulate insulin resistance [14]. Diets low in carbohydrates and, subsequently, often high in protein have beneficial effects on insulin sensitivity, especially when applied with energy restriction for weight loss or maintenance [4]. For diabetes prevention, lifestyle interventions have been of great interest [15]. Diabetic patients have a two to fourfold increased risk to die from CVD [16], next to other co-morbidities. Diabetes is a risk factor for hypertension and hyperlipidemia [17] and may cause macro- and microvascular complications [18,19].

Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death worldwide: with approximately 17.9 million deaths per year, CVD accounts for around 31% of all deaths worldwide [20]. CVD involves multiple disorders related to heart and blood vessel-related issues, such as coronary heart disease (CHD), cerebrovascular disease, and peripheral arterial disease [21]. As the leading underlying cause for CVD, atherosclerosis has been identified. Atherosclerosis is a chronic disease of the blood vessels causing plaque development in the arterial walls, called atherosclerotic lesions. Those lesions not only narrow the arteries, thus impairing the blood flow to vital organs, but plaques can also rupture and cause myocardial infarction or strokes. A precursor of atherosclerosis is endothelial dysfunction (discussed below), followed by lipid accumulation in the arterial vessel wall, inflammation, and finally, plaque formation [22].

Important factors contributing to a high risk for severe CVD development are increasing age, an unhealthy lifestyle, overweight, diabetes, hyperlipidemia, and hypertension. Age is one of the main risk factors for many non-communicable diseases, such as CVD and T2DM [21,23], and therefore warrants appropriate health care [24]. According to the WHO, the proportion of older adults above the age of 60 is projected to double in 2050 and health care costs are expected to increase accordingly [25]. Improving behavioral risk factors, such as an unhealthy diet, physical inactivity, and cigarette smoking may largely prevent CVD development [20]. Therefore, lifestyle-related treatments, for instance, nutritional interventions, are of great interest to lower CVD risk.

Cardiometabolic risk factors

Typical risk factors for the development of CVD are hypertension and hyperlipidemia. Hypertension, one of the strongest risk factors for developing CVD, has been defined as increased systolic and/or diastolic blood pressure (>140 mmHg, respectively >90 mmHg) [26]. Overall, approximately 30 - 45% of Europe's population suffers from elevated blood pressure levels, with a higher prevalence with increasing age. Hypertension has been related to several CV diseases and events, such as peripheral arterial disease, stroke, and heart failure [27].

In hyperlipidemia, patients have abnormally high circulating blood lipid concentrations, such as LDL-cholesterol and triacylglycerol (TAG), which are carried through the bloodstream by lipoproteins [28]. Elevated blood lipid concentrations have been associated with increased CVD risk [29].

Vascular (dys)function

Vascular function can be disrupted in multiple ways, such as stiffening of the arterial wall or dysfunction of the endothelial layer. The arterial wall consists of several layers, containing collagen fibers in the outer layer (adventitia), connective tissue and smooth muscle cells in the middle layer (media), and endothelial cells in the inner layer (intima) [30,31].

Endothelial function

The endothelium is a monolayer of endothelial cells between the vascular lumen and the vascular wall, and can be found in all sizes of blood vessels. A healthy endothelium is not only a physical layer, it also acts as a functional layer and exerts several physiological functions in response to physical or chemical stimuli. It can affect cellular adhesion, blood coagulation, inflammation of the vessel wall, and vasomotion by releasing or reacting to vasoactive substances, leading to vasodilation or constriction. One of those vasoactive substances is nitric oxide (NO), which is produced by endothelial NO synthase (eNOS) from the precursor L-arginine upon shear stress due to increased blood pressure. NO leads to a relaxation of the arterial wall causing vasodilation, which then, in turn, reduces the primary stimuli shear stress [32,33].

Bioavailability of NO can be reduced by chronic inflammation and increased levels of oxidative stress resulting in endothelial dysfunction. Besides, high levels of oxidative stress can induce a pro-thrombotic state by increasing the expression of cellular adhesion molecules and thereby initiating atherosclerotic plaque development by increasing leucocyte affinity [34]. Endothelial dysfunction increases with age, elevated LDL-cholesterol concentrations, diabetes, hypertension, and obesity [35,36].

Endothelial function can be assessed by the non-invasive, 'gold-standard' technique flow-mediated dilatation (FMD) of the brachial artery. This measurement uses an ultrasound technique to visualize the brachial artery. By analyzing the arterial diameter with and without a stressor, endothelial function can be assessed. For FMD, inflation of a blood pressure cuff on the forearm of the participant induces reactive hyperemia during the flow occlusion and shear stress after releasing the cuff. This shear stress causes nitric oxide (NO) release from the endothelium, which finally leads to a measurable dilation of the brachial artery. The percentage diameter increase during the reaction period is the final FMD value implicating that a lower FMD is related to stiffer arteries [32]. An FMD higher than 3% of the baseline diameter has been considered to be healthy [37] and each percentage point (pp) decrease in FMD has been related to an 8% increased risk on CVD [38].

Another method of assessing endothelial function is the relatively new, carotid-artery reactivity (CAR) measurement. This method evaluates the effect of a cold pressor test (CPT) on the carotid artery diameter. For the CPT, the participants' hand is placed in a bucket with ice-cold water (~4°C) to activate the sympathetic nervous system, which then affects the carotid artery diameter. CAR has also been suggested as a valuable measure for CV risk assessment [39,40].

Arterial stiffness

In a healthy condition, arteries can flexibly adjust to blood pressure changes during the cardiac cycle by distension. Conditions like hypertension, atherosclerosis, hyper-cholesteremia, diabetes, and increasing age lead to structural changes in the artery walls. Thickening or increased collagen production in the artery wall in the dispense of elastin fibers results in stiffening of the arteries [31,41]. Stiffening of the arteries is a predictor of CVD, such as stroke, heart failure, myocardial infarction, and dementia [31].

Arterial stiffness can be assessed by carotid-to-femoral pulse wave velocity ($PWV_{c\text{-}f}$), a 'gold-standard' measurement. $PWV_{c\text{-}f}$ measures the speed of the blood flow through the body by dividing the distance by the transit time between the two measurement points and is considered as a direct measure of arterial stiffness [42]. $PWV_{c\text{-}f}$ values largely depend on age, but a $PWV_{c\text{-}f}$ higher than 13 m/s has been associated with an increased risk for CV mortality [43]. A second option to assess arterial stiffness is the augmentation index (AIx). The AIx is considered as an indirect measure of arterial stiffness and provides additional information on pulse wave reflections in the arteries [42]. Both measures have been associated with a higher CV risk when increased [44].

Structure of the microvasculature

The retinal microvascular structure gives an indication for other microvascular systems in the human body and has been shown to correlate with age, blood pressure, and arterial stiffness [45-47]. It can be non-invasively assessed by retinal images where the arteriolar and venular diameters can be measured and a ratio of arteries and veins can be calculated. Beneficial effects on CVD outcomes have been related to increased arteriolar and decreased venular diameters in women [48].

Dietary intervention strategies

Modifiable risk factors cause 70% of all CVD worldwide. Among those causes, diet plays a key role as it has been shown to be responsible for 45% of all premature CVD-related deaths worldwide. Diets low in fruits, vegetables, nuts, legumes, and fibers are in the top 10 of diet-related causes [49,50]. High intakes of saturated dietary fat have been shown to enhance serum lipoprotein concentrations and to impair vascular endothelial function [51,52]. Diet and exercise intervention strategies have been shown to beneficially affect CVD and T2DM incidence or mortality rates in several long-term studies [53-56]. Many of those lifestyle interventions aim to reduce body weight, thereby reducing the impact of weight as a critical risk factor for CVD and T2DM development. Dietary strategies such as high-protein diets or plant-based diets have been discussed to affect CVD and T2DM development beneficially. However, results are inconclusive [4].

The PREVIEW study - diabetes prevention

The effects of a higher dietary protein intake on T2DM prevention have been assessed in a large international trial: the PREVIEW study (PREVention of diabetes through lifestyle Intervention and population studies in Europe and around the World) [57]. In this thesis, results from a substudy of the PREVIEW Maastricht cohort, which investigated the effects of dietary protein on appetite, energy balance, and CVD risk are presented in chapters 2 to 4. The PREVIEW intervention started with an 8-week weight-loss period, followed by a three-year lifestyle intervention. For the lifestyle intervention, participants either followed a moderate-protein, moderate-glycemic index (GI) diet or a high-protein, low-GI diet combined with either moderate or high-intensity exercise [55].

Dietary protein

Dietary protein accounts for around 15 energy % (EN%) of the macronutrients in the standard Western diet. When intakes are elevated (~20 to 30 EN% from dietary protein), dietary protein has been shown to effectively help with body weight management and body composition by affecting energy metabolism and promoting satiety via multiple pathways, such as secretion of anorexigenic hormones, circulating amino acids, and energy expenditure [4]. A higher protein content has been discussed to affect energy balance. Due to increased adenosine triphosphate (ATP) requirements for metabolization and oxidation of protein, the diet-induced thermogenesis (DIT) is elevated after consumption of high-protein diets. Additionally, energy balance has been shown to be negative with a higher protein intake, even with isocaloric diets [58]. Stimulating protein

synthesis rate by increased protein intake might help to preserve fat-free mass (FFM) and thereby maintain energy expenditure in conditions of restricted energy intake, when energy expenditure typically decreases [4,58].

A higher dietary protein content has not only been beneficial in energy-restricted diets but may also prevent overweight and obesity in energy-balanced conditions. Whether dietary protein exerts effects on CVD outcome parameters independent of weight loss remains inconclusive. In general, protein type, amino acid composition, and origin of the protein seem to largely influence specific markers [4], but the assessment of independent effects of particular protein types and amino acids is challenging. However, especially plant-based diets containing high amounts of plant protein combined with some animal-protein sources such as lean meat and fish have been suggested to minimize CVD risk [59].

Plant-based diets

Plant-based diets have been associated with a reduced risk of developing CVD [60-62]. A meta-analysis of prospective cohort studies showed that a vegetarian dietary pattern was associated with a decreased risk of 28% for coronary heart disease [61]. Plant-based diets contain high amounts of plant protein, fiber, unsaturated fatty acids (UFAs), and phytochemicals. All these components have been associated with improvements in CVD risk [61,63]. They can improve LDL-cholesterol levels by their plant sterol content but also by replacing saturated fats with unsaturated fats, viscous fibers which can enhance short-chain fatty acid (SFAs) production, and proteins, providing amino acids involved in, e.g., cholesterol metabolism and NO production or serving as vehicles for the plant sterols and fibers [61].

Plant-based diets are mainly or entirely based on plant-derived products. Typical examples of plant-based diets are the Mediterranean diet, the Nordic diet, the DASH diet (Dietary Approaches to Stop Hypertension), and vegetarian and vegan diets. The first three diets focus on high amounts of fruits and vegetables, legumes, whole grains, unsaturated fats, (fatty) fish, and low intake of red and processed meat. In contrast, the vegetarian diet allows egg and dairy products but renounce meat products entirely, and the vegan diet even abandons any sort of animal-derived product, including dairy products [61].

Soy products

The soybean (Glycine Max) forms an essential component of the traditional Asian diet and of vegetarian diets in the Western world. Soybeans are particularly rich in plant proteins, accounting for approximately 36 to 46% of the total energy (En%). Soy protein contains all essential amino acids and has been classified as high-quality protein. The protein quality can be specified by the digestible indispensable amino acid score (DIAAS) [9], which indicates that soy can be classified as a high-quality protein source [11,12]. Additionally, soybeans contain low amounts of carbohydrates and high amounts of polyunsaturated fatty acids (PUFAs), accounting for 62% of the total fat content (approximately 11.3 g per 100 g soybeans). Additional compounds are high doses of phytoestrogens, also called isoflavones [64,65], mainly daidzein (50%) and genistein (40%) [65]. Daidzein can be taken up in its natural form or can be metabolized to equol in the human intestine by around 50 - 55% of the Asian and 20 – 35 % of the Western population. Effects of equol are less understood yet, but it has been suggested to have even stronger health effects than the other isoflavones [66,67]. Another component of soy is the polyamine spermidine, a natural autophagy inducer which has been inversely associated with CVD in epidemiological studies [68].

Soybeans can be consumed in a large variety of products such as beans, soy milk, protein powder, or fermented soy products. This dissertation examines the effects of soy nuts on cardiometabolic and vascular health **(Chapter 5)**. The soy nuts were roasted, unsalted whole soybeans including the peal, to enable a combined effect of a high plant-protein and phytoestrogen content with fibers, minerals, and unsaturated fats. Unlike other soy products, soy nuts have relatively high isoflavones concentrations [69-71].

Outline of the thesis

The aim of this thesis was to investigate the effects of dietary interventions with a mixed high-protein content or soy nuts on several metabolic health and vascular function markers in adults (see **Figure 1.1**). This thesis presents the results of two human intervention trials, a substudy of the PREVIEW-cohort [57] performed in Maastricht and an intervention study with soy nuts. In **chapters 2** to **4**, results of the PREVIEW substudy are presented. In the PREVIEW substudy, post-obese, pre-diabetic participants stayed for two days in a metabolic chamber at the university after almost three years of lifestyle intervention of the original study, where participants received either a high-protein or moderate-protein diet. We have examined the effects of a higher dietary protein and soy nut intake on several measures of cardiovascular and metabolic health during these

two days. Effects on markers of appetite and gut peptides **(Chapter 2)**, the role of endocannabinoids in energy balance **(Chapter 3)**, and cardiometabolic health and vascular function **(Chapter 4)** during a high-protein diet are presented. **Chapter 5** describes the results of a longer-term human intervention trial, where the effects of soy nut consumption on cardiometabolic health and vascular function were studied in older adults. This study used multiple measures for cardiovascular health. In the last chapter **(Chapter 6)**, main findings of these studies are summarized, combined in a general discussion, and placed into a broader perspective.

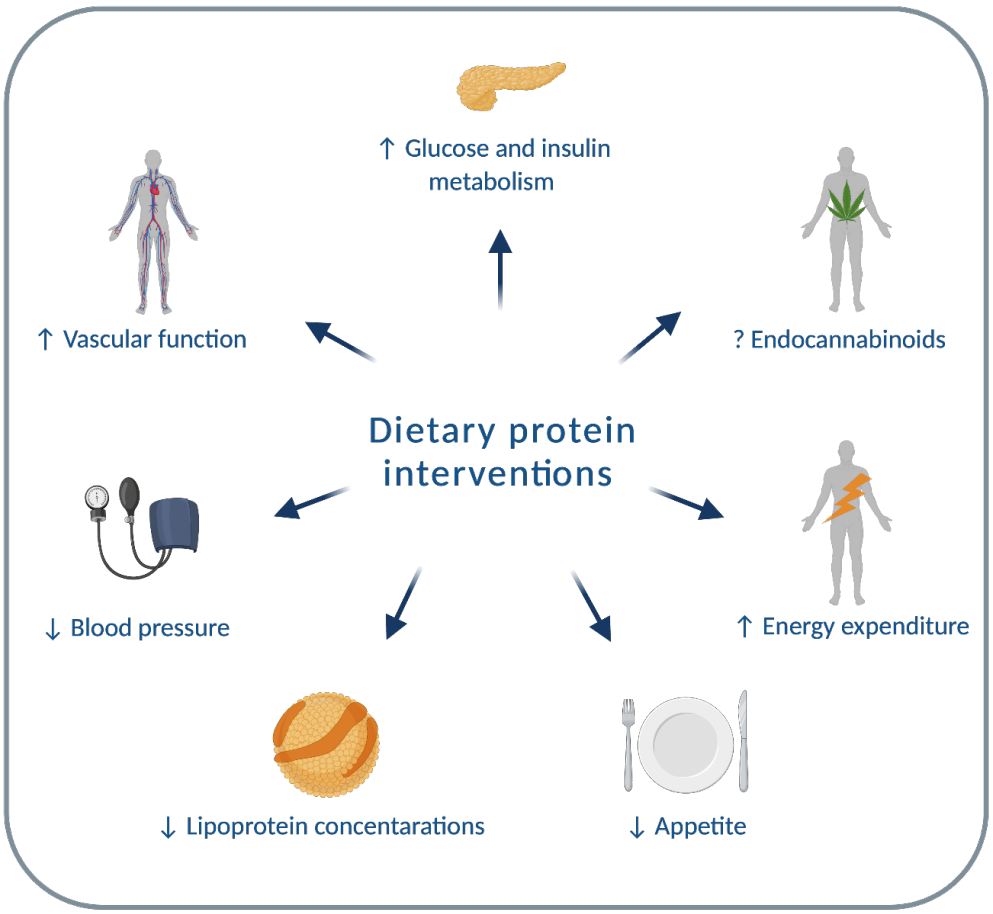

Figure 1.1: Proposed health effects of dietary protein investigated in this thesis. ↑ = increase; ↓= decrease; ? = unclear effects. Created with BioRender.com.

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CHAPTER 2

Effects of a high-protein/ moderate-carbohydrate diet on appetite, gut peptides, and endocannabinoids – a PREVIEW study

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ABSTRACT

Background: Favorable effects of a high-protein/moderate-carbohydrate (HP/MCHO) diet after weight loss on body weight management have been shown. To extend these findings, associations between perception of hunger and satiety with endocannabinoids, and with glucagon-like peptide-1 (GLP-1) and polypeptide YY (PYY) were assessed.

Design: At approximately 34 months after weight loss, 22 female and 16 male participants (mean age 64.5 ± 5.9 years; body mass index (BMI) 28.9 ± 3.9 kg/m²) completed a 48 h respiration chamber study. Participants were fed in energy balance with a HP/MCHO diet with 25%:45%:30% or a moderate-protein/high-carbohydrate (MP/HCHO) diet with 15%:55%:30% of energy from protein:carbohydrate:fat. Endocannabinoids and related compounds, relevant postprandial hormones (GLP-1, PYY), hunger, satiety, and *ad libitum* food intake were assessed.

Results: HP/MCHO versus MP/HCHO reduced hunger perception. The lower decremental area under the curve (dAUC) for hunger in the HP/MCHO diet (–56.6% compared to MP, $p < 0.05$) was associated with the higher AUC for 2-arachidonoylglycerol (2-AG) concentrations ($p < 0.05$). Hunger was inversely associated with PYY in the HP/MCHO group ($r = -0.7$, $p < 0.01$). *Ad libitum* food intake, homeostatic model assessment for insulin resistance (HOMA-IR) and incremental AUCs for gut peptides were not different between conditions.

Conclusion: HP/MCHO versus MP/HCHO diet-induced reduction in hunger was present after 34 months weight maintenance in the post-obese state. HP/MCHO diet-induced decrease of hunger is suggested to interact with increased 2-AG and PYY concentrations.

INTRODUCTION

In 2016, 39% of the adult population worldwide was considered overweight and 13% obese [1]. A positive energy balance is one of the most critical underpinnings for this development, posing a major risk for the development of chronic diseases including type-II diabetes (T2D) and cardiovascular disease [2].

While successful dieting and weight maintenance are essential to long-term improvements of metabolic disease, weight maintenance remains especially challenging. For weight reduction as well as long-term weight maintenance, dietary protein was suggested as being potentially helpful due to the notion that it appears to be more satiating than carbohydrates or fat in an acute setting [3–7], and therefore may support the reduction of food intake [8]. Additionally, a ketogenic state, induced by a short- or medium-term high-protein, low-carbohydrate condition [9,10], has been suggested as a contributor to protein-related appetite regulation. In addition to increased satiety, protein was shown to have sparing effects on fat-free mass (FFM) during weight reduction and weight maintenance [11,12], while energy expenditure increased [5,11,13,14].

The endocannabinoid system is critically involved in the regulation of energy balance and in the pathophysiology of metabolic disorders [15–17]. The system comprises endogenous lipids, the cannabinoid receptor 1 and 2 (CB1 and CB2) and the enzymatic machinery involved in the synthesis and degradation of endocannabinoids [15]. The best characterized endocannabinoids are the N-ethanolamide of arachidonic acids, known as anandamide (AEA) and the glycerol ester of arachidonic acid or 2-AG. The lipid derivatives and endocannabinoid-related compounds oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) are synthetized together with AEA and share structural similarities, but do not bind to cannabinoid receptors. AEA and 2-AG both correlate positively with markers of obesity and metabolic disorders in humans [18–20]. OEA decreases appetite and favors weight loss and lipolysis by acting through peroxisome proliferator-activated receptor-α (PPAR-α), contrasting the metabolic effects of the endocannabinoid-dependent CB1 receptor activation [21], which has been linked to obesity promotion [17]. Pregnenolone (PREG), a neurosteroid precursor, has been suggested to be a negative regulator of endocannabinoids by experimental data [22] and thereby prevents overstimulation of the cannabinoid receptor [22]. While endocannabinoids have been assessed with regard to energy balance [17,23] and fat intake [20], the role of dietary protein in endocannabinoid signaling remains unknown.

In addition, satiety-related gut peptides such as GLP-1 and PYY, have been reported to be increased [24,25] in response to high-protein intake compared to high-carbohydrate or high-fat intake. Both GLP-1 and PYY have incidentally been linked to increased satiety and reduced food intake [26]. However, based on the literature, individual perception of appetite and appetite-related gut peptides was not consistently associated [27]. Therefore, the use of gut peptides as a direct biomarker for perceived appetite alone appears insufficient [28].

The present study aimed to assess the effects of a high-protein/moderate-carbohydrate (HP/MCHO) versus moderate-protein/high-carbohydrate (MP/HCHO) diet in the post-obese state after weight loss, on the association between perception and physiology of hunger and satiety in energy balance, in a controlled respiration chamber setting. Especially, the protein-content-related differences in concentrations of endocannabinoids and related compounds as well as their potential association with hunger and satiety perception were assessed. We hypothesized that also in the post-obese phase, a high-protein/moderate-carbohydrate diet would be more satiating compared to a moderate-protein/high-carbohydrate diet, evidenced by higher satiety ratings and lower hunger ratings, possibly associated with changes in concentrations of endocannabinoids and related compounds, and with increased satiety hormone concentrations.

MATERIALS AND METHODS

The study was registered at ClinicalTrials.gov (NCT01777893), was performed in line with the Declaration of Helsinki, and was approved by the Medical Research Ethics Committee of Maastricht University Medical Centre (METC). All participants provided written informed consent for participation. The study was performed at Maastricht University from February 2017 until February 2018.

Participants

A subgroup of 40 participants was recruited from the PREVention of diabetes through lifestyle intervention and population studies in Europe and around the World (PREVIEW) cohort [29] at Maastricht University in the Netherlands, of which 2 dropped out due to a lack of time **(Supplementary Figure S2.1)**. Sample size calculation was based on energy expenditure [30]. The PREVIEW intervention study (Prevention of Diabetes through lifestyle intervention and population studies in Europe and around the World, EU seventh Framework Program, grant agreement no. 31205) is a multinational, multi-

center, 2 x 2 factorial, randomized controlled trial aimed at finding the most effective lifestyle intervention to prevent the development of T2D in predisposed individuals. Individuals were between 25 and 70 years of age and had a Body Mass Index (BMI) above 25 kg/m^2. Pre-diabetes, which was defined by a fasting plasma glucose concentration between 5.6 and 6.9 mmol/L and/or a 2 h plasma glucose between 7.8 and 11.0 mmol/L following an oral glucose tolerance test (OGTT) [29], was an inclusion criterion for participation. Exclusion criteria for this respiration chamber sub-study were claustrophobia, smoking, and previous cardiovascular events, next to the exclusion criteria for the PREVIEW study [29]. Written informed consent was obtained from all participants before starting the experiment.

Experimental design

Detailed information on the PREVIEW intervention study design, interventions, subject recruitment, primary and secondary endpoints, and baseline characteristics have been published before [29]. In short, an 8 week weight-reduction period by means of a low-energy diet was followed by 34 months of a randomized intervention comprising four treatment groups: MP/HCHO with moderate-glycemic index (GI), or a HP/MCHO diet with low-GI, combined with either moderate- or high-intensity physical activity in a parallel design. In close proximity to the last clinical investigation day (after 34 months) of the PREVIEW intervention, a subgroup of participants underwent a 48 h experiment in the respiration chamber to assess specific HP/MCHO intake-related aspects of hunger and satiety regulation. Participants arrived at the Metabolic Research Unit Maastricht (MRUM) research facilities in the morning after an overnight fast from 22:00 h the night before. The respiration chamber experiment started at 9:30 h. Participants had fixed bedtimes in the respiration chambers from 11:30 h until 7:30 h and were not allowed to sleep during the daytime or to exercise.

Anthropometric measurements

Body weight and body composition (BOD POD®, Life Measurement Inc., Concord, CA, USA) were measured before the respiration chamber experiment was started. Height was measured using a wall-mounted stadiometer during screening.

Respiration chamber

The respiration chambers are 14 m^3 airtight rooms with a controlled climate and furnished with a bed, chair, table, intercom, TV, computer, sink, and toilet. Continuous fresh air ventilation at a rate of 70 – 80 l/min was used and measured with a dry gas

meter (G6, gasmeterfabriek Schlumberger, Dordrecht, the Netherlands). O_2 and CO_2 concentrations were continuously measured by open-circuit ventilated indirect calorimetry, using dual pairs of infrared CO_2 analyzer (ABB/Hartman and Braun Uras, Frankfurt a.M., Germany) and paramagnetic O_2 analyzers (Servomex 4100, Crowborough, England and ABB/Hartman and Braun Magnos, Frankfurt a.M., Germany) [31]. Total energy expenditure (EE) was calculated according to the formula of Weir [32].

Diets and energy intake

Participants received either a MP/HCHO (15%:55%:30% of energy from protein: carbohydrate:fat) or a HP/MCHO (25%:45%:30% of energy from protein:carbohydrate: fat) diet, according to their previous intervention group during the PREVIEW study. To keep menus as comparable as possible, the basis of all meals was the same between groups, combined with either carbohydrate- or protein-rich food items. Participants were asked to pay special attention to their prescribed study diet during the week before the experiment. All meals were provided in energy balance according to individual energy requirements during the respiration chamber session. The daily energy requirement was estimated by calculating the basal metabolic rate (BMR) with the use of fat mass (FM) and fat-free mass (FFM), which was then multiplied by a physical activity index of 1.35, based on previous respiration chamber experiments [33]. Dietary intake was divided over three meals with 20% of the daily energy requirement for breakfast (at 9:15 h) and 40% for both lunch (at 13:00 h) and dinner (at 17:45 h). No other food products were allowed for consumption. Water consumption was allowed throughout the study at the participants' convenience and unsweetened coffee or tea were served at several time points. For the *ad libitum* brunch after the 48 h in the respiration chamber, participants received a buffet style meal. This meal was the same for the two intervention groups and comparable to the chamber breakfast from the days before, but not diet-specific and including choices of carbohydrate- or protein-rich options. Total energy and macronutrient intake were evaluated afterwards.

Appetite profile

Subjective perception of appetite comprising hunger, fullness, and satiety was measured by 100 mm anchored visual analogue scales (VAS) from "not at all" to "very" during day 2 in the respiration chamber [34]. Questionnaires were scored before and 30 minutes after each meal, as well as once in between the meals. In the case of a simultaneous blood draw, questionnaires were done before the blood draw. The incremental area

under the curve (iAUC) was calculated for satiety and fullness perception, and the dAUC was calculated for hunger perception using the trapezoidal rule [35].

Metabolic parameters

Fasting blood samples were taken from an antecubital vein by venipuncture on the first and last day of the respiration chamber study for the analysis of fasting glucose, insulin, β-hydroxybutyrate, and triacylglycerol. On the second day, a venflon catheter (Becton, Dickinson and Company, Franklin Lanes, NY, USA) was placed in the antecubital vein for fasted and postprandial blood collections throughout the day for collection of endocannabinoids and related compounds, glucose, insulin, GLP-1, and PYY. Blood samples were drawn directly before and 30, 60, 90, and 120 minutes after all three meals. Except for serum samples, all samples were immediately stored on ice, centrifuged for 10 minutes at 1500 g at 4 °C, immediately distributed in aliquots, and stored at −80 °C until analysis at the end of the study, enabling all samples from one participant to be analyzed in the same run. Serum samples were kept at room temperature for 30 minutes to allow clotting before centrifugation (10 minutes at 1500 g at 4 °C). For endocannabinoid and related compounds measurements, samples were collected immediately before, 60 minutes after meals, and 120 minutes after dinner. The AUC was calculated for the endocannabinoids and related substances. The iAUC was calculated for GLP-1, PYY, glucose, and insulin using the trapezoidal rule [35].

Endocannabinoids and endocannabinoid-related compounds

For endocannabinoid and endocannabinoid-related compound analysis, ethylene-diaminetetraacetic acid (EDTA) tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA) were used. Syringes and EDTA tubes were ice-chilled before blood collection and storage cups were prepared with 1% phenylmethylsulfonyl fluoride (PMSF) solution (10 mg PMSF in 1 ml methanol) and 5% 1N hydrochloric acid at final concentration. Samples were snap frozen in liquid nitrogen immediately.

The extraction, purification, and quantification of AEA, PEA, OEA, and 2-AG from blood require a set of different biochemical steps as described previously [19,36]. Samples were then subjected to isotope-dilution liquid chromatography-chemical ionization-tandem mass spectrometric analysis. Mass spectral analyses were performed on a TSQ Quantum Access triple quadrupole instrument (Thermo-Finnigan, San Jose, CA, USA) equipped with an APCI source (atmospheric pressure chemical ionization) and operating in positive ion mode [37]. Pregnenolone was extracted from plasma by a simple solid-phase extraction method using reverse-phase C18 columns according to the method

described in Vallée et al. [22] and analyzed with a GC-MS/MS (gas chromatography-tandem mass spectrometer) XLS Ultra Thermo mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) via an AS3000 II autosampler.

GLP-1 and PYY

For analyses of GLP-1 and PYY concentrations, EDTA-aprotinin tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA) with added dipeptidyl peptidase IV inhibitor (10 ml/L blood) were used. Syringes and EDTA-aprotinin tubes were ice-chilled before blood collection and samples were snap frozen in liquid nitrogen immediately. Total GLP-1 [38] and PYY3-36 concentrations were both determined using a radioimmunoassay method. For the PYY assay, a 125-Iodine label was used and data were analyzed with RIACALC (Pharmacia, Freiburg, Germany).

Glucose and insulin

Plasma for colorimetric glucose analysis (Roche Diagnostic Systems, Woerden, the Netherlands) was collected in sodium fluoride tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA). Serum for insulin analysis was collected in serum separator tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA). Samples were used to analyze fasting and postprandial insulin concentrations with a human insulin-specific radioimmunoassay (Linco Research, St Charles, MO, USA). Insulin sensitivity was estimated by calculating the HOMA-IR [39].

β -Hydroxybutyrate

EDTA vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA) were used to collect plasma samples for β-hydroxybutyrate concentrations analysis with gas chromatography-mass spectrometry (GC-MS) quantification (Varian factor four VF-5 ms, 15 mx 0.25 mm * 0.1 µM with GCMS ms 7890A-7000c, Agilent, Santa Clara, CA, USA).

Triacylglycerol

Serum samples (Becton, Dickinson and Company, Franklin Lakes, NY, USA) were used to analyze fasting triacylglycerol (GPO Trinder; Sigma-Aldrich Corp., St. Louis, MO, USA).

Statistical analysis

All statistical tests were performed using SPSS for Macintosh (Version 25; SPSS Inc., Chicago, IL, USA). Data are presented as means ± standard deviations (SDs), unless

otherwise indicated. Significance was defined as $p < 0.05$. Differences between both groups before and during their respiration chamber stay were calculated using ANOVA, and repeated measures ANOVA with BMI or body-fat percentage as a covariate, if appropriate. Not normally distributed data, as determined with the Shapiro–Wilk test, were log-transformed and tested on normality again. Partial correlation coefficients were calculated to assess associations between protein intake, appetite, gut peptides, and endocannabinoids, adjusted for BMI and fat percentage, if appropriate.

RESULTS

Study participants

The subject characteristics for the MP/HCHO and HP/MCHO group at baseline of the respiration chamber experiment are summarized in **Table 2.1**. Twenty participants started the respiration chamber experiment in the HP/MCHO condition and 18 participants in the MP/HCHO condition. The two groups were not different with regard to age, anthropometric variables, and fasting glucose and insulin concentrations prior to the respiration chamber experiment. Only triacylglycerol (TAG) was higher in the MP/HCHO group at baseline.

Table 2.1: Subject characteristics of the moderate/high carbohydrate (MP/HCHO), and high-protein/moderate-carbohydrate (HP/MCHO) group at baseline of the respiration chamber experiment.

	MP/ HCHO (*n* = 18)	HP/ MCHO (*n* = 20)
N (f/m)	18 (9/9)	20 (13/7)
Age (year)	65.1 ± 5.8	64.0 ± 6.2
BMI (kg/m²)	29.0 ± 3.8	28.9 ± 4.0
Body-fat (%)	39.5 ± 8.1	40.7 ± 7.7
Fat mass (kg)	33.9 ± 7.1	34.8 ± 8.8
Fat-free mass (kg)	52.5 ± 10.9	50.8 ± 11.3
Glucose (mmol/L)	5.8 ± 0.4	5.7 ± 0.5
Insulin (µU/ml)	14.8 ± 7.6	14.4 ± 4.9
HOMA-IR	3.8 ± 1.8	3.6 ± 1.3
TAG (mmol/L)	1.5 ± 0.7	1.2 ± 0.6*

Values are means ± standard deviation (SD). BMI: Body Mass Index; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; TAG: triacylglycerol. *$p < 0.05$.

24-hour hunger and satiety perception

Baseline appetite profile was not different between conditions. Hunger perception (VAS) based on the decremental area under the curve during the whole day was significantly lower in the HP/MCHO diet compared to the MP/HCHO diet (−56.6%, $F = 5.89$, $p < 0.05$, Figure 2.1). Looking at the individual meals, specifically breakfast (−83%; $F = 10.30$, $p < 0.01$) seemed to have contributed to the overall difference between the intervention groups. The differences in hunger ratings could not be explained by differences in energy balance (MP/HCHO: 0.2 ± 0.9 megajoule (MJ), HP/MCHO: −0.5 ± 0.9 MJ; $F = 6.03$, $p < 0.05$) [30] between the two intervention groups. There were no differences in satiety or fullness ratings between the two groups (Figure 2.1).

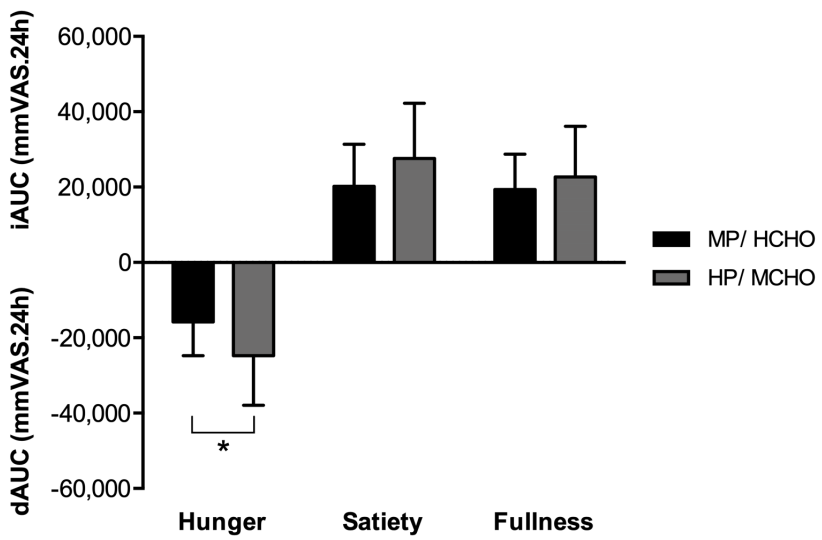

Figure 2.1: Visual analogue scales for appetite for MP/HCHO (black) and HP/MCHO (grey) groups. Hunger is presented as dAUC, fullness and satiety as iAUC. *P < 0.05. Values are means ± SD. Differences between groups were calculated with a One-Way ANOVA. MP/HCHO = moderate protein/ high carbohydrate; HP/MCHO = high protein/moderate carbohydrate; iAUC = incremental area under the curve; dAUC = decremental area under the curve; mmVAS = millimeter visual analogue scale.

Metabolic parameters

Endocannabinoids and related compounds concentrations throughout the day

2-AG showed increased concentrations 60 minutes after each meal, then decreasing back to baseline concentrations before the next meal (Figure 2.2). Around lunch, there was a significant time-by-treatment interaction for 2-AG ($F = 6.61$, $p < 0.05$). In addition,

the AUC of 2-AG was significantly higher in HP/MCHO compared to MP/HCHO (4351 ± 1616 pmol/L versus 3368 ± 1552 pmol/L, $F = 4.67$, $p < 0.05$) and the post-meal change in 2-AG during dinner was positively related to the change in hunger ($r = 0.37$, $p < 0.05$). Post-meal changes of 2-AG were not related to changes in glucose, insulin, GLP-1, or PYY during any of the meals or to body weight or body composition. The postprandial change in 2-AG after dinner was positively associated with the change in pleasantness ratings ($r = 0.425$, $p < 0.05$). There was no clear meal associated pattern or higher protein/ moderate carbohydrate intervention related effect in other endocannabinoids and related compounds. However, we observed that OEA concentrations (AUC) were inversely associated with the change in TAG **(Figure 2.3)** throughout the 48 h of the experiment ($r = -0.40$, $p < 0.05$).

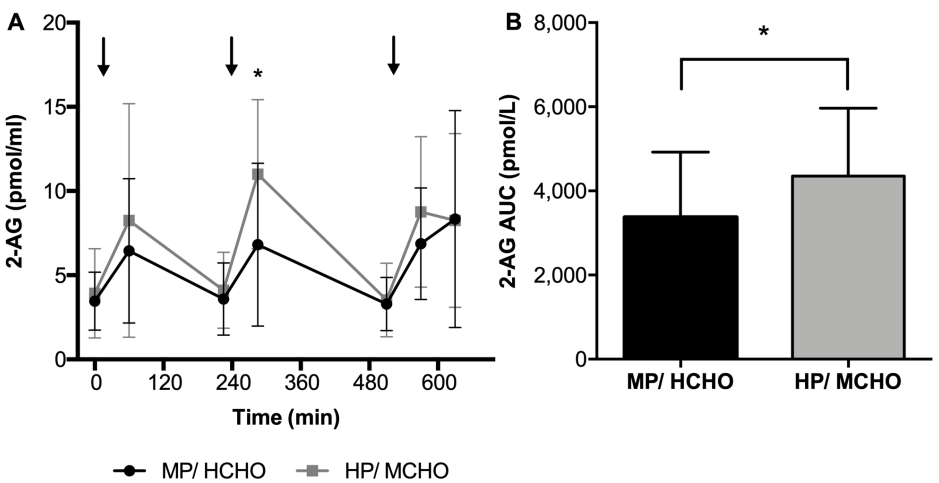

Figure 2.2: (A) Postprandial responses and **(B)** area under the curve (AUC) of 2-AG in the MP/HCHO (black) and HP/MCHO (grey) group. Arrows indicating timepoint of meals. Values are means ± SD. MP/HCHO = moderate protein/high carbohydrate; HP/MCHO = high protein/moderate carbohydrate; 2-AG: 2-arachidonoylglycerol. *P < 0.05.

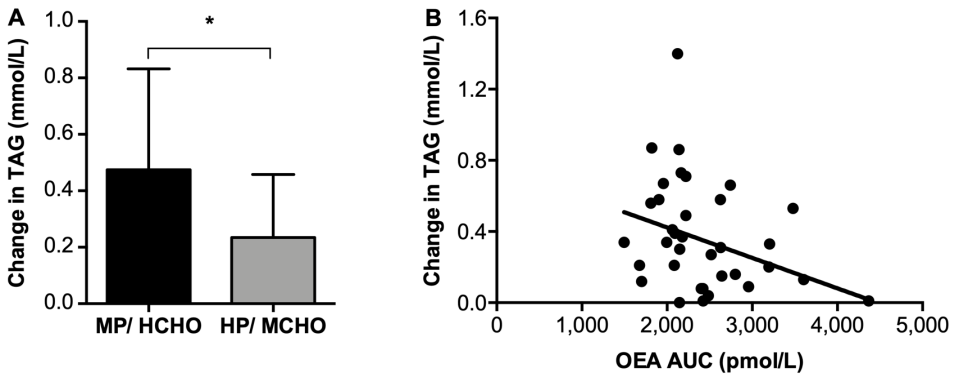

Figure 2.3: (A) AUC of change in TAG over 48 h in the MP/HCHO (black) and HP/MCHO (grey) group and **(B)** inverse association of OEA concentrations throughout the day and the change in TAG over 48h. Values are means ± SD. MP/HCHO = moderate protein/high carbohydrate; HP/MCHO = high protein/ moderate carbohydrate; TAG: triacylglycerol; OEA: oleoylethanolamide. *P < 0.05.

Gut peptides, glucose, and insulin concentrations throughout the day

Postprandial plasma GLP-1 and PYY concentrations are shown in **Figure 2.4**. No significant differences in GLP-1 and PYY response, expressed as iAUC, were found between the HP/MCHO and the MP/HCHO group **(Figure 2.4)**. In the complete group, the dAUC of hunger was inversely associated with the PYY iAUC ($r = 0.40$, $p < 0.05$) however this was especially the case in the HP/MCHO group. We found a significant interaction between group and PYY iAUC with regard to hunger ratings ($F = 7.47$, $p < 0.05$). In the HP/MCHO group, hunger (dAUC) was inversely associated with PYY (iAUC) concentrations ($r = 0.71$, $p < 0.01$; **Figure 2.5**), but not in the MP/HCHO group. GLP-1 was not associated with any of the subjective appetite assessments (hunger, fullness, and satiety). Neither PYY nor GLP-1 concentrations were associated with energy balance in any of the two intervention groups or with parameters related to glucose metabolism. Postprandial glucose concentrations were incidentally higher in the MP/HCHO group but both, glucose and insulin concentrations throughout the day **(Figure 2.4)** did not differ between the two intervention groups.

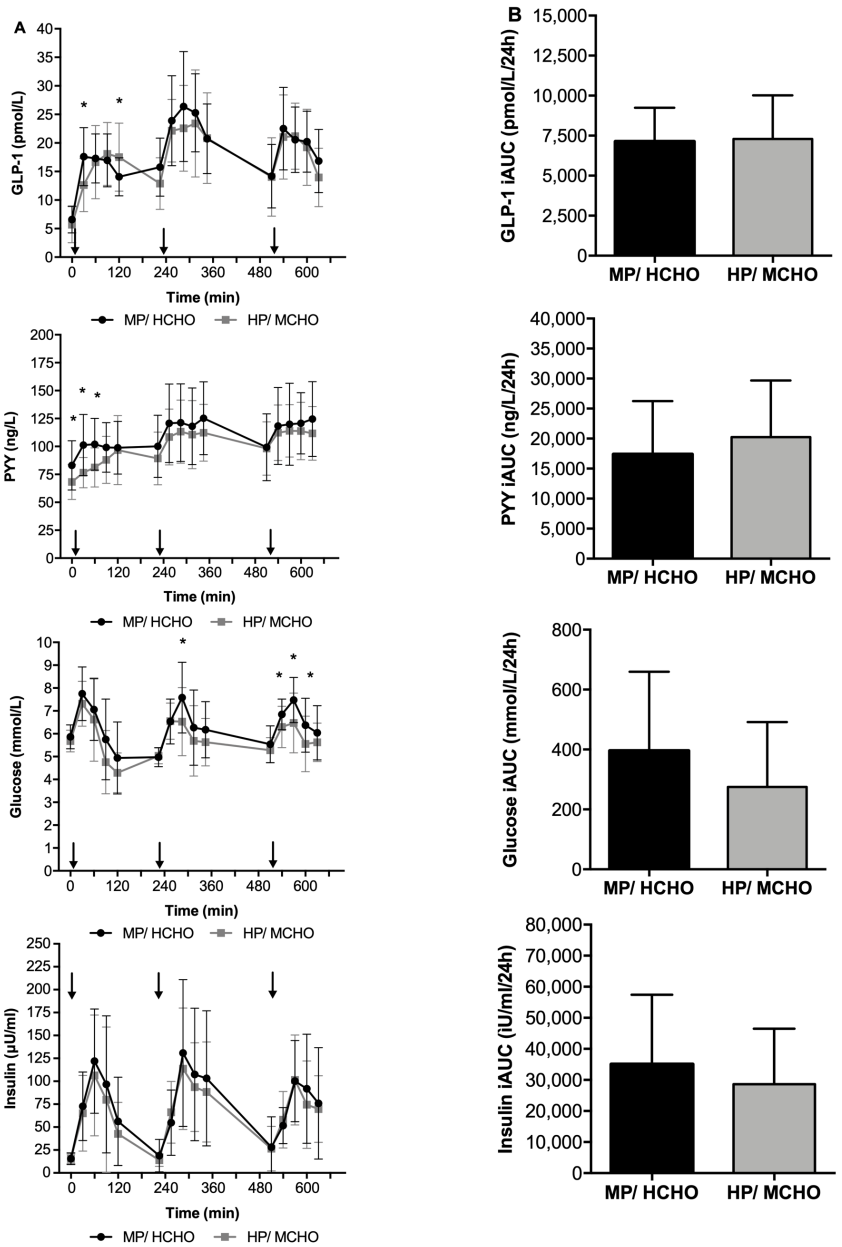

Figure 2.4: (A) Postprandial responses over time and **(B)** incremental area under the curves of GLP-1, PYY, glucose, and insulin in the MP/HCHO (black) and HP/MCHO (grey) group. Arrows indicating time-point of meals. *P < 0.05. Values are means ± SD. Differences between groups were calculated with a One-Way ANOVA. MP/HCHO = moderate protein/high carbohydrate; HP/MCHO = high protein/ moderate carbohydrate; GLP-1 = glucagon-like peptide 1; PYY = peptide YY; iAUC = incremental area under the curve.

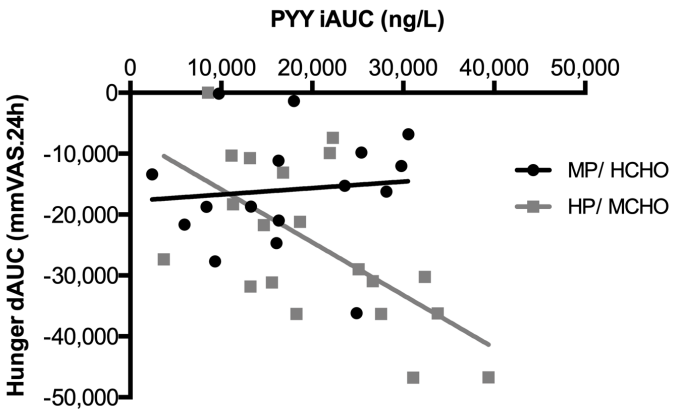

Figure 2.5: Inverse association of hunger and PYY concentrations throughout the day in the MP/HCHO (●) and HP/MCHO (■) group. Regression lines are shown in black (MP/HCHO) and grey (HP/MCHO, r = 0.710, p = 0.001). MP/HCHO = moderate protein/high carbohydrate; HP/MCHO = high protein/ moderate carbohydrate; iAUC = incremental area under the curve; dAUC = decremental area under the curve; PYY = peptide YY.

Fasted β-hydroxybutyrate and triacylglycerol concentrations

Plasma β-hydroxybutyrate concentrations, a marker for fat oxidation, were not different between dietary conditions (MP/HCHO: 90.05 ± 87.61 µg/ml and HP/MCHO: 117.57 ± 93.35 µg/ml). Baseline β-hydroxybutyrate was negatively associated with BMI ($r = -0.35$, $p < 0.05$) but was not associated with appetite perception or gut peptides. TAG concentrations increased in both intervention groups from pre- to post-respirations chamber measurement, but the increase was less pronounced ($p < 0.05$; **Figure 2.3**) in the HP/MCHO group compared to the MP/ HCHO group (0.23 ± 0.22 mmol/L versus 0.47 ± 0.35 mmol/L, respectively).

Ad libitum energy intake after leaving the chamber

Ad libitum energy intake (EI) (EI; MP/HCHO: 30.6% ± 10.4% energy requirement, HP/MCHO: 33.5% ± 12.4% energy requirement) as well as macronutrient content of meal choice (protein intake MP/HCHO: 18.5% ± 4.8% energy intake (%EI), HP/ MCHO: 17.9% ± 4.0% EI; carbohydrate intake MP/HCHO: 50.2% ± 8.1% EI, HP/ MCHO: 46.9% ± 7.5% EI; fat intake MP/HCHO: 30.3% ± 7.4% EI, HP/MCHO: 32.7% ± 6.0% EI) assessed during *ad libitum* brunch were not different between intervention groups. *Ad libitum* energy intake was not associated with excursions of gut peptides the day before or by insulin resistance, but it was negatively associated with total glucose concentrations (iAUC) of the previous day ($r = -0.40$, $p < 0.05$).

DISCUSSION

This study demonstrates that a 48 hour high-protein/moderate-carbohydrate diet fed in energy balance under controlled conditions, reduced the perception of hunger and increased 2-AG concentrations after 34 months in a post-obese condition, compared to a moderate-protein, higher- carbohydrate diet. Hunger was inversely associated with PYY concentrations in the HP/MCHO group but not in the MP/HCHO group. High-protein/moderate-carbohydrate intake led to a blunted increase in TAG compared to moderate-protein/high-carbohydrate intake, while changes in TAG were inversely associated with OAE.

Reduced hunger and increased satiety or fullness have been previously found in studies applying acute high-protein diets, even in negative energy balance [13,14,25]. The decrease in hunger in the HP/MCHO versus the MP/HCHO group in the current study, however, did not translate into a decreased energy intake in the *ad libitum* brunch. Rather large differences in appetite ratings (e.g., ~40%) were necessary to actually result in differences in energy intake of e.g., ~20% in other studies, which applied a within subject design [8].

Among the endocannabinoids and related compounds studied, 2-AG showed a consistent meal-related pattern with highest concentrations one hour post-prandially and lowest concentrations before the three meals. Interestingly, around all meals, the pattern of 2-AG concentrations was similar to that of concentrations in glucose, insulin, GLP-1, and PYY concentrations. However, in contrast to these parameters, 2-AG was the only one to be specifically affected by the higher-protein/moderate-carbohydrate content. Previous observations of plasma 2-AG dynamics around meals have been conflicting, from no meal-effect [36,40] to increased concentrations only after a hedonic meal, in both normal weight individuals [41] and in obese [42]. These data suggest that if food intake is driven by palatability of the food presented, rather than hunger, an increase in circulating 2-AG concentrations is observed, independent of the individuals' BMI [41,42]. As higher 2-AG concentrations have been observed even before the actual consumption of palatable food, the authors have proposed an anticipatory role for this endocannabinoid in signaling the pleasure of the palatable food that is going to be eaten [41]. Regarding the possible hedonic component in endocannabinoid dynamics, desire to eat was not different between the intervention groups, and meals were described as equally pleasant in our study. However, postprandial changes in 2-AG were positively related to changes in pleasantness after dinner, therefore a hedonic effect cannot be excluded. No evidence can be found for a direct postprandial association of 2-AG with

excursions of PYY and GLP-1 on an individual meal basis, suggesting a gut peptide-independent pathway of 2-AG in the current study. In contrast, 2-AG has been shown to be associated with GLP-1 [43] but not with appetite perception [41,43] previously. In the current study, however, postprandial changes of 2-AG were associated with changes in hunger after dinner. In our study, we characterized responses in participants who were still overweight, who had undergone a weight loss period for eight weeks followed by a 34 month period of weight maintenance before the current experiment was started. It is possible that the participants studied have a hedonic response different from lean, healthy-weight individuals, which in part may explain the postprandial increase in 2-AG concentrations observed in both intervention groups as 2-AG concentrations has been shown to be associated with BMI [18,19]. Finally, a likely origin of the changes in circulating 2-AG concentrations may be the intestine. Indeed, studies in rats have shown that gustatory stimulation with fat, without actual food intake, leads to an increase in both 2-AG and AEA content in the small intestine, which then further favor fat consumption through a positive feedback loop involving vagal afferents and the brainstem [44]. Accordingly, it has been shown that cheese with a specific fatty acid profile could influence plasma endocannabinoids in humans [45]. However, the role of other nutrients, especially protein on endocannabinoid concentrations, has never been studied before. To our knowledge, the current data are therefore the first evidence of a link between an increased protein/carbohydrate ratio and circulating 2-AG concentrations. Further research is needed to investigate the potential impact on weight loss and weight maintenance of this observation.

While AEA has been described as an orexigenic compound in the literature [20,36], PEA, OEA, and PREG were associated with anorexigenic effects [22,46–49]. OEA has been suggested to influence fat catabolism [50] and stimulate lipolysis [51]. While OEA was not associated with fat oxidation in the current study, the inverse relationship found between OEA and blunted TAG concentrations may be due to an increase in lipolysis.

In addition, in the present study, research participants were assessed in a controlled respiration chamber setting, and were fed in energy balance, which could over-shadow the perception of hunger and satiety that people experience in a real-life situation. Finally, the fact that 2-AG and AEA behave differently should not be surprising, as they are synthetized through different pathways, which may further support a different role for the compounds in food intake regulation [52]. As for the origin of endocannabinoids and their related compounds, we and others have proposed that prandial-related changes in plasma AEA, 2-AG, OEA and PEA may primarily reflect changes in synthesis from the gastrointestinal tract [36].

Regarding the gut peptides, we found an inverse association between hunger and PYY concentrations in the HP/MCHO group only. PYY has previously been shown to be increased in response to a HP diet and was suggested to mediate the satiating effects of dietary protein [24]. Our data supports other literature proposing a 'threshold' level of protein to be necessary to exert effects on PYY-mediated impact on hunger [53,54]. PYY and GLP-1 concentrations have been linked to satiety and food intake [26], with rather weak associations [13]. GLP-1 release on the other hand has been primarily associated with dietary carbohydrates and fat and has been shown to be lower by high protein whilst satiety was increased compared to high carbohydrate [55]. Some evidence points to a stimulation of GLP-1 by a HP condition [56,57]. In comparison, PYY appeared to be stimulated by dietary protein content to a larger extent than GLP-1 [27,57]. However, changes in gut peptides by dietary protein content in the present study did not translate into changes in appetite perception. Relations between endogenous gut peptides and appetite ratings have shown hardly any, or conflicting results in the past [27], and have often been either absent [11], or weak [13]. Despite these discrepancies in subjective and objective appetite measures, appetite ratings by visual analogue scales have been shown to be highly reproducible [34] and may therefore still be important in assessing appetite next to physiologic measures. Taken together, the current study suggests that observed effects on 2-AG, hunger, and the association of PYY and hunger are nutrient-related, since they are especially shown in the HP/MCHO group.

While changes in TAG concentrations were blunted in the high-protein/moderate-carbohydrate group, both intervention groups increased their TAG concentrations when comparing pre- and post-respiration chamber measurements. The general increase in TAG may be related to the sedentary circumstances the participants experienced throughout their 48 h stay [58,59]. High protein intake has previously been suggested to increase lipolysis [60] and inhibit lipogenesis [61], which may explain the blunted increase in TAG in the HP/MCHO condition compared to the MP/HCHO condition in the current study.

The current study investigates the effects of a diet with higher protein and lower carbohydrate content compared to a diet with lower protein and higher carbohydrate content. Although there is some evidence describing the role of protein over carbohydrate on hunger perception [11], the current study design is not sufficient to clearly allocate the effect to one of the two macronutrients. To assign the described effects on 2-AG, hunger and PYY, and TAG, conclusively to a high protein or moderate carbohydrate level, or to a combination of both, longer follow-up studies are needed.

CONCLUSIONS

In conclusion, findings from the present study confirm that a higher dietary protein/carbohydrate ratio has appetite regulating effects. Additionally, it shows that 2-AG concentrations appear to be an important contributor to appetite related effects of a high-protein/moderate-carbohydrate diet, and that PYY may be one of the mediators in this increased protein/carbohydrate ratio-induced appetite regulation with an impact on reduced hunger perception, after 34 months in the post-obese phase.

AUTHOR CONTRIBUTIONS

Conceptualization, B.G.-C., M.S.W.-P, and T.C.A.; methodology, L.T., M.D., B.G.-C., M.S.W.-P., and T.C.A.; formal analysis, L.T., M.D., and T.C.A.; investigation, L.T., M.D., and B.G.-C.; data curation, L.T., M.D., B.H., and I.M.; writing—original draft preparation, L.T.; writing—review and editing, M.D., B.G.-C., A.R., M.F., B.H., J.J.H., I.M., D.C., R.P.M., P.J.J., M.S.W.-P., and T.C.A.; visualization, L.T.; supervision, R.P.M., P.J.J., M.S.W.-P., and T.C.A.; project administration, L.T. and M.D.; funding acquisition, A.S. and M.S.W.-P.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

ABBREVIATIONS

2-AG	2-arachidonoylglycerol
AEA	anandamide
AUC	area under the curve
BHB	β-hydroxybutyrate
BMI	body mass index
BMR	basal metabolic rate
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
dAUC	decremental area under the curve
EDTA	ethylenediaminetetraacetic acid
EI	energy intake
FFM	fat-free mass
FM	fat mass
GC-MS	gas chromatography-mass spectrometry
GC-MS/MS	gas chromatography-tandem mass spectrometry
GI	glycemic index
GLP-1	glucagon-like peptide-1
HOMA-IR	homeostatic model assessment for insulin resistance
HP/ MCHO	high protein/ moderate carbohydrate
iAUC	incremental area under the curve
MJ	megajoule
mmVAS	milimeter visiual analogue scale
MP/ HCHO	moderate-protein/ high-carbohydrate
OEA	oleoylethanolamide
OGTT	oral glucose tolerance test
PEA	palmitoylethanolamide
PMSF	phenylmethylsulfonyl fluoride
PREG	pregnenolone
PYY	polypeptide YY
TAG	triacylglycerol
T2D	type II diabetes
VAS	visual analogue scale

SUPPLEMENTAL MATERIAL

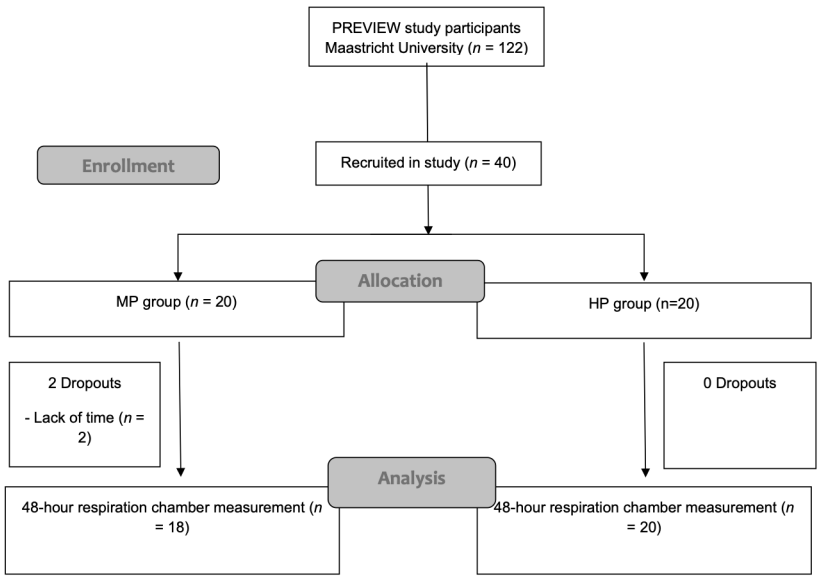

Figure S2.1: Consort flow diagram of study.

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CHAPTER 3

Role of endocannabinoids in energy balance regulation in participants in the post-obese state - a PREVIEW study

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ABSTRACT

Context: Endocannabinoids are suggested to play a role in energy balance regulation.

Objective: We aimed to investigate associations of endocannabinoid concentrations during the day with energy balance and adiposity and interactions with 2 diets differing in protein content in participants in the post-obese phase with pre-diabetes.

Design and participants: Participants (*n* = 38) were individually fed in energy balance with a medium protein (MP: 15:55:30% of energy from Protein:Carbohydrate:Fat) or high protein diet (HP: 25:45:30% energy from P:C:F) for 48-hours in a respiration chamber.

Main outcome measures: Associations between energy balance, energy expenditure, RQ and endo-cannabinoid concentrations during the day were assessed.

Results: Plasma-concentrations of anandamide (AEA), oleoylethanolamide (OEA), palmitoylethanolamide (PEA), and pregnenolone (PREG) significantly decreased during the day. This decrease was inversely related to BMI (AEA) or body-fat (%) (PEA; OEA). The lowest RQ value, before lunch, was inversely associated with concentrations of AEA and PEA before lunch. AUC of concentrations of AEA, 2-AG, PEA, and OEA were positively related to body-fat% ($p<0.05$). The HP and MP groups showed no differences in concentrations of AEA, OEA, PEA, and PREG, but the AUC of 2-arachidonoylglycerol (2-AG) was significantly higher in the HP vs. the MP group.

Conclusions: In energy balance, only the endocannabinoid 2-AG changed in relation to protein level of the diet, while the endocannabinoid AEA, and endocannabinoid-related compounds OEA and PEA reflected the gradual energy intake matching energy expenditure over the day.

INTRODUCTION

Weight gain and obesity are known risk factors for T2D. The progression of pre-diabetes to T2D in individuals with overweight or obesity can be prevented by sustained weight loss [1-3]. However, the most effective lifestyle approach to achieve this goal has not been clearly defined yet. Therefore, the main objective of the PREVIEW study (PREVention of diabetes through lifestyle Intervention and population studies in Europe and around the World) [4] was to determine whether a high-protein low-GI (glycaemic index) diet was more effective in preventing T2D compared to a moderate protein, higher-GI diet for weight maintenance. Another objective was to determine whether a shorter, but increased intensity of physical activity (PA) had an additional beneficial effect on the outcomes compared with moderate intensity PA.

The endocannabinoid system (ECS) has been suggested to play a role in the regulation of energy balance in humans [5]. The ECS encompasses the endocannabinoid type Receptor 1 and 2 (CBRs), the endocannabinoids and the pathways responsible for the synthesis and degradation of those ligands [5-7]. Endocannabinoids, of which the most studied are arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) are polyunsaturated fatty acids produced on demand from membrane phosphor-lipids to act on CBR in an autocrine or paracrine manner [8]. CB1R is widely distributed in the brain and also in peripheral tissues such as adipose tissue, liver, gastro-intestinal tract, pancreas and skeletal muscles [5-12], and is suggested to promote obesity[5]. Interestingly, by acting both peripherally and centrally and by affecting the actions of leptin and insulin, CB1R is a potential therapeutic target against obesity and T2D [7-10,13-17]. Plasma AEA is positively associated with adiposity [17] and plasma 2-AG is associated with ghrelin levels [18], while plasma oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), two endocannabinoid-related compounds, are suggested to act opposite AEA and 2-AG, and may stimulate satiety or energy expenditure [17]. Finally, the neurosteroid pregnenolone acts as a signaling specific inhibitor of CB1R, and is part of an endogenous negative feedback loop, which decreases the activity of the receptor [19,20].

The endocannabinoids and their related compounds can be modulated by the diet and in particular by polyunsaturated fatty acids content [5]. We recently observed that postprandial 2-AG concentrations were higher after a diet with increased protein-to-carbohydrate-ratio, while AEA, OEA, PEA, and PREG concentrations were not affected by the macronutrient content of the diet [21]. However, we did not explore possible associations between the ECS and energy balance or adiposity in this group of

participants. Therefore, the aim of the current study was to investigate possible associations of endocannabinoid concentrations during the day with energy balance and adiposity in a sub-group of participants with pre-diabetes in the post-obese phase of the PREVIEW study.

MATERIALS AND METHODS

The Medical Ethical Committee of Maastricht University approved the PREVIEW study, registered on clinicaltrials.gov with identifier NCT01777893, as well as the respiration chamber sub-study. The study was performed in line with the Declaration of Helsinki. All volunteers signed written informed consent.

Participants

Forty individuals were recruited from the PREVIEW study population at Maastricht University in the Netherlands, of whom two dropped out due to lack of time. For the general PREVIEW study participants underwent a screening that included anthropometric measurements as described in Fogelholm et al. [4]. Additional exclusion criteria for the respiration chamber study were claustrophobia, smoking, and previous cardiovascular events.

Study design

The PREVIEW study design was composed of a 3 year multinational, randomised trial with 4 intervention arms in a 2 x 2 factorial design in eight intervention centres (Denmark, Finland, United Kingdom, The Netherlands, Spain, Bulgaria, Australia, and New Zealand, with 2326 adults (25-70 y, BMI ≥ 25 kg/m^2) with prediabetes as defined by the ADA criteria: fasting plasma glucose 5.6-6.9 mmol/L and/or 7.8-11.0 mmol/L at 2 hours after an oral glucose tolerance test of 75 g glucose, with a fasting plasma glucose concentration <7.0 mmol/L [4]. A total of 962 participants completed the 3-year intervention starting with a two-month ≥ 8% weight reduction phase using a low-energy diet [22] followed by a randomised 34-month weight maintenance phase in one of the four treatment arms: High Protein-High Intensity Physical Activity, HP-Medium Intensity physical activity, Medium Protein-HI, and MP-MI; (MP: 15:55:30% of energy from Protein:Carbohydrate:Fat; HP: 25:45:30% of energy from Protein:Carbohydrate:Fat), with the main outcome measures being incidence of T2D over 3 years analysed by diet and PA treatment, subsequently according to diet and PA, and secondarily among others

changes in body weight, body mass index (BMI), body composition, insulin resistance (HOMA-IR) [4]. Close to the last clinical investigation day of the weight maintenance period, ~34 months after starting the PREVIEW weight maintenance intervention, participants stayed in the respiration chamber for 48 hours. The 38 participants participating in the respiration chamber measurements had lost on average 11.1 ± 3.6 kg (11.9 ± 2.5%; range 8.1% - 18.2%) during the weight loss period. After the subsequent 34-month weight maintenance phase the average body weight was still 5.5 ± 6.2 kg lower compared to baseline, corresponding with a BMI of 28.9 ± 3.9 kg/m^2 and an average regain in body weight of 5.6 kg during the weight maintenance phase. There were no differences between the two dietary intervention groups, high protein (HP) and medium protein (MP), regarding changes in body weight during the PREVIEW intervention **(Table 3.1)**.

Respiration chamber

Subjects arrived at the Metabolic Research Unit Maastricht (MRUM) research facilities in the morning having fasted overnight from 22:00h the night before. The respiration chamber session started at 09:30h and stopped 2 days later 09:30h. The respiration chamber is an airtight chamber of 14 m^3 furnished with a bed, chair, desk with computer, TV, telephone, intercom, sink, and toilet. The climate inside the chamber was controlled. O_2 consumption and CO_2 production were continuously measured by open-circuit ventilated indirect calorimetry [23]. The room was ventilated with fresh air at a rate of 70-80 l/min. Flow was measured using electronically modified dry gasmeters (G6, gasmeterfabriek Schlumberger, Dordrecht, The Netherlands). The concentrations of O_2 and CO_2 were measured with dual pairs of infrared CO_2 analyzers (ABB/Hartman&Braun Uras, Frankfurt a.M, Germany) and paramagnetic O_2 analyzers (Servomex 4100, Crowborough, England and ABB/ Hartman&Braun Magnos, Frankfurt a.M, Germany) [23]. During each 15-min period, six samples of outgoing air, one sample of fresh air, zero gas and calibration gas were measured. The gas samples to be measured were selected by a computer that also stored and processed the data [23]. Physical activity was continuously measured by use of an ActiSleep+ (ActiGraph LLC, Pensacola, FL) accelerometer worn on the hip. Subjects had fixed bed times between 23:30h and 07:30h. In the daytime, they were not allowed to sleep or to perform exercise. Meals were offered at stated times (breakfast: 9:00h, lunch: 13:00h, dinner: 17:45h), and subjects were instructed to finish these within 30 min.

Dietary intervention

Participants received either a moderate protein diet (MP: 15:55:30 from En% protein: carbohydrate:fat) or a high protein diet (HP: 25:45:30 En% from protein:carbohydrate: fat) corresponding with their dietary intervention instructions during the PREVIEW study [4]. The basis of the meals was the same between groups, combined with either carbohydrate- or protein-rich food items to keep menus as comparable as possible. The diets consisted of commercially available food items and were provided individually in energy balance. Individual daily energy requirements (DER) were calculated as the basal metabolic rate using the fat-free mass and fat mass [24] multiplied by a physical activity level of 1.35 [25]. Daily energy intake was divided over three meals, with breakfast containing 20%, and lunch and dinner 40%. During all measurements in the respiration chamber, the meals within each condition had the same macronutrient composition. Water consumption was allowed *ad libitum* between the meals; no other foods or beverages were available.

Energy expenditure and respiratory quotient

Total energy expenditure (TEE) was determined during the 48-h stay in the respiration chamber. O_2 consumption and CO_2 production were used to calculate TEE according to the formula of Weir [26]. Energy balance was calculated by subtracting TEE from energy intake. Respiratory quotient (RQ) was calculated by dividing CO_2 production by O_2 consumption as a measure of substrate oxidation.

Anthropometric measurements

Body weight and composition were determined with subjects in the fasted state before entering the respiration chamber. Body weight was measured using a calibrated scale (Life Measurement Corporation, Inc, Concord, CA, USA). Body composition was determined based on body density measured via air-displacement plethysmography with the BodPod system (BOD POD, Life Measurement Inc., Concord, CA, USA), utilizing Siri's equation for body density [27]. Height was measured using a wall-mounted stadiometer to the nearest 0.1 cm (Seca, model 222, Seca, Hamburg, Germany).

Metabolic parameters

On the first and last day of the respiration chamber experiment, fasting blood samples were taken from an antecubital vein by venipuncture for the analysis of fasted glucose and insulin. In the morning of the second day, a venflon catheter (Becton, Dickinson and Company, Franklin Lanes, NY, USA) was placed in the antecubital vein to collect fasted,

preprandial and postprandial blood for the analysis of endocannabinoid and related compounds. Blood was drawn through an airtight lock in the door, which had a plastic 'sleeve' attached on the participants' side of the lock. Participants were instructed to put their arm in the sleeve and to close it tightly around before the lock was opened. This enabled us to draw blood without affecting pressure inside the chamber. Blood samples were drawn directly before and 60 minutes after all three meals, with one sample more for the endocannabinoids and related compounds analyses at 120 min after dinner. Samples were immediately stored on ice, centrifuged for 10 minutes at 1500g at 4°C, immediately distributed in aliquots, and stored at -80°C until analysis at the end of the study, enabling that all samples from one participant were in the same analytical run.

Glucose

Plasma for colorimetric glucose analysis (Roche Diagnostic Systems, Woerden, the Netherlands) was collected in sodium fluoride tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA).

Insulin

Serum for insulin analysis was collected in serum separator tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA). Serum samples were kept at room temperature for 30 minutes to allow clotting before centrifugation (10 minutes at 1500g at 4°C). Samples were used to analyze fasting and postprandial insulin concentrations with a human insulin-specific radioimmunoassay (Linco Research, St Charles, MO, USA). Insulin sensitivity was estimated by calculating the homeostasis model assessment for insulin resistance (HOMA-IR) [28].

Endocannabinoids and endocannabinoid-related compounds

For endocannabinoid analysis, ice-chilled EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA) were used. Samples were distributed in aliquots in prepared storage cups with 1% PMSF solution (10 mg PMSF in 1 m methanol) and 5% 1N HCL at final concentration. The set of different biochemical steps for the extraction, purification, and quantification of AEA, PEA, OEA and 2-AG from plasma require are described previously [12,14,20,29,30]. Subsequently, plasma samples were subjected to isotope-dilution liquid chromatography-chemical ionization-tandem mass spectrometric analysis. Mass spectral analyses were performed on a TSQ Quantum Access triple quadrupole instrument (Thermo-Finnigan) equipped with an APCI source (atmospheric pressure chemical ionisation) and operating in positive ion mode [12]. To evaluate

between-run precision and reproducibility, quality control samples were prepared by directly supplementing a plasma pool control with endocannabinoids and run for each batch of samples analyzed. The amounts of AEA, PEA, OEA and 2-AG are expressed as pmols per ml of plasma.

Pregnenolone was extracted by a simple solid-phase extraction method using reverse-phase C18 columns from EDTA plasma, as described in Vallee et al. [19]. The steroid fraction was eluted with methanol (2 ml) into screw-cap test tubes and evaporated to dryness at 50°C under a nitrogen stream to prepare for derivatization. Dried methanol extracts of plasma and standards were then derivatized by a two-step procedure. The formation of pentafluoro-benzyloxime for negative chemical ionization detection was followed by trimethylsilyl ether formation for adequate sensitivity. Samples were then subjected to isotope-dilution gas chromatography-chemical ionization-tandem mass spectrometric analysis. The derivatized samples were injected (1 µl) directly into a GCMSMS XLS Ultra Thermo mass spectrometer (Thermo-Finnigan) via an AS3000 II autosampler. The instrument was employed in negative ion chemical ionization mode and a 15 m Rtx-5Sil MS W/Integra Guard capillary column (Restek, France) with a 0.25 mm inside diameter and 0.1 µm film thickness was employed for analyte resolution. To evaluate between-run precision and reproducibility, quality control samples were prepared by directly supplementing a plasma pool control with steroids and run for each batch of samples analyzed. The amount of pregnenolone was then expressed as ng per ml of plasma.

Urinary nitrogen

During both days in the chamber, 24h urine was collected for the analysis of urinary nitrogen excretion, as an estimation of protein metabolization (Vario Max, CN-analyzer, Elementar Analysesysteme GmbH, Langenselbold, Germany). Urine bottles were prepared with 10 ml hydrochloric acid (4 mmol/L) to prevent nitrogen degradation. Nitrogen levels were then multiplied with 6.25 to calculate the daily protein intake (g/day).

Statistical analysis

All statistical tests were performed using SPSS for Macintosh (Version 25; SPSS Inc., Chicago, IL, USA). Data are presented as means ± standard deviations (SDs). Significance was defined as $P<0.05$. Normality of the parameters was assessed using the Shapiro-Wilk test and outliers were detected with the use of box plots in SPSS. In the whole group, BMI, AEE and AUCs of activity-induced energy expenditure (AEA), AG and OEA

were not normally distributed. In the HP group, protein oxidation, fat oxidation, BMI, fat-free mass, body-fat percentage, TEE, and AEE were not normally distributed. In the MP group, AUCs of AEA, AG PEA, and OEA also were not normally distributed. For these reasons, non-parametric tests were used for the analyses. Differences between both groups were calculated using Mann-Whitney U tests. Associations between endocannabinoid concentrations were assessed using linear mixed model analysis. Changes in endocannabinoids during the day were assessed with repeated-measures ANOVA followed by LSD post-test. Repeated measures ANCOVA and Spearman correlation analysis was used to assess possible relations between endocannabinoids and anthropometric variables, energy balance, substrate oxidation, and insulin resistance.

RESULTS

Anthropometric variables, HOMA-IR, energy intake and energy expenditure did not differ significantly between the HP and MP [31]. Energy balance was significantly lower in the HP group compared to MP ($p=0.015$); respiratory quotient (RQ) also was significantly lower in the HP group [31] **(Table 3.1)**. With respect to the endocannabinoids and related compounds, AEA concentrations were positively associated with 2-AG (estimate 0.008; CI: 0.004-0.011; $p<0.001$), PEA (estimate 0.060; CI: 0.050-0.069; $p<0.001$) and OEA (estimate 0.072; CI: 0.061-0.083; $p<0.001$) concentrations, assessed with linear mixed model analysis **(Table 3.1)**. Likewise, PEA concentrations were positively associated with OEA (estimate 0.592; CI: 0.495-0.689; $p<0.001$) and 2-AG (estimate 0.269; CI: 0.081-0.457; $p<0.01$) and OEA concentrations were positively associated with 2-AG (estimate 0.072; CI: 0.061-0.083; $p<0.05$). PREG concentrations were not associated with the other endocannabinoid concentrations.

Table 3.1: Anthropometric variables in medium-protein and high-protein groups at baseline, and when entering the respiration chamber

	Baseline			Respiration chamber		
	MP (n=18)	HP (n=20)	*p*-value	MP (n=18)	HP (n=20)	*p*-value
Sex (f/m)	9/9	13/7				
Age (years)	61.5±5.7	59.4±67.7	0.443			
BMI (kg/m²)	31.2±4.1	30.0±3.4	0.553	29.0±3.8	28.9±4.0	0.942
Fat-free mass (kg)	54.7±11.9	52.7±11.9	0.654	52.5±10.9	50.8±11.3	0.553
Fat mass (kg)	39.3±9.0	36.5±6.8	0.346	33.9±7.7	34.8±8.8	0.740
Body-fat (%)	41.9±6.8	41.2±6.3	0.613	39.3±7.4	40.7±7.7	0.593
HOMA-IR	3.4±2.0	2.9±1.3	0.460	3.8±1.8	3.62±1.34	0.794
EI (MJ/d)				9.4 ± 1.7	9.3 ± 1.6	0.573
TEE (MJ/d)				9.2±1.6	9.8±1.7	0.534
EB, MJ/d				0.2 ± 0.9	-0.5 ± 0.9	0.015
RQ				0.84 ± 0.02	0.82 ± 0.02	0.004

Data are presented as mean ± SD. Differences between groups were assessed by means of Mann-Whitney U tests and the corresponding p-values are shown in the table. Different changes from baseline to respiration chamber between groups were assessed by means of analysis of covariance (ANCOVA), with baseline values as covariate; there were no significantly different changes between the groups. MP = moderate protein; HP = high protein; BMI = body mass index; EI = energy intake; TEE = total energy expenditure; EB = energy balance; RQ = respiratory quotient

The AUC from before breakfast to 120 minutes after the start of dinner, of the plasma concentrations of AEA, OEA, PEA, and PREG were not significantly different between the two groups. Differently, the AUC of the plasma concentrations of 2-AG was significantly higher in the HP compared to the MP group (4351 ± 1616 vs. 3368 ± 1552, $F=4.67$, $p<0.05$) [21]. There were no significant group*time interactions between the groups, assessed with repeated measures ANOVA **(Figure 3.1)**. Therefore, the two groups have been pulled together for further analyses.

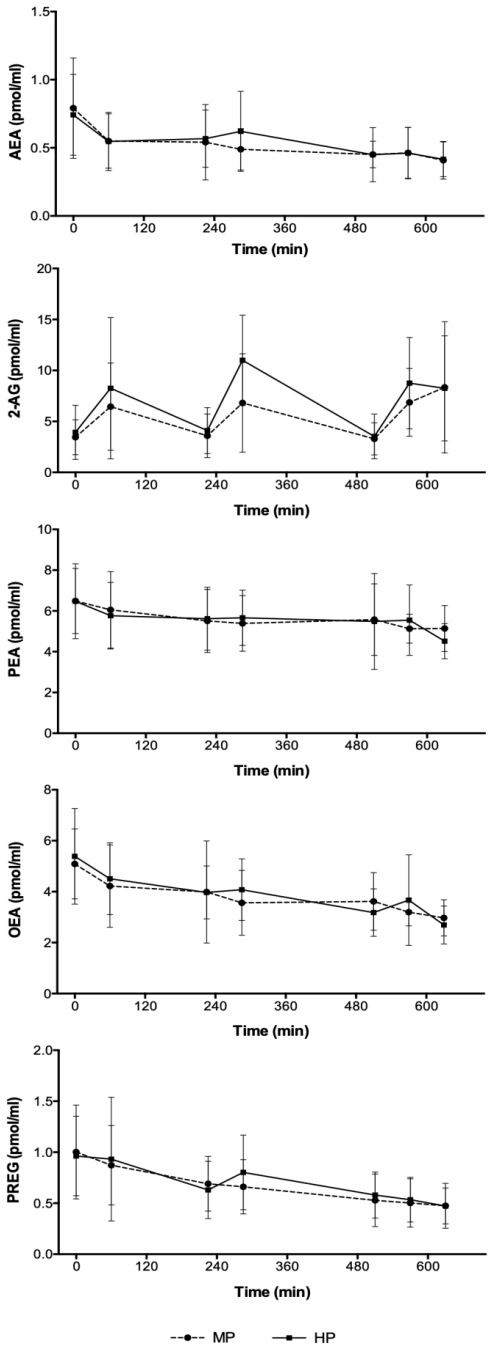

Figure 3.1: Endocannabinoid concentrations during the day in the medium protein (MP) and high protein (HP) group. AEA = anandamide; 2-AG = 2-arachidonoylglycerol; PEA = palmitoylethanolamide OEA = oleoylethanolamide; PREG = pregnenolone.

In the whole group, body-fat% was a significant predictor for AEA ($p<0.01$; $\eta_p^2=0.19$), 2-AG ($p<0.05$; $\eta_p^2=0.13$), PEA ($p<0.05$; $\eta_p^2=0.14$) and OEA ($p<0.05$; $\eta_p^2=0.16$) concentrations, assessed with repeated measured ANCOVA. These results were also reflected by significant positive associations between the AUC of the endocannabinoid concentrations of AEA (r=0.43), 2-AG (r=0.37), PEA (r=0.34), and OEA (r=0.40) and body-fat% ($p<0.05$), indicating that those subjects with a higher body-fat% had higher levels of these endocannabinoids and related compounds. Differently, the AUC of PREG concentrations was not related to body-fat%. We also assessed associations between TEE and endocannabinoid concentrations using repeated measures ANCOVA, correcting for FFM. TEE was a positive contributor to AEA ($p<0.05$; $\eta_p^2=0.17$) and OEA ($p<0.05$; $\eta_p^2=0.12$) concentrations. No relationships appeared with energy balance or HOMA-IR for any of the endocannabinoid concentrations and related compounds.

Interestingly, in the whole group the plasma concentrations of AEA, PEA, OEA and PREG were the highest in the fasted state and decreased significantly during the day **(Table 3.2)**, without showing any clear meal associated pattern. The change in these concentrations from fasting to 120 min after dinner was inversely related to BMI (AEA: r=-0.33) or body-fat% (PEA: -0.37; OEA: -0.38) **(Figure 3.2)**; changes in PREG concentrations were not related to anthropometric values. Moreover, the RQ before the lunch appeared to be the minimum RQ value over 24h, namely RQ=0.71±0.04. This RQ value was inversely associated with concentrations of AEA (r=-0.39; p=0.016) and PEA (r=-0.34; p=0.04) before lunch **(Figure 3.3)**.

Table 3.2: Concentrations of AEA (pmols/ml), 2-AG (pmols/ml), PEA (pmols/ml), OEA (pmols/ml), and PREG (ng/ml) before each meal and 60 min later, and 120 min after the start of dinner.

	B0	B60	L0	L60	D0	D60	D120
AEA*	0.77+0.33[2,3,4,5,6,7]	0.56+0.20[1,5,6,7]	0.56+0.24[1,5,6,7]	0.56+0.24[1,5,6,7]	0.45+0.16[1,2,3,4]	0.47+0.19[1,2,3,4,7]	0.41+0.13[1,2,3,4,6]
2-AG*	3.65+2.25[2,4,6,7]	7.51+5.87[1,2,5]	3.88+2.22[2,4,6,7]	9.11+5.07[1,3,4]	3.42+1.91[2,4,5,6]	7.91+4.08[1,3,5]	8.31+5.80[1,3,5]
PEA*	6.47+1.72[3,4,5,6,7]	5.89+1.76[7]	5.62+1.54[1,7]	5.55+1.38[1,7]	5.57+2.08[1,7]	5.38+1.37[1,7]	4.74+1.00[1,2,3,4,5,6]
OEA*	5.23+1.66[2,3,4,5,6,7]	4.40+1.50[1,4,5,6,7]	4.00+1.56[1,5,7]	3.86+1.26[1,2,7]	3.38+1.04[1,2,3,7]	3.46+1.36[1,2,7]	2.80+0.73[1,2,3,4,5,6]
PREG*	0.98+0.42[3,4,5,6,7]	0.90+0.52[3,4,5,6,7]	0.66+0.28[1,2,3,5,6,7]	0.74+0.33[1,2,5,6,7]	0.56+0.24[1,2,3,4,6,7]	0.51+0.23[1,2,3,4,5,7]	0.46+0.18[1,2,3,4,5,6]

B0, L0, D0: before breakfast, lunch, dinner; B60, L60, D60, D120: 60, respectively 120 minutes after the start of breakfast, lunch and dinner. Abbreviations: AEA= arachidonoylethanolamide; 2-AG = 2-arachidonoylglycerol; PEA=palmitoylethanolamide; OEA= oleoylethanolamide; PREG= pregnenolone. *Significant time interaction $p<0.001$ assessed with repeated measures ANOVA. [1]Significantly different from B0; [2]Significantly different from B60; [3]Significantly different from L0; [4]Significantly different from L60; [5]Significantly different from D0; [6]Significantly different from D60; [7]Significantly different from D120.

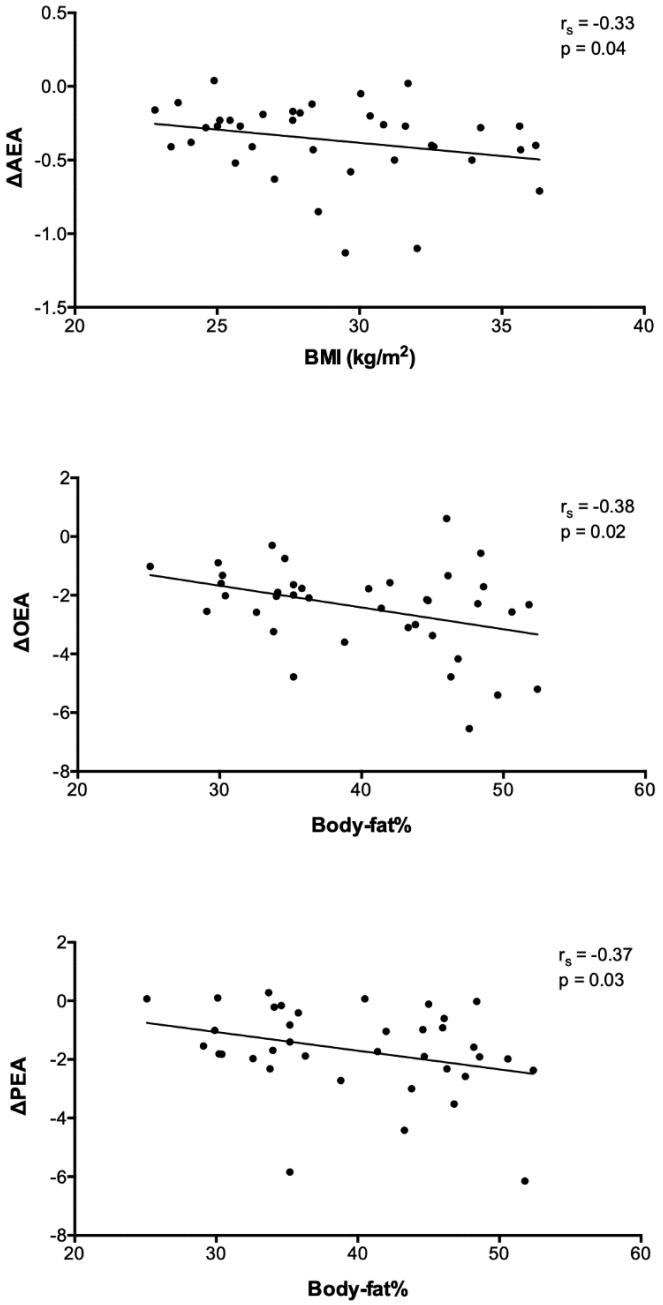

Figure 3.2: Associations of change in endocannabinoid concentration over the day and BMI (kg/m^2) and body-fat % assessed with Spearman's correlation analysis. AEA = anandamide; PEA = palmitoylethanolamide; OEA = oleoylethanolamide.

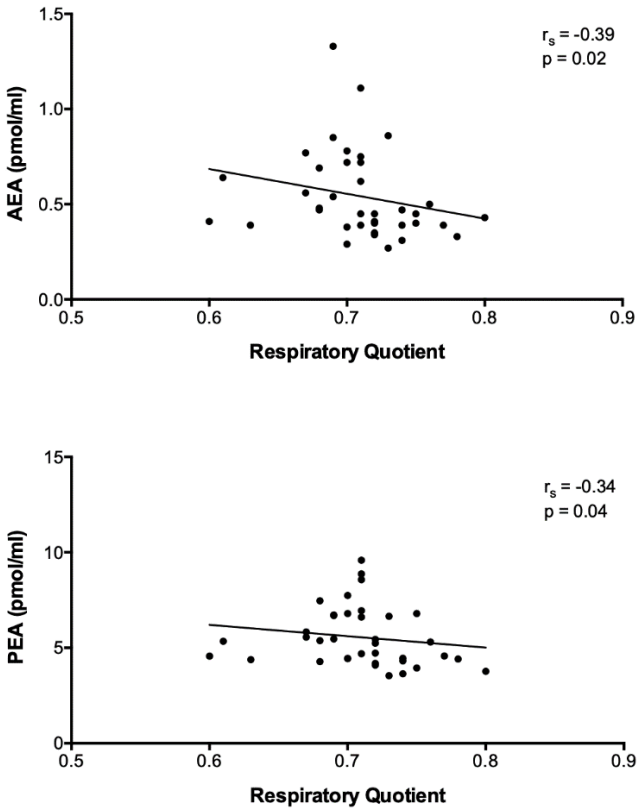

Figure 3.3: Associations of endocannabinoid concentration and respiratory quotient before the lunch assessed with Spearman's correlation analysis. AEA = anandamide; PEA = palmitoylethanolamide.

Lastly, we have assessed the relationship between change in BMI or body-fat % prior to the respiration chamber measurements and endocannabinoid concentrations during the day. Changes in BMI (%) positively predicted 2-AG concentrations (η_p^2= 0.142 $p<0.05$), but no other endocannabinoid concentrations.

Furthermore, there were no associations between body-fat% and endocannabinoid concentrations or between changes in BMI% or body-fat% and changes in endocannabinoid concentrations during the day.

DISCUSSION

The present 48h respiration chamber study, during which PREVIEW-participants in the post-obese phase were individually fed in energy balance, and in controlled macronutrient proportions, offered an excellent opportunity to investigate the concentrations of endocannabinoids and related compounds throughout the day, the changes thereof, and the possible interaction with energy balance. While we did not find associations between the endocannabinoid concentrations and energy balance or adiposity, reductions in the concentrations of AEA, OEA, PEA, and PREG during the day were inversely related with BMI and body-fat%.

In the whole group of participants, we found statistically significant decreases in concentrations from fasting to 120 min after dinner in AEA, OEA, PEA, and PREG. This may suggest that these endocannabinoids respond to a gradual energy supply to meet energy balance during the day. This is also in line with the finding that AEA and OEA were positively associated, corrected for FFM. Participants with a higher energy turn-over presented with higher concentrations of AEA and OEA, which may again be an effort to meet energy balance during the day. This was further underscored by the lowest RQ during the day, before the lunch, being inversely associated with concentrations of AEA and PEA before lunch. The significant decrease of AEA, OEA, PEA, and PREG may point toward a circadian rhythm in the secretion of these compounds, as was suggested before [32]. OEA and PEA changed in the same direction as AEA, which can be explained by the fact that these compounds, although physiologically playing different roles, are synthesized along the same intracellular pathways [9]. Again, this may be due to the controlled study design of feeding participants in energy balance, which does not imply strong feelings of craving or fullness.

Moreover, the changes in concentrations of AEA, OEA and PEA were inversely related to BMI (AEA) and body-fat% (PEA, OEA). This may indicate that those with a lower BMI or fat mass presented with a stronger energy balance regulation compared to those with a higher BMI or fat mass, who could easily live on their reserves for a longer period of time. The lack of a relationship of pregnenolone concentrations with anthropometric measurements underscores the different functionality of pregnenolone inhibiting the CB1R [19,20], which likely only takes place upon important and sustained activation of CB1R.

With respect to the effect of diets with different protein and carbohydrate compositions on endocannabinoid concentrations and interactions with energy balance, we observed no statistically significant differences in AEA, OEA, PEA and PREG concentrations

between the 2 diets used in the study. Only the AUC of 2-AG was statistically significantly different between the diets, in that it was higher in the HP diet group compared to the MP diet group [21]. Moreover, the concentrations of AEA, 2-AG, OEA, PEA and PREG were not associated with energy balance, energy expenditure, or insulin sensitivity, independently of diet group. This is in contrast with previous studies that underscore the hypothesis of AEA being an orexigenic factor showing a pattern of changes in circulating AEA concentrations around meals [29,33,34]. AEA concentrations have been observed to be increased before meals in participants with normal weight and with obesity [29] and to be decreased after food consumption [11,33,34], independently of the hedonic value of the meal in participants with normal weight [35]. Furthermore, it was observed that the endocannabinoids may affect energy balance in that AEA and 2-AG would promote energy intake and might be inhibited by PREG, while OEA and PEA would promote satiety or energy expenditure [17-20,36]. Moreover, AEA has been suggested to affect energy expenditure by mediating the effect of skeletal muscle sphingomyelins [37]. Finally, associations of peripheral endocannabinoids with energy expenditure in native Americans have been reported [38]. That we did not observe any of those effects may be due to the controlled design of the study, with participants being fed to their individual energy balance, and having to finish the food that was offered to them, without the possibility of hedonic eating or overeating [33,34]. To our knowledge, this is the first study to investigate the relationship between endocannabinoid excursion during the day, adiposity, and energy metabolism in a controlled study design during the post-obese phase, while fed in energy balance.

Interestingly, we found a positive relation between the degree of weight loss prior to the respiration chamber measurements and 2-AG concentrations during the respiration chamber, with higher weight loss being associated with lower 2-AG concentrations possibly due to a reduction in ghrelin and hunger [18]. Moreover, in the present conditions we did not observe any associations with insulin sensitivity, which have been reported before [15], although our participants still presented with the characteristics of pre-diabetes. Being previously obese, participants were in a post-obese state on average, when being assessed in the respiration chamber. While previous literature has shown links between circulating endocannabinoids and metabolic parameters in obese subjects, it is unclear how these relationships hold after people have lost weight. This relationship may depend on the timing of the measurements, i.e. during weight loss or short-term after weight loss when in fact the effect during negative energy balance is measured, or long-term after weight loss when energy balance has been established again. Studies investigating changes in endocannabinoid concentrations after bariatric surgery induced weight loss found an increase in AEA but not 2-AG levels after 2 months

after surgery [39] and a reduction in levels of 2-AG and AEA at 12 months after surgery [40]. A 16-week very low calorie diet led to a reduction in AEA levels, but not 2-AG levels [41] and a 1-year lifestyle programme including healthy eating and physical eating led to a reduction in both, 2-AG and AEA [42]. Given the rather substantial weight change after surgery, our study adds to the body of literature by assessing the post obese individuals while being fed in energy balance.

We need to point out a limitation regarding the design of the current study. We would have preferred to include the respiration chamber and endocannabinoid measurements at the start of the PREVIEW study as well. However, this was not feasible without putting too much strain on the participants. Furthermore, it has to be considered that the relationships between endocannabinoid concentrations and body-fat% and RQ that were reported in the present study, would not survive correction for multiple comparisons (e.g., Bonferroni correction). Therefore, the findings of the present study need to be confirmed in a larger group of participants. Even though meals were obtained from general supermarkets and were provided as close as possible to free living conditions, they may have slightly differed from consumed food in the home condition of the participants. Therefore, we cannot rule out that interactions between the diet group in the respiration chamber and in the free-living condition may have masked diet-specific effects. However, a possible interaction does not affect the main conclusions.

Taken together, we did not observe differences in the concentrations of endocannabinoids and related compounds due to differences in macronutrient compositions, except for 2-AG which we interpreted as possibly related to a higher satiety. We did observe a significant decrease of AEA, OEA and PEA concentrations during the day, probably indicating the gradual energy supply over the day. This decrease in endocannabinoid concentrations was inversely related to BMI or body-fat%, suggesting a stronger energy balance regulation in those with a lower adiposity. In conclusion, in energy balance, only the endocannabinoid 2-AG changed in relation with protein level in the diet, while the endocannabinoid AEA, and endocannabinoid-related compounds OEA and PEA reflected the gradual energy intake matching energy expenditure over the day.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

AUTHOR'S CONTRIBUTIONS

BG-C, TA, and MW-P designed and supervised the study. LT and MD conducted the study and analysed the respiration chamber data. DC and IM generated and analysed the endocannabinoids and related compounds data. MD, LT, DC, and MW-P wrote the manuscript; the manuscript was reviewed by all authors.

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CHAPTER 4

Effects of a high-protein diet on cardiometabolic health, vascular function, and endocannabinoids - a PREVIEW study

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ABSTRACT

Background: An unfavorable lipid profile and being overweight are known mediators in the development of cardiovascular disease (CVD) risk. The effect of diet, particularly high in protein, remains under discussion.

Objective: Therefore, this study examines the effects of a high-protein (HP) diet on cardiometabolic health and vascular function (i.e., endothelial function, arterial stiffness, and retinal microvascular structure), and the possible association with plasma endocannabinoids and endocannabinoid-related compounds in overweight participants.

Design: Thirty-eight participants (64.5 ± 5.9 (mean ± SD) years; body mass index (BMI) 28.9 ± 4.0 kg/m^2) were measured for 48 h in a respiration chamber after body-weight maintenance for approximately 34 months following weight reduction. Diets with either a HP (n = 20) or moderate protein (MP; n = 18) content (25%/45%/30% vs. 15%/55%/30% protein/carbohydrate/fat) were provided in energy balance. Validated markers for cardiometabolic health (i.e., office blood pressure (BP) and serum lipoprotein concentrations) and vascular function (i.e., brachial artery flow-mediated vasodilation, pulse wave analysis and velocity, and retinal microvascular calibers) were measured before and after those 48 h. Additionally, 24 h ambulatory BP, plasma anandamide (AEA), 2-arachidonoylglycerol (2-AG), oleoylethanolamide (OEA), palmitoylethanol-amide (PEA), and pregnenolone (PREG) were analyzed throughout the day.

Results: Office and ambulatory BP, serum lipoprotein concentrations, and vascular function markers were not different between the groups. Only heart rate (HR) was higher in the HP group. HR was positively associated with OEA, while OEA and PEA were also positively associated with total cholesterol (TC) and low-density lipoprotein (LDL) cholesterol concentrations. Vascular function markers were not associated with endocannabinoids (or endocannabinoid-related substances).

Conclusion: In conclusion, the HP diet did not affect cardiometabolic health and vascular function in overweight participants after completing a weight-loss intervention. Furthermore, our data indicate a possible association between OEA and PEA with TC and LDL cholesterol.

INTRODUCTION

Cardiovascular disease (CVD) is the most common cause of death worldwide [1]. Well-known mediators in the development of vascular damage and CVD are an unfavorable serum lipid profile and being overweight [2]. Whilst an unhealthy diet (e.g., high in salt and saturated fat) is known as a risk factor for the development of CVD [2], the effect of macronutrients, in particular the effect of dietary protein in general remains under discussion [3]. Reviews on the relationships between longer-term protein intakes with CVD [4], serum lipids, and blood pressure (BP) [5] are however inconclusive and more research is warranted in this field [6–8]. In addition to diet, it has recently been suggested that the endocannabinoid system (ECS) plays a role in the regulation of cardiometabolic health and vascular function. The ECS consists of three components: (1) the cannabinoid receptors (CB1 and CB2), (2) enzymes responsible for the metabolization of ligands, and (3) endogenous ligands. Those ligands are endocannabinoids like anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and endocannabinoid-related compounds like oleoylethanolamide (OEA), palmitoylethanol-amide (PEA), and pregnenolone (PREG).

AEA and 2-AG may affect factors directly related to cardiovascular health [9–11] including the regulation of blood pressure [11] and may exert beneficial vasoactive properties [10,12]. More specifically, a positive correlation of 2-AG with body mass index (BMI) and body fat percentage, with an unfavorable lipid profile, and a higher glycemic response has been found [9]. For AEA, results are more conflicting, as plasma concentrations vary considerably among studies [9]. Treatment with CB1 antagonists improved plasma lipid profiles [13–15], and reduced blood pressure in humans [11], but was also associated with adverse events including psychological problems [16]. Data on the impact of diet on the ECS system are limited, but it may be affected by fat intake [17]. Recently, however, we found a higher dietary protein intake had an enhancing effect on plasma 2-AG concentrations [18].

Currently, human intervention data on the role of the ECS in protein-induced effects on cardiometabolic health and vascular function are limited. Related to our findings of an increasing effect of protein intake on plasma 2-AG concentrations [18], we propose that the ECS is a possible route of action by which dietary protein affects cardiometabolic risk and vascular function markers. Therefore, this study aims to assess the effects of a high-protein (HP) versus a moderate-protein (MP) diet in overweight participants on validated markers for cardiometabolic health and vascular function. As a secondary objective, this study examines a possible association of those markers for cardio-

metabolic health and vascular function with circulating endocannabinoids and related compounds concentrations. This study was designed as a substudy of the PREVIEW intervention study [19].

MATERIALS AND METHODS

The study was performed according to the Declaration of Helsinki and approved by the Maastricht University Medical Centre's Medical Research Ethics Committee (METC). The PREVIEW study was registered on 29 January 2013 at ClinicalTrials.gov (NCT01777893). Written informed consent for participation was collected from all participants prior to the study. The substudy was performed from February 2017 until February 2018 in Maastricht, The Netherlands.

Study design

The present study was set up as a substudy to PREVIEW, a study assessing the prevention of diabetes through lifestyle intervention and population studies in Europe and around the world. The PREVIEW study was funded through the European Union (EU) 7th Framework Program (grant agreement no. 31205) [19]. Detailed information on the PREVIEW intervention and on the substudy has been published [18,19]. In brief, the PREVIEW intervention started with 8 weeks of weight loss, followed by 34 months of a randomized intervention with either a moderate-protein (MP), moderate-glycemic index (GI) or a high-protein (HP), low-GI diet, combined with either moderate- or high-intensity physical activity in a parallel design. After 34 months, a subgroup of 40 participants from the PREVIEW cohort [19] of Maastricht University in the Netherlands was recruited for the respiration chamber experiment [18]. Two participants of the 40 recruited dropped out due to a lack of time. Inclusion criteria for PREVIEW were previously described [19]. In short, participants were aged between 25 and 70 years and had to be overweight (BMI >25 kg/m^2). Adults with prediabetes were included, defined as a fasting plasma glucose concentration between 5.6 and 6.9 mmol/L and/or a 2 h plasma glucose between 7.8 and 11.0 mmol/L following an oral glucose tolerance test (OGTT) [19].

For the substudy, participants arrived fasted (from 10:00 p.m. the evening before) at the research facilities in the Metabolic Research Unit Maastricht. At baseline, fasted blood samples were drawn, and anthropometric measurements included weight and body composition (BOD POD, Life Measurement Inc., Concord, CA, USA). Vascular function

markers were assessed prior to and after their 48-h stay in the respiration chamber (in the following the terms pre- and post-respiration chamber will be used to refer to these measurement time points). In the respiration chambers energy expenditure was assessed by means of a continuous O_2 and CO_2 concentration measurement. The open-circuit indirect calorimetry was possible due to the airtight set up of the rooms with controlled climate and continuous fresh air ventilation [20]. Participants received three standardized meals per day, either with moderate-protein (MP: 15/55/30% energy from protein/carbohydrate/fat, respectively) or high-protein (HP: 25/45/30% energy from protein/carbohydrate/fat, respectively) content, which were in accordance with participants' assigned intervention group during the PREVIEW study. Physical activity intensity based on the intervention was evenly spread (n = 8 high intensity and n = 12 moderate intensity in the HP group and n = 8 high intensity and n = 10 moderate intensity in the MP group) for the respiration chamber study. Fat content and quality were comparable between the groups. At the start of the substudy, participants did not significantly differ in their habitual dietary protein intake. All meals were provided through the laboratory and the investigators to reach energy balance tailored to individual energy requirements [18].

Cardiometabolic risk markers

Ambulatory and office blood pressure

Ambulatory BP (AMBP) was measured at regular intervals during the 48 h in the respiration chamber with a portable BP monitor (Mobil-O-Graph® NG; APC Cardiovascular, Hartford, UK) as explained by Joris and colleagues [21]. In short, AMBP was measured in 15 min intervals during daytime (7:30 a.m.–11:30 p.m.) and in 30 min intervals during nighttime (11:30 p.m.–7:30 a.m.). The first measurement was discarded from the analysis and mean values for systolic blood pressure (SBP) and diastolic blood pressure (DBP), heart rate (HR), mean arterial pressure (MAP), and pulse pressure (PP) were calculated for the total 48 h period, as well as for daytime (7:30 a.m.–11:30 p.m.) and nighttime (11:30 p.m.–7:30 a.m.) separately. Additionally, systolic and diastolic dipping during the night were calculated as described [21].

Office BP was measured (Microlife, Wildnau, Switzerland) in supine position. The mean SBP and DBP were assessed, while the mean arterial pressure (MAP) and pulse pressure (PP) were calculated afterwards using the following formulae: $MAP = 1/3 \times SBP + 2/3 \times DBP$ and $PP = SBP - DBP$.

Serum lipids and lipoproteins

Fasting blood samples were taken in tubes equipped for serum separation (Becton, Dickinson and Company, Franklin Lakes, NY, USA) by venipuncture from the antecubital vein at pre- and post- respiration chamber time points. Clotting was allowed for 30 min at room temperature before centrifugation (10 min, 1500× g, 4 °C). Aliquots were stored at −80 °C until analysis. Serum aliquots were used for the analysis of total cholesterol (TC) (CHOD-PAP method, Roche Diagnostics System, Mannheim, Germany), high-density lipoprotein (HDL) cholesterol (precipitation method followed by CHOD-PAP method; Roche Diagnostics System), and triacylglycerol (TAG; GPO Trinder; Sigma-Aldrich Corp., St. Louis, MO, USA). Low-density lipoprotein (LDL) cholesterol concentrations were calculated using the Friedewald formula [22].

Vascular function measurements

Vascular function measurements were performed in a fasted state at pre- and post-respiration chamber time points, in a quiet and darkened room. The room was temperature controlled at 24 °C. Measurements were performed in supine position after an acclimatization period of at least 15 min. Detailed information has been described before by Joris et al. [23]. In brief, endothelial function was measured by brachial artery flow-mediated vasodilation (FMD) by use of ultrasound echography (Sonos 5500, Hewlett-Packard, Palo Alto, CA, USA). Pulse wave analysis (PWA; cAIxHR75) and carotid-to-femoral pulse wave velocity (PWV_{c-f}) measurements were performed in triplicate with a tonometer (SphygmoCor v9; AtCor Medical, West Ryde, Australia) to assess arterial stiffness, and retinal vascular images were obtained to determine microvascular calibers using a nonmydriatic retinal camera (Topcon TRC-NW-300; Topcon Co., Tokyo, Japan).

Endocannabinoids and endocannabinoid-related compounds

Plasma from EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA) were used for the analysis of AEA, PEA, OEA, 2-AG, and PREG as described [18]. Vacutainers contained 1% phenylmethylsulfonyl fluoride (PMSF) solution (10 mg PMSF in 1 mL methanol) and 5% 1N hydrochloric acid at final concentration. EDTA tubes and syringes for the blood sampling were ice chilled. After centrifugation (10 min, 1500 g, 4 °C) plasma was aliquoted and snap frozen immediately. AEA, 2-AG, OEA, and PEA were quantified with mass spectral analyses 5LC-M/MS (TSQ Quantum Access triple quadrupole instrument; Thermo-Finnigan, San Jose, CA, USA) [9,24,25] while PREG was quantified with GC-MS/MS (gas chromatography-tandem mass spectrometer) XLS Ultra

Thermo mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) via an AS3000 II autosampler [26]. Blood samples for endocannabinoid analysis were drawn directly before and 60 min after all three meals with one additional sample at 120 min after dinner.

Statistical analyses

Sample size was powered based on the primary outcome energy expenditure [27]. SPSS version 25 was used for statistical analyses (SPSS for Macintosh; SPSS Inc., Chicago, IL, USA). Data are presented as means ± standard deviation (SD). Data were log-transformed if not normally distributed as tested by the Shapiro–Wilk test. The trapezoidal method was used to calculate the area under the curve (AUC) for endocannabinoids and related substances [28] for the whole day. Differences at baseline between groups were determined by ANOVA and the treatment effect was calculated by an ANCOVA where the baseline variable was used as a covariate. Partial correlations with body fat % as covariate were used to assess associations between cardiovascular parameters and endocannabinoids.

RESULTS

Baseline characteristics

Participant characteristics prior to the respiration chamber stay were previously described [18]. In brief, no differences in age, BMI, body composition, and C-reactive protein (CRP) were found between the MP and HP intervention groups prior to the respiration chamber experiment **(Table S4.1)**.

Cardiometabolic risk markers

Office and ambulatory blood pressure

Mean 24 h, mean daytime, or mean nighttime ambulatory SBP, DBP, PP, and MAP were not different between intervention groups. However, 24 h and daytime HR were significantly higher in the HP group (both $p < 0.05$). No differences in nighttime SBP or DBP dipping were observed **(Table 4.1)**. Similar results were observed for the office blood pressure measurements **(Table S4.2)**.

Table 4.1: Ambulatory blood pressure measurements during 48 h of the respiration chamber stay with a moderate- or high-protein dietary diet.

		Moderate Protein (*n* = 18)	High Protein (*n* = 20)
24 h	SBP (mmHg)	133 ± 12	131 ± 12
	DBP (mmHg)	82 ± 8	79 ± 7
	MAP (mmHg)	106 ± 9	103 ± 9
	PP (mmHg)	51 ± 9	52 ± 9
	HR (bpm)	62 ± 9	67 ± 5*
Daytime	SBP (mmHg)	139 ± 13	136 ± 13
	DBP (mmHg)	86 ± 9	83 ± 8
	MAP (mmHg)	111 ± 10	107 ± 9
	PP (mmHg)	53 ± 10	53 ± 9
	HR (bpm)	63 ± 9	69 ± 5*
Nighttime	SBP (mmHg)	116 ± 13	117 ± 15
	DBP (mmHg)	68 ± 6	67 ± 8
	MAP (mmHg)	90 ± 9	90 ± 11
	PP (mmHg)	48 ± 10	48 ± 7
	HR (bpm)	58 ± 8	61 ± 7
Dipping	SBP (%)	16 ± 9	16 ± 3
	DBP (%)	21 ± 6	19 ± 7

* $p < 0.05$. Values are presented as mean ± SD. A one-way ANOVA was used to calculate differences between the groups. SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure; PP = pulse pressure; HR = heart rate; bpm = beats per minute.

Serum lipids and lipoproteins

Effects of the high- and moderate-protein diet in the respiration chamber on metabolic risk markers are presented in **Table 4.2**. Total cholesterol and LDL cholesterol concentrations were comparable between intervention groups at pre- and post-respiration chamber measurements and did not change in any of the groups. Changes in HDL cholesterol concentrations were not different between the MP and HP intervention group. The total cholesterol/HDL cholesterol ratio was not different between groups **(Table 4.2)**. As described before, changes in TAG were less pronounced in the HP group [18].

Table 4.2: Metabolic risk markers at pre- and post-respiration chamber time points with a moderate- and high-protein diet.

	Moderate Protein (*n* = 18)		High Protein (*n* = 20)		Treatment Effect
	Pre[1]	Post[1]	Pre[1]	Post[1]	Difference in Change[2]
Total cholesterol (mmol/L)	5.6 ± 1.1	5.6 ± 1.0	5.6 ± 1.0	5.5 ± 1.0	−0.1 (−0.3; 0.2)
HDL cholesterol (mmol/L)	1.4 ± 0.3	1.3 ± 0.2	1.5 ± 0.3	1.4 ± 0.3	0.0 (−0.1; 0.1)
LDL cholesterol (mmol/L)	3.5 ± 0.9	3.4 ± 0.8	3.4 ± 0.9	3.3 ± 0.8	0.0 (−0.2; 0.2)
Total cholesterol/HDL cholesterol ratio	4.0 ± 0.9	4.4 ± 1.1	3.6 ± 0.7	3.9 ± 0.7	−0.1 (−0.3; 0.1)

[1]Values are means ± SD; [2]Treatment effects (95% confidence interval (CI)) were obtained from a one-factor ANCOVA with baseline value as covariate. HDL = high-density lipoprotein; LDL = low-density lipoprotein.

Endothelial function, arterial stiffness, and retinal microvascular structure

FMD, an important marker reflecting vascular endothelial function, was not different between the groups and was also similar comparing pre- and post-respiration chamber time points, as presented in **Table 4.3**. Similar results were observed for markers of arterial stiffness, the central augmentation index adjusted for heart rate (cAIxHR75), and for $PWV_{c\text{-}f}$. Finally, retinal microvascular calibers and the arteriolar-to-venular ratio did not differ between treatment groups and did not change **(Table 4.3)**.

Endocannabinoids and endocannabinoid-related compounds

As previously described [18], plasma 2-AG concentrations increased after meals and were generally higher in the HP condition. AEA, OEA, PEA, and PREG decreased throughout the day and were not affected by dietary protein intake [18]. AEA, 2-AG, OEA, and PEA were all positively associated with body fat percentage [29].

Plasma OEA concentrations were positively associated with 24 h and daily AMBP HR **(Figure 4.1)**, total cholesterol, and LDL cholesterol **(Figure 4.1**), while PEA was positively associated with total cholesterol, LDL cholesterol, and HDL cholesterol **(Figure 4.1)**. As there were no differences in PEA and OEA between the two intervention groups, the associations are presented for the whole group. In contrast, 2-AG, AEA, and PREG showed no associations with cardiometabolic risk or vascular function markers.

Table 4.3: Vascular function measurements at pre- and post-respiration chamber time points with a moderate- or high-protein dietary diet.

	Moderate Protein (*n* = 18)		**High Protein** (*n* = 20)		**Treatment Effect**
	Pre[1]	Post[1]	Pre[1]	Post[1]	Difference in Change[2]
Vascular function					
Baseline brachial diameter (cm)	0.58 ± 0.14	0.58 ± 0.10	0.57 ± 0.12	0.56 ± 0.14	−0.02 (−0.05; 0.01)
FMD (%)	3.4 ± 2.1	3.6 ± 2.3	4.4 ± 3.2	4.4 ± 3.3	0.4 (−1.5; 2.3)
Arterial stiffness					
PWV_{c-f} (m/s)[3]	8.8 ± 1.5	8.6 ± 1.1	8.8 ± 1.3	8.8 ± 1.8	0.2 (−0.6; 0.9)
cAIxHR75 (%)	22.1 ± 8.2	21.5 ± 7.1	25.4 ± 7.0	23.3 ± 8.8	−0.7 (−4.6; 3.2)
Retinal microvascular structure					
Arteriolar width (µm)[4]	128 ± 19	127 ± 20	124 ± 23	125 ± 19	2.4 (−1.9; 6.8)
Venular width (µm)[4]	222 ± 25	220 ± 24	214 ± 29	215 ± 29	2.2 (−2.1; 6.5)
Arteriolar-to-venular ratio[2]	0.58 ± 0.09	0.58 ± 0.09	0.58 ± 0.1	0.59 ± 0.08	0.01 (−0.02; 0.04)

[1]Values are presented as means ± SD; [2]Treatment effects are mean changes (95% CI) obtained from one-factor ANCOVA with baseline value as covariate. [3]Moderate-protein group (MP): *n* = 17; high-protein group (HP): *n* = 19. [4]MP: n = 17; HP: n = 18. FMD = flow-mediated dilation; PWV_{c-f} = carotid-to-femoral pulse wave velocity; cAIxHR75 = central augmentation index adjusted for heart rate, derived from pulse wave analysis (PWA) measurement.

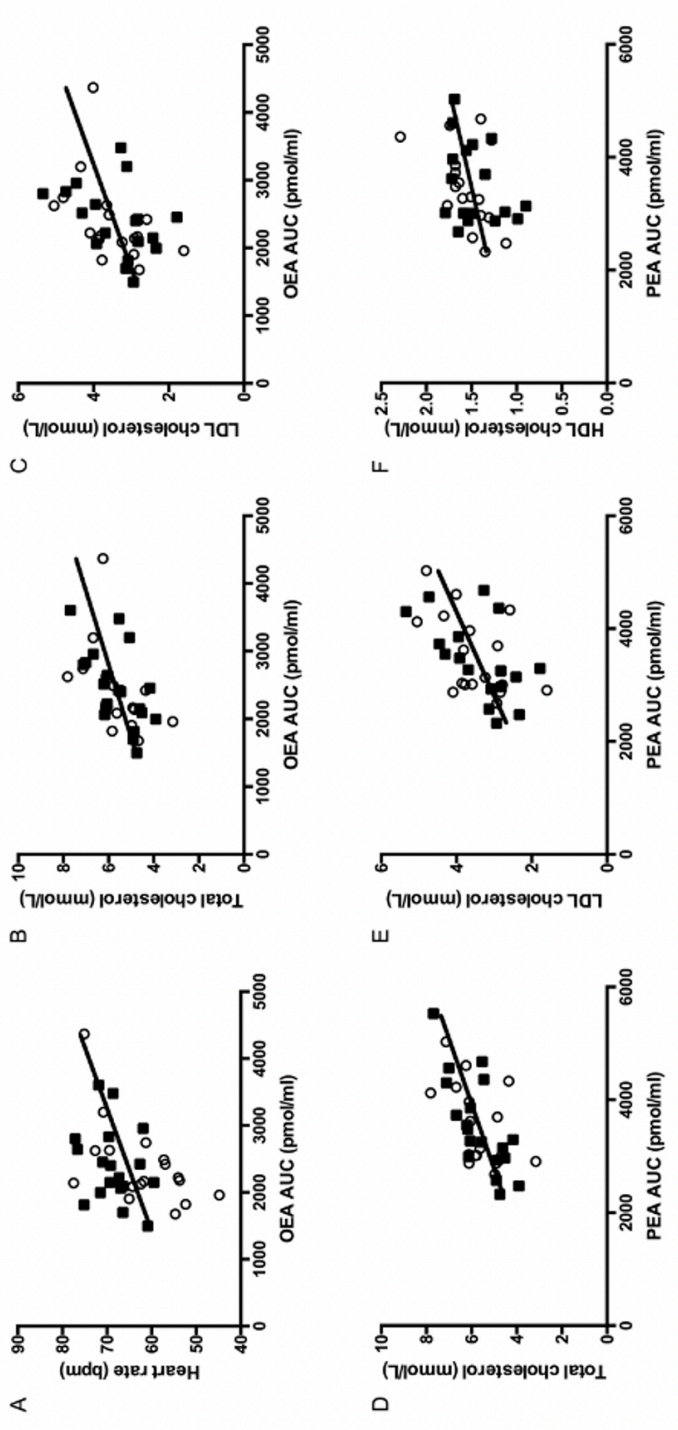

Figure 4.1: Association between OEA AUC with (A) daily heart rate (r = 0.373, p = 0.030), (B) baseline total cholesterol (r = 0.509, p = 0.002), (C) LDL cholesterol (r = 0.444, p = 0.010), and between PEA AUC with (D) total cholesterol (r = 0.594, p = 0.001), (E) LDL cholesterol (r = 0.527, p = 0.002), and (F) HDL cholesterol (r = 0.390, p = 0.025) in the whole group, as assessed with partial correlation analysis corrected for fat percentage. The regression line relates to the combined group. HP = high protein; MP = moderate protein; AUC = area under the curve; OEA = oleoylethanolamide; PEA = palmitoylethanolamide.

DISCUSSION

In the current study the effects of a high-protein diet on cardiometabolic health and vascular function markers were examined in overweight participants. The study indicates that a diet with a higher protein content did not affect markers for metabolic health and vascular function, as serum lipoprotein concentrations and BP or markers for endothelial function, respectively. Only HR was higher with a HP diet. As a secondary aim, possible associations of markers for cardiometabolic health and vascular function with endocannabinoids and endocannabinoid-related compounds were examined. Here, the endocannabinoid-related compounds OEA and PEA were positively associated with cardiometabolic risk markers, such as serum cholesterol concentrations and HR, independent of protein intake.

In general, most of the individuals in the current study can be classified as hypertensive according to the 2018 European Society of Cardiology/ European Society of Hypertension (ESC/ESH) guidelines [30]. In a previous study, a HP diet reduced DBP compared to a normal protein diet, when combined with weight loss [31]. In the present study, no effects on BP were found comparing the two protein intervention groups. However, studies reporting effects of dietary protein on the regulation of blood pressure are conflicting [32,33], possibly due to the type of protein, whether it originates from plants or animals, or due to a specific amino acid composition. In the context of the amino acid composition, sulfur-containing amino acids like cysteine or methionine may raise blood pressure, while amino acids involved in gluconeogenesis may have lowering effects on blood pressure [34]. In the current study, a mixed-protein diet was used without any focus on the origin of protein or on a particular amino acid composition, which may explain the lack of effect on BP.

In contrast, HR was higher with a higher dietary protein content. This may be an explanation for the protein-induced effects on energy balance as discussed previously [27]. A positive association between HR and energy expenditure has been demonstrated and employed to develop a prediction model for energy expenditure in free living conditions based on heart rate [35].

The link between endocannabinoids and blood pressure is still unclear and clinical studies are limited. However, pharmacological blockade of the CB1 receptor reduced BP in humans [13,14], suggesting a possible relationship of the ECS with blood pressure. The fact that the relationship could not be confirmed in the present study may be explained by the different study set up, as previous studies focused on the deactivation

of the receptor instead of investigating the relationship between BP and plasma endocannabinoid concentrations.

In our study, we observed a positive association between the endocannabinoid-related compound OEA with HR. A possible connection of cannabis use and HR was previously found, in which tetrahydrocannabinol (THC), an active compound of cannabis, increased HR without concomitant changes in BP [36]. As THC interacts to CB1, in contrast to OEA which acts CB1-independent, we suggest that effects on heart rate could also originate from a CB1 receptor independent mechanism.

Next to BP, metabolic risk markers were measured. No statistically significant differences in total cholesterol, LDL cholesterol, and HDL cholesterol concentrations were observed between the two protein intervention groups, possibly due to the short duration of the respiration chamber experiment. In addition, the total cholesterol/HDL cholesterol ratio did not differ. So far, the literature is inconclusive with regard to lipoprotein metabolism and protein intake. While beneficial effects on serum lipoprotein concentrations, independent of the amount of dietary fat intake, were only present in healthy, young individuals with a high-protein diet [37], studies in overweight and obese participants did not show a clear effect of a mixed-protein diet on serum lipoprotein concentrations [31,38,39]. In contrast, a meta-analysis investigating the effects of soy protein on lipoprotein concentrations indicated a reduction in LDL cholesterol [40,41], supporting the idea that the type of protein may be of great importance.

The ECS has been discussed to play a role in lipid metabolism [42], dyslipidemia, and lipogenesis [43]. In the current study, total cholesterol and LDL cholesterol concentrations were associated with OEA and PEA, independent of protein intake, suggesting a possible regulatory role for OEA and PEA in lipidemia. Meanwhile, 2-AG, AEA, and PREG were not related to any of the circulating risk markers.

Generally, OEA concentrations in saliva are higher in obese individuals compared to lean individuals [44] and associated with visceral fat content [45]. Animal data indicated a cholesterol lowering effect of OEA treatment [46]. While the animal data are not in agreement with the outcome of the current study, the ECS in animals may not necessarily be representative of the human ECS. PEA, in contrast, has been positively associated with total cholesterol and LDL cholesterol concentrations [47]. Interestingly, associations of PEA with HDL cholesterol were also positive in the current study whilst inverse associations were demonstrated previously [14]. The literature showed a positive association of 2-AG with TAG and a negative association with HDL cholesterol [9], while no associations were found in the current study.

No effects of a diet with a higher protein content were observed on vascular function which is in line with previous literature [48]. With regard to vascular function, most of the studies focused on the effect of specific proteins like whey or dairy products instead of a whole-protein approach. Whey protein intake was shown to improve FMD [49], whereas there was no effect of dairy intake on FMD and other markers for endothelial function [50,51], and no effect was reported by a mixed-protein approach [48]. Arterial stiffness improved after soy [52] and whey interventions [53]. However, due to the fact that this substudy was powered on energy expenditure, we cannot exclude power issues here as well. Literature regarding the relationship of vascular function and endocannabinoids is vague and barely investigated in clinical trials. In the current study, vascular functional markers did not relate to any of the endocannabinoid measurements. Similarly, OEA and PEA were not related to endothelial function in previous studies [54]. However, a connection between the ECS and vascular function cannot be ruled out completely as PEA was proposed to be a potential biomarker predicting coronary dysfunction in morbidly obese patients [54].

A limitation of the current study is the investigation of a diet with a higher protein/carbohydrate ratio compared to a diet with a lower protein/carbohydrate ratio, which omits the possibility of a clear separation between effects of an isolated higher protein concentration, a lower carbohydrate concentration, or a combination of both. In addition, the duration of the substudy was very short which makes a comparison with longer-term interventions difficult and we cannot rule out possible effects of the weight-loss intervention the participants followed before this substudy. Due to the complexity of protein quality and other factors of influence, follow-up studies are needed to confirm the relevance for 'normal life' conditions and to show whether the results can be extrapolated to overweight participants in general. The ECS is an extensive system, including different receptors, receptor variants, and ligands [11], that is associated with several anthropometric factors such as age and BMI [44,55], which further complicate interpretation of possible effects.

CONCLUSIONS

In conclusion, our data suggest that a diet with a higher dietary protein content did not affect cardiometabolic health and vascular function markers in overweight participants. Our data also indicate a possible relation between OEA and PEA and serum lipoprotein concentrations, independent of protein intake. Further research is needed to clarify the

link between endocannabinoids, their related compounds, and cardiometabolic health from a more mechanistic perspective.

AUTHOR CONTRIBUTIONS

Conceptualization, B.G.-C., M.S.W.-P., and T.C.A.; methodology, L.T., M.D., B.G.-C., M.S.W.-P., and T.C.A.; investigation, L.T., M.D., and B.G.-C.; data curation, L.T., M.D., and I.M.; formal analysis, L.T., M.D., P.J.J., and T.C.A.; project administration, L.T. and M.D.; writing—original draft preparation, L.T.; writing—review and editing, M.D., P.J.J., B.G.-C., A.R., M.F., I.M., D.C., R.P.M., M.S.W.-P., and T.C.A.; visualization, L.T.; supervision, P.J.J., R.P.M., M.S.W.-P., and T.C.A.; funding acquisition, T.C.A.; A.R., and M.S.W.-P. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

SUPPLEMENTAL MATERIAL

Table S4.1: Subject characteristics of the moderate- and high-protein group prior to the respiration chamber stay

	Moderate protein	High protein
N (f/m)	18 (9/9)	20 (13/7)
Age (year)	65.1 ± 5.8	64.0 ± 6.2
BMI (kg/m²)	29.0 ± 3.8	28.9 ± 4.0
Body-fat (%)	39.5 ± 8.1	40.7 ± 7.7
Fat mass (kg)	33.9 ± 7.1	34.8 ± 8.8
Fat-free mass (kg)	52.5 ± 10.9	50.8 ± 11.3

Values are means ± SD. BMI = Body Mass Index.

Table S4.2: Office blood pressure at pre- and post-respiration chamber time points with a moderate- or high-protein diet

	Moderate protein (*n* = 18)		High protein (*n* = 20)		Treatment effect
	Pre[1]	Post[1]	Pre[1]	Post[1]	Difference in change[2]
Brachial SBP (mmHg)	126±10	127±10	130±11	127±12	-2 [-8; 3]
Brachial DBP (mmHg)	77±7	77±6	78±6	76±7	-2 [-5; 2]
Brachial MAP (mmHg)	93±7	93±6	95±7	93±8	-2 [-5; 2]
Brachial PP (mmHg)	49±7	50±10	52±10	51±9	-1 [-5; 4]
HR (bpm)	62±9	57±6	65±7	62±6	3 [0; 6]

[1]Values are means ± SDs. [2] treatment effects [95% CI] were obtained from a one-factor ANCOVA with baseline value as covariate. BP = blood pressure; SBP = systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate; bpm.: beats per minute.

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CHAPTER 5

Longer-term soy nut consumption improves vascular function and cardiometabolic risk markers in older adults: Results of a randomized, controlled cross-over trial

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To be submitted

ABSTRACT

Background: Soy foods may contribute to the beneficial effects of plant-based diets on cardiovascular disease (CVD) risk. However, their effects on vascular function have hardly been investigated, while intervention studies on the more conventional cardiometabolic risk markers are inconclusive.

Objective: The objective of this study was to investigate if longer-term soy nut consumption improves vascular function and cardiometabolic health in older adults.

Design: Twenty-three apparently healthy participants (age between 60-70 years; BMI between 20-30 kg/m^2) participated in a randomized, controlled, single-blinded cross-over trial with an intervention (67 g of soy nuts daily providing 25.5 g soy protein) and control period (no nuts) of 16 weeks, separated by eight weeks wash-out. Volunteers followed the Dutch food-based dietary guidelines. At baseline and at 8 and 16 weeks anthropometric measurements and fasting blood samples were collected. Markers of vascular function (i.e., endothelial function, arterial stiffness, and microvascular structure) were assessed at week 16.

Results: No serious adverse events were reported and the soy nut regime was well tolerated. Body weight remained stable during the study. Serum isoflavone concentrations, which are a marker of compliance, increased after the intervention as compared with the control period (daidzein: 128.3 ng/mL; 95% CI: 72.6 to 183.9 ng/mL; p<0.001 and genistein: 439.8 ng/mL 95% CI: 246.7 to 632.9 ng/mL; p<0.001). The brachial artery flow-mediated vasodilation (FMD) response increased by 1.49 pp (95% CI: 0.03 to 2.95 pp; p=0.046) following the soy intervention, but no effect was found on the carotid artery reactivity (CAR) response. Arterial stiffness, assessed by carotid-to-femoral pulse wave velocity ($PWV_{c\text{-}f}$), was unchanged. Retinal arteriolar calibers (CRAE), a measure for microvascular structure, tended to improve by 1.42 µm (95% CI: -0.05 to 2.90 µm; p=0.059). Long-term soy nut consumption also lowered serum LDL-cholesterol concentrations by 0.17 mmol/L (95% CI: 0.02 to 0.32 mmol/L; p=0.027). HDL-cholesterol and triacylglycerol (TAG) did not change. Finally, the mean arterial pressure (MAP) decreased by 3 mmHg (95% CI: 1 to 6 mmHg; p=0.005), while office SBP and DBP decreased by 4 mmHg (95% CI: 0 to 8 mmHg; p=0.034) and 2 mmHg (95% CI: 1 to 4 mmHg; p=0.005), respectively.

Conclusions: A longer-term daily intake of soy nuts improved endothelial function, office blood pressure, and serum LDL-cholesterol concentrations, suggesting mechanisms by which an increased soy food intake beneficially affects CVD risk in older adults.

INTRODUCTION

Meta-analyses of randomized controlled trials (RCTs) have clearly shown favorable effects of plant-based diets on cardiovascular risk markers [1,2]. These diets are rich in many different nutrients, such as unsaturated fatty acids (UFAs), fibers, phytochemicals, antioxidants, and proteins [3].

Plant-based diets can be rich in soy [4,5], which provides high-quality proteins and high amounts of polyunsaturated fatty acids (PUFAs). Soy is also one of the most important sources of phytoestrogens, mainly the isoflavones daidzein (50%) and genistein (40%) [5], that may have beneficial health effects [4,5]. Finally, a meta-analysis of observational studies has indicated that soy consumption is negatively associated with cardiovascular disease (CVD) risk [6]. However, results from RCTs of soy intake on cardiometabolic risk markers are inconclusive [7-10], while long-term intervention studies with CVD events as endpoints are missing. As an alternative approach, effects on vascular function can be examined using non-invasive markers that predict the long-term risk to develop CVD [11]. For this, various techniques are available. Brachial artery flow-mediated vasodilation (FMD) in response to reactive hyperemia is a non-invasive technique to assess vascular endothelial function, while carotid artery reactivity (CAR) is a novel tool to evaluate endothelial function that depends on the effects of a cold pressor test (CPT) on the sympathetic nervous system [12,13]. Carotid-to-femoral pulse wave velocity ($PWV_{c\text{-}f}$) measures regional arterial stiffness and fundus photography is used to investigate effects on retinal microvascular structure [14].

RCTs of whole soy intake on vascular function are very scarce. Also, these trials [9,15-19] only used a limited set of markers. Therefore, the current randomized, controlled, cross-over trial aimed to investigate longer-term effects of whole soy nut consumption on different markers for vascular function and cardiometabolic risk in healthy older men and women.

MATERIALS AND METHODS

This study was approved by the Medical Ethics Committee of the University Hospital Maastricht (MUMC+) and Maastricht University (METC azM/UM), registered on clinicaltrials.gov on August 13th, 2018 as NCT03627637, and performed according to the principles of the declaration of Helsinki. All participants gave written informed consent before entering the study. The trial was performed between August 2018 and December 2019 in Maastricht, the Netherlands.

Study participants

Twenty-five healthy men and women were recruited through advertisements in local newspapers, flyers in the university, the hospital, and public buildings in Maastricht, and among people who had participated in earlier studies. Adults were invited for a screening visit when they were aged between 60 and 70 years and had a BMI between 20 and 30 kg/m^2. During a screening visit, anthropometrics and BP were measured, and fasting blood samples were drawn. Important inclusion criteria were: stable body weight (<3 kg body weight gain or loss in the past three months), an office BP below 160/100 mmHg, serum triacylglycerol (TAG) concentrations below 4.5 mmol/L, serum total cholesterol (TC) concentrations below 8.0 mmol/L, and plasma glucose concentrations below 7.0 mmol/L. Volunteers were not allowed to participate when having an allergy or intolerance to soy, when they were smoking, abusing alcohol or drugs, using medication known to affect BP, serum lipid or plasma glucose metabolism, or using an investigational study product from another trial during the past month. In addition, subjects suffering from severe medical conditions, including CVD (e.g., congestive heart failure or any CVD event in the past), diabetes, familial hypercholesterolemia, epilepsy, asthma, kidney failure, chronic obstructive pulmonary disease, inflammatory bowel diseases, auto-inflammatory diseases, and rheumatoid arthritis were not allowed to participate.

Study design and intervention

A randomized, controlled, single-blind, cross-over trial was performed comprising an intervention and control period (no nuts) of 16 weeks, separated by a wash-out period of 8 weeks (range: 6 to 12 weeks). During the 16-week intervention period, study participants consumed daily 67 g of unsalted soy nuts (Knusperkerne; Hensel, SALUS Haus, Bruckmühl, Germany). Information on the nutritional composition of the product is presented in **Supplemental Table S5.1**. The daily portion of soy nuts provided about

25.5 g soy protein, which was based on a former FDA claim when the study was initiated in 2018 of 25-30 g protein as a daily intake [20], and 174 mg of isoflavones. During the whole study, participants were requested to adhere to the Dutch food-based dietary guidelines. Therefore, intervention effects were evaluated as part of a recommended diet. Dietary advice was provided by a research assistant at baseline and throughout the study. Study volunteers were not allowed to use other soy products or dietary supplements known to interfere with the study outcomes. Participants were requested to consume the study product throughout the day.

Intervention and control periods included four visits: baseline (BL), midterm (MT; after 8 weeks), and two follow-up (FU) test days after 16 weeks (i.e., FU-A and FU-B). At each visit, anthropometric measurements were performed and fasting blood samples were collected after an overnight fast of at least 12h. Participants were also asked to abstain from alcohol and heavy exercise 48h preceding the measurement days and came to the research facilities by car or public transport to standardize measurements as much as possible. After 16 weeks, vascular function measurements were performed at FU-A (Figure 5.1).

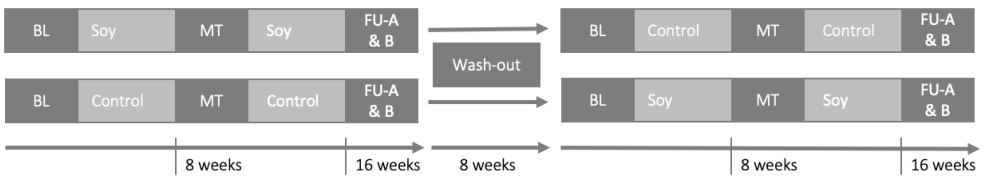

Figure 5.1: Study design. BL = baseline measurements; MT = midterm measurements; FU = follow-up measurements. BL and MT: anthropometrics and fasted blood samples; FU: anthropometrics, fasted blood samples, vascular function, blood pressure.

Between the two FU days, ambulatory blood pressure (ABP) was measured for at least 24h on the non-dominant arm (Mobil-O-Graph® NG; APC Cardiovascular, Great Britain) as described before [21]. In short, BP was automatically measured in 15 min intervals from 07:00 till 22:30 and in 30 min intervals between 22:30 and 07:00. No heavy exercise was allowed and the first measurement was discarded from the analyses. Twenty-four-hour, day- and nighttime means were calculated for systolic and diastolic BP (SBP and DBP), heart rate (HR), mean arterial pressure (MAP), and pulse pressure (PP). In addition, the variabilities (SDs) of these parameters and nocturnal BP dipping for SBP and DBP were calculated [21,22].

A validated food frequency questionnaire (FFQ) was completed after the intervention and control periods to assess energy and nutrient intakes over the past four weeks. Energy and nutrient intakes were calculated using the Dutch food composition tables (NEVO tables) [23]. Finally, participants were requested to record in diaries any protocol deviations or abnormalities regarding their health status, medication use, and alcohol intake during the whole study period.

Blood sampling and analyses

Fasting blood samples were drawn from the antecubital vein by venipuncture (BL, MT, and FU-A) or from an intravenous catheter (FU-B). Blood in serum separator tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA) was allowed to clot for at least 60 min at room temperature before samples were centrifuged (15 min at 1500g at room temperature), distributed in aliquots, snap frozen, and stored at -80°C until analysis at the end of the study. Serum was used for the analysis of C-reactive protein (CRP; immunoturbidimetric assay, Horiba ABX, Montpellier, France), TAG (Trinder; Sigma-Aldrich Corp., St. Louis, MO, USA), TC, and high-density lipoprotein (HDL)-cholesterol (CHOD-PAP method; Roche Diagnostics System, Mannheim, Germany), and isoflavone concentrations (i.e., daidzein, genistein, and equol; LC-MS/MS technique by LGC Limited, analyzed at LGC Limited, Fordham, UK) [24]. Serum concentrations of daidzein and genistein were used to measure compliance, while serum equol concentrations were determined to differentiate between equol-producers and non-producers [25]. Finally, serum low-density lipoprotein (LDL)-cholesterol concentrations were calculated using the Friedewald formula [26].

Clinical measurements

Body weight, hip- and waist circumference, and skinfold measurements for the fat percentage calculation, were measured at all visits. Height was only measured at baseline using a wall-mounted stadiometer. After an acclimatization period of at least 15 min in a supine position, vascular measurements were performed. BP levels (i.e., SBP and DBP) and HR were monitored using a semi-continuous BP monitoring device four times (Omron Intellisense M7; Cemex Medische Techniek, Nieuwegein, The Netherlands). The first measurement was first discarded and the average of the last three measurements was reported.

Measurements for arterial stiffness have been described before [27]. Briefly, the mean arterial pressure (MAP) was determined using the pulse wave at the brachial artery near the antecubital fossa with a tonometer (SphygmoCor v9, AtCor Medical, West Ryde,

Australia). Next, radial artery pulse wave analyses (PWA) were performed near the wrist of the arm using the same tonometer. The change between the first and the second systolic peak was expressed as the percentage of the pulse pressure, corrected for HR, and defined as central augmentation index (cAIxHR75). After that, $PWV_{c\text{-}f}$ was calculated by the manufacturer's program by dividing the timeframe of delay by 80% of the direct carotid-to-femoral distance [28]. For this, the program detected the delayed pulse wave arrival at the left carotid and femoral artery, which was compared to the R-wave of the electrocardiogram [28]. All arterial stiffness measurements were performed in triplicate.

Assessments of endothelial function were performed using ultrasound echography. For this, a 13-4 MHz linear transducer (MyLab^{TM}Gamma, Esaote, Maastricht, the Netherlands) in B mode was used as described before [27]. In short, the FMD measurement started with a 3-min reference period. Distal hypoxia was then induced by inflating a pneumatic cuff around the forearm to 200 mmHg for 5 min, which was followed by a 5-min post occlusive reactive hyperemia response. Brachial artery diameter throughout the FMD measurement was assessed automatically by a custom-written Matlab program (MyFMD; Prof. A.P. Hoeks, Department of Biomedical Engineering, MUMC+, Maastricht, the Netherlands). The FMD response was calculated as the maximal percentage diameter change post-hypoxia in relation to the baseline brachial diameter. CAR was assessed by recording the diameter of the left common carotid diameter close to the bulbous for a total period of 4 min. The first min was used as a baseline, followed by a CPT of 3 min. For the CPT, participants were instructed to hold their hand up to the wrist in a bucket of ice water (~4°C) without movement of the upper body and neck to enable a stable ultrasound recording of the carotid artery. For the analyses, the same program was used to assess the arterial diameter profile throughout the whole measurement, of which the CAR% response was calculated. For this, the carotid artery baseline diameter was defined as the average diameter over the first min. After immersion, the average diameter was averaged for intervals of 20 seconds. The maximal percentage change in post-immersion arterial diameter relative to the baseline arterial diameter was calculated [29].

Retinal microvascular calibers were obtained using a retinal camera (Topcon TRC-NW-300; Topcon Co.) [30]. The camera focused on the optic disc of the eye. Images were analyzed using the semi-automated interactive vessel analysis (IVAN, University of Wisconsin, Wisconsin, USA) software. Diameters of at least two, but preferably 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 [14]. The analyzed vascular segments had to be exactly the same for a particular participant at both measurements.

Statistical analyses

Randomization schedules consisting of blocks of two or four participants were generated for men and women separately using WinPepi Etcetera software. Study participants were randomly allocated to both intervention sequences within each block with a randomization ratio of 1:1. Statistical tests were performed using SPSS for Mac OS X (Version 25; SPSS Inc., Chicago, IL, USA). The study was powered on brain vascular function, while endothelial function, assessed by FMD, was another main study outcome.

Data were presented as means ± standard deviations (SDs) unless otherwise indicated. A P-value of below 0.05 was considered statistically significant using two-tailed tests. When measurements were only performed at FU, effects were examined using repeated measures analysis of variance (ANOVA) with period and sex as between-subject factors. For non-normally distributes variables, as assessed with the Kolmogorov-Smirnov test, variables are reported as the median changes. Differences in the effects between the intervention and control periods over time were examined using linear mixed modeling. Period, sex, time, intervention, and time*intervention interaction were used as fixed factors and baseline values as covariate. The interaction term was omitted from the model, if not statistically significant. When the interaction was not significant, differences in changes at weeks 8 and 16 between the intervention and control periods were compared pairwise with Least Significant Differences adjustments.

RESULTS

Study participants

A consort flow diagram of the study progress is shown in **Supplemental Figure S5.1**. Twenty-five older men and women were eligible to participate and started the study. Two women dropped out during the soy intervention. One woman due to personal reasons and one woman due to mild gastrointestinal discomfort. A total of 11 men and 12 women completed the study. Due to missing measurements or unclear images, we were unable to analyze retinal microvascular calibers and CAR of one participant and $PWV_{c\text{-}f}$ of three participants.

Study participants had a mean age of 64.1 ± 3.1 years (men: 64.0 ± 3.2 years and women: 64.2 ± 3.1 years) and their overall BMI was 25.9 ± 2.7 kg/m^2 (men: 26.8 ± 2.8 kg/m^{2}and women: 25.0 ± 2.3 kg/m^2). No serious adverse events or protocol deviations were reported in the individual diaries. Compliance based on measured isoflavone concentrations was excellent. Specifically, serum daidzein (128.3 ng/mL; 95% CI: 72.6 to 183.9 ng/mL; p<0.001) and genistein concentrations (439.8 ng/mL; 95% CI: 246.7 to 632.9 ng/mL; p<0.001) significantly increased after the intervention compared with the control period. Overall, six participants could be classified as equol producers [25] and their serum equol concentrations increased by 176.1 ng/mL (95% CI: 92.3 to 259.8 ng/mL; p=0.003; n=6) after the soy intervention **(Table 5.1)**.

Table 5.1: Serum isoflavone concentrations after 16 weeks of soy and control intervention

	Soy intervention	Control intervention	Intervention effect
Daidzein (ng/mL)	134.0 ± 114.0	2.5 ± 2.3	128.3 [72.6; 183.9]***
Genistein (ng/mL)	458.6 ± 416.3	5.1 ± 6.8	439.8 [246.7; 632.9]***
Equol (ng/mL)[1]	132.5 ± 105.1	1.5 ± 0.6	176.1 [92.3; 259.8]**

Values are means ± SDs; *n* = 23. Intervention effect (repeated-measures ANOVA with period and sex as between-subject factors): **P < 0.01, ***P < 0.001. [1]Equol producers (*n* = 6).

As expected, FFQ data indicated a higher dietary protein (3.1 En%; 95% CI: 2.3 to 4.0 En%; p<0.001) and a lower carbohydrate intake (-1.9 En%; 95% CI: -3.3 to -0.5 En%; p=0.011) during the soy intervention **(Supplemental Table S5.2)**. Total fat intake tended to be lower with the soy intervention (p=0.091). However, a lower dietary intake of saturated (SFA; -1.4 En%; 95% CI: -2.1 to -0.6 En%; p=0.001) and *cis*-monounsaturated fatty acids (*cis*-MUFA; -1.5 EN%; 95% CI: -2.3 to -0.7 En%; p=0.001) was observed, while the consumption of *cis*-PUFAs (1.9 EN%; 95% CI: 1.3 to 2.5 En%; p<0.001) was higher during the soy intervention. Additionally, the intake of cholesterol was reduced by 4.2 mg/MJ (95% CI: 1.7 to 6.7 mg/MJ; p=0.002), while the intake of dietary fibers was higher (8.7 g/day; 95% CI: 7.2 to 10.2 g/day; p<0.001) following soy intake. Although total energy intake tended to be higher during the soy period (98 kcal/day; 95% CI: -16 to 212 kcal; p=0.087), body weight, BMI, and body fat percentages did not differ. The waist-to-hip ratio, however, decreased by 0.01 (95% CI: 0.00 to 0.01; p=0.013) **(Table 5.2)**.

Table 5.2: Anthropometrics in soy and control intervention throughout the intervention trial

	Soy intervention			Control intervention			Intervention effect
	Baseline	Midterm	Follow-up	Baseline	Midterm	Follow-up	
Weight (kg)	74.6 ± 10.4	74.5 ± 10.5	74.4 ± 10.5	74.4 ± 10.0	74.2 ± 10.1	74.0 ± 9.9	0.1 [-0.4; 0.6]
BMI (kg/m²)	25.5 ± 2.7	25.5 ± 2.8	25.4 ± 2.6	25.5 ± 2.5	25.5 ± 2.5	25.4 ± 2.5	0.0 [-0.2; 0.2]
Waist circumference (cm)	86.2 ± 7.8	86.8 ± 9.1	86.0 ± 8.5	85.7 ± 9.1	85.4 ± 9.1	86.4 ± 8.5	-0.5 [-1.5; 0.6]
Waist-to-hip ratio	0.84 ± 0.07	0.84 ± 0.08	0.83 ± 0.08	0.84 ± 0.07	0.84 ± 0.08	0.85 ± 0.08	-0.01 [-0.01; -0.00]*
Body fat (%)	29.3 ± 7.0	29.2 ± 6.9	29.3 ± 6.7	29.8 ± 6.9	29.2 ± 6.5	29.2 ± 6.7	0.2 [-0.14; 0.57]

Values are means ± SDs; *n*=23. BL = Baseline; MT = Midterm (8 weeks); FU = Follow-up (16 weeks); BMI = Body Mass Index. Intervention effect (mixed-model analysis with baseline value as covariate): *$P < 0.05$.

Vascular function markers

Vascular endothelial function significantly improved, as the FMD increased by 1.49 percentage points (pp; 95% CI: 0.03 to 2.95 pp; p=0.046) following the soy intervention. As shown in **Figure 5.2**, the FMD response increased from 3.1 ± 2.7 % in the control to 4.7 ± 3.9 % in the intervention period. Baseline brachial artery diameters did not differ between periods (-0.8 mm; 95% CI: -3.7 to 2.1 mm; p=0.58). The CAR response did not differ between interventions (0.03 pp; 95% CI: -0.20 to 0.26 pp; p=0.79) **(Figure 5.2)**.

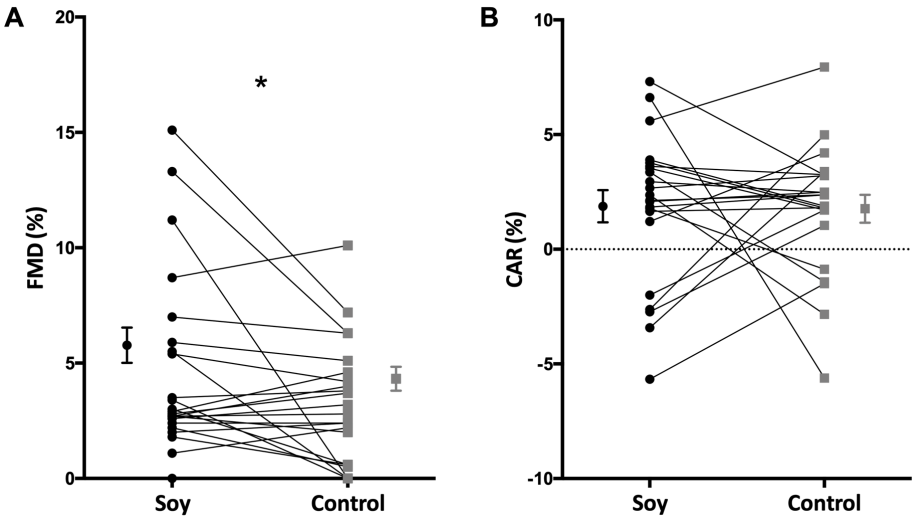

Figure 5.2: Mean (± SEM) and individual (**A**) brachial artery flow-mediated dilatation (FMD; *n* = 23) and (**B**) carotid artery reactivity (CAR) responses (*n* = 22) after 16 weeks of soy intervention and control period. Data were analyzed using repeated measures ANOVA with period and sex as between-subject factors. **P* < 0.05.

Arterial stiffness, as assessed by $PWV_{c\text{-}f}$, did not differ between intervention (7.8 ± 1.0 m/s) and control periods (8.0 ± 1.1 m/s), while the cAIxHR75 tended to increase by 1.5% (95% CI: -0.1 to 3.1 %; p=0.058) between interventions. Additionally, CRAE tended to improve by 1 µm (95% CI: 0 to 3 µm; p=0.059). The CRVE and AVR did not change **(Table 5.3)**.

Table 5.3: Vascular function measurements and blood pressure values after 16 weeks of soy and control intervention

	Soy intervention	Control intervention	Intervention effect
Vascular function			
Baseline brachial artery diameter (mm)	67.3 ± 4.7	68.1 ± 12.8	-0.8 [-3.7; 2.1]
$PWV_{c\text{-}f}$ (m/s)[2]	7.8 ± 1.0	8.1 ± 1.1	-0.3 [-0.8; 0.2]
cAIxHR75 (%)	25.2 ± 5.8	23.7 ± 8.4	1.5 [-0.1; 3.1]
CRAE (μm)[2]	113 ± 20	112 ± 20	1 [0; 3]^{T}
CRVE (μm)[2]	211 ± 21	211 ± 21	-1 [-1; 2]
AVR[1]	0.53 ± 0.08	0.53 ± 0.08	0.01 [0.00; 0.01]
Office blood pressure			
SBP (mmHg)	125 ± 9	128 ± 13	-4 [-8; 0]*
DBP (mmHg)	77 ± 7	79 ± 7	-2 [-4; -1]**
HR (bpm)	57 ± 9	57 ± 11	0 [-2; 3]
PP (mmHg)	48 ± 7	49 ± 9	-2 [-5; 1]
MAP (mmHg)	95 ± 7	98 ± 8	-4 [-6; -1]**
24h ambulatory blood pressure			
SBP (mmHg)	125 ± 10	125 ± 10	0 [-4; 3]
DBP (mmHg)	76 ± 8	76 ± 8	0 [-2; 2]
HR (bpm)	68 ± 7	68 ± 10	0 [-3; 2]
PP (mmHg)	49 ± 7	49 ± 8	0 [-3; 2]
MAP (mmHg)	98 ± 8	99 ± 8	0 [-3; 2]
SBP dipping (%)	13 ± 5	12 ± 5	0 [-2; 3]
DBP dipping (%)	15 ± 7	15 ± 6	1 [-2; 4]

Values are means ± SDs; *n* = 23. CRAE = central retinal artery equivalent; CRVE = central retinal vein equivalent; AVR = arteriolar-to-venular ratio; $PWV_{c\text{-}f}$ = carotid-to-femoral pulse wave velocity; cAIxHR75 = central aortic index corrected for heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate; PP = pulse pressure; MAP = mean arterial pressure. Intervention effect (repeated-measures ANOVA with period and sex as between-subject factors): $^{T}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$. [1]Data missing for one participant. [2]Data missing for three participants.

Cardiometabolic risk markers

As shown in **Table 5.3**, office SBP and DBP were significantly reduced by 4 mmHg (95% CI: 0 to 8 mmHg; p=0.034) and 2 mmHg (95% CI: 1 to 4 mmHg; p=0.005), respectively, while MAP decreased by 4 mmHg (95% CI: 1 to 6 mmHg; p=0.005). HR and PP did not change. In contrast, no effects were found on mean 24h **(Table 5.3)**, daytime, and nighttime ABP **(Supplemental Table S5.3)**. Only the daily DBP SD showed a higher

variability with the soy intervention (1 mmHg; 95% CI: 0 to 2 mmHg; p=0.012). The SDs of the other outcome parameters and nocturnal changes for SBP and DBP were not affected by the intervention.

Soy nut consumption lowered serum TC and LDL-cholesterol concentrations by 0.17 mmol/L (95% CI: 0.00 to 0.33 mmol/L; p=0.048) and 0.17 mmol/L (95% CI: 0.02 to 0.32 mmol/L; p=0.027), respectively **(Supplemental Table S5.4)**. Serum HDL-cholesterol and TAG concentrations **(Figure 5.3)**, as well as the TC-to-HDL-cholesterol ratio (-0.03 [95% CI: -0.14 to 0.09]; p=0.65) did not change. Finally, changes in median CRP concentrations between the intervention and control period (Δ8 weeks: -0.10 mg/L [IQR: -0.92 to 0.26 mg/L] vs. -0.02 mg/L [IQR: -0.26 to 0.18 mg/L] and Δ16 weeks: -0.04 mg/L [IQR: -0.75 to 0.22 mg/L] vs. 0.09 mg/L [IQR: -0.30 to 0.86 mg/L]) did not differ **(Supplemental Table S5.4)**.

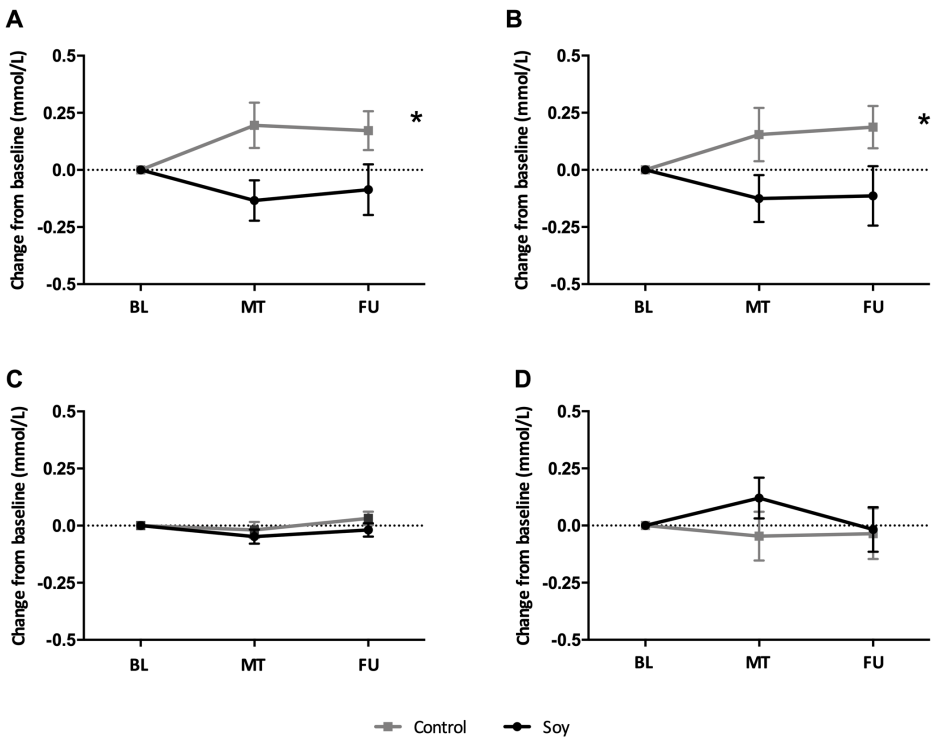

Figure 5.3: Mean changes (± SEM) in serum (**A**) LDL-cholesterol, (**B**) total cholesterol (TC), (**C**) HDL-cholesterol, and (**D**) triacylglycerol (TAG) concentrations at baseline, after 8 and after 16 weeks of soy or control intervention (*n* = 23). Data were analyzed using linear mixed model analysis with baseline value as a covariate. **P* < 0.05.

DISCUSSION

In this randomized, controlled, cross-over trial, longer-term soy nut consumption significantly improved endothelial function, office BP, and serum LDL-cholesterol concentrations, suggesting potential pathways by which an increased soy nut intake beneficially affects CVD risk in older adults. Overall, no serious adverse events were reported and the soy nut regime was well tolerated, which is in line with previous trials investigating the health effects of the consumption of whole soy nuts [7,9,10,17].

FMD significantly improved following soy intake by 1.49 pp, which is associated with a 12% decrease in CV event risk [31]. Soy nuts however did not have beneficial effects on the RHI in one study in adults at cardiometabolic risk [17]. The RHI reflects small artery reactivity, while the flow-mediated response evaluates the endothelial function of a large peripheral muscular artery that is primarily mediated by NO [32]. A possible explanation for these discrepant findings is that RHI responses are only partly mediated by the endothelial cell layer and therefore only partly depend on NO [33]. In contrast to FMD measurements, the utility and predictive value of the RHI in terms of future CVD risk are less established [34]. To the best of our knowledge, the effects of soy nuts on FMD have never been investigated before. However, a limited number of studies addressed the effect of other soy products (i.e., soy flour or cereals) on FMD, but results are contradictory. In prehypertensive or hypertensive patients [16,18], no effects were observed following a soy flour intervention, while FMD significantly improved after soy flour and soybean consumption in trials involving hypertensive, hypercholesterolemic, and renal transplant patients [15,19]. These inconsistent results may depend on differences in study duration or on the type of the consumed soy products. Besides, the focus in these trials was on specific study populations involving hypercholesterolemic patients [19], hypertensive patients [18], prehypertensive post-menopausal women [16] or renal transplant patients [15], which makes it difficult to translate these study findings to other population groups. The presence of soy components, including soy isoflavones and protein - an important source of the amino acid L-arginine - should be considered when interpreting results on FMD. A meta-analysis [35] found an intervention effect on FMD for isolated isoflavones of 1.98 pp and of 0.72 pp for soy protein [35]. Mean daily intakes of isoflavones and soy protein in these studies were between 33 to 120 mg and between 25 to 40 g, respectively. In our study, protein intake was similar (i.e., 25.5 g), but the intake of isoflavones (i.e., 175 mg) was higher. This suggests that isolated isoflavones and isoflavones from dietary compounds may behave differently, which needs, however, further be addressed in future studies. Isoflavones may activate endothelial nitric oxide synthase (eNOS) after estrogen receptor □ binding, leading to an

increased NO bioavailability [36]. Additionally, the effect on FMD can be explained by the high L-arginine content in soy protein [15], which can be converted into NO via the L-arginine-NO synthase pathway after ingestion [37]. Finally, although overall fat intake did not significantly differ, a lower intake of SFAs and *cis*-MUFAs and higher consumption of *cis*-PUFAs was observed during the soy intervention. FMD has been impaired by a diet high in SFAs [38], while beneficial effects on FMD were observed after the intake of *cis*-PUFAs [39]. In contrast, no effect was found on the CAR response. However, the CPT induces the release of catecholamines that induce not only an endothelium-mediated vasodilation in healthy persons but can also cause a catecholamine-mediated smooth muscle reaction leading to vasoconstriction [13,40]. Thus, these inconsistent results on endothelial function can be explained by the different underlying mechanisms that mediate the CAR- and FMD-responses [12,41,42].

Arterial stiffness, as measured by $PWV_{c\text{-}f}$, was not affected by the soy nut intervention. Effects of soy nut consumption on $PWV_{c\text{-}f}$ have never been investigated before. Teede and colleagues did also not observe effects of soy cereal on central PWV responses in hypertensive participants [18]. A meta-analysis of eight RCTs assessing the effects of isolated soy isoflavones on a combination of different markers of arterial stiffness observed a small but significant improvement in arterial stiffness [43]. These results, however, should be interpreted with caution because a pooled estimate was calculated from different (in)direct methods to assess arterial stiffness, including the PWV, systemic arterial compliance, augmentation index (AIx), and the cardio-ankle vascular index. As changes in $PWV_{c\text{-}f}$ result from structural changes of large elastic arterial walls, our intervention period may have been too short to find any effects at all. This is supported by a previous intervention study performed by our research group showing an improved $PWV_{c\text{-}f}$ after six months of magnesium supplementation, but not after three months [21]. Finally, we observed a non-significant trend towards an increased cAIxHR75 and improved retinal arteriolar calibers (CRAE) following soy consumption, but no effects were observed on retinal venular calibers or the AVR. Effects of soy nuts or other soy products on retinal microvascular calibers have never been investigated before in RCTs.

We did observe beneficial effects on BP levels following the intervention. Mean arterial pressure, a marker reflecting BP levels in the microcirculation that has been related to CVD risk [44], significantly improved following soy intake. Office SBP and DBP also improved, which is in line with the results of two meta-analyses [45,46] investigating the effects of a similar amount of soy protein as provided in this study in normo- and hypertensive adults and postmenopausal women. The improvements in BP were hypothesized to be induced by the isoflavones present in soy protein [46]. BP-lowering

effects were only observed in hypertensive patients but not in normotensives after a daily isoflavone dose from 65 to 153 mg/day for one to 12 months [47], or effects were only shown on SBP but not on DBP with daily ingestion of 25 to 375 mg soy isoflavones for two to 24 weeks [48]. Surprisingly, ABP was not affected in our study. In agreement, the only study assessing ABP in the meta-analyses mentioned above [46] also did not observe any effect on ABP following a 24h continuous BP measurement [16]. Inconsistent findings between office BP and ABP have been reported before by our research group [29]. It was speculated that more pronounced effects could be expected during the fasting state in the morning when blood pressure levels typically peak [29]. Due to this diurnal rhythm [49], the timing of BP assessments may be important when interpreting BP results.

Serum total and LDL-cholesterol concentrations decreased. The observed reduction of LDL-cholesterol concentrations is related to an overall CVD risk reduction [50]. HDL-cholesterol and TAG concentrations however did not change. A meta-analysis of 43 RCTs in adults, involving a variety of soy products and soy protein supplements at a median dose of 25 g soy protein per day, observed comparable effects on serum total cholesterol (effect size: 0.17 mmol/L) and LDL-cholesterol concentrations (effect size: 0.12 mmol/L) [51]. These hypocholesterolemic effects involve not only primarily extrinsic (i.e., replacement of other food products by intake of soy nuts), but possibly also intrinsic mechanisms [52]. Although there was a tendency for increased energy intake of approximately 98 kcal/day during the soy intervention, suggesting that the participants replaced only about 60% of the extra energy from the intake of the nuts, body weight remained stable during the whole trial although the WHR slightly improved with the soy intervention. In line with the food replacement theory, a lower dietary intake of SFA and *cis*-MUFAs was observed in our study, while the consumption of *cis*-PUFAs was higher during the soy intervention. An exchange of dietary SFAs for *cis*-UFAs has been shown to affect serum LDL-cholesterol beneficially [53].

Overall, 26% of our study population (i.e., six participants) could be classified as equol producers, which is in line with the literature [25]. Equol has been suggested to have a higher bioactive function [54] consistent with studies indicating that beneficial health effects of soy nuts or protein on, for example, endothelial function [55] and BP [7] may differ between equol producers and non-producers. However, our sample size was too limited to study effects of equol production status on these health outcomes.

In conclusion, longer-term daily intake of soy significantly improved vascular function and cardiometabolic risk markers in older adults, which may contribute to the beneficial effects of plant-based diets on the risk of developing CVD.

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AUTHOR CONTRIBUTIONS

The authors' responsibilities were as follows – LT, RPM and PJJ: designed the trial; LT: conducted the trial; LT and PJJ: performed the statistical analysis; PJJ: had overall responsibility for the study; LT: wrote the first version of the manuscript; all authors: interpreted the results, read and approved the final manuscript.

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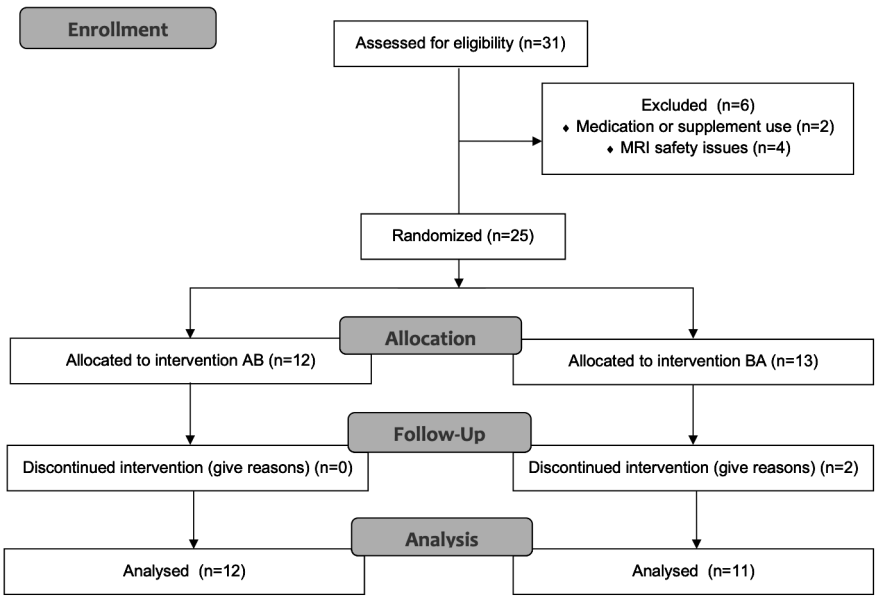
CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUPPLEMENTAL MATERIAL



Supplemental Figure S5.1: Consort flow diagram of enrollment and progress through the phases of this randomized cross-over trial with two treatment groups.

Supplemental Table S5.1: Nutrition facts of soy nuts (Knusperkerne; Hensel, SALUS Haus, Bruckmühl, Germany)

	Per 100 g	Per portion (67g)
Energy	1568 kJ/ 374 kcal	1050.6 kJ / 250.6 kcal
Protein	38 g	25.5 g
Total fat	17 g	11.4 g
Saturated fat	2.5 g	1.7 g
Monounsaturated fat	3.5 g	2.4 g
Polyunsaturated fat	11 g	7.4 g
Carbohydrates	17 g	11.4 g
Sugar	8 g	5.4 g
Dietary fiber	15 g	10.1 g
Sodium	0.02 g	0.01 g
Isoflavones	259 mg	174 mg

Supplemental Table S5.2: Average food intake over four weeks as assessed by food frequency questionnaires after 16 weeks of soy and control intervention

	Soy intervention	Control intervention	Intervention effect
Total energy intake (kcal/day)	2235 ± 361	2119 ± 440	98 [16; 212]^{T}
EN% protein	18.8 ± 1.4	15.7 ± 1.4	3.1 [2.3; 4.0]***
EN% carbohydrates	40.9 ± 5.0	42.0 ± 4.6	-1.9 [-3.3; -0.5]*
EN% fat	35.4 ± 4.9	36.5 ± 4.3	-1.2 [-2.6; 0.2]^{T}
EN% saturated fat	11.1 ± 2.0	12.4 ± 2.0	-1.4 [-2.1; -0.6]**
EN% monounsaturated fat	12.5 ± 2.5	14.0 ± 2.5	-1.5 [-2.3; -0.7]**
EN% polyunsaturated fat	8.6 ± 1.7	6.8 ± 1.9	1.9 [1.3; 2.5]***
Cholesterol (mg/MJ)	20 ± 6	24 ± 6	-4.2 [-6.7; -1.7]**
Fibers (g)	33.6 ± 5.6	24.8 ± 5.6	8.7 [7.2; 10.2]***

Values are means ± SD, *n* = 23. EN% = energy percent. Intervention effect (repeated measures ANOVA with period and sex as between-subject factors): $^{T}P < 0.1$; $*P < 0.5$; $**P < 0.01$; $***P < 0.001$.

Supplemental Table S5.3: Day and night ambulatory blood pressure values and variations (SDs) of 24h, day, and night blood pressure after 16 weeks of soy and control intervention

	Soy intervention	Control intervention	Intervention effect
Day SBP (mmHg)	129 ± 11	129 ± 10	-1 [-4; 3]
Day DBP (mmHg)	79 ± 9	79 ± 8	-2 [-2; 2]
Day HR (bpm)	71 ± 7	71 ± 11	0 [-4; 3]
Day PP (mmHg)	50 ± 8	50 ± 8	0 [-3; 3]
Day MAP (mmHg)	102 ± 9	102 ± 8	0 [-3; 2]
Night SBP (mmHg)	112 ± 9	113 ± 10	-1 [-4; 2]
Night DBP (mmHg)	67 ± 8	67 ± 7	-1 [-3; 2]
Night HR (bpm)	87 ± 8	59 ± 9	0 [-2; 2]
Night PP (mmHg)	46 ± 6	46 ± 7	-1 [-3; 2]
Night MAP (mmHg)	87 ± 8	88 ± 8	-1 [-3; 1]
SD 24h SBP (mmHg)	15 ± 3	14 ± 3	0 [-1; 2]
SD 24h DBP (mmHg)	11 ± 2	10 ± 2	1 [0; 2]^{T}
SD 24h HR (bpm)	10 ± 3	10 ± 3	-1 [-2; 0]
SD 24h PP (mmHg)	12 ± 4	12 ± 3	0 [-1; 2]
SD 24h MAP (mmHg)	11 ± 2	11 ± 2	0 [-1; 1]
SD day SBP (mmHg)	13 ± 3	13 ± 3	0 [-1; 2]
SD day DBP (mmHg)	9 ± 2	8 ± 1	1 [0; 2]*
SD day HR (bpm)	9 ± 3	10 ± 3	-1 [-2; 0]
SD day PP (mmHg)	13 ± 5	13 ± 3	0 [-2; 2]
SD day MAP (mmHg)	9 ± 2	8 ± 2	0 [0; 2]
SD night SBP (mmHg)	9 ± 3	10 ± 3	-1 [-2; 1]
SD night DBP (mmHg)	8 ± 2	7 ± 2	0 [-1; 1]
SD night HR (bpm)	5 ± 2	5 ± 2	0 [-1; 1]
SD night PP (mmHg)	7 ± 3	7 ± 3	0 [-2; 2]
SD night MAP (mmHg)	8 ± 2	8 ± 2	0 [-1; 1]

Values are means ± SD, n = 23. SBP = systolic blood pressure; mmHg = millimeter of mercury; DBP = diastolic blood pressure; HR = heart rate; bpm = beats per minute; PP = pulse pressure; MAP = mean arterial pressure; SD = standard deviation. Intervention effect (repeated measures ANOVA with period and sex as between-subject factors): $^{T}P < 0.1$, $^{*}P < 0.05$.

Supplemental Table S5.4: Fasting metabolic risk markers in soy and control intervention throughout the intervention trial

	Soy intervention			Control intervention			Intervention effect
	Baseline	Midterm	Follow-up	Baseline	Midterm	Follow-up	
TC (mmol/L)	5.91 ± 1.13	5.77 ± 0.96	5.75 ± 1.11	5.71 ± 0.94	5.87 ± 1.11	5.90 ± 1.07	-0.17 [-0.33; 0.00]*
HDL-cholesterol (mmol/L)	1.61 ± 0.46	1.54 ± 0.48	1.56 ± 0.48	1.60 ± 0.47	1.58 ± 0.50	1.63 ± 0.49	-0.03 [-0.07; 0.02]
LDL-cholesterol (mmol/L)	3.62 ± 1.13	3.48 ± 1.03	3.52 ± 1.14	3.47 ± 1.07	3.66 ± 1.13	3.64 ± 1.11	-0.17 [-0.32; -0.02]*
TAG (mmol/L)	1.13 ± 0.66	1.27 ± 0.76	1.12 ± 0.54	1.06 ± 0.64	1.01 ± 0.36	1.02 ± 0.39	0.03 [-0.08; 0.13]
TC:HDL-Cholesterol ratio	3.90 ± 1.19	4.01 ± 1.23	3.89 ± 1.00	3.78 ± 1.17	3.94 ± 1.06	3.85 ± 1.10	-0.03 [-0.14; 0.09]
CRP (mg/L)	1.01 [0.60-1.82]	0.83 [0.5-1.73]	1.27 [0.43-2.06]	1.04 [0.48-1.49]	0.85 [0.39-1.44]	1.10 [0.45-1.87]	Δ8 weeks: -0.10 vs. 0.02 Δ16 weeks: -0.04 vs. 0.22

Values are means ± SDs or medians (25-75th percentile); *n* = 23. BL = Baseline; MT = Midterm (8 weeks); FU = Follow-up (16 weeks); TC = total cholesterol; HDL = high density lipoprotein; LDL = low density lipoprotein; TAG = triacylglycerol; TC:HDL-cholesterol ratio = total cholesterol–to–HDL-cholesterol ratio; CRP = C-reactive protein. Intervention effect (mixed-model analysis with baseline value as covariate): *P < 0.05.

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CHAPTER 6

General discussion

Introduction

Type II Diabetes (T2DM) and cardiovascular disease (CVD) are largely influenced by body weight, with increased body weight being a major predicting risk factor for these age-related non-communicable diseases in humans [1,2]. Body weight is influenced by dietary intake. When energy intake exceeds energy expenditure, weight gain occurs, while an energy intake lower than the energy needs would lead to weight loss. While there are several ways to lose weight, successful weight maintenance after weight loss is challenging, as different biological responses counteracting the loss of further body weight are activated. Energy expenditure will adapt to the new body weight and a negative feedback loop regulating weight management will be stimulated, which then triggers energy intake by increasing appetite [3]. However, not only the quantity of food intake plays a crucial role, but also the composition of the diet and the quality of products is essential and should be considered. Adjusting the macronutrient composition by increasing dietary protein intake and/ or decreasing fat and carbo-hydrate content, body weight management may be supported by an increase in resting energy metabolism, diet-induced thermogenesis, and greater satiating effects of dietary protein compared with the other macronutrients [3-5]. The relevance of dietary proteins is not only limited to their beneficial effects on energy metabolism and energy intake regulation in adults but also effects of dietary proteins on metabolic health and vascular function are of great interest.

Main results – this thesis

In this thesis the effects of a high-protein diet and soy nuts on several markers of metabolic health and vascular function, which are well-known risk markers for CVD development, have been investigated. For this, two randomized controlled trials (RCTs) have been performed. The presented studies differed in their approach. One study used a mixed-protein diet with either a high- or moderate protein content in pre-diabetic participants after completing a weight-loss intervention (**Chapters 2** to **4**) and the other study investigated the longer-term effects of soy nut consumption in older participants (**Chapter 5**). The main results of these RCTs are presented in **Table 6.1**.

Table 6.1: Overview of the main findings and clinical relevance of two intervention studies presented in this thesis, investigating the effects of a high-protein diet after a weight-loss intervention and a longer-term soy nut intervention on several markers of metabolic and cardiovascular health

Chapter	Study design	Intervention and duration	Main results	Conclusions
2	***PREVIEW substudy*** Randomized controlled parallel trial, 38 overweight or obese adults, participants of the PREVIEW trial	MP: 15/55/30 En% protein/carbohydrate/fat HP: 25/45/30 En% protein/carbohydrate/fat Meals provided in energy balance 48h in the respiration chamber, at approximately 34 months after weight loss	**↓ hunger perception** **↑ 2-AG, meal-related pattern of 2-AG** **↓ TAG** = GLP-1 and PYY, glucose, insulin, β-hydroxybutyrate Hunger inversely associated with PYY in HP	**HP/MP has appetite-regulating effects: reduced hunger perception** 2-AG concentrations appear to be contributor and PYY may be one of the mediators
3			**↓ AEA, OEA, PEA, PREG during the day** Decrease inversely related to BMI (AEA) and body fat (%) (PEA, OEA) = AEA, OEA, PEA, PREG	**AEA, OEA, PEA reflected general gradual energy intake matching EE during the day** **2-AG concentrations changed in relation to protein level** Decrease inversely related to BMI and body-fat % suggesting a stronger energy balance regulation in those with a lower adiposity
4			**= BP, lipoproteins, vascular function** ↑ HR OEA associated with HR OEA and PEA associated with TC and LDL-c	**No effect of HP/MP on cardiometabolic health and vascular function markers in overweight participants** Indication for a possible relation between OEA and PEA and serum lipoprotein concentrations
5	***Soy study*** Randomized cross-over trial, 23 adults, 60-70 years, BMI between 20-30 kg/m^2	67 g soy nuts daily on top of a healthy diet (Dutch nutritional guidelines, wheel of five) 16 weeks intervention, 8 weeks washout	**↑ FMD** **↓ MAP, SBP, and DBP** **↓ total- and LDL-c;** ↑ isoflavones = CAR, $PWV_{c\text{-}f}$, cAIxHR75 = TAG, HDL-c	**Longer-term soy nut intake improved endothelial function and serum lipoprotein concentrations.** Microvascular structure and blood pressure nearly improved. **→ Beneficial effect on CVD risk in older adults. The changes in FMD has been associated with a 12% risk reduction of CV events**

MP = moderate protein; HP = high protein; En% = percent of total energy intake; GLP-1 = glucagon-like peptide 1; PYY = polypeptide YY; 2-AG = 2-arachidonoylglycerol; AEA = anandamide; OEA = oleoylethanolamide; PEA = palmitoylethanolamide; PREG = pregnenolone; EE = energy expenditure; HR = heart rate; BP = blood pressure; TC = total cholesterol; FMD = flow-mediated dilatation; CAR = carotid artery reactivity; MAP = mean arterial pressure; SBP = systolic blood pressure; DBP = diastolic blood pressure; LDL-c = low-density lipoprotein cholesterol; TAG = triacylglycerol; $PWV_{c\text{-}f}$ = carotid-to-femoral pulse wave velocity, cAIxHR75 = central aortic index corrected for heart rate; CVD = cardiovascular disease.

Chapters 2 and 3 investigated the effects of a mixed high-protein diet on metabolic markers and energy intake regulation in overweight or obese adults. This PREVIEW substudy showed that a diet with a higher dietary protein/ carbohydrate ratio could modify appetite by reducing hunger perception with PYY as a possible mediator (Chapter 2). Anorexigenic hormones were not affected by the amount of protein intake. We also found that concentrations of the endocannabinoid 2-arachidonoylglycerol (2-AG) followed a meal-related pattern, with higher concentrations after high-protein intake (Chapter 2). The other endocannabinoids and endocannabinoid-related compounds reflected gradual energy intake, which matched energy expenditure throughout the day, and were not affected by the dietary protein content as described in Chapter 3. In this chapter, it was also found that energy balance was negative after consumption of an isocaloric high-protein diet. While the first two chapters mainly focused on metabolic regulation, Chapters 4 and 5 investigated the effects of two different dietary interventions, a mixed-protein dietary intervention in overweight or obese adults after a weight-loss intervention (Chapter 4) and a longer-term soy nut intervention in older adults (Chapter 5), on cardiometabolic health and vascular function. Chapter 4 indicated a possible association of endocannabinoid-related compounds with total and LDL-cholesterol. The high-protein diet did not affect cardiometabolic health or vascular function in Chapter 4. However, a longer-term daily intake of soy nuts improved endothelial function, office blood pressure, and serum LDL-cholesterol concentrations, suggesting mechanisms by which an increased soy food intake beneficially affects CVD risk (Chapter 5).

Relevance of protein diets

Dietary protein intake is not only a fuel source but also provides the building blocks for several human tissues and body functions such as synthesis of immune system components, peptide hormones, and plasma proteins [6]. Proteins consist of non-essential and essential amino acids, which cannot be synthesized by the body and need to be consumed [7]. According to the WHO dietary recommendation, approximately 10 to 15% of the consumed energy (En%) should be protein, which is equivalent to 0.83 g/kg/day [8]. However, not only the total amount of protein is of interest, but also the quality of the protein is critical. The protein quality can be specified by the digestible indispensable amino acid score (DIAAS), a score based on individual indispensable amino acid digestibility, taking into account the bodies' requirements [9]. Most animal proteins, such as egg, dairy, or meat protein, are considered complete proteins as they contain all essential amino acids and therefore have a high score, while plant-proteins often contain

lower amounts of essential amino acids and are therefore often considered to be of less quality with lower scores. However, this does not necessarily apply to all plant-based food items. Soy is one example containing not only a high plant-based protein content but also high-quality protein with a good biological value as it contains sufficient quantities of all essential amino acids [10,11]. Soy protein has a DIAAS score of approximately 0.9 and can therefore be considered as high-quality protein (DIAAS between 0.75 and 0.99) [11,12]. For comparison, one of the highest quality proteins, whey protein isolate, has a DIAAS score of 1.09, while wheat protein has a DIAAS of 0.45 [12,13].

A combination of different products has been suggested to fulfill daily amino acid needs with plant proteins [14]. **Table 6.2** shows the amino acid content of three common protein isolates, wheat and soy as plant-protein sources, and whey as an animal-protein source as a comparison. While wheat provides with approximately 32.2% the largest portion of the total plant-based protein intake of a typical Western diet, soy provides only 2.7% [14]. In the Asian diet, the soy content is higher. A daily soy consumption of approximately 55 g has been reported in Japan compared with less than 5 g in the United States [15].

While a mixed protein diet was used in the trial reported in **Chapters 2** to **4**, providing 25:45:30% of the total energy from protein:carbohydrates:fat in the intervention (HP) and 15:55:30% of the total energy from protein:carbohydrates:fat in the control group (MP), 67 g whole soy products were supplemented daily in **Chapter 5**. The nuts provided about 25.5 g soy protein, which was based on a former FDA claim of 25-30 g protein as a daily intake when the study was initiated in 2018 [16].

The protein quality is relatively high in most Western diets due to a versatile combination of different protein types originating from animal and plant sources [17]. However, as obesity and CVD prevalence's are relatively high in the Western World, whole food products, including all dietary components, need to be considered as they may also affect health. Meat, eggs, and products high in dairy protein, for example, contain large amounts of high-quality protein but may simultaneously contain high cholesterol or saturated fat concentrations, while products rich in plant-proteins, such as soy, contain low amounts of saturated fats and high amounts of *cis*-unsaturated fats and fibers [18]. The fat content of soy consists of approximately 46% to 62% *cis*-polyunsaturated fats, 19% to 41% *cis*-monounsaturated, and only approximately 10% to 15% saturated fats [11]. The exact dietary composition of the soy products may vary depending on production processes, but the soy nuts used in **Chapter 5** contained approximately 38 En% protein and 17 En% fat, of which 15 En% were saturated, 21 En% were *cis*-

monounsaturated, and 65 En% were *cis*-polyunsaturated fats. The soy nuts used contained approximately 174 mg of isoflavones and approximately 10 g of dietary fiber.

Table 6.2: Amino acid composition of wheat, soy, and whey protein isolates

	Wheat	Soy	Whey
Threonine	1.8	2.3	5.4
Methionine	0.7	0.3	1.8
Phenylalanine	3.7	3.2	2.5
Histidine	1.4	1.5	1.4
Lysine	1.1	3.4	7.1
Valine	2.3	2.2	3.5
Isoleucine	2.0	1.9	3.8
Leucine	5.0	5.0	8.6
Total essential amino acids	18.0	19.9	34.1
Serine	3.5	3.4	4.0
Glycine	2.4	2.7	1.5
Glutamic acid	26.9	12.4	15.5
Proline	8.8	3.3	4.8
Cysteine	0.7	0.2	0.8
Alanine	1.8	2.8	4.2
Tyrosine	2.4	2.2	2.4
Arginine	2.4	4.8	1.7
Total non-essential amino acids	48.9	31.9	34.9

Values are presented in g/ 100g protein isolate. Not measured: tryptophan, aspartic acid, asparagine, and glutamine. Adapted from: Gorissen et al., 2018 [14].

The protein quality of both dietary interventions described in this thesis can be considered as high. The protein content was approximately 15 En% from protein in both control conditions, while the protein intake was highly increased to 25 En% in the high-protein diet **(Chapters 2 to 4)** and moderately, but significantly increased to approximately 19 En% with the soy intervention **(Chapter 5)**. The amount of dietary fat provided in the soy study was about 35 En% compared with 30 En% in the high-protein intervention. Carbohydrate intakes were slightly lower in the soy study with 41 En% in the soy intervention and 45 En% in the high protein diet. In both trials, the additional protein intake displaced carbohydrate intake.

Protein diets and body weight

Dietary protein may support energy homeostasis and weight management via several different pathways, including appetite regulation and energy metabolism [19].

Firstly, appetite sensation has been shown to be influenced by a higher dietary protein consumption via several mechanisms, such as anorexic gut hormones and diet-induced thermogenesis [19]. Thereby, energy intake may be limited, especially in short-term studies [19-23] but may also last up to six months [24]. However, differences in hunger could still be assessed after three years of weight maintenance in our study, as presented in Chapter 2, suggesting that longer-term effects on appetite sensation are possible. Compared to other macronutrients, dietary protein has been reported to have higher satiating properties through multiple regulatory pathways such as gut hormone secretion, effects on digestion, changed amino acid concentrations, and a ketogenic state [22]. Also the type of protein has been discussed to affect the degree of appetite regulation [25]. While results are contradictory, whey has been suggested to have higher satiating effects than casein or soy protein [26,27]. The impact on appetite sensation is partly mediated via gut hormone secretion [19]. Previous trials suggested an increase in GLP-1 and PYY secretion after high-protein intake [25,28], which could not be confirmed in the trial in Chapter 2.

Secondly, a high-protein diet during weight loss or weight maintenance may affect energy metabolism due to a higher preservation of fat-free mass (FFM) and a higher thermic effect than other macronutrients, which plays an essential role for whole-body energy expenditure [19]. The proteins' amino acid composition determines the metabolic efficiency of the protein oxidation. Proteins with a lower biological value (e.g., most plant proteins) are associated with a lower thermogenic response and a lower protein synthesis compared with proteins with a higher biological value [19]. This notion supports the assumption that not all protein types are equally effective from a metabolic perspective and that animal-based protein may not only have higher thermic effects compared with carbohydrates but also when compared with plant-based proteins [29].

Effects of protein on energy metabolism were also found in the PREVIEW substudy described in this thesis. We found a negative energy balance and an increased resting energy expenditure induced by the high-protein diet [30], as shown in Chapters 2 and 3. In addition to the effects on energy metabolism, we found a reduction in hunger perception as presented in Chapter 2, suggesting multiple mechanisms by which consumption of a diet with a higher dietary protein content may support weight management.

When comparing mixed-protein diets with plant-based diets regarding their effects on weight, cross-sectional data showed a higher energy intake and a subsequently higher BMI in people with higher meat intake [31]. However, the PREVIEW study showed that a higher mixed dietary protein content (>0.8 g/kg body weight/day) improved body weight maintenance after weight loss when compared with a lower dietary protein intake (<0.8 g/kg body weight/day) [32]. Additionally, a low-fat plant-based diet without energy restrictions led to significant weight loss and weight maintenance after one year in overweight and obese participants in an RCT [33]. According to a recent meta-analysis of RCTs, also soy products improved weight loss, most likely due to a combination of their protein, isoflavone, and fiber contents [34]. Body weight was stable during the soy intervention described in **Chapter 5**. Remarkably, as assessed by food frequency questionnaires (FFQs), energy intake tended to be higher during the soy intervention with approximately 98 kcal per day as the soy nuts were used on top of a healthy diet. Only around 60% of the extra energy due to the soy nuts were compensated with other food products.

Protein diets and T2DM

Modifying the macronutrient content of the diet has been suggested to promote glycemic control in diabetic patients [35]. While low-carbohydrate diets, which are often rich in dietary protein, have been proposed as relevant for T2DM management [36], data on the direct effects of protein on T2DM risk have been inconsistent [19]. A meta-analysis of 13 RCTs investigating the effects of a diet with at least 25 En% protein in T2DM patients found no improvements in glycemic control, represented by fasting glucose concentrations and HbA1c, a marker representing long-term changes in plasma glucose concentrations [37]. In contrast, a meta-analysis of RCTs assessing the effects of low-carbohydrate diets in diabetic patients showed that HbA1c was improved. A beneficial contribution of the increased dietary protein was suggested, probably through increased satiety and support of weight management [36]. While short-term high-protein trials suggested beneficial effects on glucose homeostasis, effects in long-term trials are less clear [19]. If protein directly affects glycemic control or indirectly via body weight management, remains questionable [19].

However, amino acids such as leucine, phenylalanine, and arginine showed insulinotropic effects when applied individually [38,39], and de novo insulin production was increased after a carbohydrate-protein hydrolysate load, resulting in a lower plasma glucose concentration [40]. Those results suggest that specific protein components may have direct effects on glucose and insulin homeostasis.

When comparing T2DM incidence in different diet groups, the highest incidence of T2DM was found in omnivores, while the lowest was found in vegans [41]. In line, a meta-analysis of eleven cohort studies showed that total and animal protein enhanced the T2DM risk, with red and processed meat as T2DM risk factors, while soy and dairy products serve as protecting factors [42]. These differential effects of different protein types may account for the variety of effects found in clinical trials. Prospective studies suggested that participants following plant-based diets had a 46 – 74% lower prevalence of T2DM [43]. In the studies described in this thesis, no effect on glucose and insulin metabolism could be found by a high-protein diet (Chapter 2) or by the soy intervention *(data not shown)*. Independent of energy restriction, a qualitative change of the macronutrients, e.g., to a plant-based diet, improved fasting insulin sensitivity and beta-cell function in overweight participants [43]. Different mechanisms have been discussed to be responsible for improvements in glucose metabolism and insulin sensitivity by plant-based diets. Possible causes are reduced oxidative stress, inflammation, gluco-toxicity, and lipotoxicity, next to enhanced incretin secretion, which has been suggested to improve beta-cell function [43]. Additionally, a reduction of visceral fat has been associated with plant-based diets before [44], which may also contribute to improved glucose homeostasis regulation [45]. Improvements in glucose homeostasis may not only protect from T2DM development but may also help to prevent long-term complications of hyperglycemia, such as macrovascular and microvascular complications [46-48]

Protein diets and CVD

Protein diets have been associated with a reduced risk of CVD [18,19]. Obesity, a central feature of the metabolic syndrome, is mechanistically linked to various cardiometabolic risk markers. These risk markers are associated with vascular dysfunction, predicting longer-term atherosclerotic disease progression and cardiovascular event rate [49]. Weight loss may be one of the mechanisms by which high-protein diets may beneficially affect CVD risk parameters. Previous data indicate that diet-induced weight loss in abdominally obese men was associated with improvements in markers of metabolic health and vascular function [50,51]. In the context of weight management, high-protein diets have been suggested to have a prominent role [52,53].

The effects of mixed high-protein diets on CVD risk factors are still under discussion. Detrimental and beneficial effects have both been suggested, depending on the type of protein consumed [54]. While intake of higher amounts of high-fat dairy products and red meat has been suggested to increase coronary heart disease (CHD) risk in one study

[55], another large cohort study showed no negative associations of a diet with a low carbohydrate and a higher protein and fat content on the risk of CHD [56]. Both studies found a marked risk reduction when vegetable sources of protein and fat, fish or low-fat dairy products were chosen [55,56].

However, the number of large RCTs with appropriate follow-up periods and cardiovascular events as endpoints is limited. Alternatively, the effect of a high-protein diet or plant-based proteins (e.g., soy nuts) on surrogate risk markers of CVD can be studied, such as markers of cardiometabolic health and vascular function **(Chapters 2, 4,** and **5)**.

Cardiometabolic markers

Effects of protein on blood pressure are still under discussion. While a meta-analysis of RCTs found no effects of protein on blood pressure [37], systolic (SBP) and diastolic blood pressure (DBP) have been inversely associated with the amount of protein intake as reported by a meta-analysis of nine cross-sectional studies [57]. Especially increased amounts of plant-protein have been shown to improve blood pressure [58], leading again to the conclusion that not only the macronutrient composition of the diet may be of great importance, but also the type, source (animal or plant-based origin), and possibly also the processing of the products [59]. When comparing soy products to other protein sources, e.g., soy milk and cow's milk, soy milk showed superior hypotensive effects [60]. In line, soy nuts improved SBP, DBP, and mean arterial pressure **(Chapter 5)**, while no effect was found during the mixed high-protein diet **(Chapter 4).** While a previous trial showed improvements in BP mediated by diet-induced weight loss [61], blood pressure lowering effects of high-protein diets has only partly been mediated by changes in body weight [62].

A distinction between dependent and independent effects of protein and other dietary components is challenging. Compared to products originating from plants, animal-based products are often high in dietary cholesterol, one of the main risk markers for atherosclerosis development [63]. According to epidemiological studies, CVD mortality and morbidity have been reduced by consumption of vegetarian diets as indicated by a meta-analysis of prospective cohort studies [64] and to lower the mortality rate of ischemic heart disease by 29% [65]. Overall, most results are generated from observational studies suggesting a negative association between vegetable protein and CVD risk, while results from RCTs are less consistent [59].

However, a meta-analysis of RCTs investigating the effects of high-protein diets suggested marginal improvements in triacylglycerol (TAG) and cholesterol concentrations [37,66]. While no effects of a high-protein diet were found on cholesterol as

described in **Chapter 2** of this thesis, TAG concentrations reacted differently in reaction to a higher dietary protein content. Even if carbohydrates are the main determinant of TAG concentrations [37,66,67], a more significant decrease in serum TAG was observed with higher protein intake before, independent of weight loss or the dietary carbohydrate content [67]. A possible underlying mechanism may be an increase in lipolysis and inhibition of lipogenesis by the dietary protein content [68,69].

When addressing the effects of specific protein sources, soy protein interventions have been shown to exert small but statistically significant hypocholesterolemic effects [70]. Concomitant, the Dutch Health Council stated that a daily isoenergetic exchange of non-soy protein with 30 g soy protein lowers LDL-cholesterol concentrations by around 0.2 mmol/L, with an even more pronounced effect in hypercholesterolemic patients [71]. The soy intake in our study **(Chapter 5)** changed LDL-cholesterol in the same ratio as suggested by the Dutch Health Council, with a decrease of 0.17 mmol/L LDL-cholesterol after intake of 25.5 g soy protein, even without instructions for compensation with other food products, indicating approximately 7% risk reduction to develop coronary events [72]. For the hypocholesterolemic effects of soy, two mechanisms have been suggested. An extrinsic mechanism by which other food products are displaced with soy products, thereby lowering saturated fat and cholesterol intake [73] and to a smaller part by an intrinsic mechanism, e.g., via LDL-receptor expression [74,75]. Several components in soy have been proposed to mediate those cholesterol-lowering effects, such as the high fiber content and isoflavones [15]. Nevertheless, not only soy is effective in improving cholesterol concentrations, but also other legumes showed hypocholesterolemic effects [76].

Vascular function markers

Vascular endothelial function and arterial stiffness are central mechanisms by which diet may affect CVD risk. Vascular function markers were assessed after a mixed high-protein diet **(Chapter 2)** and after soy nut consumption **(Chapter 5)**. While no effect on vascular function was found after the high-protein diet, longer-term soy nut consumption led to improved flow-mediated dilation (FMD). In the study presented in **Chapter 5,** FMD, an NO-mediated mechanism [77], improved after soy nut consumption, while CAR, a catecholamine-mediated mechanism [78,79], did not change. Suggesting that the beneficial effects of soy on vascular function and blood pressure deriving via the NO-mediating properties of soy. The effect on vascular function has been associated with a 12% reduced risk on CV events, as every 1 pp increase in FMD was associated with 8% risk reduction [80]. Notably, the baseline diameter did not change in this study, which

would bias the FMD outcome quantification [81]. The effects on endothelial function and probably also the effects on blood pressure may be related to the isoflavone content and the amino acid composition of soy. Soy is a good source of the amino acid arginine (Table 6.2), a precursor of NO. However, a meta-analysis showed that the effects of tree and ground nuts on FMD could only be explained partly by L-arginine. Additionally, the isoflavones, mainly daidzein and genistein in soy, can bind to estrogen receptors in the endothelial layer and in vascular smooth muscle and activate NO-synthase by phosphorylation and thereby increasing NO production as well [82]. However, no effects on FMD have been shown by a previous weight loss intervention [50]. The effects of soy nuts on FMD have never been established before and the effects of other soy products on FMD were contradictory from no observed effects [83,84] to improvements [85,86]. These inconsistent results may depend on differences in the study duration, the type of soy products used, or differences in study populations.

Peripheral arterial tonometry (PAT) is another method to assess vascular endothelial function, which was not measured in the trials described in this thesis. This method has also been shown to be modified by dietary interventions [87], but not by soy nuts [88]. Conflicting results of PAT and FMD have been reported before [89]. The primary outcome of the PAT measurement is the reactive hyperemia index (RHI), reflecting small artery reactivity, which is mediated by a complex response, partly by NO. In contrast, FMD assesses the effects in a large conduit artery and is primarily mediated by NO [89-91].

Another non-invasive measurement are retinal images to assess the microvascular structure of the eye. Interestingly, it has been suggested that an arterial diameter increase of 20 µm correlates with 17% decreased risk of CHD, respectively 16% when the retinal venular diameter was decreased, in women [92]. Dietary effects on the retinal microvascular structure have also been assessed in Chapters 4 and 5. While no effects of a high-protein diet could be found (Chapter 4), retinal arterial diameter tended to be slightly increased after soy consumption. A population-based cohort study found a positive association between isoflavone intakes and retinal arteriolar calibers [93], but the effects of whole soy products have not been investigated yet.

Arterial stiffness depends on structural characteristics of the vessel, endothelial-derived vasoactive mediators, and blood pressure [94]. A modification by dietary changes is possible, which has been reported after long-term fish-oil consumption, leading to increased PUFA intake [95]. It can be assessed by the gold-standard method pulse wave velocity ($PWV_{c\text{-}f}$), the central augmentation index (AIx), and central pulse pressure [96]. $PWV_{c\text{-}f}$ is a direct measure of arterial stiffness and is associated with a reduced CVD risk

of 14% when improving with 1.0 m/s [94]. While CAIxHR75 is an indirect measurement of arterial stiffness, which assesses the arterial pressure waveform that depends on the tone of peripheral resistance arteries [94]. In both studies, $PWV_{c\text{-}f}$ and CAIxHR75 have been assessed. No effects of the high-protein diet on both markers were found in Chapter 4 and no effect of the soy intervention was found on $PWV_{c\text{-}f}$, while AIx tended to increase in Chapter 5. However, a possible explanation is that soy nut consumption has no substantial effect on arterial stiffness and that our findings on the AIx are due to chance and should thus be interpreted with caution.

As summarized in Figure 6.1, multiple markers of cardiometabolic health and vascular function can be addressed, suggesting different mechanisms by which plant-based diets beneficially affect CVD risk.

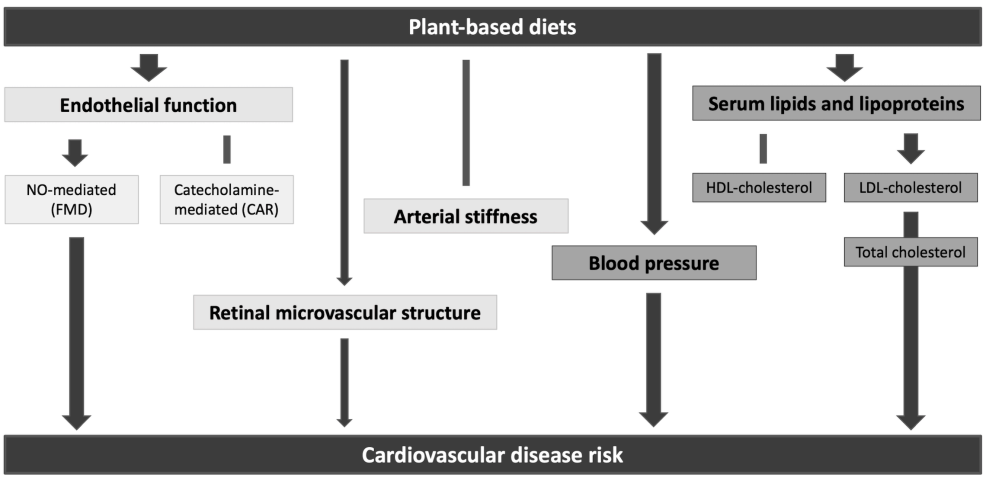

Figure 6.1: Suggested regulatory pathways by which plant-based diets may reduce the risk of cardiovascular disease development.

Possible adverse effects of protein-rich foods

Dietary protein is a critical factor of a healthy diet and appropriate amounts are compulsory for maintaining skeletal muscle mass and other tissues [6]. However, high amounts of dietary protein have been under discussion regarding their possible side effects. They have been associated with gastrointestinal complaints [66] and have been discussed in the context of renal function impairment. However, the latter has only been shown in patients with already established decreased renal function [97]. Again, the type of protein may determine the effects. While animal-based protein consumption

increased hyperfiltration acutely, plant-based protein consumption did not [98]. Soy supplementation even tended to improve renal function parameters in women with impaired renal function [99]. Due to their higher sulfur-containing amino acid content, such as methionine, animal proteins have been suggested to increase acid formation, which then might impair renal function [100]. So far, a safe upper limit for a long-term high-protein intake still needs to be investigated [19,101], but daily consumption of up to 1.66 g protein/ kg body weight has been considered safe regarding potential health risks [8,102].

Due to its isoflavone - or phytoestrogen - content, soy intake has been discussed to exert estrogen-like effects as they show structural similarities with 17-beta estradiol [103]. Increased circulating estrogen concentrations in postmenopausal women have been associated with an increased risk of developing breast cancer [104]. Due to the high isoflavone concentrations in soy, soy consumption was suspected to increase this risk [105]. Isoflavones can indeed bind to estrogen receptors (ER), but with a weaker binding affinity compared with endogenous estrogens [103]. Besides, current research shows evidence of a rather decreased risk to develop breast cancer with high isoflavone intake [106,107]. Isoflavones have a higher binding affinity to the estrogen receptor (ER) β, which is associated with a risk reduction, compared to ERα which has, in contrast, been associated with an enhanced risk [103,108]. In general, a meta-analysis of prospective cohort studies indicated that the consumption of a vegetarian diet was associated with an 18% overall reduction of cancer incidence when compared with non-vegetarians [65].

Soy has also been suspected to influence the reproductive system, but data on the effects of isoflavones on human female and male fertility are very limited. A meta-analysis of RCTs concluded that there are no effects on estradiol concentrations in premenopausal women [109], while effects on the male reproductive system are contradictory and mostly derived from animal studies to date [108].

Taken together, no health issues would be expected with the two interventions presented in this thesis based on the daily consumption of protein being lower than 1.66 g/ kg body weight/ day **(Chapters 2** to **4)**, and no indications for detrimental effects of soy could be found.

Macronutrient composition of diets

When exchanging macronutrients, e.g., carbohydrates for proteins, it remains unclear whether effects were caused by the higher protein or by the decreased carbohydrate intake. Concerning body weight loss and maintenance, a previous study investigating the effect of different macronutrient compositions on body weight management indicated that beneficial effects were caused by the high-protein content of the diet and not by the lower carbohydrate content [110]. Effects were suggested to be mainly caused by the restricted energy intake and the duration of the diet rather than by the low carbohydrate content [111]. However, a lower dietary carbohydrate content, comprising <40% of energy from carbohydrates, showed beneficial effects on CVD risk factors. A low-carbohydrate diet has also been shown to improve blood pressure and fasting TAG concentrations while a high-carbohydrate diet with displacing saturated fats may help to improve LDL-cholesterol concentrations [112]. However, the beneficial effects of high-protein diets on TAG concentrations have also been reported without changes in carbohydrate concentrations, as reported above [67]. A meta-analysis of RCTs found that low-carbohydrate diets may beneficially affect CVD risk factors, except for LDL-cholesterol, on short-term, but also stated that longer-term effects require further research [113]. Another meta-analysis additionally showed that non-energy restricted low-carbohydrate diets induced weight loss for up to one year as effectively as energy-restricted low-fat diets [114]. However, they also strengthen that possible benefits on body weight may be outweighed by potentially unhealthy changes in LDL-cholesterol concentrations [114].

In addition to the macronutrient composition itself, sex has been shown to be a mediator for the effects of specific diets [115]. While men seemed to benefit more from low-carbohydrate diets than from low-fat diets concerning weight loss, no such effects were found in women. Besides, weight loss was higher in men compared with women after the low-carbohydrate diet [115], emphasizing the need to include both sexes in these studies.

Likewise, as described in **Chapter 5** in this thesis, the effects of increased soy consumption may be caused by some soy components directly or indirectly by displacement of other products or the extrinsic effects explained above. In the case of soy protein, those extrinsic mechanisms originate from the displacement of foods with a higher saturated fat and cholesterol content with soy products, which are low in those components [73] and thereby changing the macronutrient composition of these diets.

Summary of protein quality

Taken together, an advice for the general population related to dietary protein concerning cardiometabolic and vascular health is challenging. For weight management, including the effects on appetite regulation and energy homeostasis, both mixed high-protein diets and plant-based diets appear beneficial. However, a reduction in CVD risk has mainly been associated with plant-based diets, e.g., with an increased amount of soy consumption. We found an improved vascular endothelial function (FMD), blood pressure, and LDL-cholesterol concentrations, proposing mechanisms by which an increased soy food intake beneficially affects CVD risk without effects on body weight, suggesting weight-independent mechanisms.

Comparison of dietary interventions

This thesis describes two RCTs. The different set up of these studies may explain the different results on cardiometabolic and vascular function parameters. The PREVIEW substudy **(Chapters 2 to 4)** used a mixed high-protein diet in a parallel design in overweight or obese pre-diabetics. The substudy was performed after approximately 34 months on the high- or moderate protein diet following a weight-loss period. The substudy was performed under highly controlled conditions in metabolic chambers with and the diet was provided aimed to reach energy balance. However, a downside of this controlled condition was that participants were not in a real-life situation and physical activity was remarkably reduced during their chamber stay, which may be a critical point in comparing the studies.

The soy study **(Chapter 5)** was performed in healthy older adults with a BMI between 20 and 30 kg/m^2 in a randomized cross-over design. Here, the soy nuts were consumed on top of a healthy diet based on the Dutch nutritional guidelines and only the healthy diet without any additional soy products in a randomized order for 16 weeks. Soy nuts were added as snacks to the diet; no guidelines for the displacement of other food products were given. Interestingly, we saw an increase in protein content in the dispense of carbohydrates, an improvement in fat quality, and a slightly higher energy intake of approximately 100 kcal, but no increase in body weight during the soy intervention.

Previous trials revealed that adherence to a diet with a high protein content is challenging over a longer period and that specific protein content targets are challenging to achieve [32,116,117]. The three-year PREVIEW intervention reported issues with dietary adherence to the macronutrient composition [32], which may have had also affected the results from the substudy following this period. In contrast, adherence in the soy trial seemed excellent as reported in **Chapter 5**, suggesting that adding healthy

products to the diet may facilitate adherence and improve whole diet composition without weight gain.

As whole food products were used in both trials described in this thesis, blinding of the participants during the trials was not possible. Participants of the PREVIEW trial had to prepare their meals at home during the three years of the trial, and for the soy study, no appropriate placebo for the soy nuts is available. Therefore, a placebo effect cannot be excluded in these trials. However, analyses were performed blinded to minimize these effects as much as possible.

Planetary health and sustainable nutrition

Planetary health has been defined by the Rockefeller Foundation-Lancet Commission on Planetary Health as "the health of human civilisation and the state of the natural systems on which it depends" [118]. The concept is based on the notion that human and planetary health are intensely connected. Projections indicate that by 2050, current dietary trends together with population growth will cause considerable burdens to the health systems due to increasing amounts of non-communicable diseases, but also for the Earth, due to increased water and land use, as well as greenhouse gas (GHG) emissions [119]. Plant-based diets have not only been considered lowering the risk of chronic disease development, as explained above, but they are also more sustainable due to fewer resource use compared with animal-based products for their production [120]. According to the sustainable nutrient-rich food index, plant products, e.g., soy products can be classified as a food with a favorable nutrient profile and low climate index in contrast to other dietary protein sources such as meat and dairy products, which have been classified as foods with a negative or moderate nutrient profile with high and medium climate impact [121].

Therefore, incorporating more plant-based food products into the diet may not only be supportive for a healthier life, but also in terms of environmental considerations and the respect for planetary boundaries [120]. Future recommendations for healthy diets would preferably not only be based on human health but should also integrate environmental considerations.

Concluding remarks and future perspectives

The research described in this thesis focused on the potential of a high-protein diet and a diet enriched with soy nuts to improve several metabolic health and vascular function markers. We provide further evidence that a higher dietary protein content improves body weight management and, in the case of plant-based protein, additionally improves CVD risk.

The appetite modulating effects found in this thesis **(Chapter 2)** corroborate the findings of previous trials and show once more that the dietary protein content may be a critical factor for improving weight management. We indicated that concentrations of the endocannabinoid 2-AG follow a meal-related pattern comparable to that of glucose, GLP-1, and PYY **(Chapter 2)** but with increased concentrations in the high-protein group, suggesting a mediating role in the appetite-regulating effects of dietary protein. However, literature on postprandial 2-AG concentrations is scarce and conflicting [122-125]. The potential impact of this finding on weight loss and weight management needs further investigation, especially in combination with high-protein diets, as it may indicate novel mechanistic insights into the effects of protein. Not only the effects of diet on endocannabinoid concentrations but also possible coherences of endo-cannabinoids and CVD risk are barely known. Further studies investigating the possibility of dietary modulations and if these possible modulations could provide a potential target in the combat of overweight and non-communicable diseases, would be strongly recommended.

Possible differences in effects between men and women also needs to be addressed in future studies. This has previously been questioned regarding dietary effects on weight loss [115] and needs further investigation. Especially studies assessing the effects of soy products are often conducted in postmenopausal women only. However, differential effects of soy consumption between sexes has been suggested before [126], indicating that more research is needed to address possible sex differences.

This thesis provides further evidence that an increased intake of soy products improve cardiovascular health by improving endothelial function, LDL-cholesterol concentrations, and blood pressure **(Chapter 5)**. However, this thesis only focused on the peripheral vascular effects of these dietary interventions. Obesity and impaired glucose tolerance may be next to aging an essential trigger for progressive cognitive decline, as those conditions have been related to an impaired brain vascular function [127]. A recent review summarizes the impact of specific dietary determinants on cerebral blood flow (CBF), a sensitive physiological marker of brain vascular function, in adults and discusses the relationship between these effects with potential changes in cognitive

function [128]. These effects are of particular interest since brain vascular dysfunction is a key pathological event that precedes the development of impaired cognitive function. Future research should broaden its focus on implementing new concepts for healthy lifestyles for both, peripheral and brain vascular function. Especially the interaction between periphery and brain regarding the effects of a high-protein diet and soy nuts on both vascular and metabolic health is of great importance. Analysis of CBF and cognitive function data of the soy study will be the next step investigating the effect of soy nuts on vascular health.

In the trial described in Chapter 5, participants were older, apparently healthy adults. The role of different study populations in relation to the effects of soy still needs to be investigated. Additionally, in this thesis, the effects of diets on cardiometabolic and vascular function markers are only assessed in a fasted state. As we spend most of the day in a postprandial state, future studies now also need to address effects on postprandial vascular function and metabolic health.

Furthermore, further work is needed to establish the role of equol-producing properties as a mediating factor for the health effects of soy. The ability to produce equol depends on the gut microbiota and varies between individuals. It has been suggested that equol-producing abilities are related to health improvements [129]. As described in Chapter 5, 26% of the study population could be assigned as equol producers, which is in line with the expected prevalence in the Western world [130]. If equol is the driving force in the beneficial effects of soy still needs to be determined in clinical trials focusing on differences between equol producers and non-producers while examining the links between diet and metabolic and vascular risk markers.

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APPENDICES

IMPACT

The main objective of this dissertation was to investigate the effects of dietary interventions with a mixed high-protein content or soy nuts on several metabolic health and vascular function markers in adults. This thesis support previous findings that a higher dietary protein intake lowers hunger perception and provides novel insights for the involvement of the endocannabinoid-system in energy-intake regulation. The impact of 2-arachidonoylglycerol (2-AG) concentrations in protein-related appetite regulation might be of great interest for future research as it appeared to be a contributor to the appetite-modulating effects in our study. However, the protein intervention or the endocannabinoid-system had no impact on cardiometabolic health and vascular function markers in our study. In contrast, soy nut consumption improved endothelial function and lipoprotein concentrations and therefore lowered cardio-vascular disease (CVD) risk in older adults. As the worlds' population, the proportion of the elderly, and also the diabetes incidence are expected to further increase in the coming years, it is crucial to find strategies to lower the risk for developing non-communicable diseases [1-3]. This thesis's potential impact in terms of scientific, societal and economic relevance, and implications for the translation into practice will be discussed in the following paragraphs.

Societal relevance

Malnutrition, caused by a diet providing too many or too few nutrients, represents a global threat to human health and can result in overweight and obesity or under-nutrition [4]. Worldwide, the consumption of unhealthy diets with, amongst others, high amounts of red meat increases and has been related to an estimation of 990 000 deaths in 2017 [5]. Globally, the prevalence of obesity is rapidly increasing and as it is one of the main modifiable risk factors for non-communicable diseases, those numbers are expected to concurrently increase. Diabetes incidence is enormously growing from a prevalence of 4.7% in adults in 1980 to 8.5% in 2014 and caused 1.6 million deaths in 2016. Approximately 90% of all diabetes cases are assigned to type 2 diabetes mellitus (T2DM), which is mostly associated with overweight and unhealthy lifestyle. Therefore, lifestyle improvements, such as improvements in body weight, physical activity, and a healthy diet, are useful tools in preventing disease development [1]. Even if there are several ways to reduce body weight successfully, weight maintenance after weight loss remains exceptionally challenging due to activated biological responses to prevent further weight loss, amongst other causes. Therefore, it is crucial to find strategies to

improve and simplify body weight maintenance to increase the chances of successful weight management and lower disease risk. In this context, the high-protein diet of the PREVIEW study showed promising results concerning weight management and metabolic parameters [6]. Additionally, this thesis shows that an altered hunger perception possibly contributed to these beneficial effects and weight management improvement.

Next to promoting T2DM development, obesity strongly relates to the development of CVD risk factors, such as hypertension and dyslipidemia [7]. CVD is the most common cause of death worldwide, leading to an estimated 17.9 million deaths annually [2]. As most CVD are caused by modifiable risk factors, such as an unhealthy diet and obesity, powerful prevention strategies could immensely reduce the burden of CVD. In one of our studies, we found that the addition of soy nuts to a healthy diet improved various risk factors for CVD development, such as an improvement in endothelial function, blood pressure, and the lipid profile. The effect on endothelial function, for example, can be associated with an overall 12% decreased CV event risk [8].

Another important risk factor for the above-mentioned non-communicable diseases is aging. In the last years, the aging pace of the world's population is increasing. Due to enhanced longevity worldwide, the proportion of the elderly is increasing. According to the WHO, the population of 60+ years is expected to increase from 12% to 22% between 2015 and 2050 [3]. Aging is associated with an increased risk of disease development, e.g., T2DM due to gradual cellular damage, meaning that a further increase in disease incidence can be expected in the coming years. However, healthy aging can, at least partly, be promoted by improving behavior and the environment at younger age already. This can be done by behavioral changes such as regularly eating a healthy, balanced diet and being physically active [3]. Therefore, it is of enormous importance to understand the aging process in more detail and find optimal strategies to promote healthy aging at the early onset to prevent the development of non-communicable diseases [3]. Multiple organ systems and influencing factors are involved in many age-related conditions. Those conditions often have shared co-morbidities, indicating underlying mutual causes which simultaneously implies the possibility of shared solutions.

According to the soy study in the current thesis, promising cardiovascular effects in persons aged 60 - 70 years has been shown when soy nuts were added on top of a healthy diet compared to the healthy diet without any soy product, suggesting that the simple addition of soy nuts to the diet may promote healthy aging. In this study, multiple possible working mechanisms were analyzed to understand in more detail why the consumption of plant-based foods beneficially affects health.

Economic relevance

Due to the highly increasing numbers of CVD and diabetes patients, health care costs are increasing accordingly and are expected to rise even higher in the future. The diabetes-related costs increase to USD 760 billion in 2019 and have been estimated to increase to USD 825 billion in 2030 [9]. While the global CVD-related costs added up to USD 863 billion in 2010, they are expected to rise to USD 1044 billion in 2030 [10]. Stimulating a healthier lifestyle and a healthier body weight by using dietary intervention strategies as described in the current thesis can lower the disease development risk and thereby reduce the economic burden of non-communicable diseases. A healthier diet, e.g., with more plant-based proteins, could stimulate a healthier aging process and lower the need for medical treatments while being relatively cheap and easily accessible for many people. It, therefore, contains the possibility of a substantial economic relevance by lowering the health care costs of non-communicable diseases in the aging population significantly.

Environmental relevance

Globally, more than 820 million people have insufficient access to food and even more are regularly consuming an unhealthy diet, contributing to a high food-related morbidity [11]. Currently, 462 million adults are underweight, another form of malnutrition. This number will even increase when no changes in the food production systems are made, as the world's population is further increasing to an expected population of around 10 billion people by 2050 [4,11]. It has been proposed that around 70 - 100% more foods will be required in 2050 than produced now [12]. Several adaptions are necessary to feed the increasing world's population within the planetary boundaries, such as a change to more sustainable agricultural systems. Additionally, the change to a less resource-intensive diet by reducing animal-based products and including more protein-rich plant-products instead, such as pulses and soybeans, may help to manage the increasing need for food supply in the future [13].

A shift towards a predominantly plant-based diet combined with improved food production and a reduction in food waste is necessary to provide a healthy diet, in particular for a growing world population. For both, human and planetary health, a shift from animal products to plant-based diets is vital as those diets need fewer resources for their production and are therefore more sustainable [14]. Especially the dietary protein is a huge point of discussion for shifting to those diets. However, with the increased use of nuts, soy, and legumes, protein targets can be achieved with a plant-based diet [11]. Plant-based products rich in protein, such as soy products, have a better

nutrient profile-low climate index than animal-protein sources according to the sustainable nutrient-rich food index [15]. It has been shown that water use increases with the amount of animal protein in the diet. Furthermore, around two-thirds of agricultural and one-third of arable land are currently used for livestock farming. When compared to the production of 1 kg rice or potatoes, 1 kg of beef production uses approximately 18 times more water and 163 times more land. Additionally, the CO_{2eq} production can be reduced by 53% with an entirely plant-based diet compared with a typical US diet [16].

Target groups

Both studies presented in this thesis were performed in adults and older adults. While the first study with a high-protein diet was completed in pre-diabetic, overweight and obese adults, the soy nut study was performed in persons aged 60 to 70 years with a normal to overweight BMI and no signs of impaired glucose tolerance or CVD. Multidimensional solutions are needed to tackle the medical, economic, and psychosocial consequences of a growing aging population indicating the special need for studies in the elderly to find effective, evidence-based dietary intervention strategies. However, to translate those results to a broader population, e.g., young adults or patients with established CVD, more research is needed. Next to the scientific impact for patients, the outcomes of these studies are also interesting for the food industry to launch healthy, evidence-based food concepts for e.g., plant-based products.

Translation into practice

Clinical studies in humans are critical for translating findings from cell or animal studies to a real-life setting in participants. The protein intervention investigated in this dissertation has already been used for an extended period during the PREVIEW study [17]. Following the high-protein intervention over three years turned out to be difficult, but the dietary intervention still successfully reduced T2DM risk. In contrast, the soy study's compliance was excellent, indicating that merely adding a product to the diet may be easier to follow over a more extended period. Additionally, this study showed beneficial effects of soy nuts despite a healthy diet, possibly pointing out the benefit of shifting the diet to a more plant-based origin. The soy nuts provided in this study were well tolerated, no serious adverse events occurred, and body weight remained stable even when simply adding the product to the regular diet. The daily amount of soy nuts was relatively high but feasible for the study period. For a more extended period, however, it might be an option to incorporate other soy products to promote this

intervention's long-term feasibility and success. Next to the effects on CVD risk, the role of plant-based foods in the prevention of other age-related conditions should get much more attention in future research.

This dissertation's results are or will be published in peer-reviewed scientific journals and have been presented at scientific conferences to increase awareness of this topic, share knowledge, and stimulate further research. These results may be used in a general population's advice to promote healthy dietary choices of individuals. Different patient organizations or societies could help to inform and provide awareness within specific population groups. During both studies, regular dietary guidance for the participants was provided to stimulate and promote compliance to the intervention. We strongly suggest to include dietary guidance into practice to stimulate a balanced and healthy diet and high success rate.

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SUMMARY

Metabolic health and vascular function in adults: Effects of a high-protein diet and soy nuts

The prevalence of overweight and obesity is rapidly increasing. In 2016, 39% of the world's population was overweight and 13% was obese. Excess body weight is one of the leading risk factors for the development of non-communicable diseases, such as type II diabetes mellitus and cardiovascular disease (CVD). CVD comprises multiple disorders related to heart and blood vessel-related issues and is the leading cause of mortality worldwide, with 17.9 million deaths each year. Typical risk factors for the development of CVD are cardiometabolic factors such as hypertension and hyper-lipidemia, and vascular function markers such as arterial stiffness and endothelial dysfunction.

Approximately 70% of all CVD can be related to modifiable risk factors. Therefore, maintaining a healthy diet and lifestyle is a cornerstone in the prevention of non-communicable disease due to beneficial effects on risk markers of metabolic and vascular health and improved weight management. Here, the diets' protein content may be important, especially for the control of body weight. High-protein diets have been suggested to promote satiety via multiple mechanisms. However, not only the total amount of protein is of importance, but also the quality and source of protein. Indeed, whether it originates from animal or plant sources seems to be an influencing factor in preventing or managing these non-communicable diseases. Plant-based diets, for example, have been associated with a CVD risk reduction in large cohort studies. An essential part of plant-based diets are soy products, as they are a good source of, e.g., high-quality plant proteins, polyunsaturated fatty acids, and bioactive compounds such as isoflavones.

This thesis aimed to investigate the effects of a high-protein diet and soy nuts on several markers of metabolic health and vascular function in adults.

Chapters 2 to 4 describe the results of a parallel human intervention trial investigating the effects of a mixed high-protein diet on several aspects of food intake regulation, endocannabinoids, and cardiometabolic and vascular risk factors in pre-diabetic, over-weight, or obese participants after completing three years of protein intervention. This trial was a substudy of the PREVIEW-trial (Prevention of Diabetes through Lifestyle Intervention and Population Studies in Europe and around the World), performed in respiration chambers at the Metabolic Research Unit at Maastricht University, allowing markedly close monitoring and control. For this, 38 men and women were recruited from the PREVIEW study, of which 18 participants consumed the moderate-protein (MP;

15/55/30 % energy from protein/carbohydrate/fat) control diet and 20 consumed the high-protein diet (HP; 25/45/30 % energy from protein/carbohydrate/fat). During the substudy, meals were provided in energy balance. The participants were measured for 48h in the respiration chamber after approximately 34 months of weight maintenance following an eight-week weight reduction period.

In Chapter 2, the effect of a high-protein diet on appetite regulation has been investigated. For this, appetite perception has been assessed with questionnaires and metabolic appetite regulation by analyzing anorexigenic hormones (GLP-1 and PYY). Participants from the high-protein diet group felt less hungry than those from the control group (decremental AUC -56.6%). GLP-1 and PYY concentrations were not affected by the dietary protein content, but hunger was inversely associated with the PYY concentrations in the HP group only. However, the effect on appetite perception did not affect *ad libitum* energy intake at the end of the study. In the same trial, the role of endocannabinoids in energy balance regulation has been assessed. We showed that 2-arachidonoylglycerol (2-AG) concentrations followed a meal-related pattern with the highest concentrations 60 min after the meals. Additionally, concentrations were generally higher in the high-protein group. Therefore, 2-AG has been suggested as a possible mediator in protein-mediated appetite-regulating effects (Chapter 2). However, plasma concentrations of the endocannabinoid anandamide (AEA) and the endocannabinoid-related compounds (oleoylethanolamide (OEA), palmitoylethanolamide (PEA), and pregnenolone (PREG)) were not affected by the dietary protein content as shown in Chapter 3. However, AEA, OEA, PEA, and PREG significantly decreased during the day and reflected gradual energy intake matching energy expenditure. The decrease in OEA and PEA during the day was inversely associated with body mass index and body fat percentage.

While Chapters 2 and 3 focus mainly on the metabolic components and regulation of energy intake, Chapters 4 and 5 describe the effect on cardiometabolic and vascular health parameters of two different intervention trials. Chapter 4 reports the impact of the high-protein diet, used in the previous chapters, on cardiometabolic and vascular risk markers. No effects were found on cardiometabolic markers, such as blood pressure and serum lipoprotein concentrations, and vascular function markers, such as endothelial function, arterial stiffness, and microvascular structure. When correlating these results with the endocannabinoid concentrations, OEA and PEA were positively associated with total (TC) and low-density lipoprotein (LDL) cholesterol concentrations suggesting a possible role of the endocannabinoid system in the regulation of hyperlipidemia.

Chapter 5 describes the results of a longer-term randomized cross-over trial investigating the effects of soy nut consumption on cardiometabolic and vascular health markers in healthy, older adults (60 to 70 years, with a BMI between 20 and 30 kg/m^2. Twenty-three participants consumed 67 g of soy nuts daily on top of a healthy diet or no soy products in a randomized order for 16 weeks each. The healthy diet was based on the Dutch dietary guidelines. After soy consumption, we found an improved endothelial function, office blood pressure, and serum LDL-cholesterol concentrations compared with the control condition. Endothelial function, as assessed by brachial artery flow-mediated vasodilation (FMD) response, improved by 1.49 percentage points (pp), which is related to a reduced cardiovascular risk of about 12%. In addition, soy consumption improved office blood pressure levels (SBP: -4 mmHg and DBP: -2 mmHg) and the mean arterial pressure (-3 mmHg), while LDL-cholesterol concentrations were reduced by 0.17 mmol/l. Arterial stiffness was unaffected. We concluded from this trial that a longer-term daily intake of soy improved vascular function and cardiometabolic risk markers in older adults, which may contribute to the beneficial effects of plant-based diets on the risk of developing CVD.

In conclusion, based on two human intervention trials, this dissertation provides further evidence that dietary protein may play a role in the prevention of non-communicable diseases. While a high-protein diet has been shown to affect appetite perception, possibly mediated by increased 2-AG and PYY concentrations, no effects were found on cardiometabolic or vascular health parameters. Furthermore, longer-term daily intake of soy nuts improved endothelial function, blood pressure, and serum LDL-cholesterol concentrations, representing possible mechanisms by which soy products reduce CVD risk.

SAMENVATTING

Metabole gezondheid en vasculaire functie in volwassenen: Effecten van een eiwitrijke voeding en sojanoten

De prevalentie van overgewicht en obesitas neemt toe. In 2016 had 39% van de wereldpopulatie overgewicht en 13% obesitas. Een te hoog lichaamsgewicht is één van de belangrijkste risicofactoren voor het ontwikkelen van chronische niet-overdraagbare ziekten, zoals diabetes mellitus type 2 (suikerziekte) en hart- en vaatziekten (HVZ). HVZ is een verzamelnaam voor een groot aantal aandoeningen van het hart en de bloedvaten. Deze aandoeningen zijn de hoofdoorzaak van sterfte en leiden jaarlijks tot meer dan 17.9 miljoen overlijdens. Belangrijke risicomarkers voor het ontwikkelen van HVZ zijn een verhoogde bloeddruk en ongunstige waarden van metabole risicomarkers, zoals het serumcholesterolgehalte, het plasmaglucosegehalte en non-invasieve vaatfunctiemarkers, zoals de endotheelfunctie (vasculaire endotheelfunctie) en stijfheid van de bloedvaten (arteriële stijfheid).

Risicofactoren, die kunnen worden beïnvloed, spelen een belangrijke rol bij de preventie van HVZ. Zo heeft een gezonde leefstijl gunstige effecten op de metabole gezondheid en vasculaire functie, en op het lichaamsgewicht. Het is bekend dat het eiwitgehalte in de voeding hierbij een belangrijke rol speelt, onder andere doordat het bijdraagt tot het in stand houden van een gezond lichaamsgewicht. Uit eerder onderzoek is bijvoorbeeld gebleken dat een eiwitrijke voeding verzadigend werkt. Niet alleen de totale hoeveelheid eiwit in de voeding is hierbij van belang, maar ook de kwaliteit en de bron van de eiwitten. Voor het voorkomen van chronische ziekten is het mogelijk ook belangrijk of het eiwit afkomstig is van dieren of planten. Zo is een voeding rijk aan plantaardige producten geassocieerd met een verlaagd risico op HVZ. Een belangrijk onderdeel van een plantaardige voeding zijn bijvoorbeeld sojaproducten omdat soja een bron is van hoogwaardige eiwitten, meervoudig onverzadigde vetzuren en bioactieve stoffen, zoals isoflavonen.

Het doel van dit proefschrift was nu om de effecten te onderzoeken van zowel een eiwitrijke voeding als sojanoten op verschillende markers voor metabole gezondheid en vasculaire functie in volwassenen.

In hoofdstuk 2, 3 en 4 zijn de resultaten van een humane interventiestudie beschreven. In deze studie zijn de effecten onderzocht van een eiwitrijke voeding afkomstig van zowel dierlijke als plantaardige producten op verschillende aspecten van de regulatie van de voedingsinname, endocannabinoïde concentraties, en cardiometabole en vasculaire risicofactoren. Hiervoor is gebruik gemaakt van een driejarige voedings-

interventiestudie in vrijwilligers met prediabetes en overgewicht of obesitas. Deze studie was een deelstudie van de PREVIEW-studie (Prevention of Diabetes through Lifestyle Intervention and Population Studies in Europe and around the World) en is uitgevoerd in de respiratiekamers van de Metabole Research Unit in Maastricht (MRUM). In totaal werden 38 mannen en vrouwen uit de PREVIEW-studie geworven, waarvan 18 deelnemers de controlevoeding consumeerden met een matig eiwitgehalte (MP; 15/55/30% energie uit eiwitten/ koolhydraten/ vet) en 20 deelnemers de eiwitrijke voeding (HP; 25/45/30% energie uit eiwit/ koolhydraten/ vet). Tijdens de studie waren de deelnemers in energiebalans. Na een periode van acht weken afvallen werden deelnemers na 34 maanden gedurende 48 uur in de respiratiekamer gemeten onder zeer gecontroleerde omstandigheden.

In hoofdstuk 2 werd het effect van een eiwitrijke voeding op de regulatie van de eetlust onderzocht. Hiervoor is de perceptie van eetlust beoordeeld door middel van vragenlijsten en de concentraties van de verzadigende hormonen GLP-1 en PYY gemeten. Deelnemers in de eiwitrijke voedingsgroep hadden een minder grote eetlust dan de deelnemers in de controlegroep. Concentraties van de hormonen GLP-1 en PYY werden niet beïnvloed door het eiwitgehalte in de voeding, maar het hongergevoel was wel omgekeerd geassocieerd met de PYY-concentraties in de eiwitrijke groep. Het effect op de hongerperceptie had echter geen invloed op de *ad libitum* energie-inname aan het einde van de studie. Verder hebben we in dezelfde studie de rol van endocannabinoïden onderzocht met betrekking tot de regulatie van de energiebalans. We hebben aangetoond dat de concentraties van 2-arachidonoylglycerol (2-AG) een maaltijdgerelateerd patroon volgden, waarbij de hoogste concentraties werden waargenomen 60 minuten na de maaltijden. Bovendien waren de concentraties over het algemeen hoger in de eiwitrijke groep. Daarom werd 2-AG voorgesteld als een mogelijke mediator van de eiwit-gemedieerde eetlust-regulerende effecten (Hoofdstuk 2). Zoals weergegeven in hoofdstuk 3 werden concentraties van de endocannabinoïde anandamide (AEA) en de endocannabinoïd-gerelateerde verbindingen (oleoylethanolamide (OEA), palmitoylethanolamide (PEA) en pregnenolon (PREG)) niet beïnvloed door het eiwitgehalte in de voeding. AEA, OEA, PEA en PREG namen significant af gedurende de dag en weerspiegelden de geleidelijke energie-inname, die overeenkwam met het energieverbruik. De afname van OEA en PEA gedurende de dag was omgekeerd geassocieerd met de BMI en het percentage lichaamsvet.

Hoofdstuk 2 en 3 beschrijven de metabole parameters en regulatie van de energie-inname, terwijl in hoofdstuk 4 en 5 de effecten op de cardiometabole en vasculaire gezondheidsparameters worden beschreven die onderzocht zijn in twee verschillende interventiestudies. Hoofdstuk 4 beschrijft de specifieke effecten van een eiwitrijke voeding. Er werden geen effecten gevonden op cardiometabole risicomarkers, zoals de bloeddruk en serum lipoproteïneconcentraties, en de vasculaire functiemarkers, zoals vasculaire endotheelfunctie, arteriële stijfheid en markers gerelateerd aan de micro-circulatie. Wanneer deze resultaten werden gecorreleerd met endocannabinoïde concentraties, waren OEA en PEA positief geassocieerd met de totale- en LDL-cholesterol-concentratie. Dit zou op een mogelijke rol van het endocannabinoïde systeem bij de regulering van hyperlipidemie kunnen duiden.

Hoofdstuk 5 beschrijft de resultaten van een gerandomiseerde cross-over studie naar de langetermijneffecten van sojanoten op cardiometabole en vasculaire gezondheids-markers in gezonde oudere volwassenen (60 tot 70 jaar, met een BMI tussen 20 en 30 kg/m^2). Drieëntwintig deelnemers consumeerden in een willekeurige volgorde gedurende 16 weken een voedingspatroon gebaseerd op de Nederlandse richtlijnen voor een gezonde voeding. Deze voeding werd wel of niet (controleconditie) aangevuld met dagelijks 67 g sojanoten. Na consumptie van de sojanoten vonden we een verbetering in de endotheelfunctie, bloeddruk en serum LDL-cholesterol-concentraties in vergelijking met de controleconditie. De flow-gemedieerde vasodilatatie van de arteria brachialis (FMD) verbeterde met 1.49 procentpunten (pp), hetgeen gerelateerd is aan een daling van het risico op HVZ van ongeveer 12%. Bovendien verbeterde na sojaconsumptie de systole en diastole bloeddruk (SBP: -4 mmHg en DBP: -2 mmHg), en daalde de gemiddelde arteriële druk (-3 mmHg). Serum LDL-cholesterolconcentraties namen af met 0.17 mmol/l, maar de polsgolfsnelheid in de aorta (PWV) als maat voor de arteriële stijfheid werd niet beïnvloed. Op basis van de resultaten van deze studie concludeerden we dat een dagelijkse consumptie van sojanoten de vasculaire functie en metabole gezondheid bij oudere volwassenen verbetert. Dit kan bijdragen aan de gunstige effecten van een plantaardig voedingspatroon op het risico voor het ontwikkelen van HVZ.

In conclusie, dit proefschrift levert op basis van twee humane interventiestudies verder bewijs dat eiwitten in de voeding een rol kunnen spelen bij de preventie van niet-overdraagbare chronische ziekten, zoals HVZ. Er is aangetoond dat een eiwitrijke voeding de perceptie van eetlust beïnvloedt, hetgeen mogelijk gemedieerd wordt door verhoogde 2-AG- en PYY-concentraties. Er werden echter geen effecten gevonden op de cardiometabole of vasculaire gezondheidsparameters. Een lange-termijnconsumptie

van sojanoten verbeterde de endotheelfunctie, de bloeddruk en de serum LDL-cholesterolconcentraties, hetgeen een mogelijk mechanisme is waardoor soja-producten het risico op HVZ verlagen.

ZUSAMMENFASSUNG

Stoffwechselgesundheit und Funktionalität der Blutgefäße in Erwachsenen: Auswirkungen einer proteinreichen Ernährung und Soja-Nüssen

In den letzten Jahren hat die Prävalenz von Übergewicht und Adipositas stetig zugenommen. Im Jahr 2016 waren 39% der Weltbevölkerung übergewichtig und 13% waren fettleibig. Übergewicht ist einer der Hauptrisikofaktoren für die Entwicklung nichtübertragbarer Erkrankungen wie Diabetes mellitus Typ II und Herz-Kreislauf-Erkrankungen. Herz-Kreislauf-Erkrankungen umfassen mehrere Syndrome die im Zusammenhang mit Herz- und Blutgefäßproblemen stehen und sind mit 17,9 Millionen Todesfällen pro Jahr die weltweit häufigste Todesursache. Typische Risikofaktoren für die Entwicklung von Herz-Kreislauf-Erkrankungen sind kardiometabolische Faktoren wie Bluthochdruck und Hyperlipidämie sowie Veränderungen der Marker für die Funktionalität der Blutgefäße. Dies kann zum Beispiel eine verringerte Gefäßelastizität oder eine Dysfunktion des Endotheliums sein.

Ungefähr 70% aller Herz-Kreislauf-Erkrankungen können mit modifizierbaren Risikofaktoren in Zusammenhang gebracht werden. Eine besondere Rolle spielen dabei ein gesunde Ernährung und ein gesunder Lebensstils. Dies kann sich positiv auf einige Risikomarker für die Stoffwechsel- und Blutgefäßgesundheit und auch auf ein verbessertes Gewichtsmanagement auswirken und stellt somit einen wichtigen Eckpfeiler bei der Prävention dieser nichtübertragbaren Erkrankungen dar. Insbesondere für die Kontrolle des Körpergewichts kann der Eiweißgehalt der Nahrung wichtig sein. Eine eiweißreiche Ernährung kann zum Beispiel das Sättigungsgefühl durch verschiedene Mechanismen beeinflussen. Möglicherweise ist jedoch nicht nur die Gesamtmenge des Eiweißes von Bedeutung, sondern auch die Qualität und der Ursprung. Ob es aus tierischen oder pflanzlichen Quellen stammt, scheint einer der Einflussfaktoren bei der Vorbeugung oder Behandlung dieser nichtübertragbaren Erkrankungen zu sein. Pflanzliche Ernährungsweisen wurden beispielsweise in großen Kohortenstudien mit einer Verringerung des Risikos für Herz-Kreislauf-Erkrankungen in Verbindung gebracht. Einen wesentlichen Bestandteil pflanzlicher Ernährungsweisen stellen Sojaprodukte dar, da sie eine gute Quelle für hochwertige pflanzliche Eiweiße, mehrfach ungesättigte Fettsäuren und bioaktive Stoffe wie Isoflavone sind.

Ziel dieser Doktorarbeit war es, die Auswirkungen einer eiweißreichen Ernährung und von Soja-Nüssen auf verschiedene Marker für die Stoffwechselgesundheit und die Gefäßfunktion bei Erwachsenen zu untersuchen.

In den Kapiteln 2 bis 4 werden die Ergebnisse einer parallelen Interventionsstudie bei Erwachsenen beschrieben, in der die Auswirkungen einer eiweißreichen, gemischten Ernährung auf verschiedene Aspekte der Nahrungsaufnahmeregulation, Endocannabinoide, sowie kardiometabolische und vaskuläre Risikofaktoren untersucht. Dies wurde in prä-diabetischen, übergewichtigen oder adipösen Teilnehmern im Anschluss an eine dreijährige eiweißreiche Ernährungsintervention gemessen. Diese Studie war Teil der internationalen PREVIEW-Studie (Prevention of Diabetes through Lifestyle Intervention and Population Studies in Europe and around the World), die in den Kalorimeter Räumen (respiration chambers) der Metabolic Research Unit der Universität Maastricht durchgeführt wurde, was eine sehr genaue Überwachung und Kontrolle der Studiensituation ermöglichte. Für diese Teilstudie wurden 38 Männer und Frauen aus der PREVIEW-Studie rekrutiert, von denen 18 Teilnehmer die Kontrolldiät mit mäßigem Eiweißanteil (MP; 15/ 55/ 30% Energie aus Protein/ Kohlenhydrat/ Fett) und die anderen 20 Teilnehmer die eiweißreiche Diät konsumierten (HP; 25/ 45/ 30% Energie aus Protein/ Kohlenhydraten/ Fett). Während der Teilstudie wurden die Mahlzeiten basierend auf dem individuellen Bedarf bereitgestellt. Die Teilnehmer wurden nach einer achtwöchigen Gewichtsreduktionsperiode und ungefähr 34 Monaten Gewichtserhaltung, 48 Stunden lang in den Kalorimeter Räumen untersucht.

In Kapitel 2 wurde die Auswirkung einer eiweißreichen Ernährung auf Hunger und Sättigung untersucht. Zu diesem Zweck wurde der wahrgenommene Appetit mit Fragebögen und die metabolische Regulation des Appetits durch die Analyse von sättigenden Hormonen (GLP-1 und PYY) bewertet. Teilnehmer aus der eiweißreichen Diätgruppe verspürten weniger Hunger als Teilnehmer aus der Kontrollgruppe (dekrementelle AUC -56,6%). Die GLP-1 und PYY-Konzentrationen wurden hingegen nicht durch den Eiweißgehalt in der Nahrung beeinflusst, aber das Hungergefühl war negativ mit den PYY-Konzentrationen in der HP-Gruppe korreliert. Die Auswirkung auf das Hungergefühl hatte jedoch keinen Einfluss auf die *Ad-libitum*-Energieaufnahme nach den 48 Stunden in den Kalorimeter Räumen. In derselben Studie wurde auch die Rolle von Endocannabinoid-Konzentrationen bei der Regulation der Energieaufnahme untersucht. Wir zeigten, dass die Konzentrationen von 2-Arachidonoylglycerin (2-AG) einem mahlzeitbezogenen Muster mit den höchsten Konzentrationen 60 Minuten nach den Mahlzeiten folgten. Zusätzlich waren die 2-AG Konzentrationen in der eiweißreichen Gruppe im Allgemeinen höher. Daher haben wir 2-AG als einen möglichen Mediator für die eiweißvermittelten appetitregulierenden Effekte vorgeschlagen (Kapitel 2). Die Plasmakonzentrationen des Endocannabinoids Anandamid (AEA) und der Endocannabinoid-verwandten Verbindungen Oleoylethanolamid (OEA), Palmitoylethanolamid (PEA) und Pregnenolon (PREG) wurden jedoch nicht durch den Eiweißgehalt der

Nahrung beeinflusst (Kapitel 3). In Kapitel 3 zeigen wir auch, dass AEA, OEA, PEA und PREG-Konzentrationen tagsüber signifikant abnehmen und die Energieaufnahme über den Tag verteilt schrittweise wiederspiegeln. Die Abnahme von OEA und PEA während des Tages war negativ mit dem BMI und dem Körperfettanteil korreliert.

Während sich die Kapitel 2 und 3 hauptsächlich auf die metabolen Komponenten und die Regulation der Energieaufnahme konzentrieren, beschreiben Kapitel 4 und 5 die Auswirkungen zweier verschiedener Interventionsstudien auf verschiedene kardiometabolische und vaskuläre Gesundheitsparameter. Kapitel 4 zeigt die Auswirkungen der eiweißreichen Ernährung, die auch in den vorherigen Kapiteln verwendet wurde, auf kardiometabolische und vaskuläre Risikomarker. Sowohl die kardiometabolischen Marker wie Blutdruck und Serum Lipoprotein Konzentrationen als auch die Marker für die Funktionalität der Gefäße, wie eine verringerte Gefäßelastizität, eine Dysfunktion des Endotheliums und die mikrovaskuläre Struktur, waren unverändert. Bei der Korrelation dieser Werte mit den Endocannabinoid Konzentrationen waren OEA und PEA positiv mit den Gesamtcholesterinkonzentrationen (TC) und dem Lipoprotein niedriger Dichte (LDL) assoziiert, was auf eine mögliche Rolle des Endocannabinoid-Systems bei der Regulation der Hyperlipidämie hinweisen könnte.

Kapitel 5 beschreibt die Ergebnisse einer längeren randomisierten Cross-Over-Studie, in der die Auswirkungen des Konsums von gerösteten Sojabohnen (Soja-Nüsse) auf kardiometabolische und vaskuläre Gesundheitsparameter in gesunden älteren Erwachsenen (60 bis 70 Jahre mit einem BMI zwischen 20 und 30 kg/m^2) untersucht wurden. Dreiundzwanzig Teilnehmer konsumierten 67 g Soja-Nüsse täglich, zusätzlich zu einer ausgewogenen Ernährung, oder ein kompletter Verzicht auf jegliche Sojaprodukte, in einer zufälligen Reihenfolge für jeweils 16 Wochen. Die ausgewogene Ernährung war auf den niederländischen Ernährungsrichtlinien basiert. Nach dem Verzehr des Sojaprodukts konnten wir eine verbessertes Funktion des Endothels, des manuellen Blutdrucks und der Serum-LDL-Cholesterinkonzentrationen im Vergleich zur Kontrollbedingung (ausgewogenen Ernährung ohne Soja) feststellen. Die Endothelfunktion, bewertet durch die flussvermittelte endothelabhängige Vasodilatation (FMD, „flow-mediated dilatation“) verbesserte sich um 1.49 Prozentpunkte (pp), eine Verbesserung die mit einer Verringerung des kardiovaskulären Risikos von etwa 12% in Zusammenhang gebracht wird. Zusätzlich verbesserte der Soja-Nuss Konsum den manuell gemessenen Blutdruck (SBP: -4 mmHg und DBP: -2 mmHg) und den mittleren arteriellen Druck (-3 mmHg), während die LDL-Cholesterinkonzentrationen um 0.17 mmol/l reduziert wurden. Die Gefäßelastizität blieb hingegen unverändert. Aus dieser Studie schließen wir, dass eine längerfristige tägliche Einnahme von gerösteten

Sojabohnen die Gefäßfunktion und die kardiometabolischen Risikomarker bei älteren Erwachsenen verbessert, was zu den positiven Auswirkungen einer pflanzlicher Ernährung auf das Risiko der Entwicklung von Herz- und Gefäßerkrankungen beitragen kann.

Zusammenfassend liefert diese Doktorarbeit, auf der Grundlage von zwei Interventionsstudien am Menschen, weitere Beweise dafür, dass eiweißreiche Produkte in der Ernährung eine Rolle bei der Prävention nicht übertragbarer Erkrankungen spielen können. Während gezeigt wurde, dass eine eiweißreiche Ernährung das Hunger- und Sättigungsprofiel beeinflusst, möglicherweise vermittelt durch erhöhte 2-AG und PYY-Konzentrationen, wurden keine Auswirkungen auf die kardiometabolischen oder vaskulären Gesundheitsparameter gefunden. Die längerfristige tägliche Einnahme von gerösteten Sojabohnen konnte hingegen die Endothelfunktion, den Blutdruck und die LDL-Cholesterinkonzentrationen im Serum verbessern. Diese Parameter stellen gleichzeitig mögliche Mechanismen dar, durch die Sojaprodukte letztendlich das Risiko auf die Entwicklung von Herz- und Gefäßerkrankungen verringern können.

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Lea Tischmann was born on the 10th of January 1991 in Gießen (Germany). She completed her secondary school and education as Biological Technical Assistant (BTA) at Maria-Stemme Berufskolleg Bielefeld in 2010.

In 2011, she moved to the Netherlands to study Biomedical Sciences at Maastricht University. During her under-graduate studies, she became interested in nutrition and the physiology of the human body and chose a minor in Biological Health. After completing an internship for her thesis at the Department of Human Biology at Maastricht University, she graduated in 2014. Following the bachelor's degree, Lea enrolled in the MSc Biological Sciences in Maastricht, during which she did an internship at Karolinska Institute in Stockholm and an internship in the Department of Internal Medicine at Maastricht University. In 2016, she received her master's degree with a focus on Nutrition and Metabolism and continued working on her thesis project as a research assistant in the Department of Internal Medicine.

In January 2017, Lea started her PhD in the Department of Nutrition and Movement Sciences at Maastricht University under the supervision of Prof. dr. Ronald Mensink, Dr. Tanja Adam, and Dr. Peter Joris. Her PhD research focused on the effects of a high-protein diet and soy nuts on various vascular and metabolic health markers in humans. Lea has presented her research at multiple national and international conferences. She received the Foppe ten Hoor Young Investigator Award 2020 for presenting her work on the effects of soy consumption on cardiovascular health in older adults.

LIST OF PUBLICATIONS

Published manuscripts

Drummen M*, **Tischmann L***, Gatta-Cherifi B, Cota D, Matias I, Raben A, et al. Role of endocannabinoids in energy balance regulation in participants in the post-obese state - A PREVIEW study. J Clin Endocrinol Metab. 2020. **These authors contributed equally*

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Drummen M, **Tischmann L**, Gatta-Cherifi B, Raben A, Adam T, Westerterp-Plantenga M. Reproducibility and associations with obesity and insulin resistance of circadian rhythm parameters, in free-living vs. controlled conditions during the PREVIEW lifestyle study *(Submitted)*

Tischmann L, Adam TC, Mensink RP, Joris PJ. Longer-term soy nut consumption improves vascular function and cardiometabolic risk markers in older adults: Results of a randomized, controlled cross-over trial. *(To be submitted)*

Kleinloog J, Tischmann L, Adam TC, Mensink RP, Joris PJ. Longer-term soy consumption improves cerebral blood flow and psychomotor speed: Results of a randomized, controlled cross-over trial in older males and females. *(In preparation)*