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The effect of walnut consumption on lipid and glucose metabolism, adipokines, C-reactive protein, endothelial function, body weight and blood pressure in healthy men and healthy postmenopausal women

Dissertation

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Declaration

I hereby declare that this thesis is my original work.

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ABBREVIATIONS

AA	arachidonic acid
ALA:	alpha-linolenic acid
AUC:	area under the curve
ApoB:	apolipoprotein B
BMI:	body mass index
CHD:	coronary heart disease
CRP:	C-reactive protein
CVD:	cardiovascular disease
DHA:	docosahexaenoic acid
EPA:	eicosapentaenoic acid
FMD:	flow-mediated dilatation
fRHI:	Framingham reactive hyperemia index
HDL:	high-density lipoproteins
HOMA-IR:	homeostasis model assessment estimate of insulin resistance
iAUC:	incremental area under the curve
ICAM-1:	intercellular adhesion molecule-1
IDL:	intermediate-density lipoproteins
LA:	linoleic acid
LDL:	low-density lipoproteins
MUFA:	monounsaturated fatty acids
NO:	nitric oxide
PAT:	peripheral arterial tonometry
PREDIMED:	Prevención Dieta Mediterranea
PUFA:	polyunsaturated fatty acids
QUICKI:	quantitative insulin-sensitivity check index
RHI:	reactive hyperemia index
SEM:	standard error of the mean
SFA:	saturated fatty acids
VCAM-1:	vascular cell adhesion molecule-1
VLDL:	very-low-density lipoproteins

1   INTRODUCTION

1.1   Cardiovascular disease: epidemiology, pathophysiology, and risk factors

Cardiovascular disease (CVD) is the leading cause of death worldwide. In 2008 alone, 17.3 million people died of CVD, accounting for 31% of all major causes of deaths. Over the past two decades, disparate trends in CVD mortality have emerged in different regions of the world. While there has been a decline in CVD mortality in high-income countries as a result of improvement in treatment options and preventive strategies, a staggering increase has been observed in medium- and low-income countries. What was once considered a disease of the elderly is now also plaguing a much younger population, with an astonishing 42% of CVD deaths occurring under the age of 60 in developing countries. CVD continues to be a global public health and socioeconomic issue. In most cases, CVD can be prevented, but the rise in the disease incidence indicates that adequate preventive measures are still lacking (1, 2).

The major pathological process underlying CVD is atherosclerosis, which is characterized by thickening and hardening of the arterial wall due to the formation of atheromatous plaques. The lesions develop slowly over the course of many years before any clinical symptoms become apparent. The disease process is complex and involves chronic inflammation and fibroproliferation commonly in the presence of high levels of plasma cholesterol (3). The initial step in the development of atherosclerosis is described by the widely accepted "response-to-injury" hypothesis, which postulates that the formation of atheromatous plaques is the result of some form of damage to the endothelium (4), a thin layer of endothelial cells covering the luminal surface of blood vessels. The endothelium acts as a selective permeability barrier and a metabolically active interface between the circulating blood and the arterial wall (5). It releases a number of vasoactive substances, prevents thrombosis, regulates vascular tone and growth, and modulates inflammatory responses (6).

Different forms of physical and chemical insults, including hypertension, smoking, dyslipidemia, and hyperglycemia, can promote endothelial dysfunction (7). As a result, the endothelium decreases the production of vasodilators, most notably nitric oxide (NO), increases the release of vasoconstrictors, such as endothelin-1 (5), and enhances the expression of various endothelial adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Consequently,

arteries experience prolonged constriction, and inflammatory cells bind to the endothelium via adhesion molecules, triggering a series of inflammatory processes (**Figure 1**) (7).

Early visible manifestations of atherosclerosis are known as fatty streaks (**Figure 2**), which can be detected in early childhood. Fatty streaks are patchy deposits of cholesterol and cholesterol ester in the intimal layer of arteries (8). They are formed by an aggregation of lipid-laden macrophages, also known as foam cells. Fatty streaks differ from advanced atherosclerotic lesions in that the intimal architecture and the adjacent media remain largely unaffected. However, in more advanced stages, the formation of atheromatous plaques modifies the intima, the media, and even the adventitia, leading to the narrowing of arterial lumen (**Figure 3**) (9).

Atheromatous plaques are formed by the gradual accumulation of foam cells in the subintimal layer of arteries. Both intra- and extracellular deposits of cholesterol and cholesterol ester make up the lipid core of the plaques. Various cells release inflammatory mediators that increase the adhesion of inflammatory cells, and lesions continue to grow as smooth muscle cells migrate to and proliferate in the intima and produce abundant extracellular matrix, forming the fibrous cap that provides stability to the plaques. Atherosclerosis remains clinically indolent until plaques significantly obstruct the arterial lumen, or until the fibrous cap weakens, causing plaques to rupture (**Figure 4**). Plaque rupture represents the most dangerous stage in the development of atherosclerosis. As the underlying lipid core is exposed to the blood, various procoagulant mediators are released and promote thrombus formation (4), which can lead to fatal heart attack or ischemic stroke (7).

Endothelial permeability | Leukocyte migration | Endothelial adhesion | Leukocyte adhesion
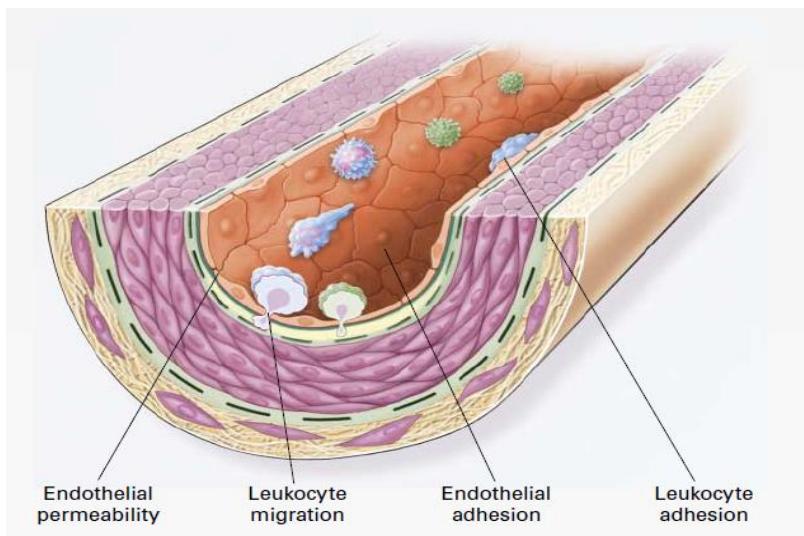
Figure 1. Endothelial dysfunction: increased endothelial permeability; upregulation of endothelial adhesion molecules; leukocyte adhesion and migration into the subintimal layer. (Source: Ross, R) (4)

Smooth-muscle migration | Foam-cell formation | T-cell activation | Adherence and aggregation of platelets | Adherence and entry of leukocytes
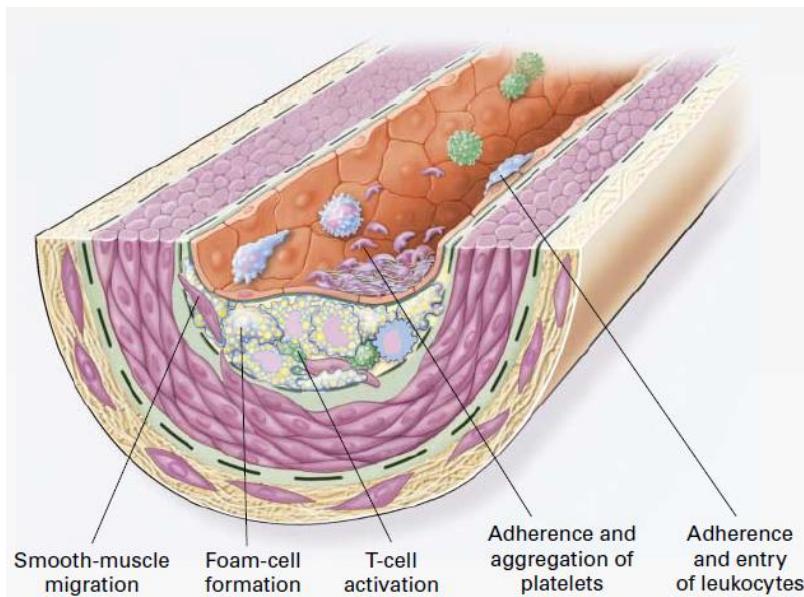
Figure 2. Fatty streak formation: aggregation of foam cells; smooth muscle cell migration. (Source: Ross, R) (4)

Macrophage accumulation   Formation of necrotic core   Fibrous-cap formation
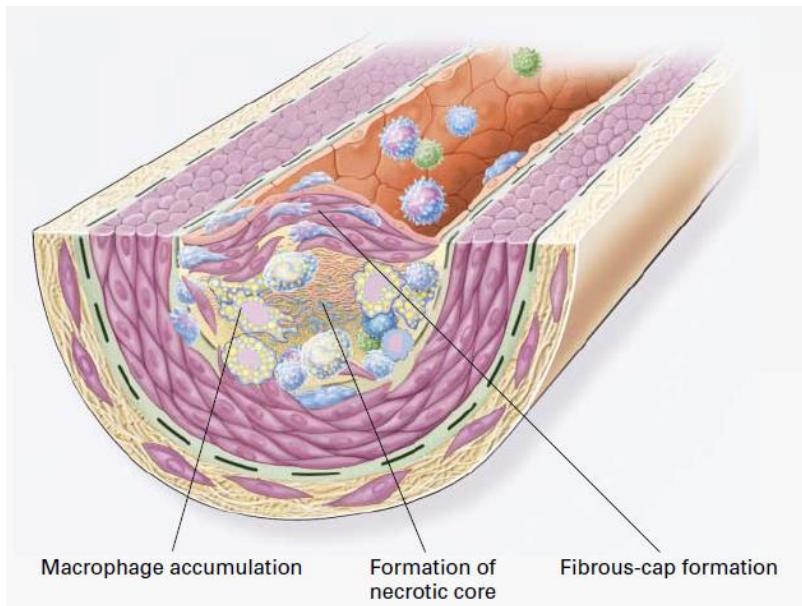
Figure 3. Advanced atherosclerotic lesions: fibrous cap and necrotic core (apoptosis, necrosis, and lipid accumulation). (Source: Ross, R) (4)

Plaque rupture   Thinning of fibrous cap   Hemorrhage from plaque microvessels
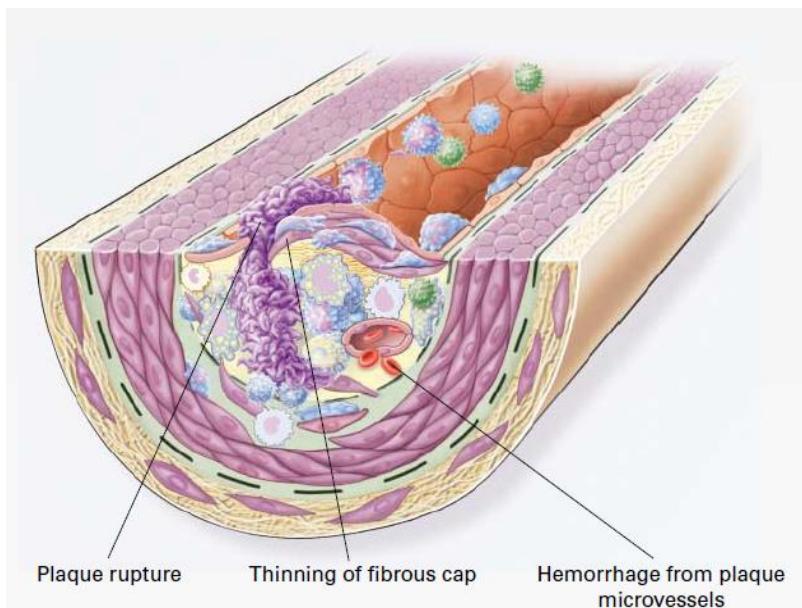
Figure 4. Unstable atheromatous plaques: thinning of the fibrous cap and plaque rupture. (Source: Ross, R) (4)

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Major risk factors of the atherosclerotic disease include positive family history, smoking, obesity, diabetes, hypertension, and dyslipidemia (10). The clustering of central obesity, impaired fasting glucose, hypertension, and dyslipidemia has been termed the metabolic syndrome, which is defined as a constellation of three or more of the conditions listed in **Table 1** (11)**.**

Table 1. Components of the metabolic syndrome.

Risk Factor	Defining Level
Abdominal Obesity	Waist Circumference
Men	>102 cm
Women	>88cm
Triglycerides	≥150 mg/dL
HDL cholesterol	
Men	<40 mg/dL
Women	<50 mg/dL
Blood pressure	≥130/85 mmHg
Fasting glucose	≥110 mg/dL

(Source: National Cholesterol Education Program) (11)

Among the common risk factors, dyslipidemia is an extensively studied and well-established independent risk factor of CVD. It is a condition in which plasma levels of lipids and lipoproteins are abnormal. Considering the central role lipids play in the pathogenesis of atherosclerosis, dyslipidemia is a crucial treatment target in the management of CVD (11).

Due to the hydrophobic nature of lipids, they are carried by specific transport vehicles known as lipoproteins in the circulatory system. There are several classes of lipoproteins, which differ from one another in their triglyceride and cholesterol content, protein components, and metabolic pathways. Major transporters of triglycerides are very-low-density lipoproteins (VLDL) and chylomicrons. While VLDL are synthesized in the liver, chylomicrons are derived from the intestine after fatty meals and carry postprandial triglycerides via the lymphatic system to the blood. The triglycerides carried by chylomicrons are released by the action of lipoprotein lipase, an enzyme present on the surface of the endothelium. This leads to the formation of chylomicron-remnant particles, which are smaller in size but still triglyceride-rich. The remnant particles are cleared from the circulation by binding to specific receptors in the liver. VLDL also undergo a series of delipidation, which results in the

formation of intermediate-density lipoproteins (IDL) and ultimately low-density lipoproteins (LDL) (12).

LDL are major plasma transporters of cholesterol and cholesterol ester. Normally LDL are removed from the bloodstream by binding to LDL receptors, which are located in the liver and extrahepatic tissues. LDL not removed by LDL-receptors can bind to scavenger receptors on macrophages and induce the formation of foam cells (13).

High-density lipoproteins (HDL) are another major group of lipoproteins synthesized in the intestine and the liver. A key function of HDL is reverse cholesterol transport, which describes the transport of cholesterol from peripheral tissues back to the liver (14). Epidemiological studies have consistently demonstrated an inverse relationship between HDL-cholesterol levels and CVD. Reducing the cholesterol load in peripheral cells is one of many potential cardioprotective effects of HDL. HDL may also show antioxidative, anti-inflammatory, and endothelium-dependent vasodilatory effects (15).

While high levels of HDL may reduce the risk of CVD, an increase in all other lipoproteins (VLDL, IDL, LDL, triglyceride-rich remnants, and lipoprotein(a)), which are collectively known as non-HDL, is considered proatherogenic. A common feature of all non-HDL particles is that each carries an apolipoprotein B (apoB) (16). There are two different apoB molecules: apoB-100 and apoB-48. ApoB-100 is synthesized in the liver and is present on all non-HDL particles except for chylomicrons, which carry apoB-48 synthesized exclusively in the intestine. ApoB-100 mediates the binding of non-HDL to LDL receptors for clearance from the bloodstream (17). Among the circulating non-HDL, LDL have the strongest link to atherosclerosis. Their atherogenic properties have been confirmed by studies examining individuals with genetic disorders causing very high levels of LDL (familial hypercholesterolemia). It has been shown that lowering LDL levels does not only prevent the formation of atherosclerotic plaques but also confer more stability to existing plaques (11).

It was long believed that triglycerides are not directly implicated in the development of atherosclerotic lesions; however, triglyceride-rich lipoproteins, which also carry apoB, may induce the development of atherosclerotic lesions similar to LDL. Moreover, humans spend most of the day in a non-fasting state; non-fasting triglycerides act as a surrogate marker for non-fasting remnant cholesterol (18). Varbo et al. examined the causal relationship between

increased remnant cholesterol levels and risk for coronary heart disease (CHD) using data from three large Danish studies. The analysis showed that for every 1 mmol/L increase in non-fasting remnant cholesterol, the risk of ischemic heart disease was increased by 2.8 fold, independent of HDL reduction (19). Two epidemiological studies have shown that low levels of non-fasting triglycerides as a result of genetic mutations are associated with a decreased risk of CHD (20, 21).

Lipid abnormalities do not commonly appear as isolated metabolic disturbances but are frequently accompanied by other CVD risk factors such as obesity and diabetes. Obesity is a prevalent health issue that has reached epidemic proportion. The Framingham Heart Study was among the first epidemiological studies showing a causal relationship between obesity and CVD (22); however, the underlying mechanisms, which involve diverse cellular pathways, are complex and only partially understood. Obesity is closely associated with the other risk factors of the metabolic syndrome. In fact, it has been regarded as the predominant risk factor in the development of the metabolic syndrome (11). The association between obesity and type-2 diabetes has been extensively studied. The risk of developing type-2 diabetes is positively associated with body mass index (BMI). The disease risk increases exponentially at BMI > 25 mg/m^2 (23, 24). Type-2 diabetes is in itself an independent risk factor of CVD (25).

The link between obesity and CVD appears to relate to the secretory function of adipose tissue. Adipose tissue cannot simply be regarded as an inert storage of triglycerides; it is also a secretory organ. In addition to releasing free fatty acids, adipocytes synthesize and secrete numerous proteins that function as enzymes, cytokines, growth factors, and hormones. Collectively, these protein factors are known as adipokines, which participate in the regulation of diverse metabolic processes and the overall energy balance (26). A number of adipokines and their key metabolic functions are listed in **Table 2** (27)**.**

Many adipokines exhibit proinflammatory properties and antagonistic functions in the insulin signaling pathways, inducing endothelial dysfunction and insulin resistance. Adiponectin is an adipokine with anti-inflammatory effects and is inversely related to insulin resistance and C-reactive protein (CRP) level. While the level of most adipokines increases in the presence of excess adipose tissue, the production of adiponectin has been shown to decrease in the obese state (26). This imbalance between the secretion of pro-inflammatory and anti-

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inflammatory adipokines in obesity contributes to a chronic, low-grade inflammatory state, which causes disturbances in metabolic homeostasis and triggers CVD. This is not only observed in visceral adipose tissue but also in perivascular adipose tissue, which is an emerging novel factor that modulates vascular function. The upregulation of pro-inflammatory adipokines and the downregulation of anti-inflammatory adipokines in perivascular adipose tissue have been shown to promote the migration and activation of inflammatory cells and the migration and proliferation of vascular smooth muscle cells across the endothelium (28).

Table 2. Adipokines and their metabolic functions.

Adipokines	Metabolic Functions
Adiponectin	Suppression of hepatic gluconeogenesis Stimulation of fatty acid oxidation in liver and skeletal muscle Stimulation of glucose uptake in skeletal muscle Stimulation of insulin secretion Modulation of food intake and energy expenditure
Leptin	Repression of food intake Promotion of energy expenditure Stimulation of fatty acid oxidation in liver, pancreas, and skeletal muscle Modulation of hepatic gluconeogenesis Modulation of pancreatic β-cell function
Chemerin	Enhancement of insulin-stimulated glucose uptake and insulin receptor substrate-1 phosphorylation in 3T3-L1 adipocytes
Visfatin	Stimulation of insulin secretion in mice Uncertain effect on insulin resistance in rodents and humans
Vaspin	Improvement of insulin sensitivity in mice Uncertain effect on insulin sensitivity in humans

(Source: modified Rabe, K) (27)

Inflammation plays a key role in the formation of atherosclerotic lesions. Chronic inflammation is closely associated with the development and progression of CVD. CRP is an acute phase reactant that is mainly produced by hepatocytes in response to the production of proinflammatory cytokines. CRP forms a complex with atherogenic oxidized LDL and can be found within atheromatous plaques. Although CRP is a nonspecific marker of inflammation, it has been regarded as a novel biomarker of CVD. However, due to the inconclusive data, its clinical utility in the risk stratification of CVD still remains unclear (29).

Excluding genetic factors, most of the above-mentioned CVD risk factors are modifiable through lifestyle intervention. Dietary modification is an indispensable part of CVD prevention and management. The amount and the types of food eaten have great influence on a person's health. In the last half-century, there has been a growing interest in identifying healthy dietary patterns and finding so-called "functional foods". The Western-type diet is commonly regarded as unhealthy due to the high intake of red and processed meats, refined grains, and sugary foods and beverages. This dietary pattern has been associated with increased risk of obesity, type-2 diabetes, CVD, and certain types of cancer (30, 31). In contrast, a traditional Mediterranean diet is widely recognized as a healthy dietary pattern. It is characterized by large amounts of vegetables, fruit, legumes, nuts, and olive oil, as well as moderate consumption of fish, poultry, dairy products, and wine, with low consumption of red meat (32). Close adherence to a Mediterranean diet is linked to reduced CVD incidence and mortality (33). This finding has prompted many studies to closely examine specific components of the Mediterranean diet. Among the most studied dietary components are nuts. In the past two decades, ample studies have explored the potential health benefits of nut consumption (34, 35). Walnuts are among the most studied tree nuts because of their high nutritional value and unique composition.

1.2   Walnuts: historical relevance, nutritional value, and health benefits

Walnuts are among the oldest tree nuts known to mankind. Records of their existence date back as far as 7000 BC (36). Walnuts belong to the genus Juglans of the botanical family Juglandaceae. Of all walnut species, Juglans regia is the most cultivated (37).

The ancient Greeks were the pioneers of walnut cultivation. They did not only regard walnuts as a source of nutrients but also used them as medicine and dyes. In ancient times, walnuts were thought to have many healing properties. Some believed that walnuts had astringent and stomachic effects and could stimulate appetite and improve digestion. Even walnut husk and bark could be used to treat inflammation, pain, and bowel ailments. Also, walnut milk was a nutritious alternative to dairy products (36). Perhaps the historical importance of walnuts is best described by the meaning of their scientific name: "Juglans" is the Latin term for "nut of Jupiter" and "regia" means "royal". In Greek mythology, Jupiter is the king of the gods. The name reflects the ancient belief that walnuts were "the choice of the gods" (38). Casas-Agustench et al. mentioned an old tale: "in ancient days when men lived upon acorns, the gods lived upon walnuts" (36).

In modern times, the potential health benefits of walnuts have not been overlooked either. For decades, scientists have been trying to determine the favourable effects of walnut consumption and identify the components of walnuts that elicit these effects. Walnuts are considered energy-dense food and provide approximately 654 kcal/100g serving (39); however, the actual metabolizable energy content of walnuts is lower than the estimated energy content (40). Although the main constituent of walnuts is fat, the fatty acid composition is considered healthy, because unsaturated fatty acids predominantly constitute the total fat content, whereas the amount of saturated fatty acids (SFA) is low. Walnuts have a unique fatty acid distribution that distinguishes them from other nuts. While most nuts are high in monounsaturated fatty acids (MUFA), walnuts are primarily composed of polyunsaturated fatty acids (PUFA: 47% of total weight), mainly linoleic acid (LA) and alpha-linolenic acid (ALA) (39). Of all edible plants, walnuts may have the highest content of ALA (9%), and compared with other nuts, they are the only nuts with a significant amount ALA (**Figure 5**) (41).

There is evidence showing an inverse relationship between ALA intake and death from CHD. Pan et al concluded in a meta-analysis that each increment of 1 g/d intake of ALA was associated with a 10% risk reduction for CHD death (42). Contrary to existing data showing a consistent inverse relationship between ALA intake and CVD, Marklund et al. observed in a prospective cohort study involving 60-year-old Swedish men and women without prior CVD that plasma ALA level determined at baseline was linked to moderately elevated CVD risk in women. The authors discussed that this finding does not conclude that ALA intake raises CVD for several reasons: Serum ALA in cholesterol esters may not be a strong biomarker of dietary consumption when compared with other PUFA in part due to its extensive metabolism via β-oxidation and conversion to long-chain PUFA. Also, the results may be confounded by the high amount of trans-fat or saturated fat found in margarine and spreads that were sources of ALA (43).

ALA is a plant-based essential omega-3 fatty acid that has also been shown to elicit anti-inflammatory and anti-atherogenic effects (44, 45). It is also the precursor of endogenous synthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA are long-chain n-3 PUFA that are typically found in oily fish. Intake of long-chain n-3 PUFA has been associated with a decrease in triglyceride levels (46). Meta-analyses of randomized

control trials have shown that EPA and DHA may have antihypertensive effects (47) and may improve endothelial function (48). Moreover, an inverse relationship between serum EPA and DHA intake and all-cause mortality was observed in senior Swedish men and women (43).
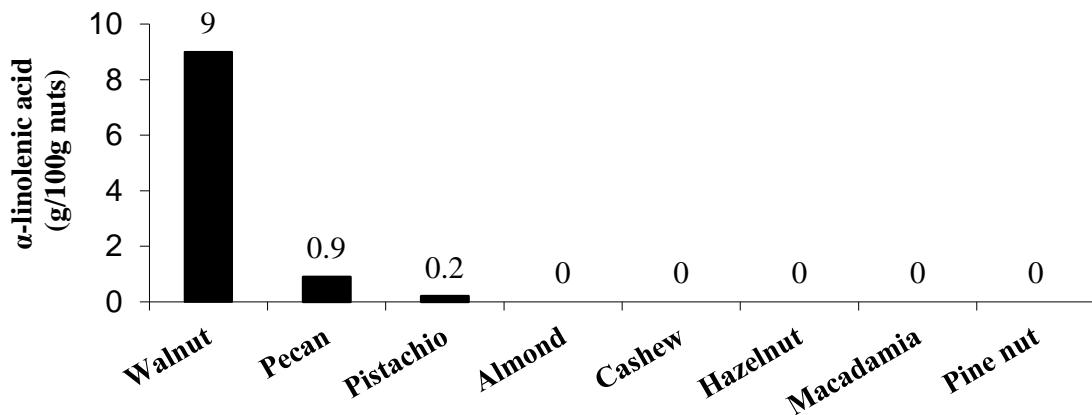
Figure 5. Alpha-linolenic acid content in 100 g nuts. (Data from source: Robbins KS) (41)

Like all plants, walnuts are cholesterol-free. However, they contain compounds that are structurally similar to cholesterol known as phytosterols. Phytosterols are non-nutritive, and their intestinal absorption is poor (35). Yet there is evidence indicating that phytosterols may inhibit the cholesterol uptake in the intestine (49).

In addition to the favourable lipid profile, walnuts also contain many other potentially cardioprotective nutrients. The major nutrients in walnuts are listed in **Table 3** (39). Walnuts are a good source of plant protein and dietary fiber (35). They contain relatively high quantities of L-arginine, an amino acid that acts as the precursor for the biosynthesis of NO (50). A number of vitamins needed for proper cellular function are also found in walnuts. For example, folate, also known as vitamin B_9, is essential for various metabolic functions. Humans need to obtain the required amounts of folate through diet because de novo synthesis is not possible. Folate plays a vital role in DNA synthesis and repair (51). Moreover, it controls the plasma homocysteine levels, an amino acid produced in the metabolism of methionine and linked to increased cardiovascular risk (52).

Walnuts also contain sizable amounts of γ-tocopherol, which is a form of vitamin E that exhibits antioxidant activities and inhibits the proliferation of smooth muscle cells, an important process in the development of atherosclerosis. Furthermore, γ-tocopherol elicits anti-inflammatory effects by inhibiting the activity of cyclooxygenase-2. Although

γ-tocopherol may be much less studied than α-tocopherol, some evidence suggests an inverse relationship between γ-tocopherol concentration and CVD (53).

Table 3. Major nutrients in 100 g of walnuts.

Nutrients	Amount
Energy (kcal)	654[a]
Carbohydrate (g)	14
Protein (g)	15
Arginine (g)	2.3
Lipid (g)	65
Saturated fatty acids (g)	6
Monounsaturated (g)	9
Polyunsaturated (g)	47
Minerals	
Na (mg)	2
K (mg)	441
Ca (mg)	98
Mg (mg)	158
Fe (mg)	2.91
Se (μg)	4.9
Vitamins	
α-tocopherol (mg)	0.7
γ-tocopherol (mg)	20.83
Folate (μg)	98
Phytosterols (mg)	92
Dietary fiber (g)	6.7

(Source: US Department of Agriculture National Nutrient Database for Standard Reference, Release 24) (39); [a] newer data indicate that metabolizable energy may be lower (40).

Walnuts are also rich in polyphenols, which are antioxidants with the ability to scavenge free radicals and regulate metal-chelating reactions. Studies have shown that polyphenols may protect LDL from oxidative modifications (54) and prevent endothelial dysfunction by modulating NO and endothelin-1 synthesis (55), reducing the risk of atherosclerosis. Interestingly, polyphenols can also increase the antioxidant activities of other compounds such as vitamin C, vitamin E, and β-sitosterol. Compared with other tree nuts and peanuts, walnuts have the highest amount of polyphenols (56).

In addition, the composition of minerals in walnuts is also considered beneficial. Walnuts have a low sodium content (35). Excess sodium intake is associated with high blood pressure and a number of other adverse health conditions. Approximately 75% of the daily salt consumption is obtained from processed foods. Intake of foods with high sodium content can displace foods containing other valuable minerals such as calcium, potassium, and magnesium (57). In walnuts, the relation of sodium to the other minerals (calcium, potassium, and magnesium) is considered favourable (35).

Walnuts with their complex mixture of macronutrients and micronutrients have been shown to have beneficial effects on many aspects of health. In animal models, walnuts have been demonstrated to improve cognitive functions (58, 59) and reduce the risk of certain types of cancer (60, 61). The potential cardioprotective effects of walnuts have been most extensively researched in humans.

Epidemiological studies have consistently shown an inverse relationship and dose-dependent effects with respect to nut consumption and risk of CVD. A pooled analysis of four large cohort studies (Adventist Health Study, Iowa Women's Health Study, Nurses' Health Study, and Physicians' Health Study) conducted in the U.S. demonstrated that subjects with the highest nut consumption had a 37% reduction in the risk of CHD mortality. Furthermore, the study showed that the risk of CHD mortality decreased by 8.3% for each weekly serving of nuts (approximately 30 g) (62).

The Prevención Dieta Mediterranea (PREDIMED) study is a landmark primary prevention trial that examined the cardioprotective effects of a Mediterranean diet supplemented either with nuts (30g mixed nuts/d, 50% walnuts) or olive oil. This multicenter study conducted in Spain included 7447 subjects between the age of 50 and 88 years who were at high risk of developing CVD but did not have CVD at the start of the study. After a median follow-up of 4.8 years, the Mediterranean diets supplemented with mixed nuts or olive oil decreased cardiovascular events by about 30%, and the nut-enriched diet decreased the risk of stroke by 49% when compared with a low-fat control diet (63). In an earlier randomized, controlled, secondary prevention trial, the Lyon Heart Study, a Mediterranean diet rich in ALA, which is present in a significant amount in walnuts, was shown to significantly reduce recurrent coronary events in subjects with a first myocardial infarction (64).

Perhaps the most widely studied CVD risk factors are plasma lipids and lipoprotein levels (56). One of the first clinical studies that investigated the effects of walnuts on plasma lipoprotein levels was published by Sabaté et al in 1993. Eighteen healthy men between the age of 18 and 43 years followed two experimental diets for a total of eight weeks. Both diets had the same composition except that one diet replaced some foods with walnuts (20% of total calorie intake). Results showed that the walnut diet reduced total cholesterol by 12% and LDL-cholesterol by 16%. Although HDL-cholesterol also decreased, the total cholesterol to HDL-cholesterol ratio decreased as well (65). Subsequent clinical studies have consistently shown that walnuts can lower LDL-cholesterol levels by 9-16% in a dose-dependent manner. Beyond the improvement of plasma lipoprotein levels, short-term feeding trials have also demonstrated that frequent walnut consumption improves other traditional CVD risk factors such as insulin resistance and hypertension as well as novel risk factors such as oxidative stress, inflammation, and vascular reactivity (56).

1.3   Aims of the study

The majority of previous feeding trials with walnuts were conducted in subjects at increased risk of developing CVD (risk factors such as metabolic syndrome, type-2 diabetes, and hypercholesterolemia) (66). A few studies involving healthy individuals were selected from a younger age group (< 50 years) (65, 67-69), and in some cases, there was an underrepresentation of women (65, 69). It is important to note that the metabolism of premenopausal women differs from that of postmenopausal women. There is evidence showing a sudden rise in CVD incidence after menopause. Thus, premenopausal women may have a different risk profile than postmenopausal women (70). In addition, many studies focused primarily on the effect of walnuts on fasting lipid and glucose metabolism. There is a scarcity of evidence concerning postprandial metabolism (66). Therefore, this study aimed to examine whether the favourable changes in fasting lipid and glucose metabolism observed in previous studies would also apply to healthy Caucasian men and women of a higher age group ($\geq$ 50 years). Furthermore, the present study also aimed to analyze the effect of walnut consumption on postprandial lipid and glucose metabolism, circulating levels of adipokines and CRP, as well as endothelial function, body weight, and blood pressure.

2 MATERIALS AND METHODS

2.1 Study subjects

The study protocol was approved by the Ethics Committee of the Medical Faculty of Ludwig-Maximilians-University of Munich and was registered on https://clinicaltrials.gov (NCT01188902).

A total of 96 healthy men and healthy postmenopausal women aged 50 and above were interviewed and examined. All subjects were recruited through posters on hospital bulletin boards and in pharmacies, or via an article in a local newspaper and the hospital magazine "LMU Klinikum Aktuell". The screening evaluation involved obtaining subjects' medical history, a physical examination, and a fasting blood test. Inclusion criteria (**Table 4**) and exclusion criteria (**Table 5**) were used to determine the eligibility of each subject. Fifty-seven eligible men and women were recruited in the study.

Table 4. Inclusion criteria.

Inclusion Criteria
▪ healthy Caucasian men and healthy Caucasian postmenopausal women ▪ age ≥50 ▪ written informed consent prior to study participation

Table 5. Exclusion criteria.

Exclusion Criteria
▪ Evidence of alcohol (women >70 g/week, men >140 g/week), tobacco or drug abuse ▪ Obesity ≥35 kg/m^2 ▪ Diabetes mellitus ▪ Hypertension >140/90 mmHg or history of hypertension ▪ LDL- cholesterol >190 mg/dL, Triglycerides >350 mg/dL ▪ History of atherosclerotic disease ▪ Liver disease of any etiology ▪ Kidney disease of any etiology (GFR ≤50 ml/min/1.73) ▪ Uncontrolled thyroid disease or other endocrine diseases ▪ Acute or chronic inflammatory diseases ▪ Active malignancy ▪ Current or previous (within 3 months) treatment with antidiabetic drugs, hypolipidemic drugs, antihypertensive drugs, anti-inflammatory drugs, vitamin E, hormonal replacement therapy ▪ Known allergy to nuts and milk proteins; lactose intolerance

2.2 Study design

The study employed a randomized, controlled, prospective, cross-over design (**Figure 6**). Each subject followed a nut-free, Western-type diet during a two-week run-in period. Thereafter, subjects were randomized to two different diet phases, each lasting for eight weeks and separated by a two-week wash-out period. A group of 28 subjects first followed a diet with walnuts and then switched to a control diet. Another group of 29 subjects followed the two diets in reverse order.
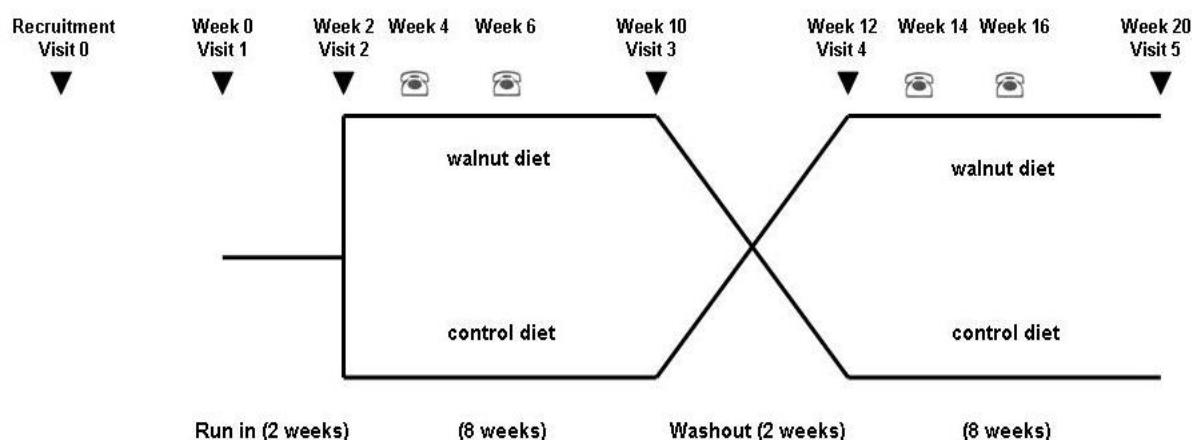
Figure 6. Study design.

2.2.1 Visit 0

Potential study subjects were interviewed in the outpatient clinic Medizinische Poliklinik II in Klinikum Großhadern. After obtaining subject's medical history, a physical examination was performed. Each subject received detailed information regarding the procedures of the study and the possible risks involved: bruise and slight pain at the site of venipuncture, temporary paresthesia in the arm after the assessment of endothelial function, and first manifestation of nut allergy. After obtaining informed consent, fasting blood samples were drawn to determine blood count and parameters of fasting lipid and glucose metabolism, liver, kidney, and thyroid function as well as CRP. Test subjects were contacted by phone to inform them of their eligibility. They were instructed to keep a three-day diet protocol and submit it prior to the next visit. The purpose of this protocol was to assess each subject's dietary habits and to allow subjects to familiarize themselves with the process of completing a diet protocol.

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2.2.2 Visit 1

Test subjects attended a consultation session with a nutritionist, who evaluated each subject's dietary habits using the three-day diet protocol completed prior to visit 1. Strict calorie restrictions were not suggested due to the differences in the individual daily energy output, which largely depends on age, gender, BMI, and the level of physical activity. However, adherence to a balanced Western-type diet consisting of 35 % fat (15 % SFA), 15 % protein and 50 % carbohydrate was requested. Subjects were instructed to complete a four-day diet protocol and submit it prior to each of the subsequent visits.

2.2.3 Visit 2, 3, 4, 5

Subjects arrived at the site after fasting for at least eight hours. Upon arrival, a brief medical history was obtained. Thereafter, subjects underwent a series of tests consisting of a physical examination, a mixed meal test and a noninvasive assessment of the endothelial function. An indwelling venous cannula was inserted to draw fasting blood samples (at 0 minute) and postprandial blood samples at 15, 30, 60, 120, 180, 240, 360, and 480 minutes after drinking the test drink. The analyzed parameters are shown in **Table 6**. A noninvasive assessment of the endothelial function using EndoPat 2000 was performed 240 min postprandially at noon to avoid potential confounding by the early morning blunting in endothelial function as reported previously (71). Subjects met with the nutritionist again on each visit. Dietary adherence was assessed using a four-day diet protocol.

Table 6. Analyzed blood parameters.

Fasting blood parameters	Postprandial blood parameters
▪ **lipid metabolism:** total cholesterol, total triglycerides, LDL-C, HDL-C, VLDL-C, VLDL-triglycerides, free fatty acids, apoB ▪ **glucose metabolism:** glucose, HbA1c, insulin, QUICKI, HOMA-IR	▪ **lipid metabolism** (at 60 120, 240, 360, 480 min): total cholesterol, total triglycerides, LDL-C, HDL-C, VLDL-C, VLDL-triglycerides, Chylomicron-triglycerides, ▪ **glucose metabolism**: (at 15, 30, 60, 120, 180 and 240 min.), glucose, insulin

apoB: apolipoprotein B; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment estimate of insulin resistance; LDL-C: low-density lipoprotein cholesterol; QUICKI: quantitative insulin-sensitivity check index; VLDL: very-low-density lipoprotein cholesterol.

2.2.4 Dietary consultation

The nutritionist contacted subjects by phone in week 4, 6, 14 and 16 to inquire about their current health and medication status, possible problems involved with dietary adherence and the completion of diet protocols. Dietary compliance was assessed using the four-day diet protocol.

2.3 Nutrition

2.3.1 Diets

Subjects were asked to follow a Western-type diet (35 % fat, of which 15 % saturated fatty acids, 15 % protein, and 50 % carbohydrate) throughout the study. They were also requested to discontinue the consumption of products containing nuts, the use of fish oil and Vitamin E supplements, and to maintain their body weight within 2 kg of their baseline value. In the walnut phase, subjects were provided with a daily allowance of 43 g of shelled, prepackaged walnuts, the amount of which contained about 30 g of fat. Subjects were instructed to replace 30 g of saturated fat with the walnuts. Subjects were provided with handouts to assist them with the integration of walnuts into their diets. In the control phase, subjects were requested to continue the Western-type nut-free diet. Dietary adherence was closely monitored using a four-day (on three weekdays and one weekend day) diet protocols.

2.3.2 Mixed meal test

Two different test drinks (A and B) were provided to the test subjects. Fourteen subjects received test drink A, which contained 25 g olive oil and 200 ml Fresubin® original drink (Fresenius Kabi: 200 kcal, 15 % protein, 55 % carbohydrate, and 30 % fat). The drink had a total of 427 kcal which consisted of 67 % fat (absolute amount 32 g), 7 % protein, and 26 % carbohydrate. Thirty-two subjects received test drink B which consisted of 90 g Calshake® powder (Fresenius Kabi), 120 ml 30 % cream, 120 ml 3.5 % milk and 25 g corn oil. The drink had a total of 1104 kcal and consisted of 72 % fat (absolute amount 88 g), 4 % protein and 24 % carbohydrate.

2.4 Endothelial function

In this study, the endothelial function was assessed using a non-invasive peripheral arterial tonometry (PAT) device (EndoPat). EndoPat is a device that uses plethysmographic

bio-sensors to measure the arterial pulsatile volume changes (PAT Signal) in the distal two-thirds of the fingers. The bio-sensors measure changes in peripheral arterial tone elicited by a five-minute occlusion of the brachial artery in one arm using a standard blood pressure cuff. Reactive hyperemia is induced by the release of the pressure cuff. During this period of time, an endothelium-dependent flow-mediated dilatation (FMD) in the finger arteries is captured by the bio-sensors as an increase in the PAT Signal amplitude. The contra-lateral arm serves as a control to assess the non-endothelial mediated changes in vascular tone (72).

The examination took place 240 min after the mixed meal test in a quiet, thermoneutral room (20-25 °C). Subjects were asked to remove watches, rings, jewelry, and restrictive clothing that could impede blood circulation in the arms. If necessary, fingernails were trimmed to avoid damaging the internal membrane of the bio-sensors. Prior to the study, subjects were allowed to rest in a bed for at least 15 minutes to attain a relaxed cardiovascular steady-state and acclimatize to room temperature. The occlusion cuff was placed around the upper arm without the indwelling needle, and the same arm was used on all visits. The bio-sensors were placed on the same finger in both hands, either on the index or middle finger. During the 15-minute test, subjects were asked to refrain from speaking and moving. The baseline PAT signal was measured for 5 min. Thereafter, a pressure cuff on the test arm was inflated to 200 mmHg, or at least 60 mmHg above the systolic blood pressure, to occlude the brachial artery. The cuff was deflated after 5 min. The bio-sensors measured changes in PAT signal for a further 5 min. The reactive hyperemia index (RHI) (**Figure 7)** and the Framingham RHI (fRHI) were determined using methods previously described (73).

$$RHI= \frac{A}{B} / \frac{C}{D} \times Baseline\,Correction\,Factor$$
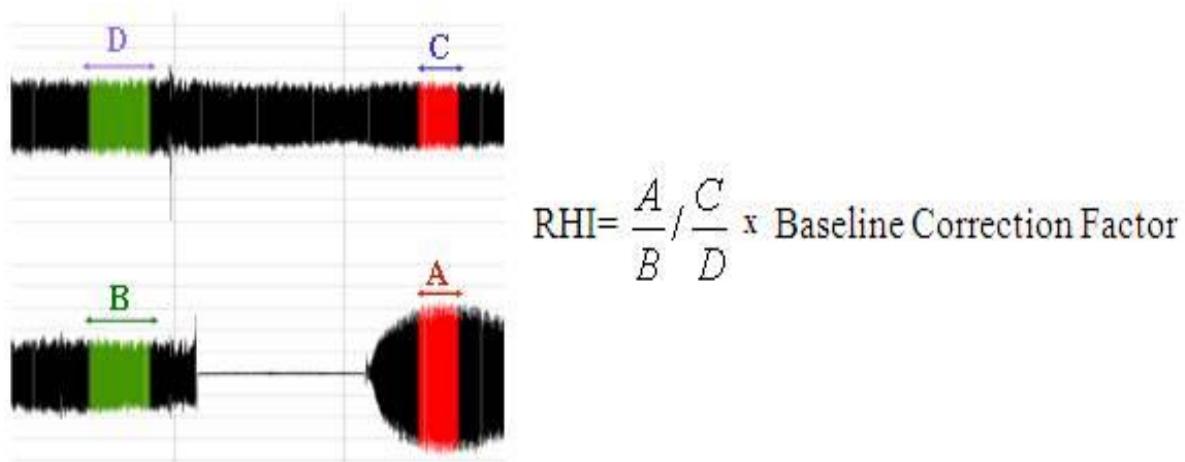
Figure 7. Reactive hyperemia index (RHI) calculation.
A: Mean PAT amplitude between 90s-150s post occlusion of the test arm. B: Mean PAT amplitude from the baseline period of the test arm. C: Mean PAT amplitude between 90s-150s post occlusion of the control arm. D: Mean PAT amplitude from the baseline period of the control arm. (Source: http://www.itamar-medical.com/EndoPAT™/FAQ) (74). PAT: peripheral arterial tonometry.

2.5   Laboratory analysis of blood samples

2.5.1   Lipid parameters

EDTA-coagulated blood samples were used to analyze lipid parameters. Plasma was obtained by centrifugation at 3.000 rpm for 10 minutes. Cholesterol and triglycerides in plasma as well as in lipoproteins were determined using enzymatic colorimetric methods at 37°C on an Alcyon 300 analyzer with reagents obtained from Diagnostic System. Total cholesterol and total triglycerides were quantified by placing plasma directly in Alcyon 300 analyzer.

Cholesterol

Cholesterol was quantitatively analyzed using the CHOD-PAP-method. First, cholesterol esterase catalyzed the hydrolysis of cholesterol esters to cholesterol and free fatty acids. Free cholesterol was then oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The formed hydrogen peroxide oxidatively coupled with phenol and 4-aminoantipyrine in the presence of peroxidase to yield a red coloured quinoneimine dye (Trinder reaction), which was measured spectrophotometrically at 500 nm.

Triglycerides

Triglycerides were quantitatively analyzed using lipoprotein lipase to hydrolyze triglyceride to glycerol and then followed by the quantification of glycerol. In the presence of ATP, glycerol was catalyzed to glycerol-3-phosphate and ADP. Glycerol-3-phosphate was then oxidized by glycerol-3-phosphate oxidase to dihydroxyacetone phosphate and H_2O_2. The quinoneimine dye was also formed in the Trinder reaction (see analysis of total cholesterol).

Chylomicrons, VLDL, HDL, LDL, non-HDL, and apoB

The individual lipoproteins (HDL, LDL, VLDL and chylomicrons) were first isolated by ultracentrifugation using a Beckman L-60 centrifuge in a type 50.4 Ti rotor. First, chylomicrons were isolated by adding 3 ml of plasma and 3.5 ml of NaCl solution at a density of 1.006 g/ml to a Beckman centrifuge tube, which was then centrifuged at 5°C at 20.000 rpm for 20 min. The chylomicron fraction, a thin layer floating on the top of the gradient, was collected. The rest of the plasma was then placed in a new centrifuge tube and 3 ml of the same NaCl solution was added. The tubes were centrifuged at 5°C at

50.000 rpm for 18 hours. The top fraction contained VLDL while the bottom fraction contained HDL and LDL.

The subnatant obtained from the ultracentrifugation was used to quantify HDL-cholesterol. First, 20 µl heparin and 30 µl manganese (II) chloride ($MnCl_2$) were added to 500 µl subnatant in a centrifuge tube. The mixture was placed in a refrigerator for 30 min and then centrifuged at 5ºC at 12.000 rpm for 15 min. Subsequently, the supernatant was collected and placed in the Alcyon 300 analyzer to determine the cholesterol content. LDL cholesterol was calculated by subtracting HDL cholesterol from the total cholesterol in the infranatant of the VLDL-spin. Non-HDL-cholesterol was calculated using the equation: *non-HDL-cholesterol = total cholesterol (mg/dl) - HDL-cholesterol (mg/dl)*. ApoB was determined by immunoturbidimetric methods using a commercially available reagent kit (DiaSys Diagnostic Systems, Holzheim, Germany).

2.5.2 Plasma fatty acids

Dietary compliance was analyzed using the diet protocols, which were evaluated with Prodi software version 5.8. A maximum of 20 % deviation from the dietary guidelines mentioned in section **2.3.1** was tolerated. The composition of fatty acids in plasma was used as a biological marker for dietary compliance and was determined by the research team of Prof. Dr. Berthold Koletzko at Dr. von Hauner Children's Hospital in Munich using methods previously described. In brief, 100 µl of plasma, 100 µl of an internal standard (1,2-dipentadecanoyl-sn-glycero-3-phosphocholine dissolved in methanol) and 0.6 ml methanol were combined in glass tubes and shaken for 30 s. Samples were centrifuged at 900 g for 5 min. The methanolic supernatant was transferred into another glass tube. Twenty-five µl sodium methoxide solution was added to the supernatant and the tubes were shaken while selective synthesis of methyl esters from glycerophospholipid fatty acids proceeded at room temperature. The reaction was stopped after 3 min by adding 75 µl methanolic HCl. Fatty acid methyl esters were extracted by adding 300 µl hexane and shaking the tubes for 30 s. The extraction was repeated. Combined extracts were dried under nitrogen flow at room temperature and individual fatty acid methyl esters were quantified using gas chromatography (75).

2.5.3 Other parameters

Parameters of glucose metabolism (glucose, insulin, HbA1c, HOMA-IR and QUICKI) and CRP were analyzed in the Department of Clinical Chemistry in Klinikum Großhadern. Glucose was quantified using the hexokinase method performed with a multi-channel

analyzer (Beckman Coulter AU2700). Automated sandwich immunoassays based on the principle of electrochemiluminescence (Roche Diagnostics) were implemented on a Roche Cobas e411 immunoanalzyer for the quantification of insulin. HbA1c was determined using the automated HPLC (high-performance liquid chromatography) method performed on a Bio-Rad Variant II HPLC system. HOMA and QUICKI indices were calculated from fasting glucose and insulin values using the following formulas:

HOMA = glucose (mg/dL) x insulin (µU/mL)/405,

QUICKI = 1 / [log(insulin µU/mL) + log (glucose mg/dL)].

The concentration of CRP was determined using a particle-enhanced immunoturbidimetric assay performed on a multi-channel analyzer (Beckman Coulter AU2700). Fasting concentration of adipokines, VCAM-1, ICAM-1, and endothelin-1 were determined using commercially available ELISA kits (adiponectin and leptin: Millipore, Billerica; chemerin, visfatin, and vaspin: BioVendor, Asheville; VCAM-1, ICAM-1 and endothelin-1: R&D Systems Europe, Abingdon, UK).

2.6   Area under the curve (AUC) and incremental area under the curve (iAUC)

Total area under the curve (AUC) and incremental area under the curve (iAUC) were used to analyze postprandial lipid and glucose metabolism. The trapezoidal rule was used to approximate the AUC (**Figure 8).** The iAUC was calculated by subtracting the baseline area from the total AUC. The baseline area is defined as the area under the fasting level. Two different calculations were used to determine the baseline area, depending on whether the fasting value was higher or lower than the postprandial value at 8h (**Figure 9** and **Figure 10**).
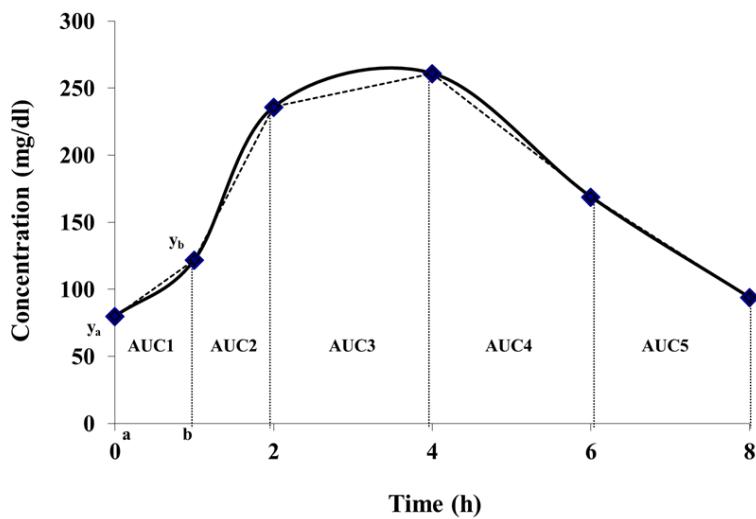
Figure 8. Area under the curve (AUC). The trapezoidal rule: *AUC 1 = (b-a) x (y$_a$+ y$_b$)/2,*
Total AUC = AUC 1 + AUC 2 + ACU 3 + AUC 4 + AUC 5
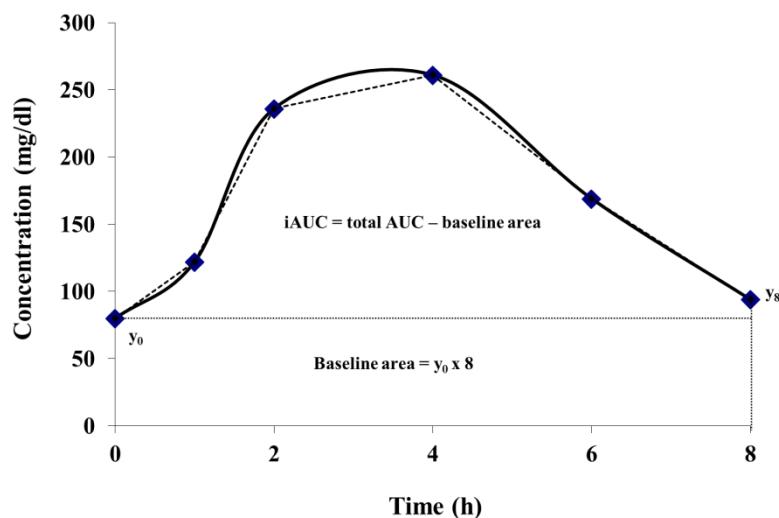
Figure 9. Incremental area under the curve (iAUC), if y$_0$ ≤ y$_8$:
iAUC = Total AUC – (y$_0$ x 8)
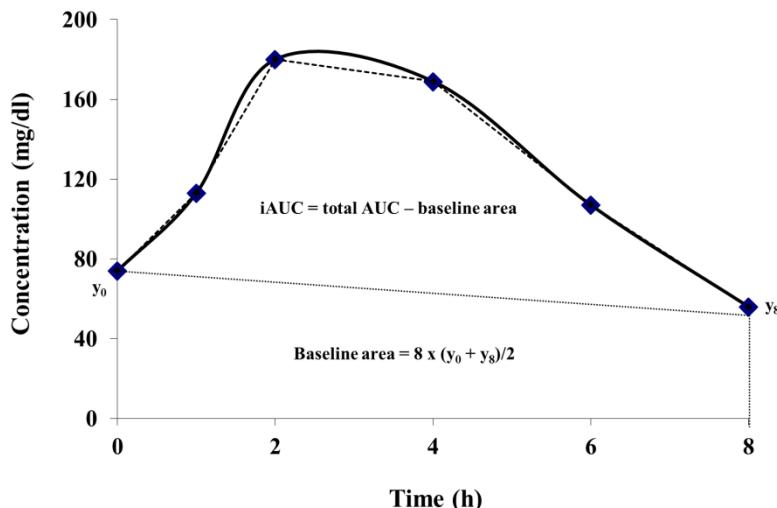
Figure 10. Incremental area under the curve (iAUC), if $y_0 > y_8$:
$iAUC = Total\ AUC - 8x\ (y_0 + y_8)/2$

2.7 Statistics

The primary outcome measure was non-HDL-cholesterol. Secondary outcome measures included: fasting total cholesterol, triglycerides, VLDL-cholesterol, VLDL-triglycerides, LDL-cholesterol, HDL-cholesterol, apoB, glucose, insulin, HOMA-IR, QUICKI, HbA1c, adiponectin, leptin, visfatin, vaspin, chemerin, ICAM-1, VCAM-1, endothelin-1, CRP, and fatty acid profiles; postprandial glucose, triglycerides, chylomicron-triglycerides, VLDL-triglycerides and endothelial function; blood pressure, BMI, waist circumference; dietary composition. An estimated sample size of 40 subjects would be required to ensure an overall power of 90% to detect a mean difference of 20 mg/dL in non-HDL-cholesterol for comparing walnut diet with control diet, assuming a drop-out rate of 33%. Power calculations were based on the data of Sabate et al. (65). The statistical analysis was conducted with the help of statistician Dr. Renee Stark (Helmholtz-Zentrum Munich). Results are reported as mean ± standard error of mean (SEM), unless otherwise stated. Dietary components were compared using two-tailed paired t-test or the Wilcoxon signed-rank test. Postprandial lipid and glucose measurements were evaluated using AUC and iAUC and a two-step process to assess changes in the shape of postprandial curves: first, pre-control vs. post-control and pre-walnut vs. post-walnut curves were compared at each time point; second, a model assessing the interaction between treatment and time was used. A mixed model was used to adjust for gender, age, BMI, diet sequence, and repeated measures for all comparisons (except for dietary components). Statistical significance was set at $p < 0.05$. Subject randomization (using a complete block design) and statistical analysis were performed on SAS 9.2.

3 RESULTS

3.1 Subject characteristics

Of 96 screened subjects, 72 fulfilled all inclusion criteria (**Figure 11**). Fifteen subjects declined participation prior to randomization. Fifty-seven subjects were randomized. During the two-week run-in period, five subjects were excluded from the study due to illness. In the first diet phase, two subjects with control diet declined further participation after visit two due to personal reasons, and one subject with walnut diet was excluded in week eight due to uncontrollable eating attacks with excessive calorie intake recorded in the diet protocols. In the washout phase, one subject, after exiting the control phase, was excluded due to excessive alcohol consumption (> 20 g/d) recorded in four consecutive diet protocols, and another subject in the same diet group terminated the study due to illness. Another subject, after exiting the walnut diet phase, was also removed from the study due to persistent hypertension measured hourly on visit two and three.
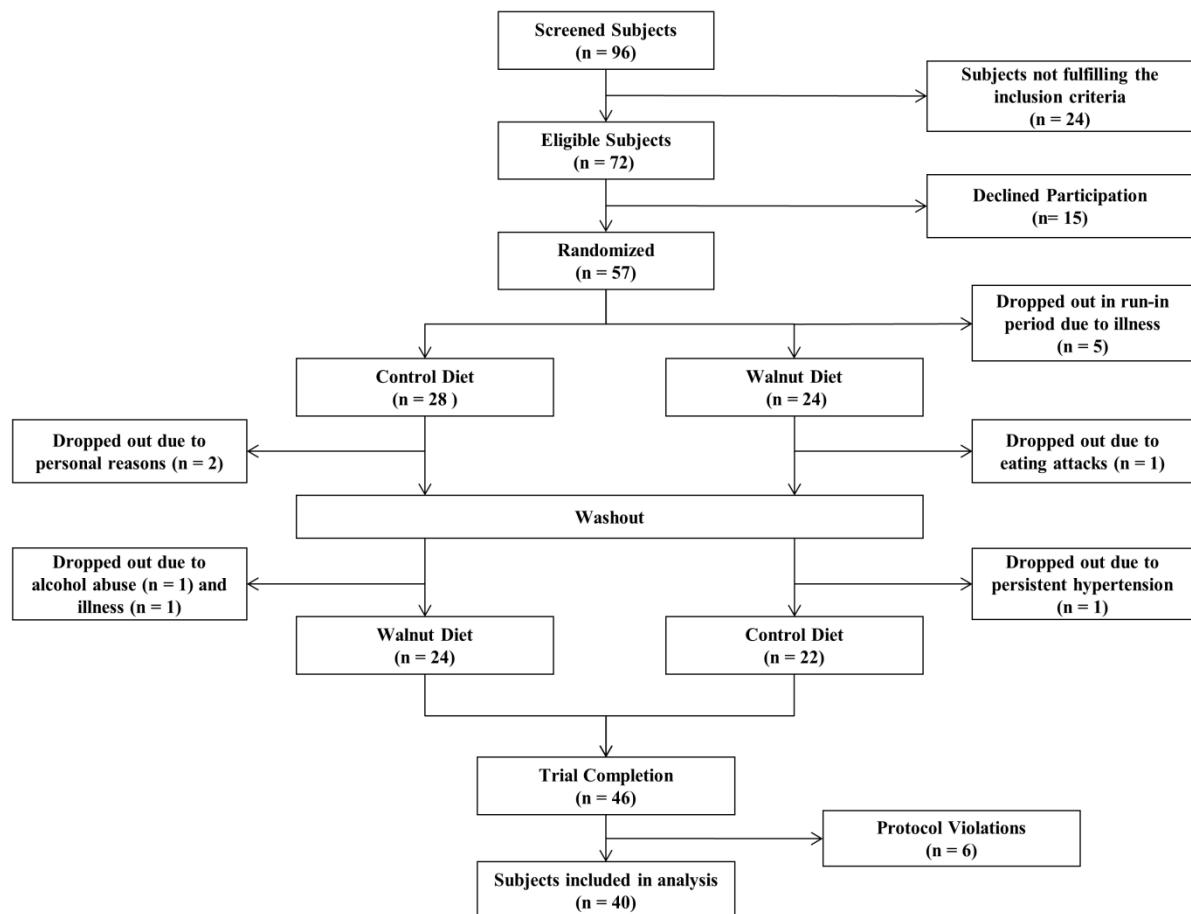
Figure 11. Flowchart of study subjects.

A total of 46 subjects (11 men and 35 women) completed all three phases of the study. Nine subjects had hypothyroidism that was controlled with L-thyroxine. Six subjects were withdrawn from data analysis due to adverse events listed in section **3.2**. In the subsequent sections, the data only refer to the remaining 40 subjects (10 men, 30 women). The characteristics of the subjects obtained at screening are described in **Table 7.**

Table 7. Demographic characteristics and baseline values of anthropometric measurements, fasting lipid and glucose parameters and CRP.

Variables	Baseline*
Gender	m = 10, w = 30
Age (y)	60 ± 1
BMI (kg/m^2)	24.9 ± 0.6
Waist circumference (cm)	86 ± 2
Systolic blood pressure (mmHg)	119 ± 2
Diastolic blood pressure (mmHg)	75 ± 2
Total cholesterol (mg/dL)	220 ± 5
VLDL-cholesterol (mg/dL)	16 ± 2
LDL-cholesterol (mg/dL)	133 ± 5
HDL-cholesterol (mg/dL)	71 ± 2
non-HDL-cholesterol (mg/dL)	149 ± 5
Total triglycerides (mg/dL)	88 ± 6
VLDL-triglycerides (mg/dL)	54 ± 7
Apolipoprotein B (mg/dL)	88.8 ± 2.4
Glucose (mg/dL)[1]	92 ± 1
Insulin (µU/mL)[1]	7.0 ± 0.6
HOMA-IR[1]	1.7 ± 0.2
QUICKI[1]	0.37 ± 0.01
HbA1c (%)[1]	5.5 ± 0.1
CRP (mg/dL)	< 0.1 (< 0.1-1.3)

*Baseline values obtained on the first study visit, prior to dietary intervention. Values are mean ± SEM or median (range), n = 40 for all parameters except those marked with [1] (n = 35). BMI: body mass index; VLDL: very-low-density lipoproteins; LDL: low-density lipoproteins; HDL: high-density lipoproteins; HOMA-IR: homeostasis model assessment estimate of insulin resistance; QUICKI: quantitative insulin-sensitivity check index; CRP: C-reactive protein.

3.2   Adverse events

Twenty-three study subjects reported the adverse events listed in **Table 8**. Eight subjects had mild symptoms of common cold. The most frequently used medications were low-dose non-opioid analgesics (aspirin, ibuprofen, paracetamol, and diclofenac), which were taken no more than four times by each of the 13 subjects throughout the entire study and with at least four days between the last use of medication and the following study visit.

Table 8. Adverse events.

Adverse event	Number of Subjects
Common cold	8
Tonsillitis	1
Medication	
Analgesics	13
Antibiotics	4
Corticosteroid	2
Operations	
Tooth extraction / implantation	3
Subtotal thyroidectomy	1

Of the 46 subjects who completed the study, six were excluded from data analysis due to the following reasons:

1. One subject developed severe tonsillitis and took an antibiotic for five days. Visit 6 had to be postponed for two weeks.
2. One subject took an antibiotic for 10 days after a tooth implantation and analgesics multiple times throughout the study.
3. Two subjects received corticosteroid injections due to orthopedic complaints. One of the subjects also had a bladder infection that was treated with antibiotics for 10 days.
4. One subject had fluctuating TSH level outside the normal reference range after receiving a subtotal thyroidectomy.
5. One subject was excluded due to violations of dietary instructions stated in section **2.3.1**.

3.3 Dietary composition

The analysis of self-reported nutrient intakes from the four-day dietary reports indicated that subjects maintained an isocaloric diet in both diet periods (**Table 9**). Compared with the control diet, subjects significantly reduced the proportion of protein and carbohydrates and increased the proportion of fat in their diet during the walnut period. The composition of dietary fat differed significantly between the two diets. The percentage of SFA in the walnut diet was 12.3 ± 0.3% compared with 14.4 ± 0.4% in the control diet. The percentage of PUFA in the walnut diet was 14.1 ± 0.2% compared with 4.6 ± 0.2% in the control diet. The walnut diet also contained a significantly higher amount of ALA. The proportion of cholesterol was significantly lower in the walnut diet while dietary fiber and vitamin E did not differ between the two diets.

Table 9. Dietary composition.

Variable	Walnut Diet	Control Diet	p*
Total Energy (kcal)	2067 ± 53	2013 ± 63	0.294
Fat (% of Total Energy)	39.2 ± 0.5	32.7 ± 0.6	< 0.001
Saturated fatty acids	12.3 ± 0.3	14.4 ± 0.4	< 0.001
Monounsaturated fatty acids	11.0 ± 0.2	11.3 ± 0.3	0.377
Polyunsaturated fatty acids	14.1 ± 0.2	4.6 ± 0.2	< 0.001
α-linolenic acid	2.4 ± 0.1	0.6 ± 0.1	< 0.001
Protein (% of Total Energy)	15.1 ± 0.3	16.0 ± 0.4	0.018
Carbohydrates (% of Total Energy)	43.5 ± 0.6	48.6 ± 0.7	< 0.001
Cholesterol (mg/d)	260.8 ± 14.3	294.2 ± 13.2	0.013
Fiber (g/d)	23.4 ± 1.1	23.5 ± 1.3	0.915
Vitamin E (mg/d)	8.5 ± 0.4	9.1 ± 0.5	0.224

Data are mean ± SEM. *Statistical significance set at $p < 0.05$ between the prescribed diets using two-tailed paired t-test or the Wilcoxon signed-rank test.

3.4 Effect on anthropometric measurements and blood pressure

Subjects maintained their baseline weight, BMI, waist circumference, and blood pressure during the study (**Table 7**).

3.5   Plasma fatty acids

The plasma fatty acid profile showed a significant reduction in SFA as well as MUFA and an increase in PUFA (notably LA and ALA) after the walnut diet (**Table 10**). The walnut diet also significantly reduced arachidonic acid (AA), EPA, docosapentaenoic acids and DHA. The changes in plasma fatty acid constitution reflected the fatty acid composition of walnuts and confirmed the self-reported changes in fatty acid intake. This shows that subjects closely adhered to the prescribed diets.

Table 10. Baseline plasma fatty acid profiles and changes from baseline.

Fatty Acid	Baseline$_w$	ΔWalnut	Baseline$_c$	ΔControl	p*
Total SFA (%)	43.54 ± 0.17	-0.69 ± 0.14	43.15 ± 0.16	0.24 ± 0.13	< 0.0001
Myristic	0.61 ± 0.03	-0.10 ± 0.03	0.54 ± 0.03	0.06 ± 0.03	0.0008
Palmitic	28.46 ± 0.18	-0.89 ± 0.16	28.12 ± 0.17	0.28 ± 0.17	< 0.0001
Stearic	13.60 ± 0.14	0.39 ± 0.11	13.68 ± 0.14	-0.11 ± 0.14	0.0003
Total MUFA (%)	14.03 ± 0.18	-2.33 ± 0.25	13.78 ± 0.21	0.10 ± 0.20	< 0.0001
Oleic	11.34 ± 0.17	-1.99 ± 0.22	11.16 ± 0.18	0.07 ± 0.18	< 0.0001
Total PUFA (%)	42.43 ± 0.26	3.02 ± 0.31	43.07 ± 0.29	-0.35 ± 0.26	< 0.0001
Linoleic	21.58 ± 0.38	4.80 ± 0.40	22.70 ± 0.43	-0.57 ± 0.34	< 0.0001
α-linolenic	0.45 ± 0.06	0.10 ± 0.06	0.41 ± 0.05	-0.02 ± 0.06	0.0020
Arachidonic	9.49 ± 0.23	-0.64 ± 0.15	9.53 ± 0.24	0.01 ± 0.15	0.0034
Eicosapentaenoic	1.49 ± 0.13	-0.27 ± 0.10	1.29 ± 0.09	0.02 ± 0.07	0.0369
Docosapentaenoic	0.23 ± 0.01	-0.03 ± 0.02	0.21 ± 0.01	-0.01 ± 0.02	< 0.0001
Docosahexaenoic	3.99 ± 0.17	-0.38 ± 0.10	3.75 ± 0.15	0.10 ± 0.12	0.0098

Fatty acid compositions are shown as percentage of the total amount of fatty acids measured.

Data are mean ± SEM. Baseline$_w$: baseline of walnut period; Baseline$_c$: baseline of control period. Δ denotes changes in each diet period; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids. * p-value of significance between walnut and controls determined using a mixed model and adjusted for age, gender, BMI, and diet sequence.

3.6   Effect on fasting lipids and apoB

Fasting lipids and apoB are shown in **Figure 12** and **Table 11**. Subjects were normolipidemic at baseline. The baseline values of lipid parameters did not differ significantly between the prescribed diets. A significant decrease in non-HDL-cholesterol was observed in the walnut period (**-**10 ± 3 mg/dL, p = 0.025) when compared with the control period (**-**3 ± 2 mg/dL). Similarly, the apoB concentration was significantly lowered by the walnut diet when compared with the control diet (**-**5.0 ± 1.3 mg/dL and **-**0.2 ± 1.1 mg/dL for walnut diet and control diet respectively, p = 0.009). The differences in treatment effect between the two diets on non-HDL-cholesterol and apoB remained significant after adjustment for gender, age, BMI and diet sequence. This analysis also indicates that age (p = 0.51), gender (p = 0.20), BMI (p = 0.80) and diet sequence (p = 0.33) did not affect the response to walnut consumption. The reduction in total cholesterol was **-**8 ± 3 mg/dL in the walnut period and **-**2 ± 2 mg/dL in the control period (difference p = 0.073). The walnut diet reduced LDL-cholesterol, VLDL-cholesterol, total triglycerides and VLDL triglycerides and increased HDL-cholesterol levels; however, these changes were non-significant when compared with those of the control period. Ratios of total cholesterol to HDL-cholesterol, LDL-cholesterol to HDL-cholesterol, and LDL-cholesterol to apoB at baseline were 3.2 ± 0.1, 2.0 ± 0.1, and 1.5 ± 0.1 respectively and remained constant in both diet periods.
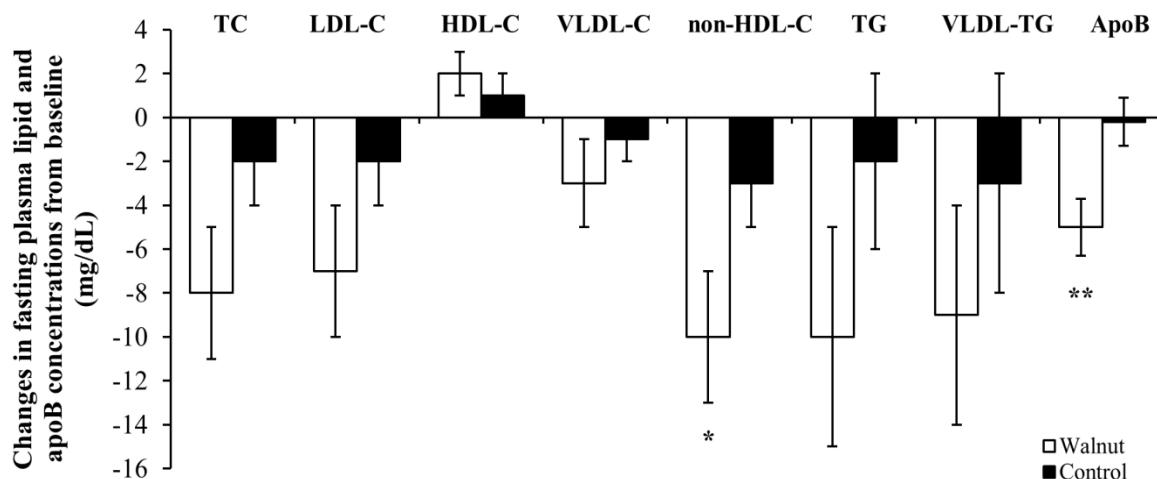
Figure 12. Changes in fasting lipids and apolipoprotein B.

Values are expressed as mean; error bars indicate SEM. *p ≤ 0.05, **p ≤ 0.01. ApoB: apolipoprotein B; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; non-HDL-C: non-high-density lipoprotein cholesterol; TC: total cholesterol; TG: total triglycerides; VLDL-C: very- low-density lipoprotein cholesterol; VLDL-TG: very-low-density lipoprotein triglycerides.

Table 11. Fasting plasma lipids and apolipoprotein B after dietary interventions.

Variable	Baseline$_w$	Δ Walnut	Baseline$_c$	Δ Control	p*
TC (mg/dL)	222 ± 4	-8 ± 3	219 ± 4	-2 ± 2	0.073
LDL-C (mg/dL)	135 ± 4	-7 ± 3	133 ± 4	-2 ± 2	0.118
HDL-C (mg/dL)	72 ± 2	2 ± 1	72 ± 2	1 ± 1	0.606
VLDL-C (mg/dL)	15 ± 2	-3 ± 2	14 ± 2	-1 ± 1	0.120
non-HDL-C (mg/dL)	150 ± 5	-10 ± 3	147 ± 4	-3 ± 2	0.025
TG (mg/dL)	89 ± 6	-10 ± 5	84 ± 6	-2 ± 4	0.323
VLDL-TG (mg/dL)	54 ± 5	-9 ± 5	51 ± 6	-3 ± 5	0.388
ApoB (mg/dL)	89.7 ± 2.4	-5.0 ± 1.3	87.4 ± 2.1	-0.2 ± 1.1	0.009

Data are mean ± SEM. Baseline$_w$: baseline of walnut period; Baseline$_c$: baseline of control period. Δ denotes changes in each diet period. ApoB: apolipoprotein B; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; non-HDL-C: non-high-density lipoprotein cholesterol; TC: total cholesterol; TG: total triglycerides; VLDL-C: very- low-density lipoprotein cholesterol; VLDL-TG: very-low-density lipoprotein triglycerides. * p-value of significance between walnut and controls determined using a mixed model and adjusted for age, gender, BMI, and diet sequence.

3.7   Effect on fasting glucose metabolism, adipokines, CRP, endothelial function and biomarkers of endothelial dysfunction

Parameters of fasting glucose metabolism (**Table 12**) were determined in a subgroup (n = 35). Fasting glucose, insulin, HOMA-IR, QUICKI and HbA1c remained stable in both diet periods (after adjustment for gender, age, BMI and diet sequence). Similarly, plasma leptin, adiponectin and chemerin concentrations as well as fasting VCAM-1, ICAM-1 and endothelin-1 remained stable in both diet periods after adjustment (**Table 12**). Plasma vaspin and visfatin concentrations were below the limit of detection of the standard assay. The median baseline CRP concentration was < 0.1 mg/dL (range: < 0.1-1.3 mg/dL). Furthermore, parameters of the endothelial function (RHI and fRHI) also did not change significantly.

Table 12. Parameters of glucose metabolism, adipokines, and endothelial function after dietary interventions.

Variable	Baseline$_w$	Δ Walnut	Baseline$_c$	Δ Control	p*
Fasting glucose (mg/dL)	92.1 ± 1.3	-0.7 ± 1.0	90.9 ± 1.4	1.3 ± 1.4	0.323
Fasting insulin (μU/mL)	7.2 ± 0.66	0.48 ± 0.45	6.83 ± 0.58	1.56 ± 0.58	0.169
QUICKI	0.363 ± 0.005	-0.003 ± 0.005	0.369 ± 0.006	-0.015 ± 0.005	0.103
HOMA-IR	1.79 ± 0.19	0.06 ± 0.13	1.65 ± 0.17	0.35 ± 0.16	0.200
HbA1c (%)	5.47 ± 0.04	0.06 ± 0.03	5.55 ± 0.04	-0.02 ± 0.04	0.232
Leptin (ng/ml)	18.7 ± 2.5	-1.5 ± 1.2	14.2 ± 2.0	-0.4 ± 0.8	0.709
Adiponectin (μg/mL)	23.3 ± 1.6	-1.0 ± 1.2	21.8 ± 1.5	1.3 ± 1.1	0.221
Chemerin (ng/mL)	220.1 ± 12.1	8.2 ± 7.0	233.8 ± 11.9	-2.8 ± 6.4	0.370
VCAM-1 (ng/mL)	605.8 ± 26.5	23.8 ± 40.9	601.3 ± 27.3	12.3 ± 24.7	0.733
ICAM-1 (ng/mL)	203.4 ± 6.6	-2.3 ± 7.2	208.1 ± 6.3	-1.3 ± 5.1	0.815
Endothelin-1 (pg/mL)	1.5 ± 0.1	-0.1 ± 0.1	1.4 ± 0.1	-0.1 ± 0.1	0.965
RHI	2.09 ± 0.10	-0.07 ± 0.10	1.92 ± 0.10	0.05 ± 0.08	0.724
fRHI	0.51 ± 0.06	-0.06 ± 0.07	0.42 ± 0.06	0.13 ± 0.07	0.174

Data are mean ± SEM. Baseline$_w$: baseline of walnut period; Baseline$_c$: baseline of control period. Δ denotes changes in each diet period. fRHI: Framingham-reactive hyperemia index; HOMA-IR: homeostasis model assessment estimate of insulin resistance; ICAM-1: intercellular adhesion molecule-1; QUICKI: quantitative insulin-sensitivity check index; RHI: reactive hyperemia index. VCAM-1: vascular cell adhesion molecule-1. * p-value of significance between walnut and controls determined using a mixed model and adjusted for age, gender, BMI, and diet sequence.

3.8   Effect on postprandial lipid and glucose metabolism

The effect on postprandial lipid and glucose metabolism was evaluated in a subgroup (n = 32). As shown in **Table 13**, changes in AUC and iAUC of total triglycerides, chylomicron-triglycerides, VLDL-triglycerides, glucose, and insulin were non-significant. Similarly, the treatment x time interaction was also non-significant for any of the parameters, indicating that the shape of the curve also did not change.

Table 13. Postprandial lipid and glucose metabolism after dietary interventions.

Variable	Baseline$_w$	Δ Walnut	Baseline$_c$	Δ Control	p*
AUC					
TG (mg x h/dL)	1023 ± 79	-30 ± 55	1066 ± 86	-52 ± 50	0.936
CM-TG (mg x h/dL)	243 ± 32	4 ± 24	246 ± 27	-12 ± 16	0.582
VLDL-TG (mg x h/dL)	572 ± 69	-5 ± 46	593 ± 71	-4 ± 44	0.831
Glucose (mg x h/dL)	370 ± 8	4 ± 8	368 ± 11	-6 ± 107	0.260
Insulin (μU x h/mL)	110 ± 15	1 ± 6	89 ± 10	15 ± 7	0.199
iAUC					
TG (mg x h/dL)	354 ± 41	17 ± 36	381 ± 34	-16 ± 22	0.386
CM-TG (mg x h/dL)	214 ± 29	-3 ± 23	213 ± 24	-8 ± 15	0.834
VLDL-TG (mg x h/dL)	192 ± 24	24 ± 25	191 ± 18	25 ± 14	0.971
Glucose (mg x h/dL)	23 ± 8	-1 ± 8	25 ± 10	-23 ± 13	0.053
Insulin (μU x h/mL)	84 ± 12	1 ± 6	64 ± 8	6 ± 7	0.528

Data are mean ± SEM. Baseline$_w$: baseline of walnut period; Baseline$_c$: baseline of control period. Δ denotes changes in each diet period. AUC: area under the curve; iAUC: incremental area under the curve. CM-TG: chylomicron triglycerides; TG: total triglycerides; VLDL-TG: very-low-density lipoprotein triglycerides. * p-value of significance between walnut and controls determined using a mixed model and adjusted for age, gender, BMI, and diet sequence.

4   DISCUSSION

The present study showed that an eight-week daily supplementation of 43 g of walnuts significantly reduced fasting non-HDL-cholesterol and apoB levels in healthy senior individuals, even after adjustment for gender, age, BMI, and diet sequence. Postprandial lipid metabolism, glucose metabolism, circulating levels of adipokines and CRP, endothelial function, body weight, and blood pressure remained unaffected. Each of the analyzed parameters will be discussed in detail in the following sections.

4.1   Diets and plasma fatty acids

During the 20-week study period, dietary adherence was closely monitored using diet protocols completed by subjects at regular intervals. The walnut diet was well tolerated and no adverse effect was reported. The amount of walnuts used in this study was selected because 43 g/d (approximately 1.5 ounces/d) is the amount needed to meet the qualified health claim issued by the U.S. Food and Drug Administration for walnuts (76).

Walnuts are energy-dense food with high satiating capacities. There is evidence supporting that nut consumption may modulate appetite and promote the feeling of fullness (77). Although subjects maintained an isocaloric diet throughout the study, macronutrient distribution differed significantly between the walnut and the control diet. Subjects consumed more fat, specifically PUFA, and less carbohydrate and protein in the walnut phase. Previous nut studies have also reported such compensatory dietary response that maintains energy balance (78-80).

Many beneficial effects of walnuts have been attributed to their high PUFA content. Compared with a traditional Mediterranean diet, a typical Western-type diet consists of a lower amount of PUFA and a higher amount of SFA. Dietary guidelines from the Food and Agriculture Organization of the United Nations and the World Health Organization recommend a consumption of SFA < 10% of total energy and a consumption of PUFA 6-11% of total energy (81). An analysis of dietary fat intake in 40 countries, mostly developed countries, showed that only 11 countries met the dietary recommendation for SFA and 20 countries met the recommendation for PUFA. In Germany, more than 80% of the studied population deviated from the recommendation on SFA intake, and close to 40% of the

population did not meet the recommendation on PUFA intake. The average PUFA intake in Germany (6.5% of total energy) was on the lower end of the recommended range, and ALA only made up 0.8% of total energy (82). In the present study, subjects did not meet the aforementioned dietary recommendations for SFA and PUFA intake in the control phase, but there was a slight but significant decrease in SFA and a marked increase in PUFA intake in the walnut phase. ALA was also raised from 0.6% to 2.4% of total energy. This confirms that walnuts are an excellent source of PUFA. Mozaffarian et al. showed in a meta-analysis that for each 5% energy derived from increased PUFA intake, the CHD risk was decreased by 10% (83).

LA is the major n-6 PUFA in walnuts. After the walnut diet, subjects of this study had significantly more LA in plasma, but the amount of AA, a downstream metabolite of LA, decreased significantly. AA is the precursor of the majority of eicosanoids. The production of proinflammatory eicosanoids has led to the speculation that higher LA intake may raise the AA level and induce a proinflammatory milieu that increases the risk of CVD and causes other health problems. As observed in this study, an increased plasma level of LA did not lead to an increase in plasma AA level. Other studies have shown that changes in LA intake appear not to significantly affect tissue AA content (84). Kinetic studies have observed that only 0.2% of LA is converted to AA (85). It is possible that a diet with increased ALA intake may hamper the conversion of LA to AA by competing for the same rate-limiting enzyme, Δ6-desaturase, which is involved in the synthesis of AA, EPA, and DHA (86, 87).

To date, clinical data have not confirmed this hypothesized risk of LA to induce inflammation and promote CVD. On the contrary, there is accumulating evidence suggesting a cardio-protective effect of LA. In a meta-analysis of prospective cohort studies, Farvid et al. showed a dose-dependent inverse relationship between LA intake and CHD. A 5% increase in energy derived from LA in place of SFA was linked to a 9% decreased risk of total CHD and a 13% decreased risk of CHD deaths (88). In a prospective cohort study involving 60-year-old Swedish men and women without prior CVD, Marklund et al. observed an inverse relationship between serum LA levels determined at baseline and all-cause mortality in men (43). These results lend support to the dietary guidelines that advocate a diet rich in PUFA (81).

4.2 Body weight

Contrary to the popular belief that increased consumption of dietary fat may lead to weight gain, subjects of this study maintained their baseline weight, most likely due to the isocaloric nature of the walnut diet. This is in line with a number of studies showing that nut consumption does not have detrimental effects on body weight and waist circumference. In fact, some studies have shown that regular nut intake may even promote weight loss (89, 90). There is some evidence suggesting that walnuts may induce fat oxidation (91), promote postprandial thermogenesis (92), and lower body fat mass (93, 94). Furthermore, the total energy content of walnuts listed in food composition tables may, in fact, overestimate the actual metabolizable energy content. Baer et al. showed that the energy value of walnuts calculated using the Atwater system, which is widely used to determine the available energy of foods, exceeded the measured value by 21% (40). An overestimation of the metabolizable energy using the Atwater system had also been previously reported for other nuts such as almonds (95) and pistachios (96). The Atwater system may be inadequate for calculating the energy value of nuts due to the lower digestibility of fat in whole nuts when compared with other food groups (95). The decreased digestibility of fat from nuts results in increased fecal fat content, which may explain, in part, the fact that the actual energy available to the body is lower than the estimated energy (40).

4.3 Lipid metabolism

The results showed that walnut consumption significantly decreased fasting non-HDL-cholesterol and apoB levels. Total cholesterol showed a trend toward reduction, and the cholesterol content of LDL, VLDL, and HDL as well as total triglycerides all improved despite statistically non-significant differences. Using a predictive model that assesses the effects of quantitative changes in dietary intake of fatty acids (SFA, PUFA, MUFA) and cholesterol on plasma total cholesterol and cholesterol fractions (97), changes in total cholesterol, LDL, and HDL were estimated to be -14.1 mg/dl, -11.0 mg/dl and -1.1 mg/dl, respectively. The calculations provide information on the relative contribution of the changes in dietary fatty acids on the observed reduction in plasma cholesterol levels. The cholesterol-lowering effect of walnuts may be largely attributed to the increase in PUFA (9.2%), since SFA intake only decreased slightly (-2.4%), and MUFA intake remained unaffected. The decreased dietary cholesterol had a negligible effect on plasma cholesterol

(97). Compared with the predicted values, the actual changes were lower. An earlier study demonstrated that the measured change in LDL-cholesterol exceeded the predicted change for a walnut-supplemented diet; however, the study subjects were hypercholesterolemic at baseline (98). Numerous feeding trials have reported a greater reduction in total cholesterol and LDL-cholesterol in hypercholesterolemic individuals. A pooled analysis of 25 feeding trials concluded that nut consumption reduced triglycerides only in hypertriglyceridemic subjects and reduced cholesterol concentration in a dose-dependent manner, with the largest decrease observed in individuals with higher baseline LDL or lower BMI as well as in those following a Western diet (compared with a Mediterranean and a low-fat diet) (34). Subjects of the current study were normolipidemic at baseline and received a comparatively lower amount of walnuts. Thus, changes in lipoprotein levels may be of smaller magnitude than those in subjects with dyslipidemia.

Currently, LDL-cholesterol remains to be the primary treatment target, while non-HDL-cholesterol and apoB are still considered secondary treatment targets. However, there is increasing evidence suggesting that non-HDL-cholesterol and apoB may be superior to LDL-cholesterol in the evaluation CVD risk (99-101). Both non-HDL-cholesterol and apoB encompass the cholesterol content of all atherogenic lipoproteins. In patients treated with statins, non-HDL-cholesterol, apoB and LDL-cholesterol all predicted future cardiovascular events, but the predictive value of non-HDL-cholesterol was greater than the other two parameters (102). Based on a meta-analysis of various lipid-modifying therapies that showed a 1:1 relationship between the percentage decrease in non-HDL-cholesterol and the risk reduction of CHD (103), the decrease in non-HDL-cholesterol for the walnut diet in the current study can be translated to a predicted 6.7% risk reduction, which resonates with the findings of the PREDIMED trial (63). The inverse relationship between walnut consumption and levels of atherogenic lipoproteins can serve as a potential explanation for the observed beneficial effects of nuts on CVD outcomes.

The cardioprotective effects of walnuts have been largely attributed to their high PUFA content. Walnuts contain abundant LA (n-6 PUFA) and ALA (n-3 PUFA), both of which are considered essential fatty acids because they cannot be made by humans. More than 50 years ago, H.M. Sinclair reported that the absence of essential fatty acids may have detrimental effects on vascular health. He proposed the hypothesis that the quantity and the structure of the dietary fatty acids may be more important than the amount of total fat ingested (104).

This idea is also reflected in the dietary guidelines that recommend replacing SFA with PUFA (81, 105).

The exact mechanisms underlying the cholesterol-lowering effects of PUFA are still unclear. It is most likely that PUFA exert their favourable effects via a number of different mechanistic actions. At the cellular level, PUFA can alter the fatty acid composition of membrane phospholipids, thus changing the microenvironments in which biological reactions take place. Changes in membrane phospholipid composition can modify the physical properties of the cellular membrane, such as its structure and fluidity, which in turn affect the structure and function of proteins and receptors embedded in the membrane. Furthermore, PUFA can act as second messengers, thus regulating intracellular signaling pathways. The PUFA released from membrane phospholipids can also function as signaling molecules, regulate gene expression by binding to transcription factors, and act as precursors for lipid mediators (106).

The favourable lipid profile of nuts cannot entirely account for the cholesterol-lowering effects observed in clinical studies. When the actual changes in lipoprotein levels were compared with estimated changes calculated using predictive equations, the actual changes exceeded predicted changes by approximately 25%. Nuts contain an array of other nonfat nutrients such as plant proteins, dietary fiber, phytosterols, tocopherols, phenolic compounds and minerals that may confer additional beneficial effects on plasma lipids. However, there is limited information on the quantity and bioaccessibility of these compounds and their relative contribution to the improvement of plasma lipid profile (107).

Phytosterols are plant-derived sterols that share structural and functional similarities with cholesterol. Unlike cholesterol, the plasma phytosterol levels are low due to poor intestinal absorption (108). Phytosterols may reduce plasma cholesterol levels by competitively inhibiting the intestinal absorption of cholesterol and by modulating the expression of hepatic and intestinal genes involved in lipid metabolism (49). The underlying mechanisms may be explained by the relatively higher hydrophobicity of phytosterols, which display a greater affinity for micelles than cholesterol, thus displacing cholesterol from micelles and lowering the intestinal absorption of cholesterol (109). In addition, dietary fiber and plant protein have also been suggested to elicit cholesterol-lowering effects (110). Walnuts contain relatively

large amounts of arginine, an amino acid that may have a beneficial effect on lipid metabolism (111, 112).

Many questions regarding the mechanisms underlying the cholesterol-lowering effects of walnuts still remain to be answered. This study showed that the improvement in lipid profile may be mediated by changes in apoB metabolism. This raises the question whether the reduction in apoB concentration is a result of decreased production or increased clearance of apoB-containing lipoproteins or a combination of both. One study showed that walnuts may facilitate the clearance of LDL. LDL particles obtained from hypercholesterolemic men during a six-week walnut-enriched diet had 50% increased association rates with LDL receptors of cultured human hepatoma cells when compared with LDL particles isolated during the control diet. The binding of LDL particles to LDL receptors was correlated with the ALA content of the LDL lipid core. However, the increased LDL association only explained about 30% of the observed LDL reduction, suggesting that additional factors may contribute to the overall cholesterol-lowering effects (113). Further research is needed to understand the biochemical processes underlying the lipid changes induced by walnut consumption.

This study did not observe a significant change in triglycerides in both the walnut and the control diet. To date, the triglyceride-lowering effect of marine-based n-3 PUFA has been well-established. Marine-based n-3 PUFA, known as EPA and DHA, differ from plant-based n-3 PUFA, ALA, in structure and effect. EPA and DHA are both long-chain PUFA that lower fasting triglyceride levels and improve postprandial lipemia. The underlying mechanisms have been suggested to involve the activation of certain transcription factors in the liver, resulting in an increased expression of genes encoding enzymes needed for beta-oxidization and a decreased expression of genes encoding enzymes involved in lipogenesis, consequently reducing the hepatic availability of fatty acids for VLDL assembly (114). In addition, data from kinetic studies have shown that long-chain n-3 PUFA decrease the rate of production and direct catabolism of non-fasting hepatic and intestinal-derived apoB (115). Long-chain n-3 PUFA do not seem to affect levels of total cholesterol and LDL-cholesterol in individuals with normal lipid levels. However, people with certain types of dyslipidemia such as elevated triglyceride levels and decreased HDL-cholesterol levels may exhibit a considerable increase in LDL-cholesterol levels after EPA and DHA supplementation. In contrast, plant-based foods rich in ALA have been shown to reduce plasma levels of cholesterol but not

triglycerides. The disparate effects between long-chain n-3 PUFA and ALA have been attributed to their difference in chain length (116). Although ALA is the metabolic precursor of EPA and DHA, the conversion in humans is rather inefficient (117), which could explain the lack of increase in long-chain n-3 PUFA levels and the lack of changes in both fasting and postprandial triglyceride levels observed in this study.

4.4   Glucose metabolism

Both experimental diets did not change parameters of fasting and postprandial glucose metabolism. Past epidemiological studies have suggested that regular nut intake may reduce the risk of developing diabetes. Data from the Nurses' Health Study I were analyzed to determine the relationship between nut intake and type-2 diabetes. The study included 83,818 women between the age of 34 and 59 years with no history of diabetes and CVD. The authors reported an inverse relationship between nut intake and risk of type-2 diabetes after adjustment for age, BMI, family history of diabetes, physical activity, smoking, alcohol consumption, and total energy intake as well as other dietary factors. Women whose nut intake was at least five times a week compared with those who never or seldom ate nuts had a relative risk of diabetes 0.74 (95% confidence interval, 0.61-0.89) (118). In the follow-up study in 2013, Nurses' Health Study II once again confirmed the inverse relationship between nut consumption and type-2 diabetes. Of note is that among the studied tree nuts, walnut consumption was associated with the lowest risk. After adjustment for BMI, women who ate more than two servings of walnuts each week in comparison with those who never or rarely ate walnuts had a hazard ratio of 0.76 (95% confidence interval, 0.62–0.94). While the other tree nuts were also associated with reduced risk of type-2 diabetes, the relationship was insignificant after adjustment for BMI (119).

Despite a clear inverse relationship between nut intake and diabetes shown in epidemiological studies, results from feeding trials have shown inconsistent findings regarding the effect of walnuts on parameters of glucose metabolism. A subanalysis of the PREDIMED trial showed that the Mediterranean diet enriched with mixed nuts (50% walnuts) demonstrated a lower incidence of type-2 diabetes in subjects who did not have the disease at enrollment but had a high CVD risk profile (120). Casas-Agustench et al. observed that a 12-week supplementation of 30 g/d of mixed nuts (15g of walnuts) decreased fasting insulin and HOMA-IR (121). Another study by Kalgaonkar showed a decrease in HbA1c and an

improvement in insulin response to an oral glucose tolerance test in women with polycystic ovary syndrome after following a walnut-enhanced diet for six weeks (122). Yet several other feeding trials reported that glucose metabolism was unaffected by walnut consumption in subjects with type-2 diabetes and metabolic syndrome (94, 123, 124). The lack of change in fasting and postprandial glucose metabolism observed in the present study may be due to the short study intervention period and the low-risk profile of the study subjects.

4.5   Adipokines

A limited number of studies have explored the effect of walnut consumption on plasma adipokines. In the present study, the adipokine levels were unaffected by the walnut diet. In contrast, previous studies have reported a positive effect on plasma adiponectin concentration in subjects who either had or were at risk of developing type-2 diabetes. Aronis et al. found that short-term consumption of walnuts (four days) resulted in a significant increase in total adiponectin level in obese individuals with metabolic syndrome (125). Kalgaonkar et al. also reported an increase in adiponectin in women with polycystic ovary syndrome after six weeks of walnut intake (122). A traditional Mediterranean diet has been shown to associate with higher plasma adiponectin level in diabetic women (126). Adiponectin exhibits insulin-sensitizing and antiatherogenic properties, and its plasma level is inversely associated with weight loss (127). Subjects in the present study did not exhibit changes in adipokine levels. This could be explained by the fact that they were non-obese at baseline, and their body weight remained stable throughout the study.

4.6   Inflammation, endothelial function and blood pressure

Inflammation plays a crucial role in the development and progression of atherosclerotic disease. Increased plasma levels of inflammatory markers are associated with elevated risk for cardiovascular events (128, 129). CRP is a well-known marker of systemic inflammation. There is evidence showing that the prognostic value of CRP level is comparable to that of cholesterol levels in assessing cardiovascular risk (130). Because walnuts contain abundant nutrients that confer anti-inflammatory effects, the present study measured plasma CRP level before and after dietary interventions. The walnut and the control diet did not have an effect on CRP level.

Previous studies have reported inconsistent findings regarding the effect of walnut consumption on CRP level. Ros et al. found that CRP level remained constant in hypercholesterolemic individuals after a four-week walnut-enriched diet (131). In another study, a high ALA diet achieved through the supplementation of walnuts, walnut oil, and flaxseed oil significantly improved CRP and markers of endothelial activation (44). In contrast to these two studies, Mukuddem-Petersen et al. reported a significant increase in CRP level after a walnut diet in subjects with metabolic syndrome; however, this trial had a parallel design, and despite a significant increase after the walnut diet, the CRP level in the walnut group was still lower compared with that of the control groups (123). It could be argued that an improvement in CRP may not be apparent in the present study because the baseline level was already very low. Despite a lack of change in CRP level, the potential anti-inflammatory effects of walnuts cannot be dismissed, since walnuts appear to reduce other inflammatory markers such as tumor necrosis factor-α (132) and interleukin-6 (133).

Inflammatory processes are accompanied by the concurrent increase in cellular adhesion molecules (4), which act as circulating markers of endothelial dysfunction (134). Zhao et al. observed that walnut consumption significantly reduced plasma levels of ICAM-1 but not VCAM-1 (135). In two other studies, both VCAM-1 and ICAM-1 decreased significantly (44, 133). Several studies have also demonstrated an improvement in endothelial function assessed using brachial artery FMD. In contrast to previous studies, the present study did not observe changes in markers of endothelial dysfunction (VCAM-1, ICAM-1, and endothelin-1). Moreover, the postprandial endothelial function assessed using PAT remained unaffected.

Lopez-Uriate et al. (136) and Berry et al. (137) also demonstrated that finger arterial pulse wave amplitude was not affected by nut consumption. Interestingly, various walnut components may exhibit different effects when compared with whole walnuts, as seen in a study that showed a favourable change in fRHI after acute consumption of walnut oil when compared with walnut skin and whole walnuts. This was attributed to the higher bioavailability of nutrients in walnut oil (138).

In the current study, there was great intraindividual variability in the PAT measurements in both the walnut and the control phase. Low interday reproducibility of RHI was observed in one study by Liu et al. who repeated the PAT measurements in 10 healthy men at fixed time

points for 3 consecutive days. It is unclear what causes the variability in PAT measurements. The endothelial response assessed by PAT is affected by many internal and environmental factors (139). Daily changes in these factors may affect the peripheral arterial response.

The PAT measurements may not be directly comparable to the results of previous studies because of the differences in study population and the methodology used. An improvement in brachial artery FMD was previously observed in hypercholesterolemic subjects after a four-week walnut diet and even after just a single walnut-enriched meal (131). Although both FMD and PAT are non-invasive methods of assessing the endothelial function, there is a lack of consistent correlation between PAT and FMD measurements. This suggests that each method may assess different changes in vascular function (140). Despite some studies showing that NO plays a central role in both methodologies (141, 142), it is believed that PAT is a measure of microvascular function, which is mainly endothelium-independent, whereas FMD is a measure of macrovascular function (143).

Because subjects of this study were at low risk of developing CVD, the effect of walnuts on inflammation, endothelial function, and blood pressure may not be apparent. Subjects of this study had blood pressure within normal range. The PREDIMED study showed in individuals at high risk of developing CVD that long-term Mediterranean diet with olive oil or nuts improved blood pressure. The improvement in diastolic pressure was greater in the Mediterranean diet groups than in a low-fat control group (144). A meta-analysis of 21 randomized controlled trials with a total of 1652 individuals showed that nut intake did not change systolic blood pressure significantly. However, subgroup analyses showed that nut consumption significantly reduced systolic blood pressure in non-diabetic individuals (145). Long-term studies are needed to assess the preventive effects of walnuts on inflammation, endothelial dysfunction, and hypertension.

4.7   Strengths and limitations

Among the major strengths of this study is its randomized, cross-over design with the addition of a wash-out period. The study systematically assessed the effect of walnut consumption on both fasting and postprandial lipid and glucose metabolism, circulating levels of adipokines and CRP, endothelial function, blood pressure, and body weight in healthy senior individuals. The study also has several limitations including a small sample

size, a short intervention period, and the underrepresentation of men. The majority of the study subjects were women so that an inter-gender comparison was not possible. Moreover, this study only included Caucasians. Different populations may respond to the dietary interventions differently. This may limit the generalizability of the results in this study. However, cholesterol-lowering effects of walnuts have also been observed in Japanese individuals (67). Furthermore, the background diet of this study was not standardized. The study also did not include an ad libitum dietary intervention, since walnuts replaced other foods. The influence of other food items (either added or omitted) cannot be completely ruled out. There was large variability in the response of study subjects to the dietary interventions, with some showing a large reduction in cholesterol and triglyceride levels while others exhibiting only small changes. The individual responsiveness to dietary interventions may be affected by other factors such as genetic makeup. It is important to note that subjects of this study were at low risk of developing CVD, thus other beneficial effects of walnuts may have been masked. Another limitation of this study is that only a subgroup analysis of postprandial metabolism was conducted. Due to the lack of elevation in postprandial triglyceride levels after using the initial test drink A (32 g of fat), the fat content was raised to 88 g in test drink B. One of the challenges of conducting postprandial trials is that no standardized test meal is available (146). The relatively small sample size in the postprandial test reduced the power of the postprandial analysis.

4.8   Conclusions

Previous studies have consistently demonstrated that walnut consumption improves lipid profile in hypercholesterolemic subjects. Results of the present study extend this finding to healthy senior individuals. Dietary modification is an integral component in the prevention of dyslipidemia, a well-established independent risk marker of CVD. This study shows that supplementing 43 g of walnuts for eight weeks favourably changed plasma lipid profile by lowering the concentration of non-HDL-cholesterol and apoB, which may explain, in part, the epidemiological observation that walnut consumption reduces the risk of CVD.

Currently, the mechanistic actions of walnuts are still unclear. Information regarding the bioavailability and bioaccessibility of the micronutrients in walnuts is scarce. The cardioprotective effects of walnuts are most likely not the product of a single nutrient but rather different nutrients working synergistically. Further research is warranted to explore the composition of walnuts and the underlying mechanisms of their beneficial effects.

5   SUMMARY

Cardiovascular disease (CVD) is the leading cause of death worldwide. Although there has been a trend showing a decline in CVD mortality in developed countries, a staggering increase has been observed in medium- and low-income countries. Excluding genetic factors, most CVD risk factors are modifiable. Dietary modification is an important component of CVD management and prevention. The amount and the types of food eaten have great influence on a person's health. In the last half-century, there has been growing interest in identifying healthy dietary patterns and finding so-called "functional foods". Nuts, especially walnuts, have garnered wide attention.

Walnuts are nutrient-dense foods with a unique fatty acid profile. While most nuts are high in monounsaturated fatty acids, walnuts are predominantly composed of polyunsaturated fatty acids (47% of total weight), mainly linoleic acid and alpha-linolenic acid. Walnuts are also the only nuts with a significant amount of alpha-linolenic acid, which has been shown to elicit anti-inflammatory and anti-atherogenic effects. Beyond the favourable fatty acid profile, walnuts also contain other potentially cardioprotective nutrients such as plant protein, dietary fiber, polyphenols, phytosterols, and tocopherols. Epidemiological studies have consistently shown an inverse relationship between nut consumption and CVD. Previous clinical studies involving walnuts were mostly conducted in individuals at increased risk of developing CVD.

This randomized, controlled, and cross-over study investigated the effect of walnut consumption on fasting and postprandial lipid and glucose parameters, adipokines, CRP, blood pressure, body weight, and endothelial function in healthy men and healthy postmenopausal women. Data from 40 subjects (mean ± standard error of mean: age 60 ± 1 years, BMI 24.9 ± 0.6 kg/m^2; 30 females) $\geq$50 years old were analyzed. Two test diets were used. The background diet was composed of a Western-type diet (35 % fat, of which 15 % saturated fatty acids, 15 % protein, and 50 % carbohydrate). The two diets were identical except that subjects were asked to replace 30 g of saturated fat with 43 g of shelled, prepackaged walnuts in the walnut phase. Diet protocols were completed at regular intervals. After a two-week nut-free run-in period, each diet lasted eight weeks, and the two diets were separated by a two-week wash-out. At the beginning and end of each diet phase, a physical examination, a mixed meal test, and a non-invasive assessment of postprandial endothelial function were conducted. Blood was drawn at 0 (fasting), 15, 30, 60, 120, 180, 240, 360, and

480 minutes. Total cholesterol, LDL-cholesterol, HDL-cholesterol, total triglycerides, VLDL-triglycerides, apoB, glucose, insulin, HOMA-IR, QUICKI, HbA1c, adipokines, biomarkers of endothelial dysfunction (VCAM-1, ICAM-1 and endothelin-1) and CRP were determined in fasting samples. Fasting non-HDL-cholesterol was calculated using the equation *total cholesterol - HDL-cholesterol*. Area under the curve (AUC) and incremental AUC (iAUC) were calculated for postprandial total triglycerides, VLDL-triglycerides, chylomicron-triglycerides, glucose, and insulin.

Compared with the control diet, the walnut diet significantly reduced non-HDL-cholesterol (walnut vs. control: -10 ± 3 vs. -3 ± 2 mg/dL; p = 0.025) and apoB (-5.0 ± 1.3 vs. -0.2 ± 1.1 mg/dL; p = 0.009). These findings remained significant after adjusting for age, gender, body mass index, and diet sequence. Total cholesterol showed a trend toward reduction (p = 0.073). Fasting VLDL-cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides and glucose, insulin, HOMA-IR, QUICKI, and HbA1c did not change significantly. Similarly, fasting adipokines, CRP, biomarkers of endothelial dysfunction, postprandial lipid and glucose metabolism (AUC and iAUC), and endothelial function were unaffected.

This study showed that supplementing 43 g of walnuts for eight weeks favourably changed plasma lipid profile by lowering the concentration of non-HDL-cholesterol and apoB. The short-term walnut consumption did not affect glucose metabolism, circulating levels of adipokines and CRP, as well as endothelial function, body weight, and blood pressure. The favourable changes in plasma lipid profile may explain, in part, the epidemiological observation that walnut consumption reduces the risk of CVD.

6   ZUSAMMENFASSUNG

Kardiovaskuläre Erkrankungen sind die weltweit führende Todesursache. Obwohl in den Industrieländern die kardiovaskuläre Mortalität abnimmt, wurde ein deutlicher Anstieg der Mortalität in Mittel- und Niedrigeinkommensländern beobachtet. Außer den genetischen Faktoren sind die meisten kardiovaskulären Risikofaktoren modifizierbar. Dietätische Modifikation ist ein wichtiger Bestandteil der Therapie und Prävention von kardiovaskulären Erkrankungen. Art und Menge der verzehrten Nahrung haben großen Einfluss auf die Gesundheit. In den letzten fünfzig Jahren ergab sich ein wachsendes Interesse bei der Ermittlung von gesunden Ernährungsmustern und bei der Suche nach so genanntem "Functional Food". Nüsse, insbesondere Walnüsse, haben hierbei große Aufmerksamkeit erregt.

Walnüsse sind nährstoffreiche Lebensmittel mit einem einzigartigen Fettsäureprofil. Im Vergleich zu anderen Nüssen, die überwiegend reich an einfach-ungesättigten Fettsäuren sind, enthalten Walnüsse große Mengen an mehrfach-ungesättigten Fettsäuren (47% des Gesamtgewichts), vorwiegend Linolsäure und alpha-Linolensäure. Walnüsse sind die einzigen Nüsse mit signifikanten Mengen an α-Linolensäure, welche antientzündliche und antiatherogenen Wirkungen aufweisen. Zudem liefern Walnüsse weitere potentielle kardioprotektive Inhaltsstoffe wie pflanzliches Protein, Ballaststoffe, Polyphenole, Phytosterine, und Tocopherole. Epidemiologische Studien zeigen eine inverse Beziehung zwischen Nusskonsum und kardiovaskulären Erkrankungen. Bisherige klinische Studien mit Walnüssen wurden meist bei Personen mit einem erhöhten kardiovaskulären Risiko durchgeführt.

Diese randomisierte, kontrollierte Cross-over-Studie untersuchte den Effekt des Walnusskonsums auf nüchtern und postprandialen Lipid- und Glukosestoffwechsel, Adipokine, CRP, Blutdruck, Körpergewicht sowie die Endothelfunktion bei gesunden Männern und gesunden postmenopausalen Frauen. Daten von 40 Probanden (Mittelwert ± Standardfehler: Alter 60 ± 1 Jahre, BMI 24.9 ± 0.6 kg/m^2, 30 Frauen) ≥ 50 Jahre wurden analysiert. Zwei Testdiäten wurden verwendet. Die Hintergrunddiät bestand aus einer westlichen Diät-Typ (35 % Fett, davon 15 % gesättigte Fettsäuren, 15 % Proteine and 50 % Kohlenhydrate). Die zwei Testdiäten waren bis auf die Tatsache identisch, dass die Probanden in der Walnuss-Phase 30 g gesättigte Fettsäuren durch 43 g geschälte,

vorverpackte Walnüssen ersetzten. Ernährungsprotokolle wurden in regemäßigen Abständen ausgefüllt. Nach einer zweiwöchigen, nussfreien „Run-in"-Periode, dauerte jede Diät acht Wochen und beide Diäten wurden durch eine zwei-wöchige Auswaschphase getrennt. Zu Beginn und am Ende jeder Diätphase erhielten die Probanden eine körperliche Untersuchung, eine standardisierte Testmahlzeit und eine nichtinvasive, postprandiale Endothelfunktionsmessung. Blutentnahmen erfolgten nüchtern, nach 15, 30, 60, 120, 180, 240, 360, und 480 Minuten. Gesamtcholesterin, LDL-Cholesterin, HDL-Cholesterin, Gesamttriglyceride, VLDL-Triglyceride, ApoB, Glukose, Insulin, HOMA-IR, QUICKI, HbA1c, Adipokine, biochemische Marker der endothelialen Dysfunkktion (VCAM-1, ICAM-1 und Endothelin-1) und CRP wurden im Nüchternblut bestimmt. Das nüchtern Non-HDL-Cholesterin wurde über die Gleichung *Gesamtcholesterin - HDL-Cholesterin* berechnet. Die „Area Under the Curve" (AUC) und die inkrementelle AUC (iAUC) wurden für die postprandialen Gesamttriglyceride, VLDL-Triglyceride, Chylomikronen-Triglyceride, sowie für Glukose und Insulin bestimmt.

Im Vergleich zur Kontrolldiät, wurde das Non-HDL-Cholesterin (Walnuss vs. Kontrolle: -10 ± 3 vs. -3 ± 2 mg/dl; p = 0.025) und ApoB (-5.0 ± 1.3 vs. -0.2 ± 1.1 mg/dl; p = 0.009) durch die Walnussdiät signifikant reduziert. Diese Ergebnisse blieben nach der Adjustierung von Alter, Geschlecht, BMI und Diät-Sequenz signifikant. Das Gesamtcholesterin (p = 0.073) zeigte einen Trend zur Reduktion. Nüchtern Gesamttriglyceride, VLDL-Cholesterin, LDL-Cholesterin, HDL-Cholesterin, Glukose, Insulin, HOMA-IR, QUICKI und HbA1c zeigten keine signifikante Veränderung. Nüchtern Adipokine, CRP, biochemische Marker der endothelialen Dysfunktion, postprandiale Lipide und Glukosewerte (AUC und iAUC) sowie die Ergebnisse der Endothelfunktionsmessung veränderten sich ebenfalls nicht signifikant.

Die vorliegende Studie zeigte, dass die achtwöchige Supplementierung von 43 g Walnüssen eine günstige Wirkung auf das Plasmalipidprofil hat, in dem sie das Non-HDL-Cholesterin und Apolipoprotein B senkt. Die kurzfristige Walnusskonsum hatte keinen Einfluss auf den Glukosestoffwechsel, den Blutspiegel von Adipokine und CRP, sowie die endotheliale Funktion, das Körpergewicht und den Bludruck. Die günstige Wirkung auf das Plasmalipidprofil könnte zum Teil die epidemiologische Beobachtung der Reduktion der Häufigkeit von kardiovaskulären Erkrankungen durch Walnusskonsum erklären.

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Ernährungsprotokoll

Name:

vom: bis:

Anzahl der Tage:

Abkürzungen:

Bech. = Becher
Port. = Portion
St.= Stück
EL = Eßlöffel
TL= Teelöffel
Sch.= Scheibe

Lebensmittel	Einheit		Anzahl	Σ
Brot				
Brötchen (Semmel)	St.	45 g		
Croissant (Blätterteig)	St.	50 g		
Graubrot (Roggenmischbrot)	Sch.	45 g		
Hefezopf	Sch.	45 g		
Knäckebrot	Sch.	10 g		
Toastbrot	Sch.	20 g		
Vollkornbrötchen	St.	55 g		
Vollkornbrot	Sch.	50 g		
Weißbrot	Sch.	35 g		
Zwieback	Sch.	10 g		
Laugenbrezel		55 g		
Brotbelag				
Butter für 1 Scheibe Brot	TL	5 g		
Margarine für 1 Scheibe Brot	TL	5 g		
Margarine halbfett s. o.	TL	5 g		
Edelpilzkäse (Blauschimmel)	Sch.	30 g		
Frischkäse	EL	30 g		
Schmelzkäse	Port.	30 g		
Schnittkäse 30% F.i.Tr.	Sch.	30 g		
Schnittkäse 50% F.i.Tr.	Sch.	30 g		
Weichkäse 45% F.i.Tr.	Sch.	30 g		
Weichkäse 60 % F.i.Tr.	Sch.	30 g		
Bierschinken	Sch.	25 g		
Corned Beef	Port.	25 g		
Fleischwurst	Sch.	20 g		
Fleischkäse (Aufschnitt)	Sch.	30 g		
Fleischsalat	Port.	50 g		
Leberwurst	Port.	30 g		
Mettwurst	Port.	30 g		
Teewurst	Port.	30 g		
Salami/Cervelatwurst	Sch.	20 g		

Lebensmittel	Einheit		Anzahl	Σ
Schinken roh, geräuchert	Sch.	15 g		
Schinken gekocht	Sch.	30 g		
Speck (Schwein Bauchspeck)	Port.	30 g		
Honig	EL	20 g		
Konfitüre	EL	20 g		
Nuß-Nougat-Creme	EL	20 g		
vegetarischer Brotaufstrich	Port.	30 g		
Frühstücksallerlei				
gekochtes Ei	St.	55 g		
Cornflakes	EL	4 g		
Cornflakes gezuckert/geröstet	EL	6 g		
Haferflocken	EL	10 g		
Müsli mit Trockenobst, ohne Zucker	EL	15 g		
Milch / Milchprodukte				
Buttermilch	Glas	200 g		
Joghurt natur fettarm (1,5%F.)	Bech.	150 g		
Joghurt natur vollfett (3,5% F.)	Bech.	150 g		
Joghurt mit Frucht fettarm (1,5%F.)	Bech.	150g		
Joghurt mit Frucht vollfett (3,5% F.)	Bech.	150 g		
Kakao/Trinkschokolade	Gl	200 g		
Milch vollfett (3,5% F.)	Gl	200 g		
Milch fettarm (1,5% F.)	Gl	200 g		
Quark Magerstufe	EL	20 g		
Quark Halbfettstufe	EL	10 g		
Kondensmilch (7,5% F.)	Port.	12 g		
Sahne (30 % F.)	EL	10 g		
Fettakäse (Schafsmilch)	Sch.	20 g		
Mozzarella	Sch.	20 g		
Obst				
Brombeere, Erdbeere, Him-beere, Johannisbeere, Heidel-beere (Beerenobst)	Port.	125 g		
Weinraube	Port.	150 g		
Apfel, Birne, Quitte,... (Kern-obst)	Port.	150 g		
Aprikose, Kirsche, Mirabelle, Pflaume, Pfirsich,... (Steinobst)	Port.	150 g		
Banane	St.	120 g		
Ananas, Kiwi, Mango, Maracu-ja,... (Südfrüchte)	Port.	150 g		
Grapefruit, Mandarine, Oran-ge, Zitrone (Zitrusfrüchte)	Port.	150 g		

Lebensmittel	Einheit		Anzahl	Σ
Rosinen, Trockenobst	Port.	50 g		
Sonstiges				
Cornichons, saure Gurken	St.	50 g		
Nüsse	Port.	100 g		
Oliven	Port.	100 g		
Erdnüsse gesalzen	Tasse	100 g		
Chips	Tasse	50 g		
Erdnußflips	Tasse	30 g		
Salzstangen	Port.	30 g		
Suppen / Eintöpfe				
als Vorsuppe				
Suppe klar	Port.	200 g		
Suppe gebunden	Port.	200 g		
Cremesuppe	Port.	200 g		
Gulaschsuppe	Port.	200 g		
Nudelsuppe m. Huhn	Port.	200 g		
als Hauptgericht				
Gemüsesuppe	Port.	400 g		
Kartoffelsuppe	Port.	400 g		
Linseneintopf	Port.	400 g		
Fleisch / Fisch				
Hackfleisch (frisch)	Port.	100 g		
Kalbfleisch	Port.	200 g		
Rindfleisch	Port.	200 g		
Schweinefleisch (mittelfett)	Port.	200 g		
Schweinekotelett	Port.	170 g		
Innereien (Leber, Herz, Niere)	Port.	170 g		
Schnitzel paniert	Port.	200 g		
Würstchen (Konserve)	Port.	100 g		
Brathähnchen (1/2)	Port.	370 g		
Geflügel (Brust)	Port.	170 g		
Ente (mit Haut)	Port.	170 g		
Fisch	Port.	175 g		
Fischfilet paniert	Port.	200 g		
Fischkonserve abgetropft	Port.	65 g		
Beilagen				
Kartoffeln (Salzkart.)	Port.	200 g		
Pellkartoffeln	Port.	200 g		
Bratkartoffeln	Port.	200 g		
Kartoffelbrei	Port.	200 g		
Kartoffelknödel/-klöße	St.	100 g		
Kartoffelpuffer	St.	75 g		
Kartoffelsalat (+Mayonnaise)	Port.	250 g		

Figure A1. Diet protocol page 1.

Figure A2. Diet protocol page 2.

Pommer Frites	Port. 200 g
weißer Reis gekocht	Port. 180 g
Natur-Reis gekocht	Port. 180 g
Nudeln eifrei gekocht	Port. 180 g
Vollkornnudeln gekocht	Port. 180 g
Semmelknödel	St. 100 g
Schupfnudeln	Port. 400 g
Spätzle, Eiernudeln gekocht	Port. 200 g

Soßen und Fette

Joghurt-Salat-Soße	Port. 40 g
Essig-Öl-Marinade	Port. 20 g
Bechamelsoße	Port. 75 g
Grundsoße	Port. 75 g
Hackfleischsoße	Port. 100 g
Jägersoße	Port. 75 g
Käsesoße	Port. 75 g
Grüne Soße	Port. 200 g
Tomatensoße	Port. 75 g
Grillsauce	Port. 20 g
Tomatenketchup	Port. 20 g
Tomatenmark	Port. 30 g
Senf	TL 6 g
Mayonnaise (80% F.)	EL 12 g
Bratfett	EL 10 g
Pflanzenöl	EL 10 g
Pesto	Port. 30 g

Gemüse / Salate

Blattsalat mit Dressing	Port. 60 g
Rohkostsalat mit Sahnedressing	Port. 180 g
Bleichsellerie, Mangold, Spinat (Blattgemüse)	Port. 200 g
Grüne Bohnen	Port. 200 g
Aubergine, Gurke, Paprika, Tomate, Zucchini (Fruchtgemüse)	Port. 200 g
Gemüsemais	Port. 200 g
Blumenkohl, Broccoli, Kohl (Rot-, Grün-, Weiß-), Kohlrabi, Rosenkohl, Wirsing	Port. 200 g
Sauerkraut	Port. 150 g
Fenchel, Lauch, Spargel, Zwiebel (Sprossengemüse)	Port. 200 g
Möhre, Radieschen, Rettich, Rote Bete, Rüben, Sellerie, Schwarzwurzel (Wurzel- und Knollengemüse)	Port. 200 g

Pilze	Port. 120 g

Fertig- und Schnellgerichte

Nudelsalat (mit Mayonnaise)	Port. 250 g
Wurstsalat	Port. 250 g
Griechischer Salat	Port. 300 g
Italienischer Salat	Port. 300 g
Bratwurst ohne Brötchen	St. 150 g
Currywurst ohne Brötchen	St. 150 g
Hamburger	St. 100 g
Cheeseburger	St. 120 g
Big Mac	St. 200 g
Maultaschen / Ravioli	Port. 200 g
Pizza (Käse, Salami)	St. 400 g
Pfannkuchen	Port. 300 g

Hülsenfrüchte

Bohnen	Port. 200 g
Erbsen	Port. 200 g
Linsen	Port. 200 g

Dessert/Kuchen / Süßes

Rührkuchen	St. 60 g
Cremetorte	St. 150 g
Obstkuchen, Rührteig	St. 120 g
Eiscreme	Kugel 50 g
Pudding	Port. 150 g
Schokolade (Riegel)	Port. 20 g
Plätzchen, Kekse	St. 10 g
Bonbon, Hartkaramelle	St. 3 g
Praline	St. 10 g
Fruchtgummi/ -bärchen	Port. 50 g
Zucker	TL 5 g

Getränke

Kaffee, ohne Milch und Zucker	kl. Tasse 150 g
Tee, schwarz	k.Tasse 150 g
Kräutertee	k.Tasse 150 g
Cappuccino	250 g
Mineralwasser	Glas 200 g
Limonade	Glas 200 g
Colagetränke	Glas 200 g
Obst- Fruchtsaft	Glas 200 g

Obst- Fruchtnektar	Glas 200 g
Bier, alkoholfrei	Glas 330 g
Bier, hell	Glas 330 g
Weizenbier	Glas 500 g
Weißwein	Glas 200 g
Rotwein	Glas 200 g
Sekt	Glas 100 g
Likör	Glas 40 g
Schnaps, Branntwein	Glas 20 g

Hier haben Sie noch einmal die Möglichkeit nichtgelistete Lebensmittel, die Sie verzehrt haben, aufzuschreiben!

9   ACKNOWLEDGEMENTS

Eidesstattliche Versicherung

Wu, Liya
Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

The effect of walnut consumption on lipid and glucose metabolism, adipokines, C-reactive protein, endothelial function, body weight and blood pressure in healthy men and healthy postmenopausal women

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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München, den 07.07.2016
Ort, Datum

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