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Stoffwechseleffekt von Walnüssen im Austausch für verschiedene Nahrungsbestandteile in gesunden Männern und Frauen –

Der Einfluss von regelmäßigem Walnusskonsum auf Blutlipide und das Darmmikrobiom: eine prospektive, randomisierte Studie

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
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Charlotte Bamberger
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1. Zusammenfassung

Aktuelle Studien suggerieren einen Zusammenhang zwischen regelmäßigem Walnusskonsum und einer Verbesserung des Lipidprofils. Bisher ist jedoch unklar, ob sich der lipidsenkende Effekt durch den Ersatz von Makronährstoffen durch Walnüsse, sowie der Zeitpunkt des Nussverzehrs erklären lässt. Daher wurden in dieser Studie der Effekt von Walnüssen auf Nüchternlipide im Austausch für verschiedene Makronährstoffe (Kohlenhydrate, Fett, oder beides) und der Einfluss des Zeitpunkts des Nussverzehrs (zu einer Hauptmahlzeit, als Zwischenmahlzeit) untersucht. Ergänzend dazu wurde der Einfluss eines regelmäßigen Walnussverzehrs auf das Darmmikrobiom analysiert.

In einer randomisierten, kontrollierten, prospektiven Cross-Over-Studie wurden 194 gesunde Männer und postmenopausale Frauen > 50 Jahre (134 Frauen, 63±7 Jahre, BMI 25.1±4.0 kg/m²) eingeschlossen. Die Probanden folgten zuerst einer 4-wöchigen nussfreien Periode auf Basis einer vollwertigen (Western-Typ) Ernährung. Anschließend wurden die Teilnehmer in 2 Interventionsgruppen randomisiert. Eine Gruppe (n=96) befolgte eine mit geschälten Walnüssen (43 g/Tag) substituierte, die andere (n=98) eine nussfreie Western-Typ Ernährung. Nach 8 Wochen der Intervention durchliefen die Probanden eine 4-wöchige Auswaschphase und folgten dann 8 Wochen lang der jeweils anderen Ernährung. Zusätzlich wurden die Probanden in 3 Ernährungsgruppen eingeteilt, in welcher Form sie den Energiegehalt der Walnüsse einsparen sollten. Während der Walnussphase sollte die erste Gruppe Kohlenhydrate (n=62), die zweite Fett (n=65) und die dritte beides (n=67) einsparen. Zusätzlich wurden die Probanden in weitere 2 Gruppen randomisiert, in welcher sie die Walnüsse entweder zu den Hauptmahlzeiten oder als Zwischenmahlzeit konsumieren sollten. Als primärer Endpunkt wurde das non-HDL-Cholesterin bestimmt. Daneben wurden Gesamtcholesterin, LDL-Cholesterin, HDL-Cholesterin, Triglyzeride, Apolipoprotein B und Lipoprotein (a) im Nüchternplasma untersucht. Zu Beginn und Ende der Interventionsphasen wurden bei 135 Studienteilnehmern Stuhlproben gewonnen und mittels 16S rRNA Amplikon Sequenzierung analysiert. Zur Bestimmung phylogenetischer interindividueller Unterschiede wurde eine multivariate UniFrac-Analyse durchgeführt. Dabei wurden Sequenzen mit einer 97% Sequenzidentität in operationalen taxonomischen Einheiten gruppiert. Signifikante Unterschiede auf Taxonomie-Ebene wurden mit dem

Kruskal-Wallis Test bestimmt. Alle Analysen wurden mit der R-Pipeline Rhea durchgeführt.

Verglichen mit der Kontrolle konnte unter Walnussverzehr eine signifikante Reduktion von non-HDL-Cholesterin (-9.14±20.66 vs -1.29±19.15; p≤ 0.0001), Gesamtcholesterin (Walnuss vs Kontrolle in mg/dl: -8.14±24.42 vs -1.02±22.55; p=0.0025), LDL-Cholesterin (-7.18±20.27 vs -1.82±17.94; p=0.0035), Apolipoprotein B (-6.56±14.00 vs -0.60±12.48; p≤ 0.0001) und der Triglyceride (-4.52±30.34 vs +4.03±42.8; p=0.0162) gezeigt werden. Zwischen den einzelnen Makronährstoff - und Mahlzeitengruppen konnte kein signifikanter Unterschied zwischen den Lipidparametern ermittelt werden.

Die UniFrac-Analyse deutete auf eine signifikante Veränderung in Komposition und Diversität der Darmmikrobiota hin. Mittels einer multidimensionalen Skalierung wurde ein Unterschied von 5% zwischen Walnuss- und Kontrolldiät ermittelt (p=0,02). Ein fortlaufender Gruppenvergleich zeigte eine signifikante Zunahme von Spezies der Familie *Ruminococcaceae* und *Bifidobacteriaceae* (p<0,02) während der Walnussdiät, wohingegen Clostridium Cluster XIVa Spezies (*Blautia*, *Anaerostipes*) signifikant weniger auftraten (p<0,05).

Ein täglicher Verzehr von 43 g Walnüssen über 8 Wochen führte zu einer signifikanten Reduktion von non-HDL-Cholesterin, Gesamtcholesterin, LDL-Cholesterin und Apolipoprotein B. In welcher Form die Probanden die zusätzliche Energie der Walnüsse einsparten, beeinflusste diesen Effekt nicht. Außerdem zeigte sich ein signifikanter Anstieg probiotisch wirksamer und Buttersäure-produzierender Spezies unter dem Walnussverzehr. Noch ist jedoch unklar, inwiefern ein langfristiger Verzehr das Darmmikrobiom beeinflusst und in welchem Zusammenhang dies mit der Verbesserung der Blutlipide steht.

2. Summary

Previous studies indicate an association between regular walnut intake and improvement in the plasma lipid profile. However, it is yet unclear whether macronutrients replaced by walnuts and time point of consumption influence this improvement. Therefore, we evaluated the effect of the isocaloric replacement of macronutrients (carbohydrates, fat or both) with walnuts and the time point of consumption (meal, snack) on fasting plasma lipid profile. Gut microbiome was evaluated in the same study population.

We included 194 healthy Caucasian men and postmenopausal women over the age of 50 (134 females and 60 males, age 63±7 y, BMI 25.1±4.0 kg/m²) in a randomized, controlled, prospective, cross-over study. Subjects followed a nut-free Western-type diet during a 4-week run-in period. Thereafter, subjects were randomized to 2 different diet phases, each lasting for 8 weeks (separated by a 4 week wash-out). One group (n=96) first followed a walnut-enriched diet (43 g of shelled walnuts/d) and then switched to a nut-free control diet. The other group (n=98) followed the diets in reverse order. Subjects were also divided into 3 different diet groups in which they were advised to reduce the intake of either carbohydrates (n=62) or fat (n=65) or both (n=67) during walnut diet. Furthermore, the groups were randomized to consume walnuts either during a meal or as snack. As primary endpoint, non-HDL-cholesterol was measured in fasting samples, Furthermore, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, apolipoprotein B and lipoprotein (a) were determined in fasting samples. Fecal samples were collected from 135 participants at the end of each intervention period for microbiome analyses. Based on 16S rRNA amplification and an OTU (Operational Taxonomic Unit) threshold of 97% similarity UniFrac distances were calculated to evaluate beta-diversity. Differential abundance was evaluated using the Kruskal-Wallis Rank Sum test. All analyses were performed using the R-pipeline Rhea.

Compared with the control diet, the walnut diet resulted in a significant reduction in fasting non-HDL-cholesterol (walnut vs control: -9.14±20.66 vs -1.29±19.15 mg/dl; p=< 0.0001), total cholesterol (-8.14±24.42 vs -1.02±22.55 mg/dl ; p=0.0025), LDL-cholesterol (-7.18±20.27 vs -1.82±17.94 mg/dl; p=0.0035), triglycerides (-4.52±30.34 vs +4.03±42.8 mg/dl; p=0.0162) and apolipoprotein B (-6.56±14.00 vs -0.60±12.48;

p=< 0.0001). There was no significant difference between the diet groups and time points of walnut consumption.

Weighted UniFrac analysis shows that walnut consumption significantly alters microbiome composition and diversity. Multidimensional scaling (metric and non-metric) indicates dissimilarities of approximately 5% between walnut and control (p=0.02). Serial group comparisons indicate that abundance of *Ruminococcaceae* and *Bifidobacteria* increased significantly (p<0.02) while Clostridium cluster XIVa species (*Blautia*; *Anaerostipes*) decreased significantly (p<0.05) during walnut consumption compared to the control diet.

Daily intake of 43 g of walnuts over 8 weeks significantly reduced non-HDL-cholesterol, LDL-cholesterol and ApoB, independent of the recommended macronutrient replacement and the time point of consumption. Furthermore, daily walnut intake significantly affected gut microbiome enhancing probiotic and butyric acid producing species in healthy individuals. Whether these changes are sustained during longer walnut consumption and how these changes are linked to the observed changes in lipid metabolism is yet unclear and requires further evaluation.

3. Einleitung

3.1 Epidemiologie kardiovaskulärer Erkrankungen

Kardiovaskuläre Erkrankungen gehören zu den Krankheitsbildern mit weltweit großer epidemiologischer Bedeutung und stellen die häufigste Todesursache der westlichen Welt dar. Im Jahr 2012 lag die geschätzte Zahl an Todesfällen, welche auf kardiovaskuläre Erkrankungen zurückzuführen war, bei 17,5 Mio. Menschen, was 31 % aller Todesfälle weltweit repräsentiert [1]. Laut der World Health Organization (WHO) könnten 80 % vorzeitiger Todesfälle durch kardiovaskuläre Erkrankungen vermieden werden, wenn man betrachtet, dass sich die hohe Mortalitätsrate aus einer Kombination vermeidbarer Risikofaktoren ergibt. Zu diesen zählen Adipositas, Hypertonie, körperliche Inaktivität, Nikotin- und Alkoholmissbrauch, falsches Ernährungsverhalten, sowie Dyslipoproteinämien [1, 2]. Global betrachtet werden diejenigen Todesfälle, welche auf eine Erhöhung des Cholesterins zurückzuführen sind auf etwa 2,6 Mio. geschätzt (Abbildung 1) [1].

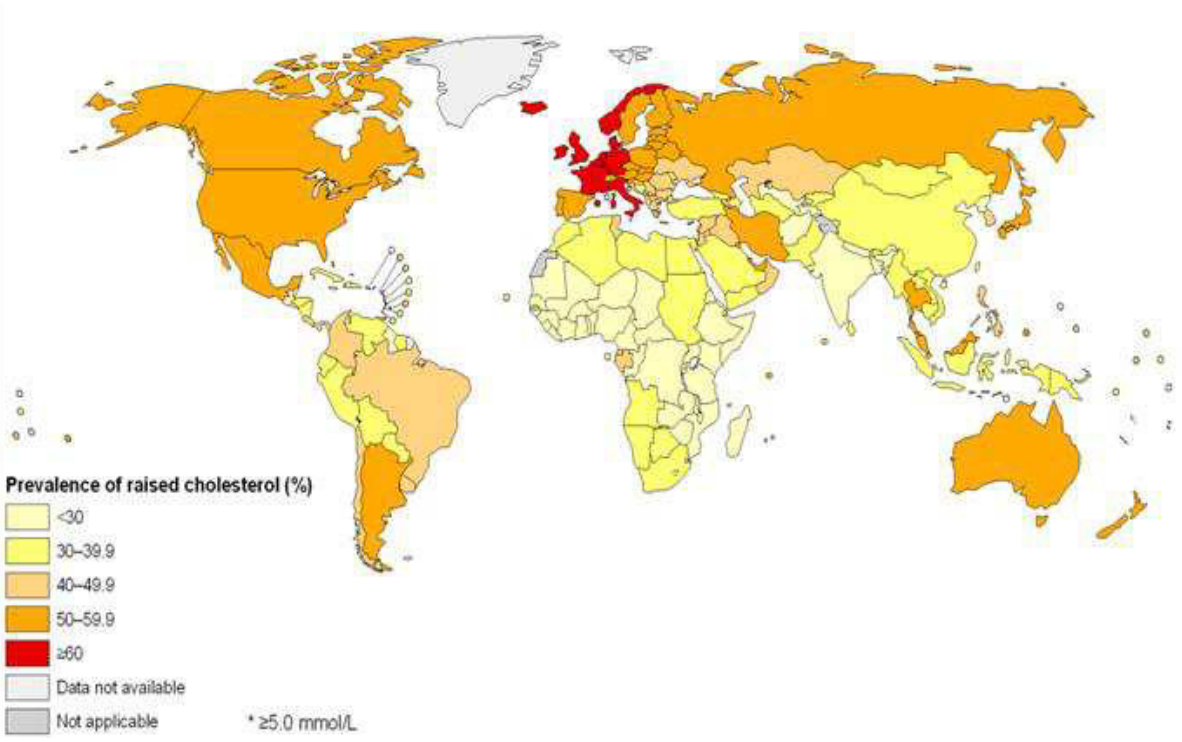

Abbildung 1 Prävalenz von Hypercholesterinämie > 25 Jahre im Jahre 2008 (altersstandardisierte Mortalität beiderlei Geschlechts) [WHO, 2011] http://gamapserver.who.int/mapLibrary/app/searchResults.aspx

3.2 Grundlagen und Klassifizierung der Lipoproteine

Cholesterin gilt als ein globaler Marker für kardiovaskuläre Erkrankungen, wobei Veränderungen des Cholesterinspiegels Veränderungen verschiedener Lipoproteine und Lipide repräsentieren. Im menschlichen Plasma lassen sich verschiedene Lipidklassen finden, welche aufgrund ihrer Hydrophobie an Proteine gekoppelt ins Gewebe transportiert werden und so für zahlreiche Funktionen (u.a. Energienutzung, Hormonproduktion, Bildung von Gallensäuren) für den Körper zur Verfügung stehen. Hinsichtlich ihrer physiochemischen Eigenschaften werden Lipoproteine nach ihrer (steigenden) Dichte in Fraktionen klassifiziert. Von besonderer Bedeutung sind dabei vor allem das low-density Lipoprotein (LDL), das very-low-density Lipoprotein (VLDL), das intermediate-density Lipoprotein (IDL) und das high-density Lipoprotein (HDL), wobei der größte Anteil des Cholesterins in Form von LDL transportiert wird [3]. Als Hauptproteinbestandteil der Lipoproteine geringer Dichte und als Ligand des LDL-Rezeptors ermöglicht das Apolipoprotein B (ApoB) die Cholesterinabgabe an das umliegende Gewebe [4]. Eine strukturelle Sonderform des LDL stellt das Lipoprotein (a) (Lp(a)) dar, welches in der Leber synthetisiert wird, allerdings unabhängig von der Synthese der anderen ApoB-haltigen Lipoproteine [5]. Die Plasmakonzentration dieses Lipoproteins (normal < 30 mg/dl) ist hauptsächlich genetisch bedingt, wobei sowohl die Ernährung als auch andere Lebensstilinterventionen keine klinisch relevanten Einflussfaktoren darstellen [6].

3.3 Pathogenese der Atherosklerose

Werden Lipoproteine oxidiert oder anderweitig modifiziert, führt dies direkt oder über eine Entzündungsreaktion zu einer Endotheldysfunktion. Man geht heute davon aus, dass dies der Ausgangspunkt für die Entwicklung atherosklerotischer Veränderungen ist (*„[…] eine variable Kombination von Intimaveränderungen der Arterien, die aus einer fokalen Anhäufung von Lipiden, komplexen Kohlenhydraten, Blut und Blutprodukten, fibrösem Gewebe und Kalkablagerungen bestehen und mit Mediaveränderungen einhergehen“*) [7, 8]. Dabei werden vornehmlich der LDL-Fraktion und dem Lp(a) atherogene Eigenschaften zugeschrieben, wohingegen mit der HDL-Fraktion protektive antiatherogene Funktionen assoziiert werden. Durch eine subintimale Anreicherung proatherogener Lipoproteine kommt es zu einer Abfolge entzündlicher Reaktionen, was zu einer zunehmenden Rekrutierung und

Infiltrierung von Immunzellen und letztendlich zu einer Entzündung und Verdickung der Gefäßwand zur Folge hat (Abbildung 2) [9].

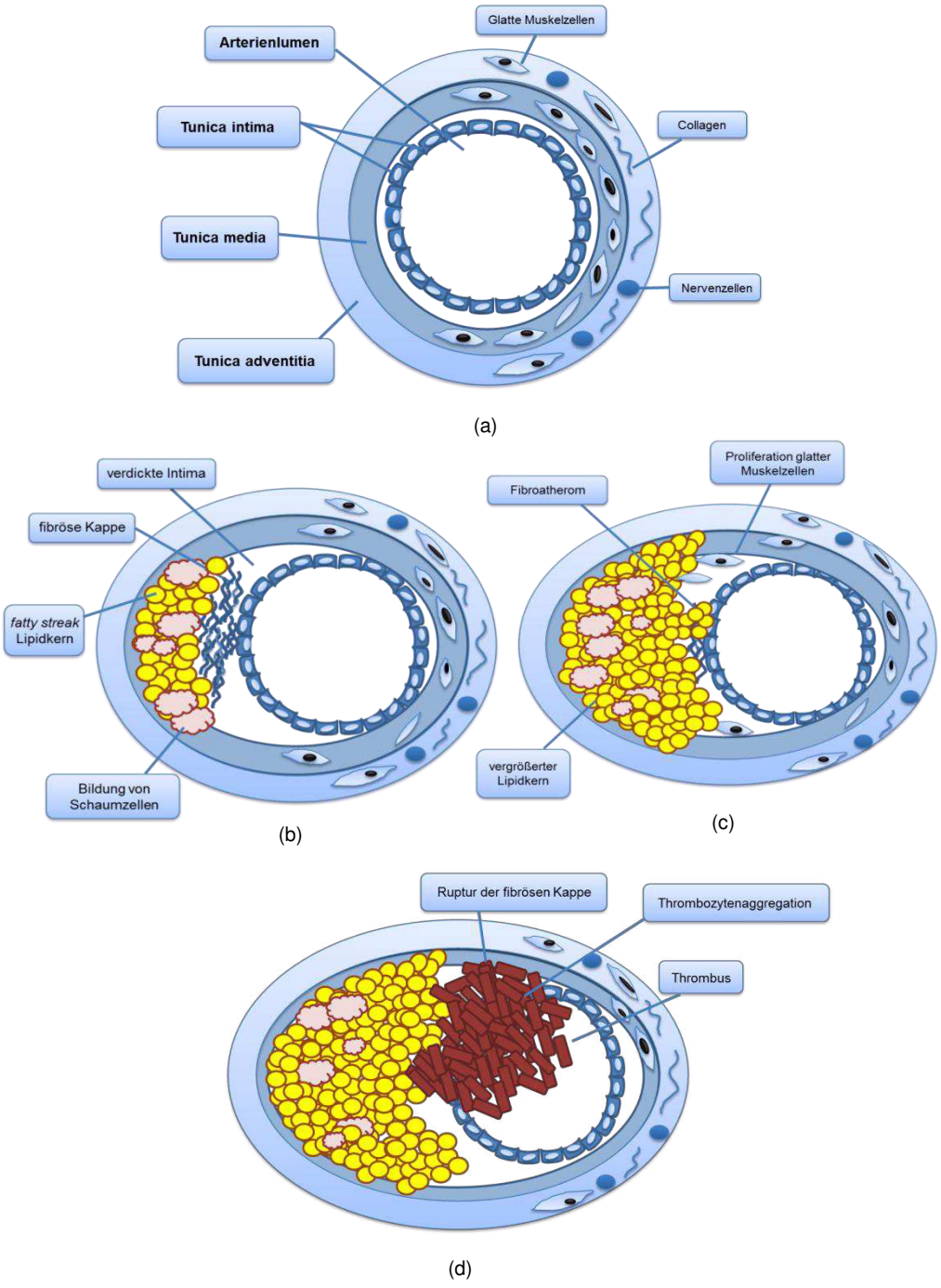

Abbildung 2 Atherogenese: (a) gesunde Arterie (b) Stabiler Plaque (c) Instabiler Plaque (d) Plaqueruptur

Eine Erhöhung der Konzentration der atherogenen Lipoproteine kann die fortschreitende Entwicklung der Atherosklerose und damit einhergehend die Inzidenz

atherosklerotischer Folgeerkrankungen (wie Herzinfarkt, Schlaganfall oder periphere arterielle Verschlusskrankheit) enorm beeinflussen [10].

3.4 Medikamentöse Therapieoptionen bei Hyperlipidämien

Das Hauptziel der lipidsenkenden Therapie umfasst vornehmlich eine Verminderung des Risikos von Folgeerkrankungen. Dabei richtet sich der anzustrebende LDL-Cholesterinwert nach dem Gesamtrisiko. Das Gesamtrisiko setzt sich zusammen aus den Risikofaktoren Geschlecht, Alter, Gesamtcholesterin, LDL-Cholesterin, HDL-Cholesterin, genetische Prädisposition, Diabetes Mellitus Erkrankung, arterieller Hypertonus, sowie Nikotinkonsum. Gemäß den Richtlinien der European Atherosclerosis Society (ESC/EAS Guidelines) für die Behandlung von Dyslipidämien steht neben der Optimierung anderer Risikofaktoren der Hypercholesterinämie-Therapie die Senkung des LDL-Spiegels im Vordergrund (Tabelle 1) [10].

Tabelle 1 Therapieempfehlungen bei Hyperlipidämie (modifiziert nach [10])

	LDL-Cholesterin-Spiegel				
Kardiovaskuläres Gesamtrisiko (SCORE) %	< 70 mg/dl (< 1,8 mmol/l)	70 bis < 100 mg/dl (1,8 bis < 2,6 mmol/l)	100 bis < 155 mg/dl (2,6 bis < 4,o mmol/l)	155 bis < 190 mg/dl (4,0 bis < 4,9 mmol/l)	≥ 190 mg/dl (≥ 4,9 mmol/l)
< 1%	Keine Intervention notwendig	Keine Intervention notwendig	Keine Intervention notwendig	Keine Intervention notwendig	Lebensstilmodifikation medikamentöse Therapie erwägen
≥ 1% bis < 5%	Keine Intervention notwendig	Keine Intervention notwendig	Lebensstilmodifikation medikamentöse Therapie erwägen	Lebensstilmodifikation medikamentöse Therapie erwägen	Lebensstilmodifikation medikamentöse Therapie erwägen
≥ 5% bis < 10%	Keine Intervention notwendig	Lebensstilmodifikation medikamentöse Therapie erwägen	Lebensstilmodifikation unmittelbare medikamentöse Therapie	Lebensstilmodifikation unmittelbare medikamentöse Therapie	Lebensstilmodifikation unmittelbare medikamentöse Therapie
≥ 10% oder Höchstrisiko	Lebensstil-modifikation, medikamen-töse Therapie erwägen	Lebensstilmodifikation unmittelbare medikamentöse Therapie	Lebensstilmodifikation unmittelbare medikamentöse Therapie	Lebensstilmodifikation unmittelbare medikamentöse Therapie	Lebensstilmodifikation unmittelbare medikamentöse Therapie

Bei Vorliegen einer sekundären Ursache (klinisch bedeutend sind hier z.B. Diabetes Mellitus, Erkrankungen der Niere oder Leber, Hypothyreose) sollte primär die Grunderkrankung therapiert werden. Ist eine sekundäre Hyperlipoproteinämie ausgeschlossen oder soweit wie möglich behandelt, sollte das LDL-Cholesterin-Ziel entsprechend dem in Abbildung 3 gezeigten Algorithmus angestrebt werden.

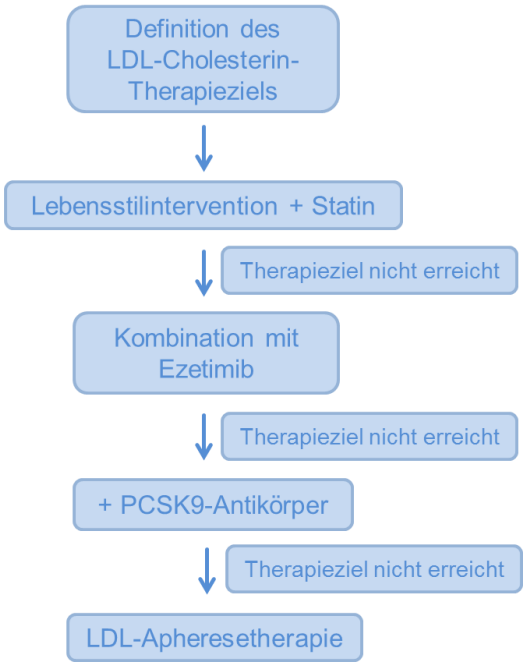

Abbildung 3 Therapiealgorithmus zur Erreichung des LDL-Ziels (modifiziert nach [57])

Der Übergang von einer Behandlungsstufe zur nächsten setzt voraus, dass die Zielwerte nach 6 - 8 Wochen (bei Lebensstilumstellung bis zu 3 Monaten) nicht erreicht oder die Medikation nicht vertragen wurden [57]. Als Mittel der ersten Wahl für eine **medikamentöse Primär- und Sekundärprävention** bei Hochrisikopatienten gilt die Statin-basierte LDL-Senkung (als Monotherapie Senkung um 30 – 40%). Dessen Wirkung vornehmlich auf der Inhibition der Cholesterinbiosynthese basiert, was eine erhöhte Aufnahme von Cholesterin über spezifische Rezeptoren und somit eine Erniedrigung des LDL-Spiegels bewirkt [11]. Unterstützend und bei Unverträglichkeit können Therapien mit anderen Wirkstoffen in Erwägung gezogen werden. Sollten eine Lebensstilintervention mit medikamentöser Kombinationstherapie nicht zu einer ausreichenden Senkung führen (z.B. im Falle einer schweren Hyperlipoproteinämie oder ausgeprägter Atherosklerose), so stellt die Lipidapherese die effektivste Therapie dar [12].

3.5 Nicht-medikamentöse, ernährungsspezifische Behandlungsmaßnahmen

Die Basis der **nicht-medikamentösen Behandlung** von Fettstoffwechselstörungen bildet sowohl eine kaloriengerechte Optimierung der Lebensmittelauswahl als auch regelmäßige körperliche Aktivität, sowie Nikotinverzicht, Blutdruck- und Gewichtsnormalisierung [13]. Dabei nimmt eine langfristige Ernährungsumstellung einen besonderen Stellenwert ein. Die Maßnahme zur Senkung der LDL-Konzentrationen besteht im Wesentlichen im Austausch gesättigter Fettsäuren durch mehrfach ungesättigte Fettsäuren (PUFAs). Die unterschiedlichen Klassen der Fettsäuren besitzen unterschiedliche nutritive Bedeutung. PUFAs werden aufgrund ihrer physiologischen Wirkung kardioprotektive Eigenschaften zugeschrieben [14]. Unterschieden werden hierbei Omega-3 (n-3) - und Omega-6 (n-6)-Fettsäuren, wobei als wichtigste Vertreter die alpha-Linolensäure (n-3) und die Linolsäure (n-6) zu nennen sind. Die Hauptnahrungsquellen mehrfach ungesättigter Fettsäuren bilden vor allem marine Quellen (fetter Seefisch), Nüsse, Samen und pflanzliche Öle.

Das kardioprotektive Potential der n-3-PUFA kann vornehmlich auf ihre antiarrhytmische, antihypertensive, antiinflammatorische und antithrombotische W**i**rkweise zurückgeführt werden [15-18]. n-3-PUFA-Präparate werden mit der Indikation einer Hypertriglyceridämie oder in Kombination mit Statinen (z.B. im Falle gemischter Hyperlipidämie) therapeutisch eingesetzt, wobei eine Dosis von 2 – 4 g/Tag empfohlen wird [19, 20]. Als Therapiemaßnahme finden n-3-PUFAs sowohl in der Primär- als auch in der Sekundärprevention kardiovaskulärer Erkrankungen ihren Einsatz. So zeigte u.a. die PREDIMED Studie, eine klinische randomisierte und kontrollierte Langzeit-Multicenter-Studie, dass die mediterrane Ernährung, ergänzt durch natives Olivenöl oder Nüsse (vornehmlich Walnüsse), einen positiven Einfluss auf den arteriellen Blutdruck, Blutfette, Blutzuckerwerte und Entzündungsparameter hat. Insgesamt konnte die Ereignisrate für Herzinfarkte, Schlaganfälle sowie kardiovaskulär bedingte Todesfälle um 30 % reduziert werden [21]. Ebenso konnte der positive Effekt der mediterranen Ernährung in der Sekundärprevention kardiovaskulärer Erkrankungen belegt werden. So zeigte die in den 90er Jahren durchgeführte Lyon-Heart-Studie, dass eine mediterrane Ernährungsform das Wiederauftreten kardiovaskulärer Ereignisse in Risikopatienten deutlich vermindern kann, wobei sowohl die primären Endpunkte (Tod oder Reinfarkt) als auch sekundäre Endpunkte (z.B. erstmaliger Myokardinfarkt, Schlaganfall) signifikant seltener erreicht

wurden, was etwa einer Halbierung des Risikos für kardiovaskuläre Ereignisse entspricht [22, 23]. Eine nachhaltige Veränderung des Ernährungsverhaltens steht somit im Fokus sowohl in der Prävention als auch in der Therapie kardiovaskulärer Erkrankungen.

3.6 Kardioprotektive Wirkung von Nüssen

Eine mediterrane Ernährungsform basierend auf dem vermehrten Verzehr mehrfach ungesättigter Fettsäuren aus marinen Quellen, Olivenöl und Nüssen ist aufgrund ihrer antioxidativen und kardioprotektiven Wirkung von ernährungsphysiologischer Bedeutung in der Primärprevention koronarer Erkrankungen. Der positive Einfluss des Verzehrs von Nüssen auf kardiovaskuläre Risikofaktoren und kardiovaskuläre Mortalität wurde bereits in zahlreichen epidemiologischen Studien demonstriert. So fasst eine aktuelle Meta-Analyse das kardioprotektive Potenzial von regelmäßigem Nussverzehr zusammen und schlussfolgert, dass ein erhöhter Konsum eine Risiko-reduktion für sowohl kardiovaskuläre Erkrankungen, als auch Krebsmortalität und Gesamtmortalität mit sich bringt [24] (Abbildung 4a).

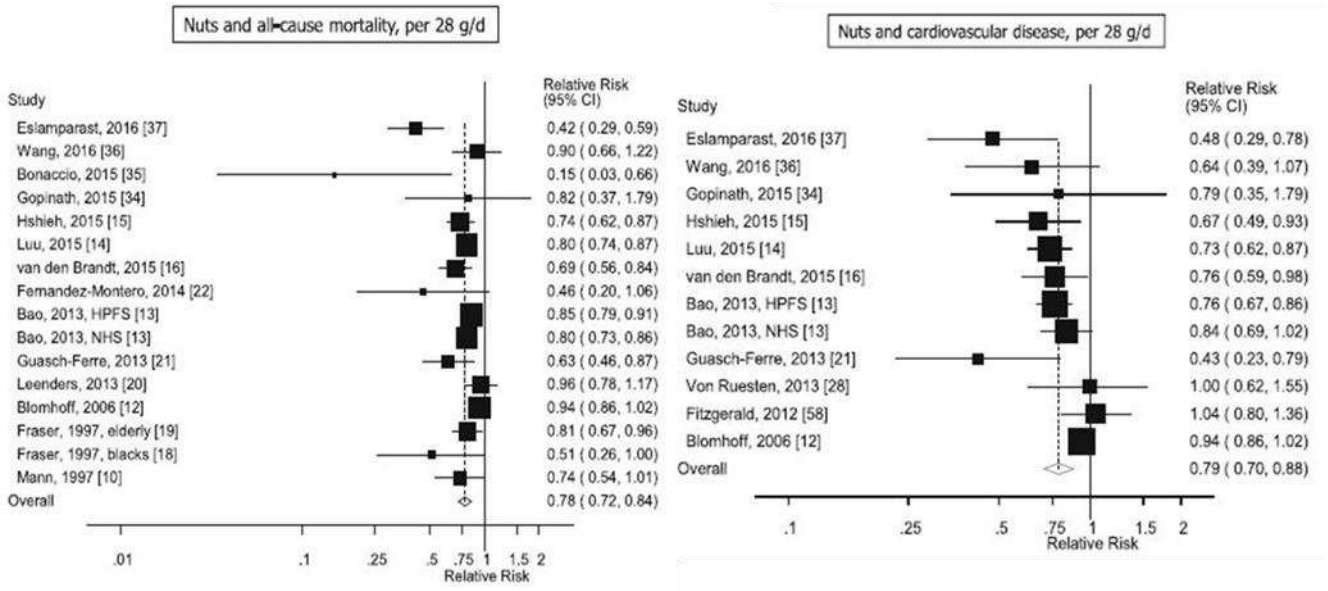

Abbildung 4a Nussverzehr (28g/d) und Risikoreduzierung von Gesamtmortalität und kardiovaskulären Erkrankungen (nach Aune et al., 2016) [25]

Eine zusammenfassende Analyse von vier großen US amerikanischer Kohortenstudien (Adventist Health Study n = 31 208, Iowa Women's Health Study n = 34 000, Nurses' Health Study n = 86 016 und Physicians' Health Study n = 22 071) konnte ähnliche Ergebnisse zeigen: ein erhöhter Nusskonsum (> 5 mal pro Woche) ging mit einer Risikoreduktion für koronare Herzerkrankungen von 40 % einher, was einer Risikominderung der kardiovaskulären Mortalität von durchschnittlich 6,3 % entsprach (Abbildung 4b) [25].

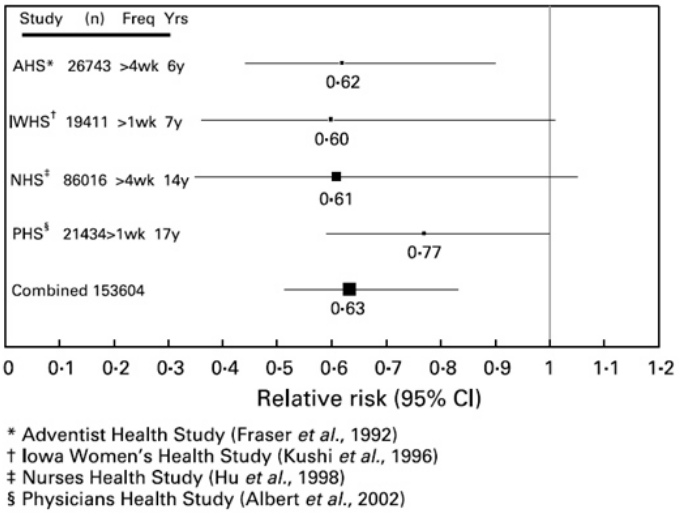

Abbildung 4b Prospektive Kohortenstudien über Nusskonsum und koronare Herzerkrankungen (Kelly und Sabaté, 2006) [24]

Der Zusammenhang zwischen einem erhöhten Nussverzehr und einer Risikoverminderung kardiovaskulärer Ereignisse wurde aus verschiedenen Blickwinkeln betrachtet. Untersucht wurde dabei der Einfluss auf inflammatorische Marker in Stadien atherosklerotischer Plaquebildung [26], Endothelfunktion [27] und oxidativen Stress [28], Insulinresistenz [29], Gefäßreagibilität [30], Blutdruck [31] und Plasmalipid- und Lipoproteinkonzentrationen [32]. Ein Großteil der Studien untersuchte den Effekt von regelmäßigem Nussverzehr als Teil einer Ernährung, welche u.a. fettarm [33, 34] und reich an Kohlenhydraten [35] war, oder bei welcher die mediterrane Ernährungsform dominierte [27, 36]. Insgesamt zeigten die klinischen Studien stets einen Cholesterin-senkenden Effekt bedingt durch regelmäßigen Nussverzehr, wenngleich die Kontrolldiäten variabel waren [37]. Vor diesem Hintergrund stellt sich die Frage, ob ein isokalorischer Austausch der Hauptmakronährstoffe (Kohlenhydrate, Fett) durch Nüsse in Bezug auf ihr lipidsenkendes Potenzial von Relevanz ist.

3.7 Lipidsenkende Eigenschaften von Walnüssen

Vor diesem Hintergrund nimmt die **Walnuss** (*Juglans regia*) innerhalb der Nuss-Familie eine wichtige Rolle ein [38]. Auf Grundlage wissenschaftlicher Untersuchungen hat die Europäische Behörde für Lebensmittelsicherheit (EFSA) folgende Aussage zum gesundheitlichen Nutzen von Walnüssen als Health Claim zugelassen: „*Supportive but not conclusive research shows that eating 1,5 oz* [42,5243 g] *of walnuts per day, as part of a low saturated fat and low cholesterol diet, and not resulting in increased caloric intake may reduce risk of coronary heart disease*" [39, 40]. Walnüsse zeichnen sich vor allem durch ihr günstiges Fettsäureprofil und ihren hohen Anteil an PUFAs aus. Walnusskerne weisen einen Fettgehalt von durchschnittlich etwa 65% auf, was sich aus 6% aus gesättigten Fettsäuren (SFAs), 9% aus einfach ungesättigten Fettsäuren (MUFAs) und bis zu 47% aus mehrfach ungesättigten PUFAs zusammensetzt. Besonders hervorzuheben ist hierbei der auffällig hohe Gehalt an alpha-Linolensäure (9,1 g pro 100 g Walnüsse), welcher einzigartig innerhalb der Nuss-Familie ist (Tabelle 2).

Tabelle 2 Nährstoffzusammensetzung der Walnuss (*Juglans regia*)

Nährstoff	Pro 100 g
Energie [kcal]	654
Kohlenhydrate [g]	13,7
Ballaststoffe [g]	6,7
Fett gesamt [g] SFA gesamt [g] MUFA gesamt [g] PUFA gesamt [g] ALA (18:3n-3) [g] LA (18:2) [g]	65,2 6,1 8,9 47,2 9,1 38,0
Magnesium [mg]	158,0
Calcium [mg]	98,0
Y-Tocopherol [mg]	20,8

Des Weiteren hebt sich die Walnuss durch einen verhältnismäßig hohen Ballaststoffanteil von bis zu 7% hervor, ebenso wie durch einen hohen Proteingehalt von bis zu 30% und einer hohen Menge an Spurenelementen, Pflanzensterinen und anderen sekundären Pflanzenstoffen [41].

3.8 Prebiotische Eigenschaften der Walnuss

Neben ihren lipidsenkenden Wirkungsweisen werden der Walnuss sowohl probiotisch als auch prebiotisch wirksame Eigenschaften zugesprochen [42]. Eine Beeinflussung des Darmmikrobioms in Komposition und Diversität durch die Ernährung stellt einen wichtigen therapeutischen Ansatz in der Prävention und Behandlung entzündlicher Darmerkrankungen dar. Eine Fehlbesiedelung des Darms (Dysbiose) wird diversen Krankheitsbildern einschließlich Adipositas, Diabetes Mellitus Typ 2, Kolonkrebs, kardiovaskulären - und chronischer Erkrankungen in Verbindung gebracht [43, 44]. Das Darmmikrobiom eines erwachsenen gesunden Menschen umfasst geschätzt 10^{14} ansässige Mikroorganismen und zeichnet sich durch eine Vielzahl an Gattungen und Spezies aus [45]. Grob eingeteilt dominieren vier bakterielle Stämme (Phyla) das Darmmikrobiom: *Firmicutes*, *Bacteroidetes*, *Proteobacteria* und *Actinobacteria*. Im Dickdarm können fast ausschließlich obligate anaerobe Bakterien (z.B. *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium* spp., *Ruminococcus* spp.) gefunden werden, während im Dünndarm hauptsächlich fakultative Anaerobier (z.B. *Lactobacillus* spp.) angesiedelt sind [46]. Tatsächlich lassen sich gut 57 % aller Variationen der mikrobiellen Strukturen im Darm durch eine Veränderung des Ernährungsverhaltens erklären [47]. So führt eine akute Ernährungsumstellung (z.B. der Wechsel zu einer rein pflanzlichen Ernährung) innerhalb der ersten 24 h zu einer Veränderung der mikrobiellen Gemeinschaften, welche jedoch bei Abbruch dieser Ernährungsform binnen 48 h wieder in ihren Ursprungszustand umgekehrt werden kann [48]. Die prebiotische Wirkung von Nüssen (insbesondere Mandeln, Pistazien und Walnüsse) konnte in aktuellen Studien nachgewiesen werden, indem regelmäßiger Nusskonsum zu einer deutlichen Verschiebung zu Gunsten probiotisch wirksamer Bakteriengemeinschaften führte [42, 49-51], wobei als wichtigste Veränderung eine Zunahme von Buttersäure-produzierenden Mikroben beobachtet werden konnte. Buttersäure zählt zu den kurzkettigen Fettsäuren (short-chain fatty acids, SCFAs), welche als Fermentationsprodukt unverdaulicher Kohlenhydrate entstehen und als wichtige Metaboliten für intestinale Zellen fungieren [52, 53]. Bisher ist jedoch noch unklar, welche genauen molekularen Mechanismen sich hinter der prebiotischen Wirkung von Nüssen verbergen. Es wird vermutet, dass nicht-verdauliche Polyphenole und Polysaccharide (Fasern, Ballaststoffe) die Aktivität intestinaler mikrobieller Enzyme

beeinflussen und so möglicherweise das Wachstum probiotischer Bakterien zusätzlich fördern [54-56].

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4. Studienziele

Im Rahmen der Studie wurde der Effekt eines regelmäßigen Walnuss-Konsums (43 g/d über 8 Wochen) auf diverse Stoffwechselparameter bei gesunden Männern und (postmenopausalen) Frauen untersucht. Dabei wurde untersucht, ob der lipidsenkende Effekt von Walnüssen auf einen gleichzeitigen Austausch von Makronährstoffen (Kohlenhydrate, Fett) oder auf den Zeitpunkt des Verzehrs (Hauptmalzeit, Zwischenmalzeit) zurückzuführen ist.

Studienziel 1 - Überprüfen der Hypothese, dass regelmäßiger Konsum von Walnüssen die Plasmakonzentrationen von non-HDL-Cholesterin (primärer Endpunkt) und anderen wichtigen Lipidfraktionen reduziert, unabhängig davon, welche Makronährstoffe (Kohlenhydrate vs. gesättigte Fette) gegen die Walnüsse ausgetauscht werden.

Studienziel 2 - Überprüfen der Hypothese, dass regelmäßiger Konsum von Walnüssen die Plasmakonzentrationen von non-HDL-Cholesterin (primärer Endpunkt) und anderen wichtigen Lipidfraktionen reduziert, unabhängig davon, ob die Walnüsse zu den Hauptmalzeiten oder als Zwischenmalzeit verzehrt werden.

Des Weiteren sollte der Einfluss des Walnuss-Konsums auf das menschliche Darmmikrobiom untersucht werden.

Studienziel 3 - Überprüfen der Hypothese, dass regelmäßiger Konsum von Walnüssen einen Einfluss auf die Diversität mikrobiotischer Bakteriengemeinschaften im menschlichen Magen-Darm-Trakt hat.

5. Eigenanteil

Mein Eigenanteil an dieser Studie umfasste neben der Probandenrekrutierung, – aufklärung und – betreuung vornehmlich das Abdecken ernährungsphysiologischer Aspekte einschließlich der individuellen Ernährungsberatung, der Vorbereitung von Studieninformationsmaterial, der Auswertung von Ernährungsprotokollen und Labordaten, der Pflege von Labor- und Protokolldatenbanken, sowie die Kontaktpflege mit den Studienteilnehmern. Des Weiteren habe ich die Ergebnisse unserer Studie auf mehreren wissenschaftlichen Kongressen vorgestellt (American College of Cardiology 2017 [Postervortrag], Deutsche Gesellschaft für Innere Medizin 2017 [Postervortrag inkl. Posterpreis und Reisestipendium], Deutsche Gesellschaft für Ernährung 2017 [Vortrag]).

6. Publikationsliste

Publikationen

Bamberger, C., Rossmeier, A., Lechner, K., Wu, L., Waldmann, E., Stark, R.G., Altenhofer, J., Henze, K., Parhofer, K.G. *A walnut-enriched diet reduces lipids in healthy Caucasian subjects, independent of recommended macronutrient replacement and time point of consumption: A prospective, randomized, controlled trial.* Nutrients 2017, **9**, 1097, doi:10.3390/nu9101097

Bamberger, C., Rossmeier, A., Lechner, K., Wu, L., Waldmann, E., Fischer, S., Stark, R.G., Altenhofer, J., Henze, K., Parhofer, K.G. *A Walnut-Enriched Diet Affects Gut Microbiome in Healthy Caucasian Subjects: A Randomized, Controlled Trial.* Nutrients 2018, **10(2)**, 244, doi:10.3390/nu10020244

Kongressteilnamen

54. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung (DGE) e.V. (1.-3. März 2017, Kiel): *Vortrag*

66. American College of Cardiology (ACC) Scientific Sessions (17.-19. März 2017, Washington, DC): *Poster*, MedScape Deutschland (https://deutsch.medscape.com/artikelansicht/4905891)

123. Kongress der Deutschen Gesellschaft für Innere Medizin (DGIM) (29. April-2. Mai 2017, Mannheim): *Poster, Posterpreis, Reisestipendium*

American Heart Association (AHA) Scientific Sessions (11.-15. November 2017, Anaheim, Kalifornien): *Poster*

Abstracts

Bamberger, C., Rossmeier, A., Lechner, K., Wu, L., Waldmann, E., Fischer, S., Stark, R.G., Altenhofer, J., Henze, K., Parhofer, K.G. *Walnüsse als Teil der täglichen Ernährung senken Blutlipide, unabhängig davon, ob sie als Snack oder Hauptmalzeit konsumiert werden.* Internist 2017, **58(1)**; https://doi.org/10.1007/s00108-017-0235-y

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Bamberger, C., Rossmeier, A., Lechner, K., Wu, L., Waldmann, E., Fischer, S., Stark, R.G., Altenhofer, J., Henze, K., Parhofer, K.G. *Walnüsse als Teil der täglichen Ernährung senken Blutlipide bei gesunden Männern und Frauen, unabhängig davon, welche Makronährstoffe dadurch ersetzt werden*. Proc. Germ. Nutr. Soc. Abstractband zum 54. Wissenschaftlichen Kongress 2017, **Volume 23**

Bamberger, C., Rossmeier, A., Lechner, K., Wu, L., Waldmann, E., Fischer, S., Stark, R.G., Altenhofer, J., Henze, K., Parhofer, K.G. *A Walnut-Enriched Diet Beneficially Alters Gut Microbiome in Healthy Caucasian Subjects.* Circulation 2017, **136(1)**. 136:A15055

7. Publikation I

A walnut-enriched diet reduces lipids in healthy Caucasian subjects, independent of recommended macronutrient replacement and time point of consumption: a prospective, randomized, controlled trial

Charlotte Bamberger [1], Andreas Rossmeier [1], Katharina Lechner [1], Liya Wu [1], Elisa Waldmann [1], Renée G. Stark [2], Julia Altenhofer [1], Kerstin Henze [1] and Klaus G. Parhofer [1]

Nutrients 2017, **9**, 1097; doi:10.3390/nu9101097 (Special Issue: *Nut Consumption for Human Health*)

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Article

A Walnut-Enriched Diet Reduces Lipids in Healthy Caucasian Subjects, Independent of Recommended Macronutrient Replacement and Time Point of Consumption: a Prospective, Randomized, Controlled Trial

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Abstract: Studies indicate a positive association between walnut intake and improvements in plasma lipids. We evaluated the effect of an isocaloric replacement of macronutrients with walnuts and the time point of consumption on plasma lipids. We included 194 healthy subjects (134 females, age 63 ± 7 years, BMI 25.1 ± 4.0 kg/m^2) in a randomized, controlled, prospective, cross-over study. Following a nut-free run-in period, subjects were randomized to two diet phases (8 weeks each). Ninety-six subjects first followed a walnut-enriched diet (43 g walnuts/day) and then switched to a nut-free diet. Ninety-eight subjects followed the diets in reverse order. Subjects were also randomized to either reduce carbohydrates (n = 62), fat (n = 65), or both (n = 67) during the walnut diet, and instructed to consume walnuts either as a meal or as a snack. The walnut diet resulted in a significant reduction in fasting cholesterol (walnut vs. control: −8.5 ± 37.2 vs. −1.1 ± 35.4 mg/dL; p = 0.002), non-HDL cholesterol (−10.3 ± 35.5 vs. −1.4 ± 33.1 mg/dL; $p \leq 0.001$), LDL-cholesterol (−7.4 ± 32.4 vs. −1.7 ± 29.7 mg/dL; p = 0.029), triglycerides (−5.0 ± 47.5 vs. 3.7 ± 48.5 mg/dL; p = 0.015) and apoB (−6.7 ± 22.4 vs. −0.5 ± 37.7; $p \leq 0.001$), while HDL-cholesterol and lipoprotein (a) did not change significantly. Neither macronutrient replacement nor time point of consumption significantly affected the effect of walnuts on lipids. Thus, 43 g walnuts/d improved the lipid profile independent of the recommended macronutrient replacement and the time point of consumption.

Keywords: walnuts; nuts; lipids; cholesterol; fat; cardiovascular disease; n-3-PUFA; macronutrient replacement; carbohydrate

1. Introduction

Although a number of factors are causally linked to cardiovascular disease (CVD), alterations in lipid metabolism, and specifically an elevated concentration of apolipoprotein B (apoB)-containing lipoproteins are considered major risk factors [1]. Therapeutic interventions aiming to reduce LDL cholesterol (LDL-C) levels are of great clinical relevance in the treatment and prevention of CVD.

While statins are the cornerstone of drug therapy and should be used in all patients with established CVD [2], lifestyle changes play an important role in primary prevention.

There is strong epidemiologic and clinical evidence that diets rich in omega-3 (n-3) fatty acids are protective and may reduce cardiovascular and overall mortality [3–5]. In this context, nuts—especially walnuts—play a key role due to their unique fatty acid composition with high content of unsaturated fatty acids, specifically polyunsaturated fatty acids (PUFA). It has been shown that walnut consumption can affect clinically relevant endpoints (such as cardiac death or endothelial dysfunction), and that this may be mediated through effects on oxidative stress, inflammation, and lipids [6–8]. Most of the studies examined the effect of nuts as part of a diet compared with nut-free control diets, which were either low in total fat [9,10] and high in carbohydrate [11], high in fat [12], as part of a Mediterranean diet [13,14], or on a habitual diet [15,16]. Although dietary controls have been variable, the overall results of these clinical trials have consistently shown a cholesterol-lowering effect of regular nut consumption [17].

In a previous study, we investigated the effect of daily consumption of 43 g (1.5 oz) walnuts within a Western-type diet over eight weeks in healthy Caucasian men and postmenopausal women on lipid and glucose metabolism, adipokines, and endothelial function [18]. When compared with a control diet without walnuts, the walnut diet significantly reduced non-HDL cholesterol (non-HDL-C) and apolipoprotein B. Other fasting lipid parameters, as well as biomarkers of endothelial function, postprandial lipids, and glucose metabolism showed no significant changes.

Against this background, the question arises whether it is of any relevance which kind of macronutrient is substituted by the walnuts. Subjects will reduce carbohydrates, fat, or both when they consume walnuts, which may influence the lipid modifying effect. Furthermore, we wanted to investigate whether the time point of consumption (meal or snack) has an effect on the lipid profile, as it has been shown for almonds (which have a more pronounced effect when taken as a snack) [19].

The aim of the current study was therefore to investigate the effect of walnut consumption in subjects who isocalorically replaced fat, carbohydrates, or both with walnuts as a snack or as a meal.

2. Subjects and Methods

2.1. Subjects

Study subjects were recruited by advertisement in local newspapers and through posters in the outpatient clinic. We included healthy non-smoking subjects older than 50 years (men and postmenopausal women) with LDL-C <190 mg/dL, triglycerides (TG) <350 mg/dL, and a body mass index (BMI) <35 kg/m^2. We excluded persons with a history of cardiovascular and atherosclerotic disease, a known allergy to tree nuts, a vegan or ovo-lacto vegetarian lifestyle, and patients on regular medication (except stable treatment of thyroid disease and hypertension). A total of 268 subjects were screened. After the initial screening, 204 subjects were randomized and included in the trial. Ten subjects did not complete the study. In total, 194 subjects (60 men and 134 women) completed the study and were included in the main data analysis (complete case analysis). However, an intention to treat analysis was also performed.

2.2. Study Design

The study was designed as a randomized, controlled, prospective, cross-over study. Each subject followed a nut-free Western-type diet during a 4-week run-in period. Thereafter, subjects were randomized to two different diet phases, each lasting for 8 weeks (separated by a 4-week washout period, during which study subjects again followed a nut-free Western-type diet). One group (n = 96) first followed a walnut-enriched diet (43 g of shelled walnuts/d) and then switched to a nut-free control diet. The other group (n = 98) followed the diets in reverse order (Figure 1).

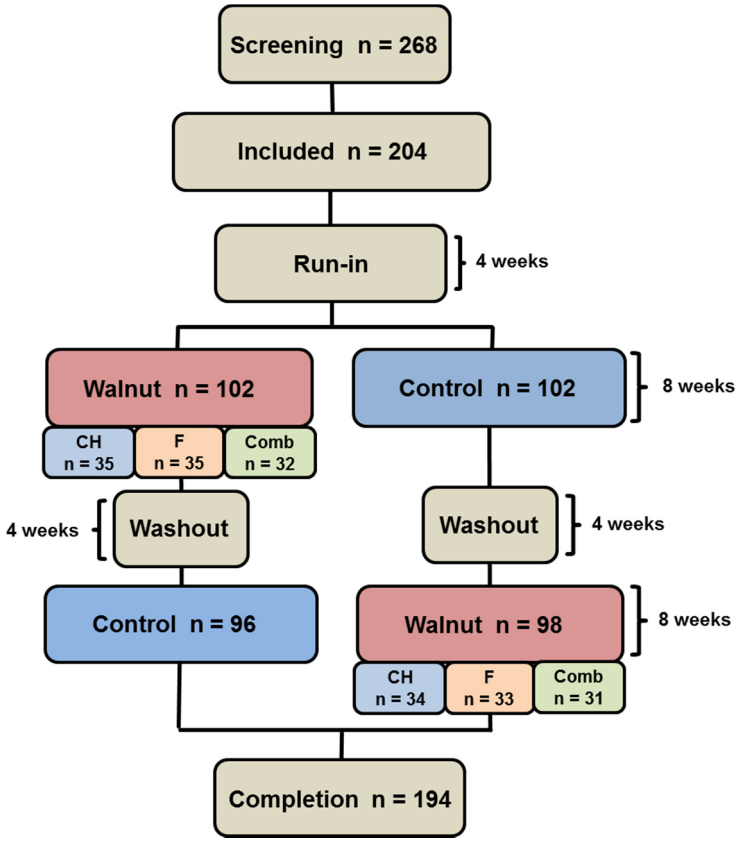

Figure 1. Flowchart of study subjects. In total, 204 subjects were randomized. Ten subjects dropped out due to disease ($n = 2$), medication ($n = 1$), personal reason ($n = 5$), protocol violation ($n = 2$). A total of 194 subjects were included in statistical evaluation. CH: carbohydrate restriction, F: fat restriction, Comb: combined carbohydrate and fat restriction.

Study duration was six months (24 weeks) for each subject (Figure 2). Before starting the run-in period, subjects underwent counseling by a nutritionist, who evaluated dietary habits using a four-day dietary report that had been prepared by the participants during the preceding days. At the beginning and end of each diet phase, subjects were evaluated in the study center. Subjects met with a nutritionist at each follow-up visit, and a telephone follow-up was conducted regularly once during each diet phase. Prior to each visit, subjects were instructed to keep a four-day food record.

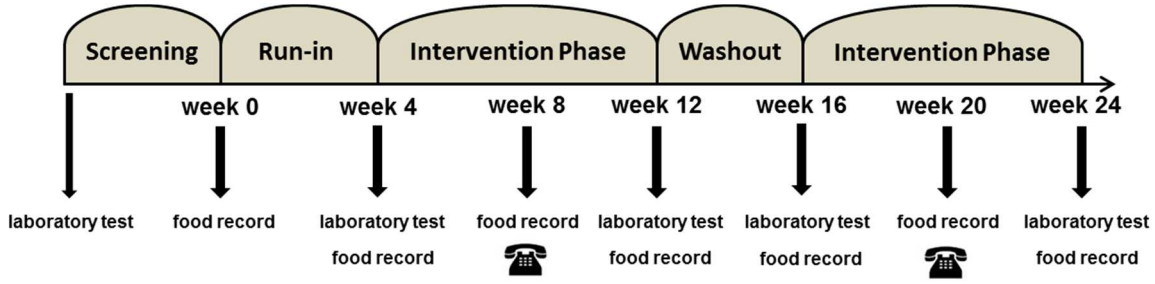

Figure 2. Flowchart of study procedures.

The recommended background diet during the run-in and the washout phases was a nut-free Western-type diet consisting of 50% carbohydrates, 35% fat (15% saturated fat), and 15% protein. Subjects were instructed not to consume dietary supplements containing omega-3 fatty acids (such as fish oil and linseed oil) during the entire study. At the beginning of the walnut diet, each subject was provided with a daily serving of 43 g of shelled, prepackaged walnuts (this corresponds to approximately additional 300 kcal/day mostly as fat). The subjects were randomized into three different diet groups, in which they were advised to reduce the intake of either carbohydrates (CH, *n*

= 62), fat (n = 65), or both (n = 67) during the walnut diet. They were instructed to replace either 70 g carbohydrates or 30 g of (saturated) fat with the walnuts. Subjects assigned to the third group were advised to replace both macronutrients (35 g carbohydrates and 15 g fat) with the daily walnut serving. These recommendations were food based, i.e., on the basis of individual food reports (free text), a nutritionist recommended specific measures to adjust the diet. Furthermore, the groups were randomized to consume walnuts either during a meal or as snack. Study subjects who were randomized to eat walnuts during a meal were instructed to eat unprocessed walnuts with main dishes. As outlined in Figure 2, each individual completed seven food reports to give recommendations and to monitor compliance. Dietary reports were evaluated using PRODI® 6.2 Nutri-Science GmbH (Nutri-Science GmbH, Freiburg, Germany). Consumption of the daily serving of walnuts was controlled by evaluating the food records and by asking study participants. Study participants received detailed study material that helped them assess their individual caloric intake. The same material showed how to replace carbohydrates or fat sources in their daily diet, i.e., by reducing the intake of bread or pasta to save carbohydrates.

2.3. Laboratory Measurements

EDTA (Ethylenediaminetetraacetic acid)-containing blood samples were used to analyze fasting lipid parameters. Very low density lipoproteins (VLDL) (d < 1.006 g/mL) were separated by ultra-centrifugation (Beckman L-60 centrifuge with 50.4 Ti rotor). Total cholesterol (TC), TG, VLDL-cholesterol (VLDL-C), apoB, lipoprotein (a) (Lp (a)) concentrations, fasting glucose, and C-reactive protein CRP were directly measured on an autoanalyzer (Respons® 910, DiaSys Diagnostic Systems, Holzheim, Germany) by using ready-to-use reagent kits (DiaSys Diagnostic Systems, Holzheim, Germany). HDL cholesterol (HDL-C) was measured after precipitation with heparin and manganese (II) chloride (polyanion precipitation). LDL-C was calculated by subtracting HDL-C from the total cholesterol in the infranatant of the ultracentrifugation. Non-HDL-C was calculated by subtracting HDL-C from TC. Hemoglobin A1c (HbA1c) concentrations were analyzed in the Department of Clinical Chemistry at the University of Munich Medical Center using routine laboratory tests. Endothelial activation markers sVCAM-1 and endothelin-1 were determined using commercially available ELISA kits (Human sVCAM-1/CD106 Quantikine ELISA Kit DVC00; Endothelin-1 Quantikine ELISA Kit DET100; R&D Systems, Minneapolis, MN, USA).

2.4. Statistical Analysis

Our primary outcome measure was fasting non-HDL-C. Secondary outcome measures included: fasting TC, TG, VLDL-C, VLDL-TG, LDL-C, HDL-C, apoB, Lp (a), fasting plasma glucose, HbA1c, hsCRP, BMI, and waist circumference. Based on our previous study [18], we estimated an average difference between the control and the walnut diet of 10 mg/dL in the primary endpoint (change in non-HDL-C) in each of the three subgroups. The power calculation indicated that 40 subjects per group were needed to complete the treatment periods to detect the indicated mean difference in non-HDL-C. We also advised subjects to consume walnuts either as snacks or with meals. The power to detect a 20% difference between subjects receiving walnuts as a snack vs. walnut as a meal was 30% for each subgroup. However, if all of the subjects receiving walnuts as snacks are compared to those receiving walnuts as meals (independent of the macronutrient being substituted), then the power increases to >50%. Changes in mean fat, protein, carbohydrate, and calorie consumption in the different dietary periods were examined with a two-tailed unpaired t-test or non-parametric Wilcoxon as appropriate. Results were arranged so that our dataset contained one observation for findings in the control phase—including baseline, post and change in the lipid values—and another observation for the findings in the walnut phase with corresponding values for each subject. For the analyses, we used a mixed generalized linear model accounting for these repeated measures and additionally adjusted for the baseline parameter values, age, gender, baseline BMI, and treatment sequence, type of diet reduction (fat, carbohydrate or both) and ingestion of walnuts as a meal or a snack. Comparisons between the effect of walnuts and controls on changes in the parameters of lipid metabolism and glucose metabolism were thus examined with the generalized linear regression models described

above, adjusting for the factors described above. The effect of macronutrient replacement and walnuts as meal or snack in the walnut phase on lipid parameters were analyzed using a model that adjusted for baseline parameter value, age, gender, baseline BMI, treatment sequence, type of diet reduction (fat, carbohydrate or both) and ingestion of walnuts as a meal or snack. For the comparison of treatment to the control phase for macronutrient replacement and walnuts as a meal or snack, an interaction term was included in separate regression analyses (either treatment*type of diet reduction or treatment*meal/snack). Statistical significance was set at $p \leq 0.05$. Primary analyses were performed in 194 subjects completing the study (complete case analyses). In addition, we also performed intention to treat analyses (ITT) of all 204 randomized subjects with all of the missing values imputed using single Markov chain Monte Carlo (MCMC) imputation. ITT analyses studying the effect on fasting non-HDL-C (primary outcome measure) were also performed with five imputation data sets. Randomization (blocking of 12; SAS proc factex) and statistical analysis were performed using SAS 9.3. Data were blinded for laboratory analysis.

2.5. Ethics

The study was conducted according to the guidelines in the Declaration of Helsinki and the ICH Harmonized Tripartite Guideline for Good Clinical Practice. The study protocol was approved by the ethics committee of the Faculty of Medicine of the University of Munich. After adequate information, all of the subjects provided written informed consent. The study was registered at ClinicalTrials.gov (NCT02329067) and performed between February 2015 and May 2016 at the University of Munich Medical Center. Walnuts were provided by the California Walnut Commission (Folsom, CA, USA).

3. Results

In total, 134 females and 60 males (mean age 63 ± 7 years) completed the trial. Baseline characteristics were measured after the run-in phase, and are shown in Table 1. Baseline characteristics did not differ significantly between groups.

Table 1. Baseline characteristics of all study subjects

Demographic and Anthropometric Values (*n* = 194)	**Baseline**	**(Min–Max)**
Gender	m = 60 f = 134	
Age (years)	63 ± 0.54	(50–86)
BMI (kg/m^2)	25.4 ± 0.29	(17.2–35.3)
Waist circumference (cm)	83.5 ± 0.81	(62–108)
Fasting Metabolic Parameters		
TC (mg/dL)	231.6 ± 2.7	(119–330)
Non-HDL-C (mg/dL)	161.7 ± 2.6	(71–243)
LDL-C (mg/dL)	146.3 ± 2.3	(54–206)
HDL-C (mg/dL)	68.6 ± 1.2	(35–111)
Triglycerides (mg/dL)	101.2 ± 4.0	(31–296)
Glucose (mg/dL)	91.4 ± 0.7	(74–118)
HbA1c (%)	5.4 ± 0.02	(4.0–6.4)

Values are mean ± SEM and median (range) for lipoprotein (a); TC, total cholesterol; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol.

Walnut consumption significantly reduced non-HDL-C, TC, LDL-C, VLDL-C, TG, VLDL-TG and apoB when compared with the control period (Table 2, Figure 3), but did not significantly change HDL-C and Lp (a). Intention to treat (ITT) analysis with the imputation of five datasets also shows that treatment with walnuts results in a decrease in non-HDL cholesterol, which is −7.61 (STD err: 1.77; *p*-value: <0.0001) lower in the walnut phase than in the control phase. Further ITT analyses are shown in Supplemental Table S1.

Table 2. Adjusted effect on fasting lipids, apolipoprotein B (apoB) and lipoprotein (a) (Lp (a)) (mg/dL) of intake of walnuts compared with controls in 194 subjects completing the study.

Parameter	Baseline[w]	ΔWalnut	Baseline[c]	ΔControl	*p* *
TC (mg/dL)	231.7 ± 2.7	−9.5	231.6 ± 2.5	−2.2	0.0003
LDL-C (mg/dL)	146.3 ± 2.3	−7.3	146.0 ± 2.1	−1.9	0.0009
HDL-C (mg/dL)	68.6 ± 1.1	0.5	68.8 ± 1.2	−0.1	0.297
non-HDL-C (mg/dL)	163.1 ± 2.6	−9.4	162.8 ± 2.4	−1.5	<0.0001
VLDL-C (mg/dL)	16.7 ± 1.1	−2.2	16.8 ± 1.1	0.5	0.0021
TG (mg/dL)	101.2 ± 3.4	−5.5	102.8 ± 3.5	3.4	0.0043
VLDL-TG (mg/dL)	74.3 ± 3.6	−3	77.8 ± 3.7	3.9	0.0355
Lp (a) (mg/dL)	12 (1–139)	−0.4	11.5 (2–173)	−0.6	0.8079
apoB (mg/dL)	109.9 ± 1.6	−6.8	109.5 ± 1.5	−0.9	<0.0001

Values are mean ± SEM and median (range) for lipoprotein (a); * *p*-value refers to comparison between ΔWalnut vs ΔControl; change in parameters and *p*-value calculated with multivariate regression adjusting for age, gender, baseline parameter, baseline BMI, treatment sequence, type of diet reduction, and walnuts as a snack or meal. The intention to treat analysis in all 204 subjects randomized showed that the same parameters changed significantly, with only marginally different *p*-values; TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; VLDL-C, very low density lipoprotein-cholesterol; TG, triglycerides; Lp (a), lipoprotein (a); apoB, apolipoproteinB.

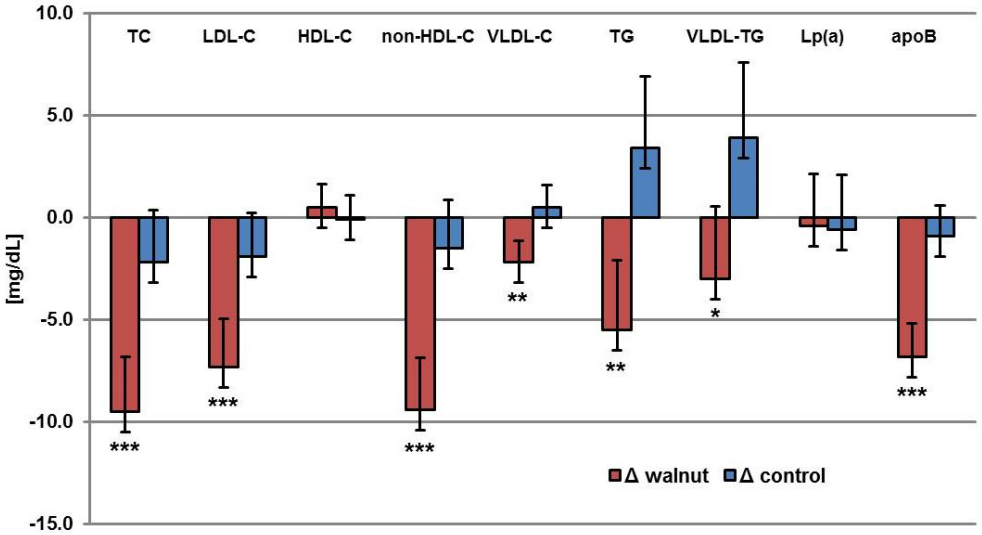

Figure 3. Changes in fasting plasma lipid levels and apoB concentrations from baseline (mg/dL) during the walnut diet phase and control diet phase. (n = 194) Values are mean ± SEM. *p*-value refers to differences between walnut phase and control phase; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; VLDL-C, very low density lipoprotein-cholesterol; TG, triglycerides; Lp (a), lipoprotein (a); apoB, apolipoproteinB.

The effect of walnut consumption on lipid parameters was independent of the macronutrient replaced by walnuts (CH vs. fat vs. combined) (Table 3, Figure 4). ITT analysis with the imputation of five datasets showed that there is no difference in the change in non-HDL cholesterol according to type of diet reduction when looking at the walnut group: Δcarbohydrate vs. Δfat: −0.69 (STD err: 3.45; *p*-value: 0.8419); Δcarbohydrate vs. Δcomb: −3.42 (STD err: 3.35; *p*-value: 0.3072); Δfat vs. Δcomb: −2.73 (STD err: 3.29; *p*-value: 0.4062), and the comparison of walnut to controls (Δcarbohydrate vs. Δfat: −2.69 (STD err: 4.23; *p*-value: 0.5267; Δcarbohydrate vs. Δcomb: −4.67 (STD err: 4.23; *p*-value: 0.2716). Further ITT analyses are shown in Supplemental Table S2.

Table 3. Effect of walnut consumption on fasting lipids, apoB and Lp (a) when walnuts replace carbohydrates, fat, or both.

	Walnuts				Difference between Walnuts and Control			
Parameter	**ΔCarbohydrate**	**ΔFat**	**ΔComb**	***p***	**ΔCarbohydrate**	**ΔFat**	**ΔComb**	***p***
TC (mg/dL)	−11.9 ± 2.7	−9.7 ± 2.6	−6.2 ± 2.8	0.3158	−8.5 ± 3.5 (0.0148)	−9.2 ± 3.4 (0.0070)	−4.1 ± 3.3 (0.2202)	0.5113
LDL-C (mg/dL)	−9.0 ± 2.3	−6.9 ± 2.3	−5.6 ± 2.4	0.5681	−6.8 ± 2.8 (0.0166)	−6.4 ± 2.8 (0.0210)	−3.0 ± 2.7 (0.2677)	0.5657
HDL-C (mg/dL)	0.1 ± 0.8	0.7 ± 0.8	1.5 ± 0.9	0.4817	0.1 ± 1.1 (0.9185)	0.4 ± 1.1 (0.7153)	1.5 ± 1.1 (0.1863)	0.6688
non-HDL-C (mg/dL)	−11.2 ± 2.3	−9.6 ± 2.3	−6.7 ± 2.4	0.3786	−8.6 ± 3.1 (0.0059)	−10.0 ± 3.0 (0.0011)	−5.1 ± 3.0 (0.0886)	0.4947
VLDL-C (mg/dL)	−2.8 ± 1.1	−2.7 ± 1.1	−0.8 ± 1.1	0.3232	−2.6 ± 1.5 (0.0871)	−3.4 ± 1.5 (0.0236)	−2.1 ± 1.5 (0.1591)	0.8191
TG (mg/dL)	−4.9 ± 3.8	−5.5 ± 3.7	−4.1 ± 3.9	0.9622	−5.0 ± 5.5 (0.3603)	−10.5 ± 5.3 (0.0509)	−11.1 ± 5.3 (0.0371)	0.6853
VLDL-TG (mg/dL)	−1.3 ± 3.9	−1.2 ± 3.8	−4.1 ± 4.0	0.8343	−3.5 ± 5.7 (0.5446)	−6.6 ± 5.6 (0.2383)	−10.3 ± 5.5 (0.0645)	0.6951
Lp (a) (mg/dL)	−1.4 ± 1.0	−0.3 ± 1.0	0.7 ± 1.0	0.3405	−1.2 ± 1.3 (0.3669)	0.7 ± 1.3 (0.5798)	1.0 ± 1.3 (0.4590)	0.4471
apoB (mg/dL)	−8.3 ± 1.6	−6.5 ± 1.6	−4.8 ± 1.6	0.2939	−6.6 ± 2.1 (0.0019)	−7.1 ± 2.0 (0.0007)	−4.2 ± 2.0 (0.0392)	0.5654

Values are mean ± SEM. Change in parameters and *p*-value calculated with multivariate regression adjusting for age, gender, baseline parameter value, baseline BMI, treatment sequence, walnuts as snack or meal; *p*-value refers to differences between groups; values in brackets are *p*-values referring to the comparison walnut vs. control within the diet group. TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; VLDL-C, very low density lipoprotein-cholesterol; TG, triglycerides; Lp (a), lipoprotein (a); apoB, apolipoproteinB.

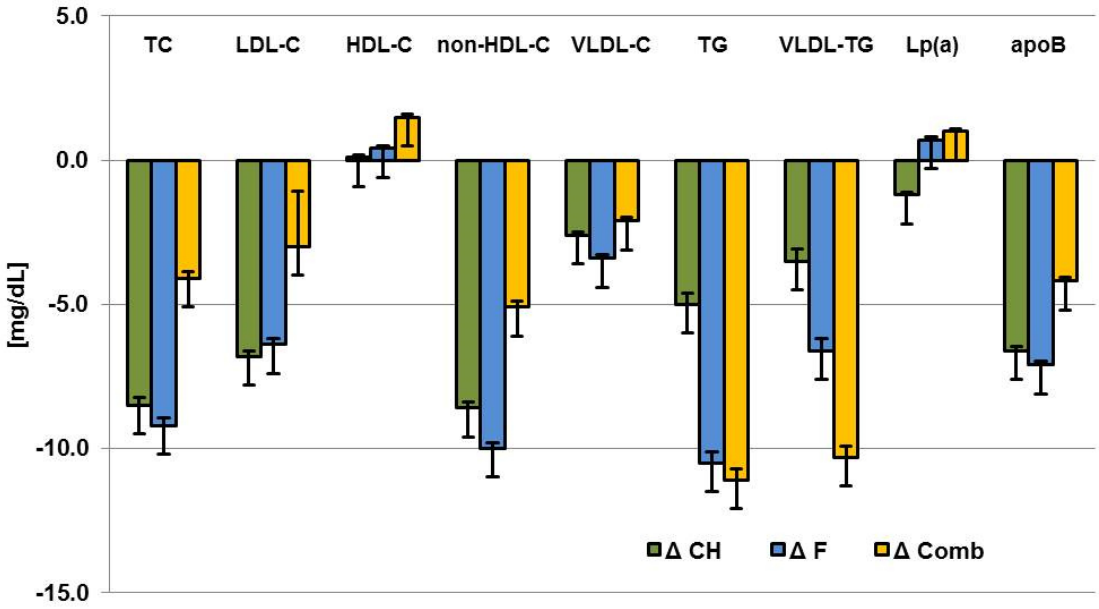

Figure 4. Changes in fasting plasma lipid levels and apoB concentration from baseline in subgroups (mg/dL). Values are mean ± SEM. CH: carbohydrates reduced in walnut-phase (n = 62); F: Fat reduced in walnut-phase (n = 65); Comb: both fat and carbohydrates reduced in walnut-phase (n = 67). TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; VLDL-C, very low density lipoprotein-cholesterol; TG, triglycerides; Lp (a), lipoprotein (a); apoB, apolipoproteinB.

Similarly, the effect was independent of whether walnuts were consumed as a snack or with a meal (Table 4). ITT analysis with the imputation of five datasets shows that walnuts given as a meal versus a snack results in a change in non-HDL cholesterol, which is −5.15 (STD err: 2.70; p-value: 0.0587) and lower when given with meals than as a snack (only looking at the walnut phase). Comparing the results of both phases shows a change in non-HDL cholesterol, which is −2.05 (STD err: 3.51; p-value: 0.5595) lower when given with meals than as a snack. Further ITT analyses are shown in Supplemental Table S3.

Table 4. Effect of walnut consumption on fasting lipids, apoB, and Lp (a) when walnuts were consumed with meals or as snack.

	Walnuts			Difference between Walnuts and Control		
Parameter	**Meal**	**Snack**	p	**Meal**	**Snack**	p
TC (mg/dL)	−11.6 ± 2.2	−7.0 ± 2.3	0.1277	−7.3 ± 2.7 (0.0077)	−7.1 ± 2.8 (0.0126)	0.9515
LDL-C (mg/dL)	−8.3 ± 1.9	−6.0 ± 2.0	0.3948	−5.2 ± 2.2 (0.0222)	−5.7 ± 2.3 (0.0150)	0.8714
HDL-C (mg/dL)	0.6 ± 0.7	0.9 ± 0.7	0.7889	1.4 ± 0.9 (0.1058)	−0.2 ± 0.9 (0.8646)	0.212
non-HDL-C (mg/dL)	−11.3 ± 1.9	−7.0 ± 2.0	0.1039	−8.6 ± 2.4 (0.0005)	−7.1 ± 2.5 (0.0056)	0.6538
VLDL-C (mg/dL)	−3.2 ± 0.9	−1.0 ± 0.9	0.0648	−3.8 ± 1.2 (0.0017)	−1.5 ± 1.2 (0.2254)	0.1809
TG (mg/dL)	−7.5 ± 3.0	−2.1 ± 3.2	0.2057	−12.9 ± 4.3 (0.0029)	−4.7 ± 4.4 (0.2919)	0.182
VLDL-TG (mg/dL)	−4.5 ± 3.2	−0.1 ± 3.3	0.2964	−9.2 ± 4.5 (0.0427)	−4.4 ± 4.6 (0.3461)	0.4596
Lp (a) (mg/dL)	−0.3 ± 0.8	−0.3 ± 0.8	0.9745	0.4 ± 1.1 (0.7208)	−0.02 ± 1.1 (0.9841)	0.7927
apoB (mg/dL)	−8.6 ± 1.3	−4.5 ± 1.3	0.024	−6.5 ± 1.6 (0.0001)	−5.3 ± 1.7 (0.0022)	0.6024

Values are mean ±SEM. Change in parameters and p-values calculated with multivariate regression adjusting for age, gender, baseline parameter value, baseline BMI, treatment sequence, walnuts as snack or meal; p-values refer to differences between groups; values in brackets are p-values referring to the comparison of walnut-phase vs. control within the group. TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; VLDL-C, very low density lipoprotein-cholesterol; TG, triglycerides; Lp (a), lipoprotein (a); apoB, apolipoproteinB.

Analysis of self-reported nutrient intakes from the four-day dietary reports showed that the study subjects did not fully maintain an isocaloric diet as recommended during both intervention periods, but increased caloric intake during walnut consumption (+423 kJ (101 kcal) vs. +88 kJ (21 kcal), $p = 0.042$). In the CH group, the proportion of fat increased significantly (+20.4 g vs. +1.6 g, $p < 0.001$), whereas the amount of carbohydrates decreased significantly (−23.7 g vs. +0.6 g, $p = 0.004$) during walnut consumption. In the fat group, the intake of carbohydrates did not significantly differ between the walnut and control diet (−9.0 g vs. +2.8 g, $p = 0.067$), but fat intake significantly increased during walnut consumption (+21.6 g vs. +7.2 g, $p < 0.001$). In the third group (Comb), a significant increase of fat during the walnut diet could be observed (+21.4 g vs. +0.1 g, $p < 0.001$), while carbohydrate consumption did not change significantly. A detailed analysis of the dietary reports indicates that subjects did not fully comply with the recommended substitution (Figure 5). Thus, the CH group reduced carbohydrates, but did not fully compensate for the additional walnuts. Instead, other fat was also reduced, and overall energy intake increased. Similarly, the fat group did not adequately reduce other fat during walnut consumption, but also reduced carbohydrates and increased overall energy consumption.

When the three walnut diets were compared with each other, no significant difference was detected, which indicated that subjects of all of the diet groups ate a similar diet during the walnut phase, despite different recommendations. A subgroup analysis of those subjects who properly followed the dietary instructions (10–30 subjects in each group) showed no significant difference in the decrease of blood lipids during walnut consumption between the different diet groups.

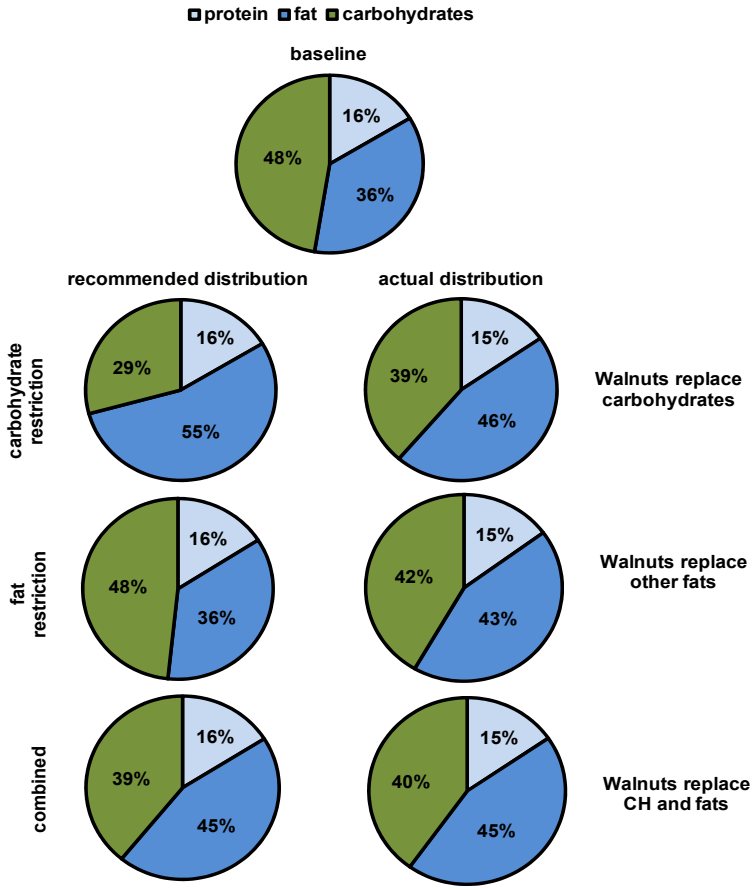

Figure 5. Adherence to recommended macronutrient replacement during walnut consumption. Percentage of daily total calories at baseline, as well as the recommended and the actual distribution of calories, were calculated by analyzing dietary reports.

Walnut consumption did not affect hsCRP, endothelial markers, and fasting glucose, but was associated with a significant increase in HbA1c (+0.07, $p = 0.0057$).

4. Discussion

Our study demonstrates that supplementing 43 g of walnuts daily for eight weeks significantly decreases fasting lipid parameters, including non-HDL-C, apoB, TC, LDL-C, VLDL-C, TG, and VLDL-TG in healthy individuals, irrespective of whether the subjects are instructed to replace carbohydrates, fat, or both. We did not observe changes in hsCRP, biomarkers of endothelial dysfunction and anthropometric parameters, including waist circumference and body weight, but observed a minimal increase in HbA1c (no effect on fasting glucose). The observed effects did not change materially after adjustment for gender, age, BMI, and diet sequence. Furthermore, the results were virtually identical when an intention to treat analysis was performed.

4.1. Lipid-Lowering Effect

Within the tree nut family, walnuts have been found to be particularly promising in cardioprotective health. As one of the first, Sabaté et al. demonstrated the cholesterol-lowering effect of daily walnut consumption [20]. Our results showed a significant reduction in LDL-C (−7.3 mg/dL), which represents a reduction of 5.0%. These findings resonate with the results of our previous study [18], in which a similar pattern was observed (non-HDL-C: −5.8%, TC: −3.9%, apoB: −6.2%, VLDL-C: −13.2%, TG: −5.4%, VLDL-TG: −4.0%). Our results suggest that the increased n-3-PUFA intake (15.0%) was principally responsible for the cholesterol-lowering effect of walnuts. There is evidence that a high n-3-PUFA intake provides cholesterol-lowering effects through several potential mechanisms [21]; however, the exact underlying mechanisms are still not fully understood.

4.2. Macronutrient Replaced and Time Point of Consumption

In our previously published study, we asked subjects to reduce saturated fatty acid intake while eating walnuts, but observed that carbohydrates and fat were reduced. This led us to question whether replacing different macronutrients had a differential effect on the plasma lipid profile. Several lines of evidence indicate that the effect could be different if walnuts replace carbohydrates or fat. The reduction of carbohydrate intake has been shown to decrease plasma TG levels and to increase HDL-C without affecting LDL-C levels [22]. On the other hand, the replacement of saturated fat with monounsaturated fat has been associated with decreased TC, LDL-C, and HDL-C [23]. The replacement of saturated fat with carbohydrates has been reported to result in lower TC, LDL-C, and HDL-C levels, but also a slight increase in TG [24,25]. A replacement of saturated fat with both monounsaturated fat and carbohydrates effectively lowered LDL-C; however, the replacement with monounsaturated fat was associated with lesser reductions in HDL-C and lesser increases in TG concentrations [26]. Replacing saturated fat with polyunsaturated fat and/or monounsaturated fat has been shown to be equally efficacious at reducing lipoprotein levels [27]. Based on these findings, we hypothesized that the greatest lipid-lowering effect would be seen in the group that replaced fat by walnuts. However, there was no statistically significant difference in lipid reduction between the diet groups.

Although we invested considerable time and effort (frequent visits with a nutritionist; detailed analysis and discussion of food protocols), our subjects did not fully comply with the recommended diet (i.e., substitution of carbohydrates or fat or both for walnuts). In fact, there was no statistical difference between the three diets, indicating that subjects had a very similar diet, despite very different recommendations. This probably reflects the "real world", and indicates that most people will cut down on carbohydrates and fat (non-walnut fat) if they consume walnuts (irrespective of recommendations). To ensure better compliance of the dietary recommendations, a much more controlled setting is needed, such as an in-patient setting or at least a setting where all of the meals are provided. However, this setting would introduce other confounders (e.g., restricted physical activity). On the other hand, it is questionable whether the results would be different, as a subgroup analysis of those subjects who properly followed the dietary instructions also showed no significant difference concerning the effect of walnut consumption on lipids. This indicates that the effect is independent of whether walnuts replace carbohydrates, fat, or both. This supports the notion that

walnuts improve the lipid profile, not only independent of the recommendations, but also independent of the actually executed diet.

In our study, the effect of walnut consumption on lipids did not depend on whether walnuts were consumed with meals or as snacks. In a previous study describing the effect of almonds, it has been demonstrated that the beneficial effect on various metabolic parameters was most pronounced when almonds were taken as snacks [19]. It can be assumed that nuts when consumed as a snack alone may result in enhanced acute satiety responses, thus keeping the balance to the overall energy intake [28,29]. On the other hand, almonds have been shown to significantly reduce post-prandial glucose excursion by slowing digestion, in addition to lowering serum cholesterol levels when consumed with a meal [30]. Further trials may be necessary to validate whether the time and form of consumption influence the effect on blood lipid levels.

4.3. Other Findings

The secondary parameter hsCRP and the endothelial markers VCAM-1 and ET-1 remained unaffected during the walnut diet. These findings are consistent with the results of our previous study [18], as well as other studies focusing on the effect of a walnut or n-3 PUFA-enriched diet on endothelial and inflammatory markers [7,14].

To our surprise, we observed a slight, clinically irrelevant but significant increase in HbA1c (5.4 ± 0.3 vs. 5.5 ± 0.3). These findings contrast with epidemiological studies that show an improvement in glucose parameters after nut consumption in subjects with or at risk of diabetes [28,31–33]. The antidiabetic effects of n-3 PUFAs are expected to result from enhanced insulin sensitivity due to an increased expression of insulin receptors [34]. Several potential explanations can be offered for the observed change in glucose metabolism. First, our study subjects consumed more calories during the walnut phase, which may affect glucose metabolism and HbA1c levels. Second, statin therapy, which dramatically decreases LDL-C, and genetic variants leading to lower LDL-C, both also negatively affect glucose levels [35,36]. Thus, theoretically, the small decrease in LDL-C could be linked to the increase in HbA1c. Third and most likely, it could be a chance finding. Nevertheless, this aspect deserves further investigation.

Body weight and BMI remained stable during both walnut and control diet. These findings are consistent with our prior research, where daily walnut consumption did not lead to a significant change in anthropometric parameters. Indeed, data from both observational and clinical studies show that supplementing nuts to habitual diets does not cause weight gain [37,38], despite the fact that subjects often seem to consume more energy when eating nuts. However, recent data indicate that the metabolizable energy content of walnuts is lower than predicted [39]. According to these data, one 43 g serving of walnuts can be estimated to contain about 942 kJ (225 kcal, thus 5.22 kcal/g metabolizable energy), about 314 kJ (75 kcal) less than previously assumed.

4.4. Strengths and Limitations

A major strength of our study is its prospective, randomized, cross-over design with washout periods to ensure good basic conditions. Furthermore, our study set the focus on nutritional replacement to investigate whether the exchange of macronutrients for walnuts and the time point of consumption may have an impact on the lipid-lowering effect of walnuts. On the other hand, the interpretation is limited by the fact that the study relied on self-reported food records completed by the participants. These data may be highly susceptible to recall bias. Furthermore, dietary intake was not monitored daily, but rather recorded for four consecutive days. These were in turn interrupted by non-representative conditions (e.g., public holidays). Finally, in order to standardize baseline diet, patients had to follow a Western-type diet during the run-in period, which by itself represented a dietary change for many subjects.

5. Conclusions

Our study demonstrates that supplementing 43 g of walnuts for eight weeks favorably changes plasma lipids by lowering total cholesterol, LDL-C, non-HDL-C, triglycerides, and apoB. Thus, embedding walnuts in a healthy subject's nutrition may be an effective strategy for reducing the risk for cardiovascular disease. It seems irrelevant which macronutrients are replaced and whether walnuts are consumed in meals or as snacks.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/9/10/1097/s1, Table S1: Intention to treat analysis examining differences between treatment with walnuts and controls with all missing values imputed using single MCMC imputation; Table S2: Intention to treat analysis examining effect of walnut consumption on fasting lipids, apoB and Lp (a) when walnuts replace carbohydrates, fat or both with all missing values imputed using single MCMC imputation; Table S3: Effect of walnut consumption on fasting lipids, apoB and Lp (a) when walnuts were consumed with meals or as snack in an intention to treat analysis.

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Author Contributions: The authors' responsibilities were as follows: K.G.P. designed the study. C.B., A.R., L.W., K.L. and E.W. were responsible for subject recruitment, testing and data collection. K.H. and J.A. conducted sample analysis for plasma lipid profile. C.B. and J.A. were responsible for the dietary consultations and the analysis of the nutritional protocols. R.G.S., A.R. and K.G.P. analyzed the data. C.B. and K.G.P. drafted the manuscript. K.G.P. had primary responsibility for the final content. All authors read and approved the final manuscript.

Conflicts of Interest: C.B., A.R., K.L., L.W., E.W., R.S., J.A. and K.H. declare no conflict of interest. The research was supported by a grant from the California Walnut Commission (Folsom, CA) to K.G.P.. The Walnut Commission had no role in study performance, data analysis and or manuscript writing.

Abbreviations

apoB	apolipoprotein B
BMI	body mass index
CH	carbohydrates
comb	combined
CVD	cardiovascular disease
ET-1	endothelin 1
F	fat
HbA1c	hemoglobin A1c
HDL-C	high-density lipoprotein cholesterol
hsCRP	high-sensitive C-reactive protein
LDL-C	low-density lipoprotein cholesterol
Lp (a)	lipoprotein a
n-3	omega-3
PUFA	polyunsaturated fatty acids
TG	triglycerides
TC	total cholesterol
VCAM-1	vascular cell adhesion molecule-1
VLDL-C	very-low-density lipoprotein cholesterol

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8. Publikation II

A walnut-enriched diet affects gut microbiome in healthy Caucasian subjects: a randomized, controlled trial

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A Walnut-Enriched Diet Affects Gut Microbiome in Healthy Caucasian Subjects: A Randomized, Controlled Trial

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Abstract: Regular walnut consumption is associated with better health. We have previously shown that eight weeks of walnut consumption (43 g/day) significantly improves lipids in healthy subjects. In the same study, gut microbiome was evaluated. We included 194 healthy subjects (134 females, 63 ± 7 years, BMI 25.1 ± 4.0 kg/m^2) in a randomized, controlled, prospective, cross-over study. Following a nut-free run-in period, subjects were randomized to two diet phases (eight weeks each); 96 subjects first followed a walnut-enriched diet (43 g/day) and then switched to a nut-free diet, while 98 subjects followed the diets in reverse order. While consuming the walnut-enriched diet, subjects were advised to either reduce fat or carbohydrates or both to account for the additional calories. Fecal samples were collected from 135 subjects at the end of the walnut-diet and the control-diet period for microbiome analyses. The 16S rRNA gene sequencing data was clustered with a 97% similarity into Operational Taxonomic Units (OTUs). UniFrac distances were used to determine diversity between groups. Differential abundance was evaluated using the Kruskal–Wallis rank sum test. All analyses were performed using Rhea. Generalized UniFrac distance shows that walnut consumption significantly affects microbiome composition and diversity. Multidimensional scaling (metric and non-metric) indicates dissimilarities of approximately 5% between walnut and control ($p = 0.02$). The abundance of *Ruminococcaceae* and *Bifidobacteria* increased significantly ($p < 0.02$) while *Clostridium* sp. cluster XIVa species (*Blautia*; *Anaerostipes*) decreased significantly ($p < 0.05$) during walnut consumption. The effect of walnut consumption on the microbiome only marginally depended on whether subjects replaced fat, carbohydrates or both while on walnuts. Daily intake of 43 g walnuts over eight weeks significantly affects the gut microbiome by enhancing probiotic- and butyric acid-producing species in healthy individuals. Further evaluation is required to establish whether these changes are preserved during longer walnut consumption and how these are linked to the observed changes in lipid metabolism.

Keywords: walnuts; nuts; diet; gut microbiome; lipids; cholesterol; prebiotic; probiotic; butyric acid

1. Introduction

The human gut microbiome encompasses approximately 1014 resident microorganisms, mainly consisting of bacteria, and corresponds to 1000 distinct species with a collective genome containing at least 100 times as many genes as the human genome [1]. The establishment of high-throughput sequencing allows the metagenome to be studied for broad analyses of intestinal microbiota composition [2]. These microbial communities contribute to host health through various functions including probiotic properties, biosynthesis of vitamins and essential amino acids, as well as production of metabolic byproducts from indigestible dietary constituents. Butyrate, a short chain fatty acid which is produced by bacterial fermentation of non-digestible carbohydrates in the colon, acts as a major energy source for intestinal epithelial cells, enhances intestinal epithelial barrier function and modulates immune function [3,4].

The fact that there is considerable variation in the constituents of the gut microbiota among apparently healthy individuals strengthened the hypothesis that there is a clear link between health, disease and diversity of the human gut microbiome. Indeed, a dysbiosis of the gut microbiota is associated with the pathogenesis of both intestinal and extra-intestinal disorders including inflammatory bowel disease, metabolic diseases such as obesity and diabetes mellitus type 2, and cardiovascular diseases [5]. The impact of environmental factors, including aspects of lifestyle or drug therapy on the microbiota is of major clinical interest. Diet is one of the main determinants of the microbial composition in the gut influencing diversity, distribution and abundance of microbial populations from the early stages of life [6]. Indeed, diet changes are thought to explain 57% of the total structural variation in the gut microbiota [7]. An acute change in diet has been shown to alter microbial composition within just 24 h of initiation (e.g., switching to a completely plant-based diet), with reversion to baseline within approximately 48 h of diet discontinuation [8]. According to this, there is growing interest in modifying the gut microbiota for long-term health benefits.

The microbiome analysis was part of our previously published study in which we investigated the effect of regular walnut consumption (43 g/day) on the lipid profile in healthy subjects, resulting in a significant reduction of LDL-cholesterol, apoB, triglycerides and non-HDL-cholesterol after eight weeks of intervention [9]. Evidence from recent animal and human feeding studies shows a correlation between regular nut consumption and a shift within the gut microbiome, indicating prebiotic properties of members of the tree nut family. However, the exact mechanisms by which nuts offer their prebiotic effects on microbial diversity is not fully understood [10,11]. Another issue to be addressed is, how these changes might be associated with the observed changes in lipid metabolism.

The aim of this sub study was to investigate the effect of walnut consumption on the gut microbiome composition and microbial diversity.

2. Materials and Methods

2.1. Study Design

The study comprises a randomized, controlled, prospective, cross-over design as previously described [9]. Each subject followed a nut-free Western-type diet consisting of 50% carbohydrates, 35% fat (15% saturated fat), and 15% protein during a 4-week run-in period. Thereafter, subjects were randomized to 2 different diet phases, each lasting for 8 weeks (separated by a 4-week washout). One group (n = 96) first followed a walnut-enriched diet (43 g of shelled walnuts/day) and then switched to a nut-free control diet. The other group (n = 98) followed the diets in reverse order (Figure 1). Study duration was 6 months (24 weeks) for each study subject. During the walnut diet the subjects were randomized into three different diet groups, in which they were advised to reduce the intake of either carbohydrates (CH, n = 62; 44 with stool samples), fat (n = 65; 47 with stool samples), or both (n = 67; 46 with stool samples). They were instructed to replace either 70 g carbohydrates or 30 g of (saturated) fat with the walnuts. Subjects assigned to the third group were advised to replace both macronutrients (35 g carbohydrates and 15 g fat) with the daily walnut serving. These recommendations were food

based, i.e., on the basis of individual food reports (free text), a nutritionist recommended specific measures to adjust the diet. Stool samples were collected at the end of each diet phase.

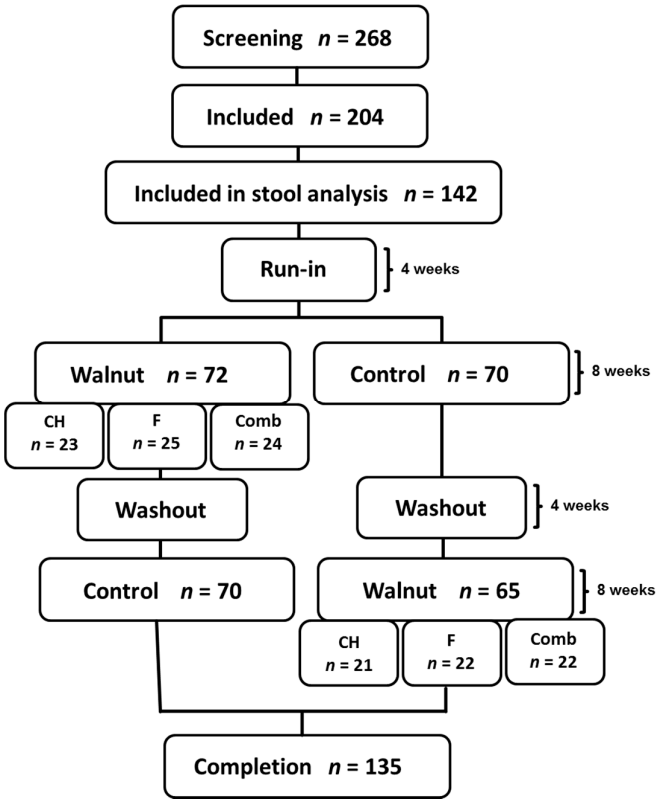

Figure 1. Flowchart of study subjects. In total, 204 subjects were randomized. 142 subjects were included in stool analysis. 7 subjects were excluded due to antibiotic therapy. In total, stool samples from 135 study subjects were included in statistical evaluation. CH: carbohydrate restriction; F: fat restriction; Comb: combined carbohydrate and fat restriction.

2.2. Study Subjects

Study participants (n = 204) were healthy Caucasian men and postmenopausal women older than 50 years (134 females and 60 males, age 63 $\pm$ 7 years, BMI 25.1 $\pm$ 4.0 kg/m^2) [9]. We included healthy non-smoking subjects older than 50 years (men and postmenopausal women) with LDL-C < 190 mg/dL, triglycerides (TG) < 350 mg/dL, and a body mass index (BMI) < 35 kg/m^2. We excluded persons with a history of cardiovascular and atherosclerotic disease, a known allergy to tree nuts, a vegan or ovo-lacto vegetarian lifestyle, and patients on regular medication (except stable treatment of thyroid disease and hypertension). Stool samples were only available in 142 of the original 204 subjects. Further 7 study subjects were subsequently excluded due to antibiotic therapy. In total, 270 stool samples (2 samples from each of the 135 study subject) were analyzed.

2.3. Stool Sample Collection

Subjects were instructed to collect stool samples within 24 h before the next study visit and refrigerate them until the visit. The required materials for a hygienically safe stool collection were provided by the study center. The study kit comprised disposable gloves, a sample-catching paper (Süsse Labortechnik MED AUXIL 150 $\times$ 470 mm #S1000), sample-collecting tubes including 8 mL of stool DNA stabilizer (stratec molecular #1038111100), transport bags, as well as instructions for use and a questionnaire (Bristol Stool Chart) for recording sampling conditions and sample quality [12].

Samples were immediately frozen at −20 °C, transported on dry ice and then stored at −80 °C until further analysis.

2.4. Sample Processing and Sequencing

The identification and comparison of microbial communities was evaluated by using high-throughput sequencing of the V3/V4 region of the 16S rRNA gene [2]. The method is based on the isolation of genomic DNA and its duplication produced by Polymerase Chain Reaction (PCR), followed by sequencing of the PCR amplicon by using a specific primer that binds to highly conserved sequences on the 16S rRNA gene [13]. Sample processing has been divided into DNA isolation, library construction by PCR, amplicon cleaning and dilution, and sequencing. DNA was isolated with a modification of the protocol by Godon et al. [14]. After isolation, DNA was purified using a silica-membrane based NucleoSpin gDNA Clean-up Kit (REF 740230.250 Machery-Nagel). The PCR was performed in duplicate and the PCR products of duplicates were pooled prior to cleaning [13]. For quality control, a selection of samples was analyzed by electrophoresis. PCR purification was performed by using AGENCOURT AMPure XP Beads (Beckman Coulter, Brea, CA, USA). The 16S rRNA gene amplicon libraries were sequenced in paired-end modus using an Illumina MiSeq.

2.5. Data Analysis

The resulting dataset was processed through taxonomic classification against a database of reference 16S rRNA gene sequences. After sequencing, raw data reads were assigned to their corresponding sample via demultiplexing using previously assigned barcode pairs which are unique for each sample. The demultiplexing was performed by using an in-house developed Perl script. After demultiplexing, data were analyzed using the IMNGS platform (www.imngs.org), which is based on the UPARSE approach for sequence quality check, chimera filtering, and cluster formation [15]. Output was an Operational Taxonomic Units (OTU) analysis calculating and visualizing the relative abundance of the bacterial taxa present in each sample. For quantifying alpha-diversity, the intra-sample variation is calculated. Richness gives the value of present OTUs within one sample while the diversity index estimates the number of equal species within one sample. Simpson effective counts out more weight on dominant species while Shannon is based on richness and evenness. To avoid incorrect estimation of species richness due to differential sequencing depth, only normalized counts that are above 0.5 were considered. Based on an OTU threshold of 97% similarity, UniFrac distances (a distance metric for microbial community comparison) were calculated to evaluate beta-diversity (diversity between the samples). Beta-diversity was determined by Principal Coordinate Analysis (PCoA) using both unweighted and weighted UniFrac metrices. Metric Multidimensional Scaling (MDS) and non-metric multidimensional scaling (NMDS) projections of the generalized UniFrac distances were produced and a PERMANOVA test was performed to determine statistical significant differences. Differential abundance was evaluated performing the Kruskal–Wallis rank sum test. To determine differences between groups, based on the relative abundance of occurring OTUs, a non-parametric ANOVA (Kruskal–Wallis rank sum test) was applied. Significant differences based on prevalences between groups are calculated by Fisher exact test. For downstream processing of intermediate files generated by IMNGS, a fully modular R-based pipeline (Rhea) was developed for analysis of microbial profiles [16]. Statistical significance was set at $p \leq 0.05$. Any p-values less than 0.05 are shown.

2.6. Ethics Statement

The Study was conducted according to the guidelines in the Declaration of Helsinki and the ICH Harmonized Tripartite Guideline for Good Clinical Practice. The study protocol was approved by the ethics committee of the Faculty of Medicine of the University of Munich. After informing subjects about the study, the intervention, and side effects, all study participants provided written informed consent. The study was registered at ClinicalTrials.gov (NCT02329067) and performed between February 2015 and May 2016 at the University of Munich Medical Center. Walnuts were

provided by the California Walnut Commission (Folsom, CA, USA). Sample analysis was performed at the Chair of Nutrition and Immunology (Core Facility Microbiome) of the Institute for Food and Health (ZIEL) at the Technical University of Munich (Freising, Bavaria, Germany).

3. Results

Alpha-diversity for the walnut and control diets is shown in Figure 2. Supplementing walnuts in the diet did not significantly affect bacterial diversity measured by Shannons effective (walnut vs. control 68.189 vs. 70.118, $p = 0.3789$) and Simpsons effective (33.138 vs. 35.405, $p = 0.0861$) counts. According to this, there was no significant difference in evenness as well as in richness (179.326 vs. 179.393, $p = 0.8522$) for the walnut diet compared to the control diet.

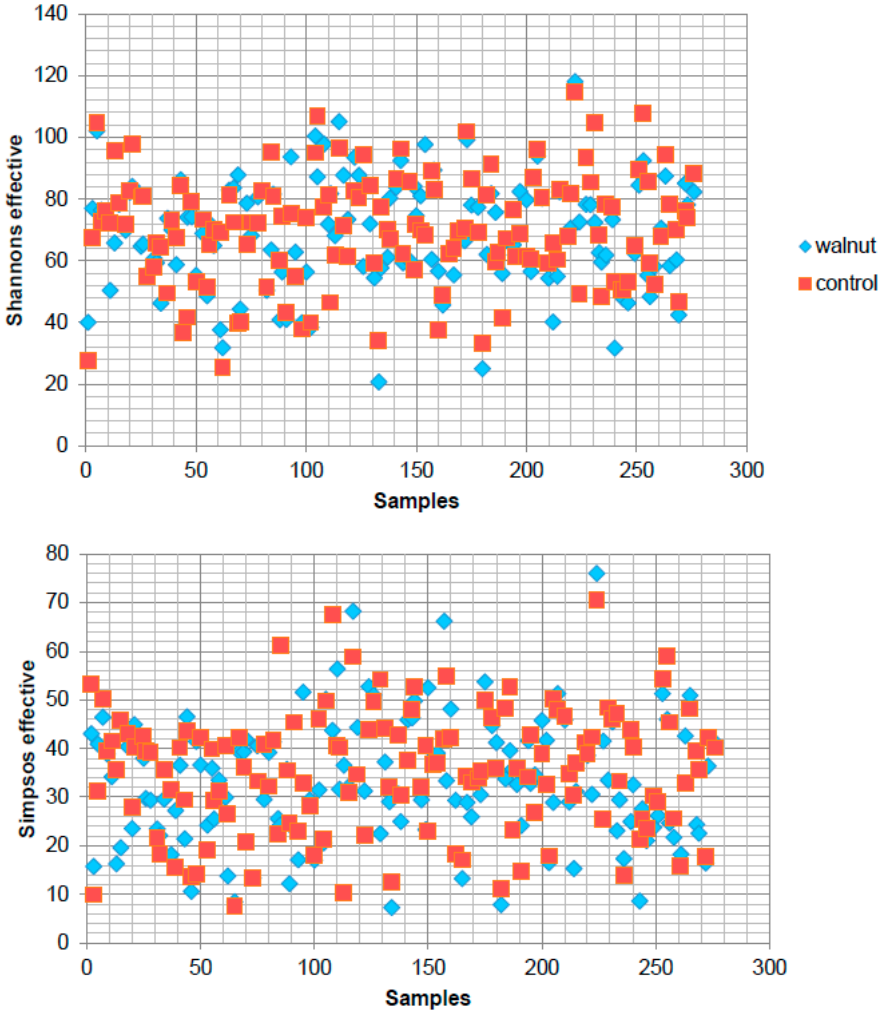

Figure 2. Within-sample alpha-diversity of stool of each subject collected at the end of each diet phase. Calculation of the alpha-diversity for each sample (blue: walnut diet, red: control diet) for evaluating species richness and diversity by using Shannon and Simpsons effective indices. The diversity of a microbial profile for a certain index is the number of different species related to abundance and richness.

By using generalized UniFrac distances considering the phylogenetic distance between OTUs, a multidimensional distance matrix in a space of two dimensions has been visualized by MDS and NMDS. Beta-diversity for walnut and control diet is shown as Principal Coordinate Analysis plot in Figure 3a. Generalized UniFrac analysis demonstrated a clear clustering between the walnut and the control group. MDS (metric and non-metric) indicated significant dissimilarities of approximately 5% between walnut and control ($p = 0.02$).

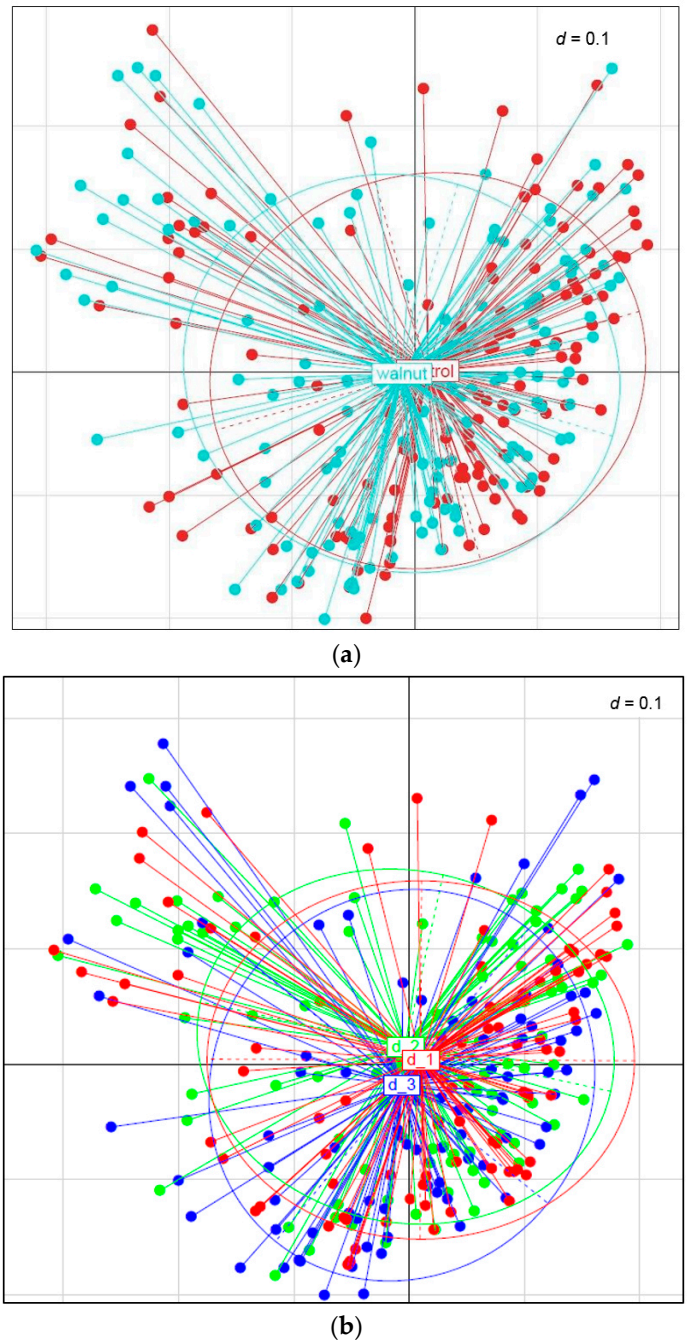

Figure 3. (**a**) Beta-diversity between walnut and control diet. Distinct clustering was observed between the walnut and the control diet. By using generalized UniFrac distances considering the phylogenetic distance between Operational Taxonomic Units, MDS plot indicates significant dissimilarities of approximately 5% between walnut and control (p = 0.02). The multidimensional distance matrix in a space of two dimensions is visualized as MDS plot. Subject's clustering and coloring were done according to the diet type. Each dot end indicates a sample position in the microbiota dataset (blue: walnut diet, red: control diet). (**b**) Beta-diversity between three different diet types during walnut consumption. Distinct clustering was observed between the diets. By using generalized UniFrac distances considering the phylogenetic distance between Operational Taxonomic Units, MDS plot indicates significant dissimilarities of approximately 5% between the different diets (p = 0.026). The multidimensional distance matrix in a space of two dimensions is visualized as MDS plot. Subject's clustering and coloring were done according to the diet type. Each dot end indicates a sample position in the microbiota dataset (red: d_1: carbohydrate restriction; green: d_2: fat restriction; blue: d_3: both).

Generalized UniFrac analysis demonstrated a clear clustering between the different diet groups during walnut consumption (Figure 3b). Again, MDS (metric and non-metric) indicated significant dissimilarities of approximately 5% between the three diet types ($p = 0.026$).

Although walnut consumption shifted the predominant phyla from Firmicutes (61.2% after walnut consumption vs. 63.9% after control) to Bacteroidetes (30.8% vs. 27.4% respectively), these changes in abundance were not significant. Relative abundance was calculated from the relative abundance of 16S rRNA gene sequences for each bacterial community by using the IMNGS platform. The relative changes in OTUs for the bacterial phyla are shown in Figure 4a.

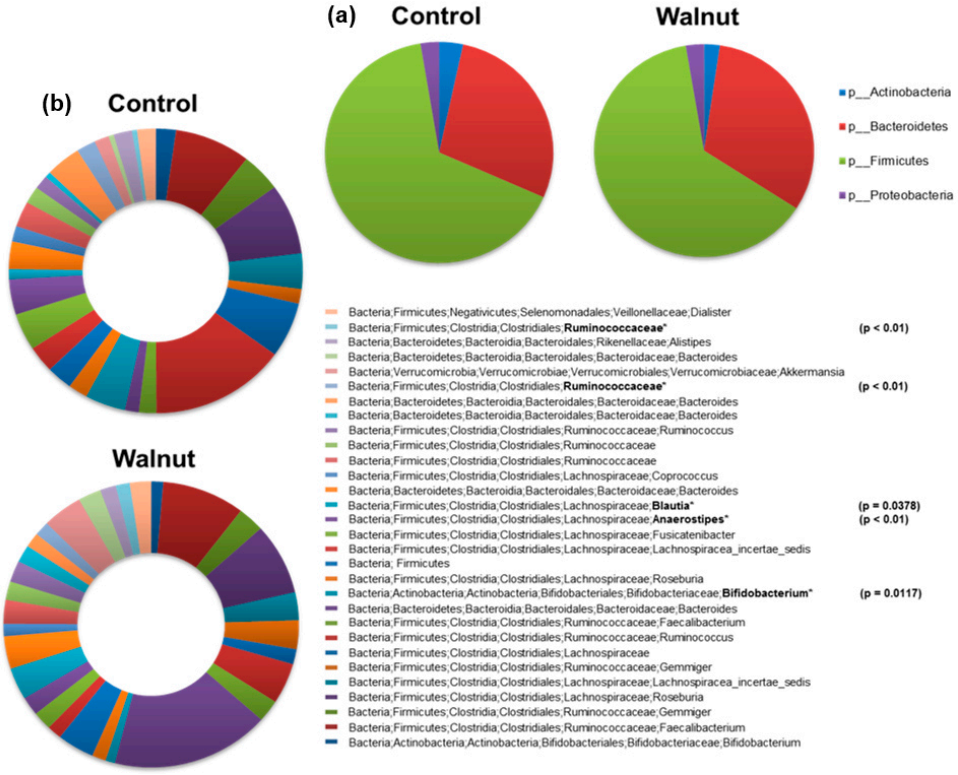

Figure 4. (**a**) Relative abundance of the 4 dominating bacterial phyla between the walnut and the control diet. Walnut consumption shifted the predominant phyla from *Firmicutes* (61.2% after walnut consumption vs. 63.9% after control) to *Bacteroidetes* (30.8% vs. 27.4%). Relative abundance was calculated from the relative abundance of 16S rRNA gene sequences for each bacterial community by using the IMNGS platform. (**b**) Most abundant Operational Taxonomic Units for both walnut and control phase at genus level. Significant different OTUs are marked with by using * and *p*-values. *p*-values were calculated using a pairwise Fisher test.

The predominant bacteria at genus level (Figure 4b) were assigned to four different phyla (*Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Verrucomicrobia*), five classes (*Clostridia*, *Bacteroidia*, *Actinobacteria*, *Verrucomicrobiae*, *Negativicutes*), 5 orders (*Clostridiales*, *Bacteroidales*, *Bifidobacteriales*, *Verrucomicrobiales*, *Selenomonadales*) and seven families (*Ruminococcaceae*, *Bacteriodaceae*, *Lachnospiraceae*, *Bifidobacteriaceae*, *Veillonellaceae*, *Rikenellaceae*, *Verrucomicrobiaceae*).

After walnut consumption, significant shifts in the relative abundance of four members of the phyla *Firmicutes* and in one member of the phyla *Actinobacteria* could be observed (Figure 5A). A significant increase could be identified in two unknown species of the genus *Ruminococcaceae* spp. (*Clostridium* Cluster IV) ($p < 0.02$) and in the species *Bifidobacterium* of the genus *Bifidobacteriaceae* spp. ($p < 0.02$). In parallel, a significant decrease was observed in the relative abundance of two *Lachnospiraceae* species (*Clostridium* Cluster XIV) (a) *Anaerostipes* ($p < 0.01$) and (b) *Blautia* ($p = 0.04$).

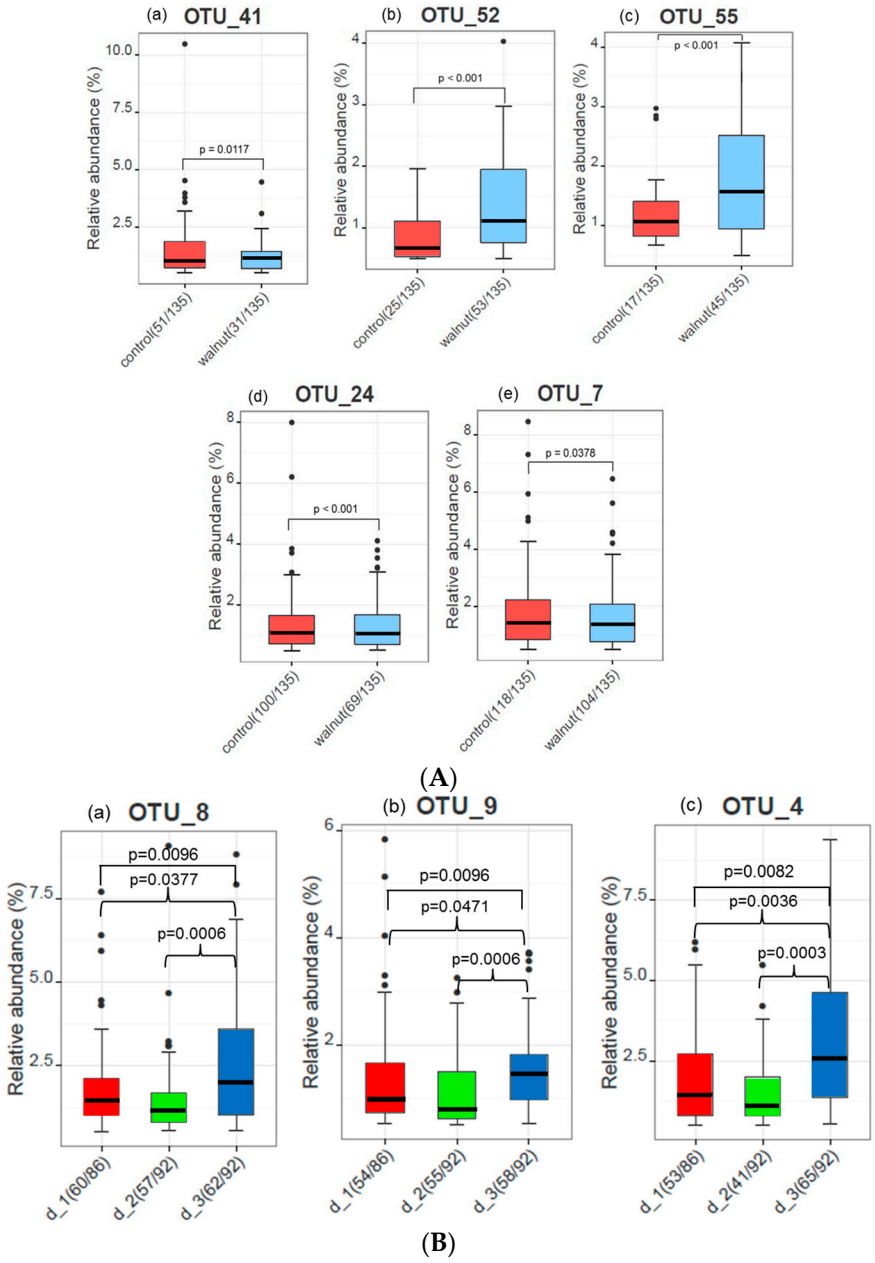

Figure 5. (**A**) Serial-group-comparisons between walnut and control diet. Boxplots of all significant comparisons. Since the data is not normally distributed, a non-parametric Kruskal–Wallis rank sum test and a Fisher's exact test has been used performed in Rhea. (**a**) OTU_41: *Bacteria*; *Actinobacteria*; *Actinobacteria*; *Bifidobacteriales*; *Bifidobacteriaceae*; *Bifidobacterium*; (**b**) OTU_52: *Bacteria*; *Firmicutes*; *Clostridia*; *Clostridiales*; *Ruminococcaceae*; (**c**) OTU_55: *Bacteria; Firmicutes*; *Clostridia*; *Clostridiales*; *Ruminococcaceae*; (**d**) OTU_24: *Bacteria*; *Firmicutes*; *Clostridia*; *Clostridiales*; *Lachnospiraceae*; *Anaerostipes*; (**e**) OTU_7: *Bacteria*; *Firmicutes*; *Clostridia*; *Clostridiales*; *Lachnospiraceae*; *Blautia*. (**B**) Serial-group-comparisons between three different diets during walnut consumption. Boxplots of all significant comparisons. Since the data is not normally distributed, a non-parametric Kruskal–Wallis rank sum test and a pairwise Wilcoxon rank sum test has been used performed in Rhea. d_1: carbohydrate replacement, d_2: fat replacement, d_3: both. (**a**) OTU_8: *Bacteria*; *Firmicutes*; *Clostridia*; *Clostridiales*; *Ruminococcaceae*; *Gemmiger*; (**b**) OTU_9: *Bacteria*; *Firmicutes*; *Clostridia*; *Clostridiales*; *Lachnospiraceae*; *Fusicatenibacter*; (**c**) OTU_4: *Bacteria*; *Bacteroidetes*; *Bacteroidia*; *Bacteroidales*; *Bacteroidaceae*; *Bacteroides*.

Since subjects were advised to reduce either fat or carbohydrates or both during walnut consumption we also evaluated whether this affects microbiome. Comparing these three diet types during walnut consumption revealed significant shifts in the relative abundance of two members of the phyla *Firmicutes* and in one member of the phyla *Bacteroidetes* (Figure 5B). Over all groups, a significant difference could be identified in a species of the genus *Ruminococcaceae* spp. ($p < 0.01$), in one *Lachnospiraceae* species ($p < 0.01$) and in one species of the genus *Bacteroidaceae* spp. ($p < 0.01$). Pairwise testing showed significant differences between the diet types.

4. Discussion

Daily consumption of 43 g walnuts resulted in significant changes in composition and diversity in the gut microbiome by enhancing probiotic- and butyric acid-producing species in healthy individuals.

Obviously, diet is an important factor determining the composition of the gut microbiota. In healthy adults, bacterial clusters within the phyla *Bacteroidetes* and *Firmicutes* usually dominate the intestinal microbiota, whereas the proportions of *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* are relatively small [17]. In animal models, the ratio of *Bacteroidetes* and *Firmicutes* is altered in response to dietary changes [18]. However, although diet-induced shifts in the gut microbiota occur within a short period of time (between 1–4 days after a change in diet), these changes have been shown to be reversed just as rapidly [19,20]. Both genomic sequencings of bacterial rRNA from mice and humans indicate that a high-fat diet promotes a reduction of *Bacteroidetes*, while a fat-restricted diet results in the opposite scenario [21–23]. On the other hand, a high-fat Western-type diet in mice resulted in an increased abundance of *Firmicutes* and a decrease in *Bacteroidetes* [19,24,25]. In contrast, no relationship was observed between the ratio of *Bacteroidetes* and *Firmicutes* and diets low in carbohydrates [26]. Since sufficient and conclusive data from human feeding trials are missing, it is difficult to determine the mechanisms by which walnuts, as part of a Western-type diet, may confer their modulating effects on microbial distribution and changes in the ratio of the major bacterial phyla.

In our study, generalized UniFrac distances demonstrated a distinct clustering between the walnut and the control groups as well as between the different diet types, demonstrating that beta-diversity was altered by walnut consumption. MDS plotting indicated significant dissimilarities of approximately 5% between bacterial clustering during the walnut and the control diets after eight weeks of intervention ($p = 0.02$).

Overall, we identified five OTUs that were significantly associated with walnut consumption. In particular, we found an enrichment of members of the genus *Ruminococcaceae* spp. and *Bifidobacteriaceae* spp. Members of the genus *Bifidobacterium* spp. are proven to exert positive health benefits on their host due to their probiotic properties. *Bifidobacterium* spp. are the normal inhabitants of a healthy human gut, thus, a shift in their relative abundance and composition is one of the most frequent features present in various gastrointestinal diseases including inflammatory bowel disease and colorectal cancer [17,27–29]. *Ruminococcaceae* spp. are an abundant fraction of the human gastrointestinal microbiota and are associated with several important metabolic functions within the *Clostridiales* order (*Clostridium* sp. cluster IV) [1] due to the production of butyric acid. The short chain fatty acid butyric acid is generated from fermentation of indigestible polysaccharides [30] and provides energy for intestinal epithelial cells and contributes to host health by facilitating maintenance of colon epithelial integrity and controlling inflammatory processes [31,32]. Our findings are consistent with other studies investigating the effect of a walnut-enriched diet on the gut microbiome (consumption of 42 g/day walnuts over a period of three weeks) indicating a significant ($p < 0.05$) increase in the relative abundance within the *Clostridiales* order. [33]. Comparable results could be observed in a trial in rats showing significantly greater species diversity after ten weeks on walnut diet by increasing the abundance of probiotic-type bacteria including *Lactobacillus* spp., *Ruminococcus* spp. and *Roseburia* spp. [34].

Besides the significant increase of members of *Ruminococcaceae* spp. and *Bifidobacteriaceae* spp., our findings also showed a significant decrease of two representatives from the *Lachnospiraceae* family under

walnut consumption. These butyric acid-producing microbes account for a great proportion of the *Clostridia* class (*Clostridium* spp. Cluster XIVa) and are highly abundant within the human microbiome. This contrasts with a trial evaluating the effect of walnut consumption on colon carcinogenesis in mouse models which observed an increased abundance of *Lachnospiraceae* spp. during the walnut diet [35]. However, the recommended daily serving of walnuts was higher and intervention period longer. This discrepancy must be evaluated in further trials.

While eating walnuts, subjects were instructed to either reduce fat, carbohydrates or both. In a subgroup analysis, we evaluated whether this also affects the gut microbiome. This analysis showed significant differences in the relative abundance of three microbial representatives (*Ruminococcaceae* spp., *Lachnospiraceae* sp., *Bacteroidaceae* spp.) between the different diet types, whereby no distinct tendency could be observed after pairwise comparison. Thus, it is difficult to make a clear statement about possible different effects as a consequence of macronutrient restriction. As previously mentioned, our subjects did not fully comply with the recommended diet (i.e., substitution of carbohydrates or fat or both for walnuts), indicating that subjects had a similar diet, despite different recommendations [9]. Since our study was designed as "free-living-study" it has to be kept in mind that there are probable discrepancies in the intake of further phytonutrients (including flavonoids, carotenoids, polyphenols, etc.) and dietary fiber intake, which may also induce changes in the gut microbiome (although we did not observe any change in overall fiber intake). However, the study did not focus on changes in these components, particularly since the study relied on self-reported food records making it difficult to correctly estimate phytonutrient intake. The effect of these components can only be addressed by a different study design.

The exact mechanisms by which walnuts may exert their beneficial health effects have not yet been sufficiently investigated. The short chain fatty acid butyrate may beneficially affect metabolic and inflammatory processes and, thus, obesity, diabetes and inflammatory bowel diseases [36,37]. However, only few feeding trials have examined the prebiotic effect of nuts, especially walnuts. Thus, the exact mechanism by which they shift the relative abundance of microbial communities and modulate fluctuations in the microflora composition in the gut in favor of butyrate-producing microbes is unknown. Furthermore, non-digestible material from nuts, mainly polyphenols and polysaccharides including dietary fiber seem to have a prebiotic effect by increasing *Lactobacillus* spp. and *Bifidobacterium* spp. growth and fermentation of indigestible components to short-chain fatty acids including butyrate [10,38,39] that may also alter activity of intestinal microbial enzymes [40].

In contrast to a higher beta-diversity, the change in alpha-diversity was not significantly different between the walnut and the control groups. This indicates that, under walnut consumption, the gut microbiota showed a slightly lower diversity than under the control diet; however, this difference was not significant. Walnut consumption shifted (not significantly) the predominant phyla from *Firmicutes* (61.2% after walnut consumption vs. 63.9% after control) to *Bacteroidetes* (30.8% vs. 27.4%) (Figure 4). As mentioned above, the correlation between specific diets and shifts within the *Firmicutes*/*Bacteroidetes* ratio is a matter of controversy [21–26]. Our data do not agree with the results of two previous animal feeding studies demonstrating that walnuts significantly altered the relative abundance of these two major gut bacterial phyla independent of the length of walnut consumption [34,41]. However, study conditions are hardly comparable due to the different walnut serving sizes and varying study durations. To date, no human feeding trials are available to discuss the effect of walnut consumption on the *Firmicutes*/*Bacteroidetes* ratio.

As a first conclusion, our data indicate a correlation between walnut consumption and a shift within the gut microbiome, suggesting that a regular supplementation might offer prebiotic and probiotic benefits by improving the microbiome composition and diversity.

Recently, three papers described the prebiotic properties of other members of the tree nut family in human clinical feeding trials. One study determined the effects of almond and pistachio consumption on gut microbiota composition in humans. The effect of pistachios was much stronger than that of almonds and resulted in an increase in potentially beneficial butyrate-producing bacteria in the

phylum *Firmicutes* [42]. Comparable results have been demonstrated by another human feeding trial with a similar initial hypothesis but over a much shorter intervention period of only 18 days. The effect of pistachio consumption on gut microbiota composition was again much stronger compared to that of almond consumption. It was concluded that almonds and especially pistachios can affect the composition of the fecal bacterial microbiota [43]. In vitro and in vivo studies analyzed the prebiotic effect and fermentation properties of raw and roasted almonds, as well as almond seed and almond skin [40,44–47]. Both raw and roasted almonds showed potential prebiotic effects on intestinal bacteria and metabolic activities, showing a stimulatory effect on fecal *Lactobacillus* spp., and *Bifidobacterium* spp. [40]. Significant increases in the abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. could also be observed in fecal samples as a consequence of both raw almond and almond skin supplementation [47].

Although our findings are only observational, the results indicate that nuts (especially walnuts) may represent an important dietary supplement not only to positively influence blood lipids but also to improve gut microbiome health. It is unclear if and how the changes in the microbiome are linked to the observed changes in fasting lipid metabolism [9]. The study design and the high variability in the observed changes in the microbiome preclude any valid conclusions at this point. Interestingly, there are only very few studies investigating the effect of statins on gut bacteria. It has been hypothesized that gut bacteria may cause inherent differences in the way subjects metabolize and benefit from therapeutic agents due to higher levels of bacterial-derived bile acids [48]. Furthermore, gut microbiota analysis in mice treated with hypolipidemic drugs revealed a modification in composition in favor of probiotic-type bacteria from *Lactobacillus* spp. [49]. However, the exact mechanisms by which cholesterol-lowering substances may interact with the human gut microbiome have not been sufficiently investigated. Another interesting aspect that should be considered for further investigations is the finding that certain metabolites strongly correlate with microbial community structures which would allow gaining insights into microbiome–host interactions, also in context of certain diseases and therapeutic interventions [50,51]. The analysis of metabolic fingerprints might be useful to understand how microbial structures are influenced by regular walnut consumption.

More interventional nutritional studies might be required to quantify the underlying mechanisms by which walnut components influence microbiome composition and how the abundance of butyrate-producing bacteria is increased. Furthermore, further evaluation regarding whether these observed changes are preserved during longer walnut consumption is required.

5. Conclusions

Current study results show a shift within the composition of microbial communities in the human gut under nut consumption. This shift is characterized by an increase in the relative abundance of potentially beneficial butyrate-producing bacteria. In our study, we showed that daily intake of 43 g walnuts over eight weeks significantly affected the gut microbiome by enhancing probiotic- and butyric acid-producing bacteria in healthy individuals. It is unclear whether these changes are preserved during longer walnut supplementation and how these changes are associated with the observed changes in lipid metabolism. More human intervention trials investigating different servings of nuts over a longer time period might be useful to further evaluate the prebiotic properties of walnut consumption.

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Author Contributions: The authors' responsibilities were as follows: K.G.P. designed the study. C.B., A.R., L.W., K.L. and E.W. were responsible for subject recruitment, testing and data collection. K.H. and J.A. conducted sample analysis for plasma lipid profile. S.F. was responsible for stool sample analysis. R.G.S., A.R., S.F., C.B. and K.G.P. analyzed the data. C.B. and K.G.P. drafted the manuscript. K.G.P. had primary responsibility for the final content. All authors read and approved the final manuscript.

Conflicts of Interest: C.B., A.R., K.L., L.W., E.W., S.F., J.A. and K.H. have nothing to disclose. The research was supported by a grant from the California Walnut Commission (Folsom, CA, USA) to KGP. The Walnut Commission had no role in study performance, data analysis and or manuscript writing.

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10. Abkürzungsverzeichnis

ApoB	Apolipoprotein B
BMI	Body-Mass-Index
EAS/ESC	European Atherosclerosis Society Guidelines
EFSA	Europäische Behörde für Lebensmittelsicherheit
HDL	High Density Lipoprotein
IDL	intermediate density lipoprotein
KHK	Koronare Herzkrankheit
LDL	Low Density Lipoprotein
Lp(a)	Lipoprotein (a)
MUFA	Monounsaturated Fatty Acid (einfachgesättigte Fettsäure)
OTU	Operational Taxonomic Units
PCSK-9	Proprotein Convertase Subtilisin Kexin Type
PREDIMED	Prevención con Dieta Mediterránea / Prevention with Mediterranean Diet)
PROCAM	Prospective Cardiovascular Münster Heart Study
PUFA	Polyunsaturated Fatty Acid (mehrfachgesättigte Fettsäure)
SCFA	Short Chain Fatty Acids
TC	Total Cholesterol
TG	Triglyceride
VLDL	Very Low Density Lipoprotein
Wa	Walnut/Walnuss
WHO	World Health Organization
16s rRNA	16S ribosomale RNA

11. Abbildungsverzeichnis

12. Tabellenverzeichnis